ROLES OF THE PHOSPHATIDYLINOSITOL 3-KINASE REGULATORY SUBUNIT P55GAMMA ON REGULATING SURVIVAL SIGNALING IN BREAST CANCER CELLS

A Dissertation
submitted to the Faculty of the
Graduate School of Arts and Sciences
of Georgetown University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Tumor Biology

By

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Washington, DC
December 21, 2009
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ABSTRACT

Phosphatidylinositol 3-kinase (PI3K), specifically its catalytic subunit, is involved in tumorigenesis. However, the roles of the regulatory subunit of PI3K in cancer are largely unknown. Our aim is to determine the roles of the PI3K regulatory subunit p55gamma on regulating the survival signaling of breast cancer cells.

Silencing of p55gamma significantly reduces cell growth and induces apoptotic cell death. As a result, breast cancer cells are less capable of forming colonies in vitro. Morphologically, p55gamma reduction induces multipolar mitosis, as indicated by immunostaining of alpha-tubulin. Silencing of p55gamma also causes cell cycle arrest in G2/M phase, and the G2/M checkpoint proteins are correspondingly modulated after p55gamma reduction. The up-regulation of cyclin B1 and phosphorylated Cdc2, as well as down-regulation of Chk-1 protein, confirm an increased proportion of cells lingering in mitosis phase and indicates that these cells might die from prolonged mitosis phase retention. Moreover, silencing of p55gamma results in down-regulation of the survival signaling protein-survivin, further supporting our hypothesis that p55gamma is involved in survival signaling. These data indicate that expression of p55gamma is necessary for cell growth; the silencing of p55gamma results in significant M phase retention,
increased apoptosis, mitotic defects, and profound cell death in some cells. Interestingly, the survival signaling mediated by p55gamma might involve not only the conventional but also the non-conventional PI3K/Akt pathway, because not all cells with p55gamma silencing show reduced activation of Akt.

Upon activation of PI3K through epidermal growth factor stimulation, the localization of p55gamma, which is shown by a GFP-p55gamma fusion protein, shifted from the cytoplasm to not only the cellular membrane, but also the nuclear region. This suggests that a novel role of p55gamma might exert by interaction with the nuclear proteins. Furthermore, we demonstrate the formation of a protein complex consisting of calmodulin, p55gamma and Rb, and we show the change in the relative binding in the presence of calcium. These observations indicate that p55gamma may regulate cell signaling through interaction with these proteins.

In conclusion, our study has revealed for the first time that p55gamma plays a significant role in the survival signaling of breast cancer cells.
ACKNOWLEDGEMENTS

I would like to extend my deepest appreciation to my mentor, Dr. Michael D. Johnson, for his inestimable patience, and his enduring support and guidance for my thesis study. It has been a privilege for me to have him as a mentor after the tremendous trauma in my scientific life, the loss of my late mentor, Dr. Robert B. Dickson (1952-2006). It has been a great experience working with Dr. Johnson because of his extraordinary enthusiasm in scientific research and mentorship, which will serve as a guide for me in my future scientific endeavors. I would also like to thank my other thesis committee members, Drs. Todd Waldman, Anton Wellstein, Vicente Notario, C. Richard Schlegel, Dean Rosenthal, for their helpful suggestions and constructive criticisms.

I would like to extend my thanks to Drs Susette C. Mueller and Xuehua Xu, for their insightful discussions for the microscopic imaging study of my thesis work, and the service at the microscopic imaging core facility.

I would also like to extend my thankfulness to the past and current members of Dickson/Johnson lab, Drs. Tushar B. Deb, Chenyong Lin, Linda Barlow, Jiankang Wang, Mingxue Lee, Lilian Chen, Christine M. Corticchia, Radmila Jancovic, Chetana Revenka, Mr. Philip Fengpai Chou, Mr. I-Chu Tseng, Ms. Deborah Carbott, Mr. Daniel Kyle, Ms. Eva Andersson, Ms. Annie Hong Zuo, Mr. Robert Barndt. I would like to remember their help and friendship.
Finally I would like to acknowledge the Department of Defense for the DOD DPRTA Predoctoral Traineeship Award (W81XWH-04-1-0408). The funding I received provided me with more than just the opportunity for quality training. It was instrumental in allowing me to design a research experience that suited my career interest.
DEDICATION

To

My mother and father, Huilan Liu and Yanjin Wang,

Who instilled in me a love for science, and made all of this possible.

Also to

My husband, Mitchell Ho

For his lasting patience and encouragement.
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ABBREVIATIONS

ATM, Ataxia telangiectasia mutated
ATR, Ataxia telangiectasia and Rad3 related
BCA, bicinchoninic acid
BCR, breakpoint cluster region
Cdc2, Cell division cycle 2
Cdk-1, Cyclin dependent kinase 1
Chk-1, Checkpoint Kinase-1
DMEM, Dulbecco’s Modified Eagle Medium
EGF, epidermal growth factor
ER, estrogen receptor
F-actin, actin filaments
FBS, fetal bovine serum
GBM, glioblastoma
HER2, Human Epidermal growth factor Receptor 2
IGF-1 or 2, insulin-like growth factor-1 or 2
IGF-1R, insulin-like growth factor-1 receptor
IRS-1, insulin receptor substrate-1
IMEM, Improved Minimal Essential Medium
PARP, Poly ADP ribose polymerase
PDGF, platelet-derived growth factor receptor
PDK, Phosphoinositide dependent kinase
PI3K, Phosphatidylinositol-3 kinase
PI3P, Phosphatidylinositol-3, 4, 5-trisphosphate
Plk-1, Polo-like-kinase-1
qPCR, Quantitative real-time reverse transcription PCR
RNAi, RNA interference
shRNA, short hairpin RNA
SREBP-1, Sterol-regulatory element binding protein-1
TIRFM, Total Internal Reflection Fluorescence Microscopy
MAPK, Mitogen-activated protein kinase
mTOR, mammalian target of rapamycin
miRNA, microRNA
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CHAPTER 1

INTRODUCTION

1.1 Breast cancer and cancer drug development

Breast cancer is a very common malignancy. About one in eight women develop breast cancer in their lifetime. Men also develop breast cancer, although at a much lower rate than women. As shown by American Cancer Society in Breast Cancer Facts & Figures 2009 (www.cancer.org), approximately 194,280 new cases of invasive cancer were estimated to be diagnosed in 2009. Among these cases, an estimated 40,610 women and men will die from this disease without proper treatment.

The past decade has seen unparalleled discovery in the molecular and genetic bases of breast cancer. Patient’s death rate was significantly reduced by early-stage cancer detection, new chemotherapy and recurrence prevention drug development. In addition to surgery and breast radiation therapy, tamoxifen (blocking action of estrogen) adjuvant therapy or aromatase inhibitors (stopping estrogen production) are given to patients to reduce disease recurrence. The aromatase inhibitors are usually administered to postmenopausal women experiencing tamoxifen resistance. Unfortunately, many patients experience bone problems while being treated with aromatase inhibitors [1]. Trastuzumab is an antibody that can selectively bind to and inactivate the Human Epidermal growth factor Receptor 2 (HER2) protein. This makes the HER2 protein unable to induce uncontrollable reproduction of the cells in the breast, thus increases the survival of people with breast cancer [2]. FDA approved the usage of trastuzumab for
all breast cancers with HER2 overexpression as part of the adjuvant therapy in 2006 (www.cancer.gov), and the drug has improved the survival of many breast cancer patients, however, a majority of patients develop resistance to trastuzumab within one year after treatment initiation [3], the mechanisms of resistance include the disrupted interaction between antibody and target, the compensatory up-regulation of the other signaling, the altered downstream signaling, and the increase in binding partners of trastuzumab.

Recently, significant efforts have been focusing on the signaling pathways that involved in the breast cancer development. Cells receive signals from extracellular through ligand stimulation of a series of cell surface receptors such as insulin receptor, integrin receptor, epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, and others. The activated receptors then pass the stimulant to the signaling transducers and activate downstream pathways. The signaling networks involved in cell growth and survival, invasion, metastasis, and angiogenesis draw intensive interest in cancer treatment, because they are often dramatically up-regulated or altered in tumor tissues compared with normal host cells. The most frequently altered in breast cancer includes the key components of phosphatidylinositol 3-kinase pathway [4].
1.2 Phosphatidylinositol 3-kinase (PI3K)

1.2.1 PI3K signaling

PI3Ks are bifunctional proteins showing both serine-threonine and phospholipid kinase activities [4]. They belong to a conserved family that phosphorylates phosphoinositides at the 3'-hydroxyl group. This reaction is well known by its product, the phosphatidylinositol-3, 4, 5-trisphosphate (PIP3), a critical second messenger for cell signaling. Upon activation, PIP3 recruits a series of downstream target proteins such as protein kinase C [5;6], p70 S6-kinase [7], Akt/protein kinase B [8] and others. Meanwhile, PIP3 translocates to the cellular membrane and regulates many fundamental processes such as cell growth and metabolism, survival signaling, and cytoskeletal rearrangements.

There are I, II and III totally three subclasses of PI3Ks defined by structure, regulation, and substrate specificity [9], and one additional class of distantly related enzymes containing the serine/threonine kinase activity in vitro. The activity regulated through PIP3 is a unique feature of the class I PI3Ks, which includes class IA and class IB. So far only the class IA PI3Ks was reported implicated in human cancer. Class IA PI3Ks are thought to exist mainly as heterodimers formed by one regulatory subunit (p85α, p55α, p50α, p85β, p55γ) and one catalytic subunit (p110α, p110β, p110δ). The activation of PI3Ks is believed to occur through association of the Src homology 2 (SH2) domain of the regulatory subunit with the phosphorylated tyrosine residues of the
activated receptor tyrosine kinase (RTK) [10]. The binding of regulatory subunits to RTK results in both relieving the inhibition of the catalytic subunit and targeting the catalytic subunit to the plasma membranes to facilitate signaling transfer to downstream effector proteins [10]. The representation for activation of PI3K through ligand binding to the receptor tyrosine kinase is summarized in Figure 1.1.
Figure 1.1 Schematic representation of the Class IA PI3K activation pathway.

PI3K is shown being activated through phosphorylation of the Receptor Tyrosine Kinases by a ligand. The autophosphorylation of the receptor attracts the regulatory subunits p85α, or p85β, p55α, p50α, or p55γ. The regulatory subunits then recruit p110α to the plasma membrane and the complete kinase transforms PIP2 into PIP3. The downstream target protein network which can be activated by PIP3 is simplified by showing only the major target Akt, which is involved in effects such as cell differentiation, cell proliferation, survival, etc.
1.2.2 Catalytic subunits of Class IA PI3Ks

The catalytic subunits of class IA PI3Ks including p110α, p110β, and p110δ, are encoded by the PIK3CA, PIK3CB, and PIK3CD genes, respectively [11]. They share similar structure composition including an N-terminal regulatory subunit binding domain, followed by a Ras binding domain, then a C2 domain which confers binding to the plasma membrane, a phosphatidylinositol kinase domain, and finally a C-terminal catalytic domain. Many PI3K inhibitors under development today target the PI3K catalytic subunits, which are either pan or isoform specific.

Transgenic mice models with silencing of catalytic subunits of PI3K provided insights into the major functions of these proteins. In normal tissues, the expression of p110α and p110β is ubiquitous, while p110δ manifests mostly leukocyte localization. Accordingly, knockout mice with deletion of either PIK3CA [12] or PIK3CB [13] are embryonic lethal, indicating the indispensable functions of these genes in embryonic development. Mice with gene targeting p110δ were viable, but showed defective immunological phenotypes, decreased expression of regulatory subunits, as well as decreased activation of Akt signaling [14-16].

1.2.3 Regulatory subunits of Class IA PI3Ks

Five proteins encoded by three genes form the group of class IA PI3Ks regulatory subunits. Gene pik3r1 encodes p85α, and the shorter isoforms p55α and p50α are generated from alternative transcription-initiation sites different from p85α; gene pik3r2
and gene pik3r3 encode p85β and p55γ, respectively. The common domain structure of regulatory subunits includes a p110-binding domain located at the C-terminal, also called the inter-Src-homology 2 (SH2) domain, flanked by two SH2 domains. The difference of the regulatory subunits lies in the N-terminal region, while p55α and p55γ contains a shorter N-terminal domain, the longer isoform p85α and p85β contains a Src-homology 3 (SH3) domains and a BCR homology domain, and several proline-rich regions [11] (Figure 1.2 A). So far the regulatory subunits have been recognized as having three major functions: (1) to bind and stabilize p110 catalytic subunit by protecting it from degradation; (2) to maintain the PI3K activity at basal level when no stimulant is present; and (3) to bind receptor tyrosine kinase and recruit p110 to the membrane for the complete PI3K activity upon stimulation. In addition to these major functions, when overexpressed, excessive regulatory subunits p85α in monomeric form can negatively regulate PI3K signaling through occupying the adaptor proteins mediating the downstream kinase signaling [17]. So far there have been no reports on regulatory subunits other than p85α that show inhibitory effects on kinase signaling when overexpressed. Data from knockout mice models suggested that silencing one regulatory subunits may have profound effects on increasing the protein level of the other regulatory subunits, as well as the physiological readouts such as increased insulin sensitivity [18]. Moreover, the significantly different responses to insulin signals by individual regulatory subunits of PI3K have been reported in human skeletal muscles [19]. It appears that these regulatory subunits have certain compensatory functions with
each other; they are also differentially localized and react distinctly to the same stimulant, thus justifying the existence of each individual subunit.

1.2.4 Involvement of PI3K in breast cancer

Class IA phosphoinositide 3-kinase (PI3K) is specifically implicated in the pathogenesis of cancer. Most PI3K-addicted tumors contain hyper-activated PI3K signaling, which has stimulated the development of PI3K inhibitors. The frequently modulated genes include mutation of PIK3CA, AKT, and PIK3R1, loss of PTEN, or amplification of PIK3CA [10]. PI3K inhibitors currently in clinical trials competitively occupy the ATP-binding pocket of the PI3K catalytic subunit, thus preventing its activation. However, the intracellular survival and proliferation signaling networks are complicated and have both redundant and non-overlapping functions, so that hitting multiple targets activating the same signaling pathway has become a promising strategy for combination therapy. Further investigations on PI3K pathway alterations such as manipulation of regulatory subunits could complement this approach.

The pik3rl gene has been suggested to act as an oncogene in human ovarian and colon cancers [20], on the basis of evidence that expression of its exon 13 deletion mutation construct leads to increased PI3K activity, probably through interruption of the kinase accessibility of Serine608 residue on p85α. In addition, the pik3rl gene is also overexpressed in glioblastoma [21].
The high frequency of somatic mutations in the PIK3CA gene encoding the PI3K catalytic subunit gene has been revealed in a variety of cancer types [22-25]. The constitutively active mutants were found to transform primary, immortalized cells into cancerous cells, which are oncogenic in vivo in breast [26-28], ovarian [28], or colorectal cancers [24;29]. The structural basis for increased activity of PIK3CA mutants has been determined. Although the mutations could be found on other exons, they most frequently occur in the three “hotspots”, including the kinase (exon 20) domain mutation H1047R, and the helical (exon 9) domain mutations E542K, and E545K. These mutations elicit different mechanism of lipid kinase activation [27]. Comparing to the cases containing no PI3K pathway activation, Herceptin treatment results in worse prognosis with either PIK3CA activating mutations or PTEN lost-of-function mutation or deletion [30]. An earlier research using Single-Strand Conformational Polymorphism (SSCP) for mutation analysis to detect the PIK3CA mutations in 180 breast cancer tissue samples showed 26% of the tumors contain the mutation. Half of these mutations occur on exon 9, and the other half occur on exon 20 [31]. This paper also showed that the PIK3CA mutant is more frequent in more virulent histological types such as lobular and ductal carcinomas [31]. Another independent study following 1394 cases of early stage breast cancer also correlated PIK3CA overexpression with poor prognostic variables such as higher grade, nodal metastasis, etc [32]. These data suggested that the activating mutations of PIK3CA promote cancer development and indicate poor prognosis for cancer patients.
However, individual studies do not always come to the same conclusion, there have been several independent studies showing that \textit{PIK3CA} mutation may act as an indicator of good outcome, or no effect at all, and making the role of this gene event more complex and controversial. In a study on a population of 188 Japanese breast cancers, the effect of \textit{PIK3CA} mutation was determined \cite{33}. The \textit{PIK3CA} mutations were found in 29% of the cases, among these 31% were on exon 9 and 54% on exon 20. These mutations were identified only in the invasive carcinomas, and positively correlated with ER-\textit{α} and Akt activation. Interestingly, in this study 176 patients were evaluated, and the presence of mutations were found to correlate positively with a relapse-free survival rate \cite{33}. Similarly, in a recent study of 590 primary breast tumors, the H1047R \textit{PIK3CA} mutation was found to be correlated with an improved patient survival with negative HER2, low tumor stage, and the nodal negativity \cite{34}. In addition, no correlation between mutation and survival rate, or the prognostic factors such as age of the patients, staging, tumor size and location has also been reported \cite{28,35}, however, the discrepancy might be that these studies included all kinds of mutations, not only those occurring on hotspots, thus diluted the effect of the hotspot mutations, which are considered more potent in tumorigenesis \cite{36}. Therefore, the correlation of \textit{PIK3CA} mutation with patient survival rate remains undetermined, although \textit{PIK3CA} mutation is identified in invasive breast cancers.
1.3 Phosphatidylinositol-3 kinase regulatory subunit p55γ

1.3.1 Discovery and tissue-specific expression of p55γ

Human p55γ was discovered by Dey and his colleagues in 1998 [37], three years after its murine homologue p55pik was cloned [38]. In order to identify proteins bound to insulin-like growth factor-1 receptor (IGF-1R), they used a yeast two hybrid system to screen a cDNA library established from fetal brain and isolated a cDNA encoding pik3r3, which they named hp55γ, to distinguish from its murine form counterpart [38]. Human p55γ contains an inter-SH2 domain like the rest of the regulatory subunits to associate with catalytic subunits and form a functional kinase. The fully assembled kinase then activates a serial of downstream target proteins, which are involved in a host of cellular activities such as cell metabolism, cell survival, vesicle trafficking, etc. The predicted amino acid sequence of human p55γ shares 96% amino acid homology with mouse p55pik [38] and 95% homology with rat p55γ [39]. The p55γ protein also shares homology with human p85α and bovine p85β [37].

Four different p55γ mRNAs migrating at 5.8, 3.2, 2.0, and 1.6 kb, respectively, have been identified in human fetal and adult tissues [37]. The smaller mRNAs (3.2, 2.0 and 1.6 kb) seem to be exclusively expressed in humans, and are not reported in mice and rats. Moreover, human fetal tissues contain the large mRNA (5.8 kb) and lack the smaller ones, suggesting the role of the smaller mRNAs on developmental regulation in
different species [37]. Interestingly, the sizes and distribution of the p55γ mRNA are quite different from those reported on p85α and p85β [38;39].

The expression of p55γ is controlled by Sterol-regulatory element binding protein-1 (SREBP-1) [40]. The p55γ expression is decreased after incubation with cholesterol and 25-hydroxycholesterol, through blocking the cleavage of the endogenous SREBP-1; on the other hand, growth factors such as PDGF activate SREBP-1 and induces the expression of p55γ, adenovirus mediated SREBP-1 expression also induces the p55γ expression. Likewise, fasted mice refed with a high-carbohydrate diet showed activation of SREBP-1 and p55γ up-regulation in the liver [40]. Interestingly, the activity of SREBP-1 can be conversely modulated by PI3K signaling. It was shown that the lipid synthesis and cell proliferation induced by keratinocyte growth factor must be mediated through the cooperation of both PI3K and JNK signaling to activate SREBP-1 [41]. These results suggest that for the growth effect initiated by keratinocyte growth factor, the signal may be modulated by the positive feedback of p55γ towards activation of SREBP-1, as a regulatory subunit of PI3K. The phosphorylation of p55γ can be induced by growth factor stimulation, it has been reported that insulin-like growth factor-1 (IGF-1) stimulation of 293 cells overexpressing p55γ results in tyrosine phosphorylation of p55γ [37].

In normal human tissues, both fetal and adult tissues have the similar p55γ expression profile; brain and testes have the highest amount of p55γ mRNA expression,
whereas liver has the lowest expression levels [37]. The expression level of p55γ is upregulated in some tumor samples. For example, the p55γ mRNA was upregulated in hepatocellular carcinomas comparing to normal liver tissues (www.oncomine.org). Comparison of mRNA expression across 8 independent analysis showed that the invasive breast ductal carcinomas contain significantly overexpressed p55γ mRNA compared to normal breast tissues (p=0.005) (www.oncomine.org).

1.3.2 Chromosome location, splice variants and domain structures of p55γ

The gene encoding p55γ is called pik3r3, and is located in the reverse strand of human chromosome 1. Four splice variants of p55γ have been identified in humans so far. The sequences of these splice variants are compared using alignment tools (http://workbench.sdsc.edu) and the location of the deletions were identified (Figure 1.2 B and C).
(A) gene  protein

p85α
p85α
p50α
p55α
p55γ
pik3r1  pik3r2  pik3r3

Pro. rich  Pro. rich  Pro. rich
BH
SH2
p110 binding

Unique N-terminal 34 amino acids

Not drawn to scale

(B) N-terminal splice variant
Deletion of 36 amino acids (36-71)
C-terminal splice variant
Deletion of 59 amino acids in the inter-SH2 region

N-34  Proline-rich  N SH2  P110 bind  C SH2

KD93  KD90
<table>
<thead>
<tr>
<th>N-terminal splice variant</th>
<th>C-terminal splice variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deletion of 36 amino acids</td>
<td>Deletion of 59 amino acids</td>
</tr>
</tbody>
</table>

The diagram shows the sequences for both splice variants with deletions highlighted.
Figure 1.2 Comparison of the protein structures of Class IA PI3K regulatory subunits. (A) Comparison of the domain structures of the Class IA PI3K regulatory isoforms. The sequence similarity is indicated by similar shapes and shades. The approximate location of the amino terminal 34 amino acids is indicated with an arrow. (B) Domain structures of p55γ amino acid sequence. N-terminal and C-terminal splice variants as the targeting sites for shRNA KD90 and KD93 (chapter 2) are indicated. (C) Protein sequence alignment of four splice variants of p55γ. The original sequence is presented second from top. Identical sequences are highlighted in green, or otherwise highlighted in yellow color. The dashes represent gaps in the sequence alignment.
Two variants encode the same 461 amino acid sequence as the prototype protein. The third carboxyl-terminal splice variant harbors a 59-amino acid deletion between the p110 binding region and C-terminal SH2 domain at 256-314 position, resulting in a sequence of 402 amino acids in length [37]. The fourth splice variant contains an amino-terminal 36 amino acid deletion spanning 36-71, disrupting the proline-rich domain; and a carboxyl-terminal splice site 256-314 deletion, the variant is 366 amino acids in length (http://www.ensembl.org). The two variants of different sizes did not seem to have impaired protein interaction with IGF1R and IR in the yeast two-hybrid assay [37]. However, the functions of the modulated amino acids in splice variants were not clear. Interestingly, using site-directed mutagenesis, p55pik, the mouse homolog of human p55γ, has been reported to use two different translational initiation start codons from the cDNA sequence, and the resulting alternative translational initiated protein is shorter, and termed p50pik, with the N-terminal 31 amino acids missing [42]. So far there is no evidence showing whether mouse p50pik has a human homolog.

Compared to the other regulatory subunits, the domain structure of p55γ is special. It has a unique N-terminal 34 amino acid domain, which contains a pYxxM motif (codon 29-32 amino acids) that potentially binds to the SH2 domains of a cell signaling molecule [37]. Interestingly, the N-terminal 34 amino acids of p55γ share 50% identity and 62% similarity with that of p55α, the pik3r1 gene product translated from an alternate initiation site compared to p85α [43]. There are also domains in p55γ that are
highly homologous with other regulatory subunits, such as a proline-rich motif, two Src homology-2 (SH2) domains, and an inter SH2/p110 binding site. Unlike human p85, p55γ has no SH3 domain or breakpoint cluster region (BCR) domain [37]. Many investigations have explored the unique N-terminal amino acid sequence using overexpression or gene silencing approaches and some interesting results will be discussed.

1.4 Amplification/overexpression of p55γ in various tumors

1.4.1 Breast cancer and ovarian cancer

The mRNA level of p55γ is significantly up-regulated in tumors from breast and ovarian cancer patients compared to normal tissues [44]. Similarly, malignant breast tumors have higher levels of p55γ mRNA than the ductal carcinoma in situ tumor extracts [45]. In addition, the protein level of p55γ was also up-regulated in ovarian cancer tissue array samples when compared with normal tissue [44]. These observations indicate that p55γ up-regulation is probably associated with these two major gynecological malignancies.

1.4.2 Glioblastoma

High level expression of p55γ was detected in brain tissues, especially in fetal brain, suggesting p55γ may be involved in brain embryogenesis. Interestingly, in a subclass of aggressive glioblastomas (GBMs) characterized by lacking amplification or
overexpression of epidermal growth factor (EGF) receptor, but overexpressing insulin-like growth factor 2 (IGF-2), p55γ showed dramatic genomic gains [46]. On the other hand, knockdown of pik3r3 inhibited IGF2-induced growth of GBM-derived neurospheres [46]. These results suggested that p55γ may act as an effector of IGF-2 in the absence of EGF receptor, and promotes cell proliferation in GBM.

1.5 Proteins associated with p55γ

1.5.1 PI3K catalytic subunit p110α

As the binding partner of the PI3K heterodimer, p110α associates with p55γ through the inter-SH2 domain of p55γ. It has been demonstrated that the amino acids 203-217 on the inter-SH2 domain are indispensable for the binding of p55γ to the NH2 terminus region of p110α [47]. The binding of p110α and p55γ is initiated by stimulants such as growth factors and completed by the recruitment of p55γ to the receptors and the subsequent association of p110α with p55γ. The outcome of this binding is presumably the production of second messenger PIP3 and the activation of downstream targets regulating cell survival and proliferation signaling.

1.5.2 Insulin signaling proteins IGF-1R, IR and IRS-1

Dey and others found that IGF-1R, insulin receptor, and insulin receptor substrate-1 (IRS-1) interact with p55γ in vitro in a yeast two-hybrid system, and this binding
requires receptor activation [37]. The two SH2 domains of p55γ are involved in the association with IGF-1R, and the association occurred at tyrosine 1316 residue of the receptor. The interaction between p55γ and IGF-1R was also detected by co-immunoprecipitation of lysates from IGF-1 stimulated 293 cells overexpressing p55γ [37]. In 293 cells, the association of p55γ with IRS-1 and IGF-1R appeared to be dependent on PI3K, since it was increased by wortmannin, a broad range inhibitor of PI3K [47].

1.5.3 Retinoblastoma protein Rb

The Rb/p55γ interaction is identified to occur through the N-terminal 24 amino acids of p55γ and the C-terminal big pocket domain of Rb spanning amino acids 379 to 928 [48]. The 24 amino acids of the amino terminus of p55γ were found indispensable for this interaction, and the overexpression of which thwarted MCF7 cell cycle progression by inhibiting the expression of cyclin D and E and their promoter activity [48]. Several groups worked on this peptide fragment, which is considered a dominant-negative module for the endogenous p55γ that binds to and sequesters the effector proteins but conveys no further signaling. The growth inhibitory phenotype of the N-terminal 24 peptide was also observed in a gastric cancer cell model using an overexpression construct [49].

1.5.4 Tubulin
Tubulin exists as a heterodimer consisting of two tightly linked $\alpha$ and $\beta$ isoforms, and both subunits polymerize to form microtubules as part of the cytoskeleton system. In contrast, $\gamma$-tubulin is considered a centrosome specific protein which interacts with the dimer and helps nucleate the microtubules [50]. The p85 subunit of PI3K has been demonstrated to bind to both $\alpha$ and $\beta$ isoforms of tubulin constitutively and associate with $\gamma$-tubulin when stimulated by insulin [51;52]. Collective data from different groups have shown that both inter-SH2 and SH2 domains of p85$\alpha$ protein were involved in the \textit{in vitro} association with tubulin [43;51-53]. In contrast, both p55$\alpha$ and p55$\gamma$ bind tubulin more tightly through the amino-terminal 34 amino acids than through the inter-SH2 and SH2 domains; moreover, N-terminal 34 amino acid region and the inter-SH2 and SH2 domains are not competitive in their binding [43].

1.5.5 Tec

Tec is a member of the Src tyrosine kinase family. Tec receives stimulants from various cytokines such as IL-3 and IL-6, then transmits the growth signal to PI3K through binding the SH2 domains of p85$\beta$ or p55$\gamma$ and activates the PI3K signaling [54]. Thus, the tyrosine kinase Tec is considered as a mediator connecting the cytokine signaling with PI3K through interaction with p85$\beta$ and p55$\gamma$. 
1.6 Statement of Purpose

The highly frequent activation of PI3K/Akt pathway in cancer, particularly in human breast cancer, has promoted research towards the development of PI3K inhibitors. Some of these targeted therapies currently under development or are in clinical trials show great promise for the treatment of PI3K related tumors. However, these inhibitors work as single agents only in PI3K-addicted tumors with no other pathway alterations [36], so combination therapies with other gene-disruption, cytotoxic drugs or radiation therapies are needed to enhance the effectiveness.

Similar to the majority of protein kinase inhibitors, most existing PI3K inhibitors bind competitively to the catalytic domains. Recently, emerging evidence indicates that the regulatory subunits of PI3K also play a significant role in tumor development [10;39;48;55-57]. The p55γ regulatory subunit, like other regulatory subunits of PI3K, is necessary to maintain and regulate the catalytic activity by binding to the catalytic subunit. Interestingly, recent studies showed that p55γ also binds other proteins that regulate cell growth and cellular structure [48;51;54]. Thus, p55γ may exert its role independent of the catalytic subunits on the PI3K/Akt pathways in breast cancers. In this regard, extensive studies on the function of p55γ and its role in tumor development will be very useful for targeting PI3K signaling.

This dissertation explores the various functions of p55γ in breast cancer. However, the practical application of our research is beyond the scope of this dissertation. It is hoped that this study would widen our vision on designing efficient PI3K inhibitory
drugs. In addition to targeting the catalytic subunits of PI3K, regulatory subunits such as p55γ should be also considered as potential targets; the combination therapy hitting multiple targets in PI3K pathway potentially associates with increased efficacy and may eventually leads to increased patient survival.

1.7 Hypothesis/Specific Aims

Based on the roles of the N-terminal amino acid fragment of p55γ as a dominant negative form of the whole protein and a negative mediator of cell proliferation and cell cycle progression, it is hypothesized that p55γ acts as a positive regulator of cell growth and survival. Physical interactions between p55γ and other signal proteins may result in the corresponding changes on cell growth and survival, which may be independent on the traditional Akt pathway. The general objective of the present study is to determine the pro-proliferation and pro-survival effects of p55γ in breast cancer cells, and to further explore the mechanisms underlying these effects. To accomplish this objective, following aims were proposed:

1. Investigate the effects of p55γ gene silencing on cell proliferation and survival in breast cancer cells (Chapter 2).

2. Determine the effect of overexpression of p55γ in breast cancer cells on cell proliferation and survival as well as the influences on the p85α regulatory subunit, and its intracellular trafficking upon EGF stimulation (Chapter 3).

3. Characterize the interaction between p55γ and calmodulin (Appendix).
CHAPTER 2

EFFECT OF P55\textsubscript{γ} DOWN-REGULATION ON PROLIFERATION AND SURVIVAL SIGNALING IN BREAST CANCER CELLS

2.1 Abstract

Overexpression of the PI3K regulatory subunit p55\textsubscript{γ} occurs frequently in breast cancers. It has been proposed that the N-terminal dominant-negative peptide could have therapeutic effect on cancer because its delivery can reduce cancer cell proliferation. By using RNAi to specifically deplete p55\textsubscript{γ}, we investigated the loss-of-function phenotype in a range of breast cancer and immortalized human cell lines.

Silencing of p55\textsubscript{γ} by transduction of cells with lentiviral mediated shRNA resulted in significantly decreased cell proliferation and survival and more importantly, reduced clonogenic ability of breast cancer cells. Further analysis of cell cycle profiles and related proteins in these cells indicated that the reduction of cell growth by silencing of p55\textsubscript{γ} is largely due to G2/M phase arrest and alteration of the corresponding cell cycle regulatory proteins. Cyclin B1 and phospho-Cdc2 were up-regulated, and Chk1 levels were down-regulated upon p55\textsubscript{γ} silencing, indicating prolonged retention in mitotic phase and potential cell death during this phase. The mitotic catastrophe marker protein Plk-1 was down-regulated as well, strongly suggesting the presence of mitotic catastrophe induced cell death. Down-regulation of the oncogenic protein survivin
further indicated that p55γ reduction is potentially associated with anti-survival signaling.

Immunostaining showed that p55γ silencing directly results in cells with aberrant chromosome segregation, microtubule misalignment, and multi-polar mitosis. Interestingly, p55γ-regulated signaling appears independent of downstream Akt signaling in some breast cancer cells, suggesting a novel pathway mediated by p55γ in these cells. Overall, our findings demonstrate that p55γ plays an important role in cell proliferation and survival through regulating a novel signaling pathway in breast cancer cells. The results also suggest that p55γ may be used as an effective target for anti-breast cancer drug development.

2.2 Introduction

The p55γ gene has been implicated in the onset or progression of various types of cancer. However, the underlying molecular mechanisms responsible for p55γ-related malignancy remain elusive. Human p55γ is a non-kinase protein consisting of 461 amino acids. One of its established functions is binding to PI3K catalytic subunit p110α to help forming an active kinase, which then activates a cascade of effector proteins such as Akt, and to regulate cell proliferation, survival, angiogenesis, and many other cellular functions.

Transgenic mouse models and embryonic stem cells provided valuable information on the molecular balance and composition shift for the regulatory subunits of PI3K. It
has been shown that the gene products of \textit{pik}3\textit{r}1 and \textit{pik}3\textit{r}2 are the predominant isoforms in the insulin-sensitive tissues, as evidenced by the extracts from \textit{pik}3\textit{r}1/- mice showing 70\% loss of regulatory activity, and \textit{pik}3\textit{r}2/- mice showing 25\% of reduction [18]. Although no results on \textit{pik}3\textit{r}3 knockout mice have been published so far, it is tempting to predict a 5\% reduction on regulatory activity for \textit{pik}3\textit{r}3 knockout mice if they could be generated. We expected that in the insulin-sensitive tissues, the effect of p55\(\gamma\) on glucose responsive PI3K signaling would be minor. However, overwhelming evidence has shown that p55\(\gamma\) is involved in cell cycle regulation and tumorigenicity.

Since p55\(\gamma\) has been identified to associate with a cell cycle regulatory protein such as the retinoblastoma protein Rb, it may be involved in initiation of the cell cycle checkpoint. Once the cell emerges from G0, there are two checkpoints that play roles in the proper execution of the sequential events of the cell cycle, the G1/S checkpoint at late G1 and G2/M checkpoint at late G2. Premature entry into S phase or M phase with damaged DNA will eventually lead to cell death. The G2/M checkpoint is of special interest since it has been demonstrated that chemicals which either abrogate or induce the G2/M checkpoint both could synergize with chemotherapy to kill the tumor cells [58]. A number of key G2/M checkpoint modulators have been identified, such as Checkpoint kinase-1 (Chk-1), Cyclin dependent kinase 1 (Cdk1/Cdc2), and cyclin B1. In eukaryotic cells DNA damage first leads to activation of Chk-1, followed by phosphorylation/activation of Cdc25C, then the inactivation of Cdc2/cyclin B1 complex
and G2/M arrest [59]. Agents that abrogate the G2/M arrest would lead to increased mitotic defects, and increased cell death. Polo-like-kinase-1 (Plk-1) phosphorylates cyclin B1 and targets cyclin B1/Cdc2 to the nuclear fraction during mitosis [60]. It is sometimes regarded as a marker protein for G2/M checkpoint abrogation induced mitotic cell death [61;62]. To characterize the effect of p55γ on the G2/M checkpoint, these modulating proteins were analyzed in cells with reduced p55γ.

As a major downstream target protein of PI3K, Akt belongs to a serine-theronine protein kinase family including Akt1 (Akt/PKB), Akt2, and Akt3. Akt1 is involved in protein synthesis and survival signaling so Akt1 knockout mice with intact Akt2 show increased thymic apoptosis, and increased neonatal mortality. If they were born, they appear smaller in size [63;64]. Akt2 is specifically involved in glucose metabolism and insulin signaling pathways so that knockout mice lacking Akt2 display a severe diabetic phenotype [65]. The function of Akt3 is currently not very clear, but appears to be related with postnatal brain development, since Akt3 knockout mice have smaller brains [66]. The complete activation of Akt requires the sequential phosphorylation on amino acids serine 473 by mammalian Target of Rapamycin (mTOR) [67] and threonine 308 by Phosphoinositide dependent kinase 1(PDK1) [68].

Although Akt is the major downstream target of PI3K, not all downstream targets of PI3K are Akt dependent, for example, the Bruton tyrosine kinase (Btk) family kinases including Btk, Tec non-receptor tyrosine kinase [69], the serum- and glucocorticoid-regulated kinases (SGKs) [70], several PKC family members [5;6] and Rho GTPase
are independent of Akt activity. Interestingly, Tec non-receptor tyrosine kinase has been shown to transfer the signal from cytokines to p55γ through dimerization and activation of Jak1/Jak2 [54]. These results suggest that p55γ could potentially mediate cellular proliferation and survival signaling through Akt-independent pathways.

We hypothesized that reduction of p55γ may result in cell death and cell cycle deregulation at G1 and G2/M checkpoints, part of these effect might be mediated through an Akt-independent pathway. In order to investigate the function of p55γ in breast cancer, we utilized RNAi methodology to reduce p55γ protein level, and the downstream target proteins influenced by this reduction were also analyzed. Several RNAi effectors can be used for gene silencing: they are short interfering RNA (siRNA), microRNA (miRNA) and short hairpin RNA (shRNA) [72]. SiRNA is a set of synthesized RNA duplex targeting specific mRNA for degradation. It was first discovered and used to knockdown genes in plants [73], and then used to modulate gene expression in mammalian cells [74]. MiRNA is a group of naturally occurring, single stranded RNA consisting of 21-25 nucleotides and was first described in Caenorhabditis elegans [75]. Endogenous miRNAs can be isolated, concentrated and then used to target specific mRNAs. Although not used in this dissertation study, it could be used for clinical related drug development in the future. ShRNA are single-stranded RNA molecules which contain double stranded RNA domains, after expression and processing in the cells, the shRNA transforms into siRNA. Compared to synthetic siRNA-mediated gene knockdown, whose effect is fast and transient, the shRNA
delivers sustained repression of the target gene in the cells [76]. ShRNA expression plasmids targeting p55γ in this study are packaged into lentivirus and introduced into 293T cells, as a fast and easy method to produce large quantities of shRNA-containing virus. Off-target effects produced by RNAi are a problem encountered by many researchers, and needs to be addressed. When the multi-protein RNA-Induced Silencing Complex (RISC) loads the sense strand instead of the antisense strand, non-gene-specific effects occur that recognize and cleave the non-targeted mRNA sequences. As few as 11 bases of continuous identical nucleotides have been proven to be sufficient to induce off-target effects [77]. To obtain greater specificity and fewer off-target effects, a basic bioinformatic analytical tool was used. BLASTn (NCBI; www.ncbi.nlm.nih.gov/BLAST) was used to select two from the five shRNA expression plasmids obtained. I used two shRNAs targeting different sites of the p55γ sequence and compared the resultant phenotypes to minimize the possibility that the phenotype are due to non-specific effects.

2.3 Materials and Methods

Cell culture

Eighteen established cell lines including 15 breast cancer cell lines were obtained from the Tissue Culture Shared Resource of the Lombardi Comprehensive Cancer Center (LCCC) in Georgetown University Medical Center (Washington DC) to evaluate their endogenous p55γ expression level. All cells were maintained in recommended
growth media and cultured in a 37°C, 5% CO₂ environment until subconfluency was reached. MDA-MB-468, BT549, T47D, MDA-MB-361, MDA-MB-157, MDA-MB-436, MDA-MB453 and HEK293 were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Mediatech, Inc. Manassas, VA) supplemented with 10% fetal bovine serum (FBS); SKBr-3, AU565, BT474, ZR-75-1 were cultured in RPMI1640 supplemented with 10% FBS; MCF7 was cultured in Improved Minimal Essential Medium (IMEM) (GIBCO, Carlsbad, CA) supplemented with 10% FBS; Hs578T was cultured in DMEM supplemented with 10% FBS and 10 μg/ml insulin; A1N4 was cultured in IMEM supplemented with 0.5% FBS, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, and 10 ng/ml EGF; MCF10A was cultured in DMEM/F12 (GIBCO) complemented with 2.5 mM L-glutamine, 20 ng/ml EGF, 5% horse serum, 0.01 mg/ml insulin, and 500 ng/ml hydrocortisone. Cell lysates were prepared as described under Western blot analysis.

**Lentiviral production and titration**

Lentiviral shRNA constructs targeting p55γ were purchased from Open Biosystems (Huntsville, AL). Control pLKO (12 μg) or the same amount of each of the two individual lentiviral shRNA plasmids against p55γ (KD90, mature sense: GCTTTGGACAACCGAGAAATA or KD93, mature sense: GAGAAGAGTAAAGAGTATGAT) were transfected into 293T packaging cells along with 1.2 μg of VSVG envelope plasmid, and 6 μg of pCMV dR8.91 using the FuGENE 6 (Roche) transfection reagent. The culture media containing virus were collected after
48h and 72h of transduction and pooled. For viral titration, HT1080 cells were grown in 6-well plates in DMEM supplemented with 10% FBS until they reached 60% confluency. The shRNA-containing virus in 10-fold increasing serial dilutions (10^{-2} to 10^{-7}) were added to the cells along with 8 μg/ml of polybrene; the selection antibiotics puromycin (2 μg/ml) was added to the cells after 24 h, and the puromycin containing growth medium was refreshed every three days until visible colonies were formed, in 10-14 days. The colonies were stained with 0.5% crystal violet in 25% methanol and counted. The approximate titer of each virus was determined by multiplying the number of colonies in the highest dilution plates by the dilution factor.

**Viral transduction (infection)**

The cells were trypsinized the day before transduction and reached 50-60% confluency on the day of transduction. The target or control virus stocks diluted with growth media at a multiplicity of infection at 10-20 were added to the cells with 8 μg/ml of polybrene. Cells were incubated for 24 h with virus, and then changed to the fresh growth media with no virus for the designated time before western blot analysis, or trypsinized with 0.05% Trypsin-EDTA (GIBCO) and split for subsequent experiments.

**Cell proliferation assay**

Cells were transduced with shRNA containing virus and one day after transduction, were counted with a hemocytometer after trypan blue staining and same numbers of live
cells were seeded in 96-well plates at a density of 2,000-4,000 cells in 100 μl per well in phenol red-free media. On Days 1, 3, 5, 7 after plating, 10 μl of MTS/PMS-based CellTiter 96® AQueous One solution (Promega, Madison, WI) was added to each well and incubated for 1 hr at 37°C, and the absorbance was read at 490nm with a ULTRA MARK microplate imaging system (BIO RAD, Hercules, CA). Each experiment was repeated four times. The data were analyzed and plotted with PRISM software (GRAPHPAD).

Antibodies and reagents

Rabbit polyclonal antibodies against phospho-Akt (S473), phospho-Akt (T308), and total Akt, total Cdc2, phospho-Cdc2 (Thr161), phospho-H3, p38 MAPK, phospho-p38 MAPK (Thr 180/Tyr182) (3D7), and survivin antibodies were obtained from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibody against p55γ was obtained from Invitrogen (Carlsbad, CA). Mouse monoclonal antibody against Cyclin B1 was obtained from Neomarker (Fremont, CA). Rabbit polyclonal antibodies against Chk-1 and Plk-1, and goat polyclonal antibody against p55γ (N-13) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against β-actin was obtained from Sigma (St. Louis, MO). Mouse epidermal growth factor (EGF) was obtained from BD Biosciences (Bedford, MA). Mouse monoclonal antibody against α-tubulin was obtained from SIGMA (Saint Louis, Missouri).
Western blot analysis

Cells were lysed in a lysis buffer containing 10 mM Tris, pH 7.4, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 2 µg/ml each of pepstatin, leupeptin, and aprotinin. Lysates were vortexed and centrifuged at 15,000 g for 15 min at 4°C. Lysate protein concentrations were measured using the BCA protein assay kit by Pierce (Rockford, IL). Equal amounts of crude proteins were run on 4-12% SDS-PAGE gels and electrophoretically transferred to PVDF membranes. Blots were blocked with 5% nonfat dry milk in PBST buffer containing 0.05% Tween-20 for 1 h, washed in PBST three times (10, 5 and 5 min each) and incubated with primary antibody in PBS containing 2% nonfat dry milk overnight, at 4°C. Blots were washed three times (10, 5, 5 min each). Immunoreactivity was detected by sequential incubation with horseradish peroxidase-conjugated secondary antibody and ECL reagents.

Cell cycle analysis

After treatment, the cells were trypsinized and washed once with ice-cold PBS. Approximately 1x10^6 Cells were fixed with ice-cold ethanol while being vortexed. The DNA was stained with propidium iodide (PI) (3.8 mM sodium citrate, 50 µg/ml of PI (Sigma) in PBS). The RNA was removed with 10 µg/ml of RNase A and incubated for 3 h at 4°C. Then the cells were analyzed with a FACStar Plus dual laser system by Becton Dickinson (Franklin Lakes, NJ) at the LCCC Flow Cytometry Shared Facility.
Annexin-V Apoptosis assay

Both floating and attached cells were collected 72 h after shRNA transduction, and washed in PBS. Cells (1 \times 10^6) were resuspended in 100 \mu l of binding buffer (0.1 M Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4; 1.4 M NaCl; 25 mM CaCl_2) with Annexin V-Phycoerythrin and 7-Amino-actinomycin D according to the vendor’s suggestions (BD Pharmingen). Cells were then analyzed with a FACStar Plus dual laser system by Becton Dickinson (Franklin Lakes, NJ) at the LCCC Flow Cytometry Shared Facility, and further analysis was carried out using CellQuest software (BD Biosciences).

Clonogenic assays

Cells were transduced with the respective shRNA expressing virus for 24 h and then trypsinized. Live cells were seeded in 6-well plates at a density of 100, 200, and 400 cells containing 3 ml of complete medium. After 10 days of incubation at 37 °C, plates were washed with PBS and stained with 0.5% crystal violet. Images were taken using a Canon digital PowerShot A590 IS camera.

Real-time RT-PCR

Total RNA was extracted using the Trizol reagent according to the manufacturer’s protocol (Invitrogen). cDNA synthesis was carried out using hexamer random primers
and SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time reverse transcriptase PCR (qPCR) was performed with cDNA generated from 1 µg of total RNA. Primers were designed for qPCR using Primer Express software (Applied Biosystems, Foster City, CA), and sequences were available upon request. qPCRs were carried out using SYBR green PCR master mix (Applied Biosystems) in an ABI Prism 7900HT sequence detection system (Applied Biosystems). Values were quantified using the comparative threshold cycle method, and samples were normalized to GAPDH.

**Visualization of microtubule structure by confocal microscopy**

For α-tubulin staining, cells were seeded onto glass coverslips. At 48 or 72h after infection, cells were fixed in methanol for 5 min at –20°C and then washed in PBS/0.1% Triton X-100. To block nonspecific binding, the coverslips were incubated in blocking solution (PBS containing 0.1% gelatin and 10% normal donkey serum) for 15 min followed by a 15 min incubation in blocking solution containing a monoclonal anti α-tubulin (Sigma, St. Louis, MO) antibody (1:2,000 dilution) and sequential washing with PBS/0.1% Triton X-100. Cells were then incubated for 15 min at room temperature in the presence of blocking solution containing an Alexa Fluor 488–conjugated goat anti-mouse immunoglobulin G (H+L) (Molecular Probes, Eugene, OR; 1/2000). Coverslips were washed three times in PBS/0.1% Triton X-100, rinsed in PBS, and finally mounted with the Antifade mounting medium (Biomeda, Foster City, CA). Cells were
viewed using a 63x oil objective of a Zeiss LSM510/META laser confocal microscope. The images were processed with MetaMorph (Molecular Devices).

**Statistical analysis**

When needed, experimental data were analyzed using a commercial statistical software package (GraphPad Prism, version 4.03, San Diego, CA) and expressed as mean ± standard deviation (SD). Statistical significance of differences between groups was determined using unpaired t-test. Significance was declared for p < 0.05 unless otherwise indicated.

**2.4 Results**

**2.4.1 Effect of p55γ reduction on cell proliferation**

The lentiviral mediated shRNA system was used to knockdown pik3r3, and unravel the physiological role of p55γ in a panel of breast cancer cell lines. The commercially available package contained five shRNA constructs against pik3r3 (KD89-KD93), targeting different sites of the pik3r3 nucleotide sequence and one control construct pLKO.1. The first step was to screen these five constructs and pick at least two for further experiments and to exclude the off-target effects. First, a nucleotide specific basic local alignment search tool, BLASTn (NCBI;www.ncbi.nlm.nih.gov/BLAST) was used to compare the targeting nucleotide sequences of the five shRNA constructs to the nucleotide database. The specificity of these shRNA was investigated to minimize the
unwanted sequence match with non-target sequences, since as few as 11 consecutive nucleotides complementary with the RNAi effectors could lead to downregulation of a non-target gene and the off-target effects [77]. KD89 and KD92 were excluded from the list because KD89 overlaps with the sequence coding for human p85α, and KD92 barely reduced p55γ protein in a trial experiment (data not shown). After searching for the consensus sequences of KD90, KD91, and KD93 in human genomic plus transcript bank, KD90 and KD93 turned up to have consensus sequence specific for the pik3r3 gene. KD91 turned out to have a sequence of 14 nucleotides overlapping with the heavy chain of the spindle-associated protein clathrin. To avoid off-target effects on degradation of clathrin, KD90 and KD93 were used for the subsequent experiments.

Endogenous protein expression level of p55γ were examined in a panel of 18 cell lines, including 15 breast cancer cell lines, with a specific mouse antibody against p55γ (Figure 2.1 A). Western blot analysis of normalized total cell lysates showed that six breast cancer cell lines, including MDA-MB-468, BT-549, SKBr-3, MDA-MB-453, BT474, and ZR-75-1 cells contain relatively higher level of p55γ (more than 2.5 times higher than the mammary epithelial cell A1N4). This suggested that overexpression of p55γ may occur in some cases of breast cancer.

Next, MDA-MB-468, BT-549, SKBr-3, and MDA-MB-453 cells, which contain relatively high expression of p55γ, were selected as our experimental models. After transduction of lentiviral mediated shRNAs, expression of p55γ was determined by western-blot with a specific antibody against p55γ. Result showed that a marked
reduction of the level of p55γ proteins (≥ 80%) was observed in all cell lines (Figure 2.1 B). This indicated that both KD90 and KD93 shRNAs exhibited capacities to efficiently reduce p55γ expression in these cells.

To determine the effects of p55γ silencing on breast cancer cell proliferation, a MTS/PMS colorimetric assay was utilized, and the increase in the metabolically active cells were measured by the increase in the absorbance reading at the optical density of 490 nm. In the cell lines tested, different doubling times were observed. While control pLKO.1 transduced cells showed constant proliferation rates, the p55γ shRNA-transduced cells showed a significantly lower proliferation rate already detectable at 3 days of plating (4 days after transduction) (Figure 2.1 C). In order to determine the effects of p55γ silencing on cell survival, a trypan blue exclusion assay was performed to visualize the dead cells, since only the dead cells that have lost the ability to exclude the trypan blue dye will be stained. After p55γ silencing in MDA-MB-468 cells, the proportion of dead cells was examined under the microscope. Results not only showed a marked decrease in the number of attached cells, but also manifested more dead cells stained with trypan blue in those attached cells (Figure 2.1 D). In addition to these four cell lines, some cell lines expressing lower level of p55γ (MDA-MB-231, MDA-MB-436, and HEK293) have also been transduced with shRNA expressing virus, and the similar effect on cell growth and survival was also observed in these cells. The above data suggested that p55γ regulatory subunit is involved in the proliferation and survival of these breast cancer cells.
(C) Relative Proliferation of BT549, MDA-MB-468, SK-BR-3, and MDA453 over 8 days with different treatments (pLKO, KD90, KD93).

(D) Bright field and Trypan blue images of pLKO, KD90, and KD93 treatments.
Figure 2.1 Reduction of p55 is associated with decreased proliferation and survival.

(A) Protein levels of p55γ were determined in a panel of 18 cell lines including 15 breast cancer cells. Cell lysates were extracted from subconfluent, actively growing cells and blotted with a p55γ specific antibody. β-actin was probed to normalize equal protein loading. (B) Silencing of p55γ by two independent lentiviral mediated shRNAs (KD90, KD93). KD90 and KD93 were used to knock down the gene encoding p55γ in the chosen cell lines. Lysates were prepared 48 h after transfection, and blotted with a p55γ specific antibody. (C) Cell proliferation was reduced by loss of p55γ. Breast cancer cells transfected with shRNA were seeded on 96-well plates. On days 1, 3, 5 and 7 after plating, proliferation was measured by a MTS-based assay. Results represented are mean± SD of four independent experiments. (D) Increased dead cells observed after p55γ reduction in MDA-MB-468 cells. Cells were transfected and photos were taken 7 days later. Cells were either observed in light field for gross morphology (top panel) or stained with trypan blue for dead cells right on the dish (bottom panel). Cells were photographed using the 10x objective of an Olympus IX71 Inverted Epi-Fluorescent Microscope.
To eliminate the possibility that these virus-transduced cells were killed through non-specific toxic effect that may be either preexisting or inadvertently introduced in the p55γ-targeting shRNA but not in the control pLKO.1 shRNA during viral production, additional breast cancer cell lines were screened. The proliferation and survival of the MCF7 breast cancer cell line was identified to be unaffected by p55γ. MCF7 cells were transduced with shRNA-containing virus and subjected to proliferation assay following the procedure identical with the other four cell lines. The results show that shRNA successfully reduced p55γ protein (Figure 2.2 A), but did not affect the proliferation rate of MCF7 cells (Figure 2.2 B). The absence of effects on cells proliferation by p55γ reduction in MCF7 cells suggested that the non-specific toxicity unrelated with p55γ reduction is most likely not involved in the above cell proliferation experiments.
Figure 2.2 Proliferation of MCF7 cells was not affected by p55γ reduction.

(A) Western blot analysis of p55γ protein reduction after virus mediated knockdown in MCF7 cells. KD90 and KD93 were used to knock down the gene encoding p55γ in MCF7 cells. Lysates were prepared 48 h after transfection, and blotted with a p55γ specific antibody. β-actin was used as the probe for equal amount of protein loading control. (B) Cell proliferation with reduced p55γ in MCF7 cells. The experiments were conducted using the same protocol as for the other four breast cancer cell lines. The data plotted are the results from three independent experiments, shown as mean ± SD.
2.4.2 Colony formation ability is impaired by p55γ reduction

The reduced cancer cell growth and survival by p55γ silencing may also reflect the decreased ability of a single cancerous cell to form tumors. The clonogenic assays or colony formation assays are *in vitro* cell survival assays based on the ability of a single cell to grow into a colony [78]. To test our hypothesis that colony formation ability is impaired in breast carcinoma cells by p55γ reduction, a clonogenic assay was conducted in BT549 cells upon p55γ silencing. While the control lentivirus infected cancer cells had normal colony formation ability, with more visible colonies generated by plating more cells from the starting time, the cells infected with virus mediating p55γ knockdown exhibited significantly lower colony forming ability (p<0.05) (*Figure 2.3 A and B*). Interestingly, upon p55γ silencing, the number of colonies was not significantly increased when more cells were plated at the starting time, suggesting that the surviving cells not killed by p55γ reduction manifested decreased ability to form colonies. These data indicated that silencing of p55γ not only reduced breast cancer cell proliferation, but also decreased the ability of colony formation of single cancer cells.
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(B)  

![Bar chart showing colony numbers](chart.png)

- **pLKO**
- **KD90**
- **KD93**

Initial cell numbers vs. Colony numbers

*Significant difference*
Figure 2.3 Reduction of p55γ gene leads to decreased colony formation ability.

(A) BT549 cells were transfected with shRNAs and then plated the day after transfection on 6-well dishes at the indicated (top) initial cell numbers. After ten days the cells were fixed, stained and counted. (B) The normalized colony numbers from three independent experiments were plotted. Asterisk indicated p value <0.001 compared with the corresponding controls.
2.4.3 Effect of p55γ reduction on apoptosis in breast cancer

The increased number of dead cells after p55γ reduction may reflect aberrant cell death. One of the reasons is programmed cell death, also called apoptosis. Therefore, the presence of apoptosis was determined by flow cytometry. First, the subdiploid fraction of the cell cycle histogram representing the apoptotic cells was analyzed after ethanol fixation. Cells with subdiploid DNA content increased approximately 2-6 folds in BT549 cells and MDA-MB-468 cells upon p55γ reduction (Figure 2.4 A), indicating that apoptosis may be involved in reducing cell survival in these cells.

Second, to further quantify the apoptotic fraction in p55γ reduced breast cancer cells, we determined the early apoptosis markers in MDA-MB-468 cells using an Annexin-V based apoptosis assay (Figure 2.4 B). We found that at 72 h after viral transduction, while the pLKO.1 maintained relatively low amounts of early apoptotic cells, reduction of p55γ with either KD90 or KD93 had higher percentage of apoptotic cells, and the KD93 transduced cells showed a significant difference compared with controls. The same apoptosis analysis was also used to detect early apoptosis in transduced SKBr3 cells; however, no significant changes were found (data not shown). Together, loss of p55γ seemed to increase the proportion of apoptotic fraction in some breast cancer cells, a result similar to previous findings by p55γ reduction in ovarian cancers [44].
Figure 2.4 Reduction of p55γ is associated with increased sub G0/G1 population and apoptotic cells. (A) BT549 or MDA-MB-468 cells were transfected with the indicated shRNA for 72 h before being fixed with ethanol and analyzed by flow cytometry. The percentage of sub G0/G1 population is shown. Results are representative of three independent experiments with similar outcomes. (B) Apoptosis was induced by p55γ silencing in MDA-MB-468 cells. P55γ was silenced in MDA-MB-468 cells with shRNA, and 72 h later, the early apoptotic cells were identified using an Annexin-V based apoptosis assay. Results shown represent mean ± SD of three independent experiments. The respective p values are shown on the top of the bars.
2.4.4 Reduction of p55γ leads to distinct Akt activation

Activation of Akt signaling is mediated mainly through PI3K, but the involvement of regulatory subunits in this activation is largely unknown. To determine whether reduction of the p55γ using shRNA decreased cell growth and survival by inactivation of the survival signaling through Akt, cell lysates collected at 48 h after shRNA transduction was analyzed by blotting with anti-phospho Akt antibodies. Results showed that Akt activation was decreased, especially at the threonine 308 activation site after p55γ knockdown in BT-549 and MDA-MB-468 cells (Figure 2.5 A). However, no reduction in Akt activation at the threonine 308 was detected in SK-Br-3 and MDA-MB-453 cells (Figure 2.5 B). This implied that the effect of p55γ down-regulation on cell survival was not dependent on Akt activation in all cell lines. Since Akt is a major effector protein for anti-apoptosis signaling, the increased activation of Akt in SKBr3 cells and decreased activation in BT549 and MDA-MB-468 cells supported the observation in the apoptosis assays (above).
Figure 2.5 P55γ reduction induced cell death is Akt-independent. Western blot with equal amounts of cellular extracts from the treated cells were blotted at 72 h after shRNA transfection with the indicated antibodies. β-actin was used as loading control. Results shown are representative of three independent experiments with similar outcomes. Note: pAkt T308 level was downregulated in BT549 and MDA-MB-468 cells (A), but was upregulated in SK-Br-3 and MDA-MB-453 cells (B).
2.4.5  

**Cell cycle G2/M arrest and cell death induced by p55γ reduction**

Decreased cell growth and survival also suggested aberrant cell division that may reflect the changes on cell cycle. To determine whether p55γ depleted cells had abnormal cell cycle profiles, cell cycle changes after p55γ silencing were analyzed by flow cytometry. The most dramatic change in cell cycle profile was the induction of G2/M cell cycle arrest, which was strong (>75% increase) in BT549 and MDA-MB-468 cells and significant (P<0.001), while weak (8-50%) in SKBr-3 and MDA-MB-453 cells and insignificant (P>0.1) (**Figure 2.6 A and B**). In MDA-MB-468 cells transduced with KD90 shRNA, the G2 percentage increased by ∼75% compared to control cells, while in the case of KD93 shRNA, the G2 percentage increased by ∼123% compared to controls. In BT549 cells, the G2 percentage increased by ∼196% and ∼167% upon KD90 and KD93 transduction, respectively (**Figure 2.6 B**). These data indicated that the p55γ reduction changed the cell cycle profile and induced a G2/M arrest, which suggested that cell cycle arrest may be responsible for the reduced cell growth and survival after p55γ depletion.
(A)  

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<th>Dip G2 (%)</th>
<th>Dip S (%)</th>
<th>Dip G1 (%)</th>
<th>Dip G2 (%)</th>
<th>Dip S (%)</th>
<th>Dip G1 (%)</th>
<th>Dip G2 (%)</th>
<th>Dip S (%)</th>
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<td>18.3%</td>
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<td>51.9%</td>
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<tr>
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</table>

(B)  

<table>
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<tr>
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<th>Dip G2 (%)</th>
<th>P value*</th>
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</thead>
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</tr>
<tr>
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<td>KD90</td>
<td>24.0 ± 10</td>
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</tr>
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<td>&lt;0.001</td>
</tr>
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</tr>
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</tr>
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</tr>
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<td>KD93</td>
<td>KD93</td>
<td>13.0 ± 3.4</td>
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</tr>
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</table>
Figure 2.6 Effect of p55γ reduction on the G2/M arrest. (A) Reduction of p55γ induces G2/M arrest in breast cancer cells. Seventy-two hours after viral transduction, the cells were trypsinized, washed, stained with propidium iodide (PI) and analyzed for DNA content by flow cytometry. (B) The results in (A) are represented as the mean value with standard error of the mean (SEM) from three independent experiments. Student’s t-test showed two-tailed p values of p55γ deficient cells compared with control pLKO shRNA transfectant.
2.4.6 Reduction of $p55\gamma$ modulates G2/M checkpoint proteins

To reveal how reduction of $p55\gamma$ causes a G2/M phase arrest, the expression and activation of some essential proteins responsible for regulating mitosis was determined. Cdk1/Cdc2 and its associated protein cyclin B1 are required for mitosis entry and maintenance [79-81]. Cyclin B1 activates Checkpoint kinase-1 (Chk-1) during mitosis and is quickly degraded by proteolysis for mitotic exit [82]. Chk-1 is an important mediator of G2/M cell cycle checkpoint. Chk-1 inhibition leads to premature centrosome separation and Cdc2 activation [83], and the activated Cdc2/cyclin B1 complex is crucial for maintaining mitosis status. The expression of these two proteins was examined in MDA-MB-468 cells. As expected, the cyclin B1 protein was elevated in $p55\gamma$ depleted cells, with more significant elevation in the case of KD93, while Chk-1 level was reduced also, with a more significant reduction in the case of KD93 (Figure 2.7 A). The total Cdc2 level did not change, but activated phospho-Cdc2 was slightly elevated upon $p55\gamma$ reduction in both BT549 and MDA-MB-468 cells (Figure 2.7 B). The constitutive elevation of cyclin B1 and activated Cdc2 indicated that these $p55\gamma$ deficient cells are probably arrested in the M phase of cell cycle and fail to exit from mitosis. We found that the Chk-1 protein was decreased upon KD90 and KD93 transduction. Interestingly, in KD93 transduced cells where Chk-1 was more efficiently reduced, more cyclin B1 was accordingly accumulated (Figure 2.7 A). When the membrane was probed for other mitosis regulating proteins, it was found that Polo-like-kinase-1 (Plk-1) protein was downregulated in $p55\gamma$ depleted cells (Figure 2.7 A).
prolonged mitotic status was supported by the retention of phosphorylated histone H3. Histone H3 is phosphorylated on S10 during mitosis by Aurora kinase [84], and the phosphorylated H3 has been used as a mitotic marker [85]. Western blot for phosphorylated H3 showed that activation of histone H3 was increased after p55γ was reduced (Figure 2.7 A), indicating elevated level of mitosis.

To further explore the regulation mechanism of the above proteins, real-time PCR was utilized to examine them at the transcriptional level. The mRNA levels of p55γ, Chk-1, Plk-1, and cyclin B1 were determined in MDA-MB-468 cells after pik3r3 gene silencing from total RNA samples extracted from these cells. The results showed that upon p55γ reduction, Chk-1 mRNA was decreased (Figure 2.7 C), indicating the reduction of Chk-1 was probably through regulation at the transcriptional level. However, Cyclin B1 and Plk-1 mRNA level were not changed upon pik3r3 gene silencing (Figure 2.7 C), indicating that the increased Cyclin B1 and Plk-1 protein expression was probably mediated through a decrease in protein degradation. These data suggested that the regulatory effect of p55γ reduction on cell cycle regulatory proteins is mainly due to the inhibition of protein degradation.
Figure 2.7 p55γ reduction modulated G2/M checkpoint proteins. (A) Western blot with equal amounts of cellular extracts from the MDA-MB-468 cells were blotted 72 h after shRNA transfection with the indicated antibodies. β-actin was used as loading control. Data are representative of three independent experiments with similar outcomes. (B) Lysates from BT549 and MDA-MB-468 cells were blotted for total CDC2 and phosphoCDC2. β-actin was used as loading control. Data are representative of three independent experiments with similar outcomes. (C) RT-PCR for respective gene expression with mRNA samples extracted from MDA-MB-468 cells 72 h after transfection. Results from four duplicate samples for each group were shown as mean ± SEM.
2.4.7 Reduction of p55γ leads to abnormal chromosome alignment and increased multipolar mitotic spindles

Maintenance of the G2/M stage without increased cell proliferation suggests that the cells might die during or immediately after mitosis. This kind of cell death is also called mitotic catastrophe. Since decreased degradation of cyclin B1 is observed after p55γ reduction, this prevented cells from exiting mitosis properly. Thus, it was hypothesized that a fraction of the cells die during deregulated mitosis as a result of p55γ reduction. To test this hypothesis, the shRNA transduced cells were stained with DAPI to show if there was any chromosome morphological changes during mitosis. Results showed defected chromosome alignment after p55γ reduction, manifested as misalignment of condensed chromosome, and the presence of tripolar chromosome (Figure 2.8).
Figure 2.8 Misalignment of chromosomes during metaphase after p55γ down-regulation. MDA-MB-468 cells were transduced with shRNA expressing viruses as indicated. After 72 h, cells were fixed and stained with DAPI (blue). Phase contrast images (middle panel) were taken to show the cells were rounded and going through active mitosis. The pictures were taken with 60x oil objective.
Abnormalities in chromosome alignment always indicate malfunction of the microtubule apparatus. The 85 KDa subunit of PI3K has been shown directly associating with α/β tubulin, probably through the inter-SH2 domain of p85 [51]. Since the inter-SH2 domain of p85 and p55γ are homologous, it was tempting to hypothesize that p55γ also associated with tubulin through its inter-SH2 domain and was involved in microtubule functions. Interestingly, the N-terminal 34 amino acids of p55γ and p55α has been demonstrated to bind tubulin with higher affinity [43], indicating that different regulatory subunits may associate with tubulin through different functional domains. Nevertheless, it was hypothesized that since p55γ interacts with tubulin, knocking down this protein would lead to perturbation of the microtubule distribution during mitosis. We then visualized the microtubule structure in MDA-MB-468 cells by staining the cells with an antibody against α-tubulin to specifically monitor microtubule morphology during mitosis (Figure 2.9 A). Results showed that after pik3r3 gene silencing the mitotic spindle asters were significantly increased. Both p55γ shRNAs induced the development of more than two spindle asters in 50% of the mitotic cells (Figure 2.9 B), thus potentially leading to failed mitosis and cell death. On the contrary, in the control pLKO.1-transduced cells less than 2% of multiple spindle asters developed in mitotic cells. Similar microscopy experiments were conducted with MDA-MB-453 and SKBr3 cells using KD90 and KD93 shRNA, and the increased multiple spindle asters was likewise observed (Figure 2.9 C and D), although the proportions of mitotic cells were fewer than that of the MDA-MB-468 cells. These results indicated
that p55γ reduction leads to increased incidence of abnormal chromosome alignment and multipolar mitotic spindles, which might cause mitotic catastrophe.
Figure 2.9 p55γ is associated with an increased incidence of multipolar mitosis.

(A) Aberrant mitotic figures showing mitotic defects. After shRNA transfection, MDA-MB-468 cells were fixed and then stained for immunofluorescence microscopy with anti-α-tubulin (green) 72 h after transfection. Cells were counterstained with DAPI (blue). Arrows point out the location of the centrosomes as the sites from where microtubule asters originated. Microscope settings were held constant for all image acquisitions. (B) Reduction of p55γ was associated with increased incidence of multipolar mitosis.
MDA-MB-468 cells were transfected with virus as described and after 72h immunostained with antibody against α-tubulin. For each sample, more than 100 mitotic cells were scored, and the percentage of cells showing two, three, and more than three microtubule asters are shown. Data is representative of three independent experiment showing comparable outcomes. Results were analyzed by Chi-square test comparing control pLKO.1 and KD90, or control and KD93; the two-tailed P value is less than 0.0001 in both cases (GraphPad Software). Reduction of p55γ was also associated with increased incidence of multipolar mitosis as demonstrated by confocal microscopic detection in MDA-MB-453 (C) and SKBr-3 cells (D). Bar: 10μm
2.4.8 Survival signaling protein is modulated by p55γ reduction

To further delineate the survival pathway that is affected by p55γ knockdown, the level of apoptosis protein inhibitor family member survivin and the stress response protein kinase, activated p38 Mitogen-activated protein kinase (MAPK), was determined by western blot analysis. While total p38 MAPK remained constant, activated p38MAPK (pp38) increased, and survivin was down-regulated after p55γ reduction (Figure 2.10). Therefore, the signaling was modulated towards increased death signals and decreased survival signals, thus supporting the dramatic cell death observed following p55γ down-regulation. This finding strengthened our hypothesis that p55γ regulates the survival signaling.
Figure 2.10 Survival signaling protein and stress response protein are modulated by p55γ knockdown. Western blot analysis of equal amounts of cellular extracts from the viral-transduced cells with antibodies against survivin, phospho-p38, total p38 and p55γ are shown. β-actin was used as a loading control.
2.5 Discussion and conclusion

PI3K/Akt signaling is a universal pathway that regulates many important cellular events. The discovery that p55γ knockdown leads to distinct Akt phosphorylation in different cell lines adds to the complexity of the signaling pathways that p55γ involves, although the changes observed in proliferation and cell growth are very similar in the four cell lines, including increased mitotic defects, decreased proliferation, and increased cell death. Both MDA-MB-468 and BT549 cell lines showed significant G2/M arrest in addition to the profoundly increased spindle asters, which strongly suggested that some of these cells were blocked in mitotic phase.

PI3K activation is usually associated with G1 to S progression [86;87]. However, several studies also implicate that PI3K signaling involves in both S-entry and the G2-M transition [88;89]. In this study, it was observed that silencing of the p55γ regulatory subunit in some breast cancer cells caused G2/M cell cycle arrest and mitotic catastrophe, which resulted in reduced cell growth and survival. These results revealed a novel mechanism by which PI3K exerts its role on cell cycle regulation, especially on the G2/M phase of cell cycle through p55γ.

The involvement of p55γ in the G0 to G1 phase transition is thought to be mediated through binding to the cell cycle regulator retinoblastoma protein (Rb) [48]. Thus, the G2/M phase cell accumulation in some cell lines (MDA-MB-468, BT549) was probably caused by the defective Rb, since it was shown that Rb depletion could abrogate the G2/M checkpoint of U2OS cells in the presence of DNA damage [90]. Similarly, it has
been demonstrated that both in cell culture systems and in xenograft mouse tumor models, dominant-negative p55γ inhibits cell proliferation regardless of the Rb status [56]. The Rb deficient cells transfected with dominant negative p55γ showed decreased proliferation, and they were arrested in G2/M and S phase [56]. These results suggested that modulation of p55γ might be able to affect the cell proliferation independent of Rb. Indeed, results in the current study also showed that the proliferation of both Rb positive and negative/mutant cells was affected by p55γ knockdown.

In the results shown above, p55γ reduction might lead to broken chromosomes and DNA breaks by inducing the defective mitosis. Cells containing mutant Rb protein (MDA-MB-468 and BT549) were defective in G1 checkpoint, and proceeded to G2/M with the DNA breaks. Because of the damage, they could not exit mitosis to form two identical progenitor cells, and died during or immediately after mitosis. On the contrary, although the G1 checkpoint is effective in the cells containing wild-type Rb (SKBr-3 and MDA-MB-453), some cells overcome the cell cycle arrest due to Akt activation, pass the G1 checkpoint with damage and then died in later cell cycle stages. It has been shown that activated Akt can abolish HEK293 cells from going through the G2/M cell cycle arrest induced by PI3K inhibitor LY294002 [91]. In experiments conducted for this study, Akt activation was up-regulated in SKBr3 and MDA-MB-453 cells, which potentially alleviated the G2/M checkpoint induced by p55γ reduction. On the contrary, significant G2/M arrest was observed following p55γ reduction in BT549 and MDA-MB-468 cell lines, which showed less Akt activation.
Activation of Akt and its downstream signaling pathways are thought to be mainly mediated by PI3K. However, the precise contribution to Akt activation by the various regulatory subunits is largely unknown. Inhibition of cell proliferation by p55γ reduction seems to be mediated through both Akt-dependent and Akt-independent mechanisms, as the changes on the phosphorylation level of Akt do not follow p55γ depletion in all the cases. Indeed, one of the established p55γ-interacting proteins, the non-receptor tyrosine kinase Tec, has been identified as a mediator for Akt-independent PI3K signaling pathway [54]. Thus, p55γ regulated signaling appears distinct of the p85 regulatory subunit mediated traditional Akt downstream signaling in some cells. In this light, because of its nuclear localization, p55γ is more likely involved in modulating nuclear processes. Indeed, the data presented here showed that p55γ plays an essential role in cell cycle progression, especially during mitosis. Recent studies showing that silencing of p55γ in ovarian cancer resulted in decreased cell proliferation further support our results [44]. In addition, the specificity of PI3K signaling may also be determined by its regulatory subunits besides catalytic subunits, as PI3K catalytic subunit binds to the regulatory subunits with different affinity. Furthermore, the expression levels of p55γ are lower than those of the p85 regulatory subunit in most cells, suggesting that compared to p85, the contribution of p55γ to Akt phosphorylation is probably minor. In contrast, our data indicated that modulation of p55γ in breast cancer cells caused profound effects such as G2/M phase arrest and mitotic catastrophe.
through signaling pathways other than Akt. Taking all these data into account, it appears that p55γ regulated signaling may involve a novel pathway to regulate cell fate.

Among all the cell lines investigated, only MCF7 cells survived the anti-proliferation effects of p55γ knockdown. For the purpose of discussion, the key findings of the p55γ knockdown experiments were summarized, and the estrogen receptor (ER), p53, and Rb mutation status were listed (Table 1). From the existing data, it appears that the mitotic defect was common for these cell lines; in addition, apoptosis was negatively related with Akt activation, and the mutant Rb was positively related with G2/M arrest. The only characteristic shared by all the cell lines affected by p55γ reduction was the ER negativity. These data promoted us to further investigate whether other ER positive cells are resistant to the growth inhibitory effect of p55γ reduction, if so, then p55γ might play an indispensable role in the proliferation and survival of the ER negative breast cancer cell lines.
Table 1. A comprehensive survey of the cell lines used in RNAi study (Chapter 2) and a summary of the results. MDA468: MDA-MB-468, MDA453: MDA-MB-453, MDA231: MDA-MB-231, MDA436: MDA-MB-436, WT: wild type, N/A: not available.

<table>
<thead>
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<th>Rb</th>
<th>G2/M arrest</th>
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<td>+</td>
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Growth inhibition of the breast cancer cells by p55γ reduction happens mainly via the G2/M arrest, with a small contribution of apoptotic cell death. The alteration of G2/M phase is generally thought to be modulated by G2/M regulatory proteins. Indeed, cyclin B1 level is up-regulated and Chk1 and Plk-1 levels are down-regulated upon p55γ silencing. Cyclin B1 is the regulatory subunit of the Cdk1, the kinase required for mitotic initiation. Cyclin B1 accumulation begins in S-phase and continues throughout the G2 phase until mitosis [92]. Results showed that in p55γ-depleted cells, cyclin B1 was accumulated, which could result in prolonged activation of cyclin B1/Cdc2 complex, and maintained cells in G2/M. The Chk1 protein is a serine/threonine kinase preferentially expressed during S and G2 [93]. Chk1 is also responsible for the G2 checkpoint of the cell cycle. At G2, activated Chk1 phosphorylates CDC25A, -B and -C to prevent cyclin B/Cdc2 activation, resulting in G2 arrest [59]. It has been shown in mouse embryonic stem cell lines that depletion of Chk-1 resulted in premature cyclin B1/Cdc2 activation, as well as premature mitosis and DNA damage, which leads to mitotic catastrophic cell death [94]. A dramatic G2/M arrest, chromosome misalignment, kinetochore defects, as well as mitotic catastrophe was also reported in Chk-1 depleted Hela cells [95]. The mitotic catastrophe is the results of abrogated checkpoint of cell cycle, especially at G2/M phase. The down-regulation of Chk1 upon p55γ silencing abrogates the G2 checkpoint, allowing cyclin B1/Cdc2 activation to occur, which facilitates mitosis entry.
In present study some cells accumulated in G2/M upon p55γ silencing, in addition, significantly increased mitotic figures suggested that cells mainly arrested during mitosis. The DNA damage caused by p55γ depletion could lead to activation of PI3K related kinase such as Ataxia telangiectasia mutated (ATM), and Ataxia telangiectasia and Rad3 related (ATR). The activation of ATM and ATR then initiate the Chk-1 pathway. Interestingly, an earlier study showed that Chk-1 depletion could partially rescue the activation of Plk-1, regardless of the presence of DNA damage, indicating a negative regulation of Chk-1 to Plk-1 [95]. However here we demonstrated that Chk-1 and Plk-1 are both down-regulated by p55γ depletion. This apparent controversy might be due to direct down-regulation of Plk-1 by p55γ instead of indirectly through Chk-1, or cell line-specific differences, or the synchronization status of the cell cycle.

Plk-1 is the best characterized member of the polo-like kinase family and is an important regulator of cell cycle progression during M-phase, because it is essential for the functional maturation of mitotic centrosomes [96], the M phase exit and regulation of the Anaphase-Promoting-complex (APC) [97], and the activation of the Cdc2/cyclin B1 cascade [98]. Plk1 contributes to G2/M progression through phosphorylation and activation of cyclin B1-Chk1 complex on a serine residue in the middle of the nuclear export signal sequence essential for translocation [60]. The previous studies have demonstrated that depletion of Plk-1 by RNAi induces mitotic catastrophe in gastric cancer [99], and various skin cancer cells [61;100]. The phenotype of Plk-1 depletion is G2/M cell cycle arrest, decreased proliferation and survival, and increased apoptosis,
which has been demonstrated using RNAi method in prostate cancer [101;102],
glioblastoma [101], bladder cancer [103], pancreatic cancer [104], esophageal cancer
[62], cervical cancer [105], leukemia [106], and lung cancer [107]. In the present study,
Plk-1 was down-regulated as a result of p55γ silencing in breast cancer cells.
Consequently, similar phenotypes as observed in the Plk-1 depleted cancer cells were
shown, such as G2/M arrest, decreased proliferation and survival, as well as aberrant
segregation of chromosome, microtubule misalignment, multicentrosomes, or
multipolar mitosis. Subsequently, the predominance of apoptotic bodies is also
observed after p55γ depletion. Thus, upon p55γ silencing, the majority of cells may at
first arrest in G2/M stage, with a differential percentage of cells that overcome G2/M
transition checkpoint and enter aberrant mitosis, leading to catastrophic cell death.
These data indicate that mitotic catastrophe is the primary mediator of cell death upon
p55γ silencing in breast cancer cells.

The PI3K regulatory protein p55γ potentially directs its associating proteins to
achieve various biological functions. However, so far no evidence has been shown that
it could act directly as a transcription factor. Indeed, the RNA levels of cyclin B1 and
Plk-1 are not changed upon p55γ silencing, suggesting that the regulation of these
proteins is not at the transcriptional level but rather through reduced protein
degradation. So far little is know about the upstream regulation of Plk1. Therefore, how
p55γ depletion caused down-regulation of Plk1 needs to be further investigated.
In summary, our study with suppression of gene expression show that p55γ, a specific PI3K regulatory subunit isoform, has differential and essential roles in cell cycle progression through both Akt dependent and Akt independent mechanisms. Reduction of p55γ leads to defective mitosis, mitotic catastrophic cell death, and has profound effect on cancer cell survival. Based on these results, a hypothetic model of p55γ regulating cell proliferation, cell survival, and mitotic spindle structures is proposed (Figure 2.11).
Figure 2.11 Hypothetical models for the pathway of cell survival and mitotic
catastrophe regulated by p55γ in breast cancer cells. The dotted lines indicate
unknown pathways; the solid arrows represent positive regulation, and the blocked
arrows represent negative regulation.
PI3Ks pathway components have been considered promising targets for the treatment of cancer as well as other diseases. However, PI3K inhibitors currently in clinical trial need to be combined with other cytotoxic drugs or radiation to enhance the effectiveness, which limited their clinical applications. It is absolutely necessary to employ new strategies targeting PI3K/Akt pathways to improve the efficiency of PI3K inhibitors. In contrast to catalytic subunit inhibition, our studies show a new strategy for PI3K inhibition directed against regulatory p55γ subunit signaling that may block cellular proliferation and survival.
CHAPTER 3

EFFECT OF FULL-LENGTH P55γ OVEREXPRESSION IN BREAST CANCER CELLS

3.1 Abstract

The p55γ protein is a unique regulatory subunit of class IA PI3K. The effect of full-length p55γ expression on cell growth and cell cycle progression remains elusive despite the anti-proliferative effect reported for dominant-negative p55γ fragments. Here the effects of the p55γ protein on regulation of cell cycle and growth were determined using full-length overexpression cell models. First, different p55γ expression constructs with no tag, Flag tag, His tag, or GFP tag were established. Full-length p55γ expression from these constructs was validated by western blot analysis. However, no significant changes on cell cycle progression and cell growth were induced by expression of the full-length protein. Interestingly, p85α, the most abundant regulatory subunit, was found significantly down-regulated after p55γ overexpression. This suggests that the effect of p55γ on cell growth is possibly due to the balance adjustment among the regulatory subunits, to maintain a consistent biological signaling for cell growth. To summarize, this finding emphasized the importance of eliminating multiple targets activating the same signaling pathway, since the efficacy of a drug down-regulating one gene target might be compromised through up-regulation of another gene product activating the similar survival pathway.
Activation of the PI3K/Akt pathway is regulated by EGF stimulation and manifested by translocation of PI3K subunit from the cytosol to the cellular membrane. The localization of the GFP-p55γ fusion protein was determined with confocal microscopy and Total Internal Reflection Fluorescent (TIRF) microscopy. In response to EGF stimulation, GFP-p55γ fusion protein translocated from a diffuse cytosolic localization to the cellular membrane. Laser confocal microscopy also showed nuclear localization of GFP-p55γ after EGF stimulation. In addition, GFP-p55γ travels to the same subcellular compartments as Akt upon stimulation.

This study demonstrates that the biological function of the GFP-p55γ fusion protein resembles that of the p85α subunit through regulating the PI3K/Akt pathway, indicating that the functions of these two proteins in the PI3K/Akt pathway probably overlap to some extent. This study not only provides novel evidence showing compensatory modulation among regulatory subunits, but also confirms the biological function of the GFP-p55γ construct. The construct is a reliable tool for the future immunostaining research investigating the function of p55γ.

3.2 Introduction

P55γ is potentially oncogenic for breast cancer since the pik3r3 mRNA encoding for this protein is upregulated in breast cancer in contrast to normal tissues in the array analysis [45]. Comparison of pik3r3 gene overexpression across nine independent analyses of ductal breast carcinoma and normal tissues showed a significant
upregulation of p55γ in breast carcinoma (www.oncomine.org). p55γ is also found to be more intensively expressed in malignant ovarian cancer tissues compared to normal tissues [44]. Silencing of p55γ significantly reduced cell growth and induced apoptotic cell death, and the affected breast cancer cells were less capable of forming colonies in vitro (Chapter 2).

Besides the catalytic subunits of PI3K, p55γ protein is also associated with other regulatory and cytoskeletal proteins such as Rb, IGF-1R, IRS and Tubulin, and is anticipated to be involved in insulin signaling pathways [37], cell cycle regulation [48], cellular structure modulation [43], and Tec kinase signaling [54], besides PI3K signaling (Chapter 1). Further experiments also provided evidence that p55γ may exert its effects through the non-conventional PI3K-Akt pathway (Chapter 2). Compared to the other regulatory subunits of PI3Ks, the domain structure of p55γ is special. Interestingly, overexpression of the amino terminal 24 amino acids of p55γ inhibits cell cycle progression in a variety of tumor cell lines [48;49;55;56]. The 24 amino acid peptide is regarded a dominant-negative module of p55γ inhibiting tumor growth. Therefore, overexpression of the full-length p55γ protein would be expected to stimulate cell growth, and promote tumorigenesis in breast cancer cells. To test this hypothesis, we generated eukaryotic expression constructs with different tags for easier detection, expressed p55γ in breast cancer cells, and detected the effect on cell proliferation and cell cycle progression. In order to precisely measure the effect of protein expression, cells expressing p55γ were first synchronized in the G0/G1 phase of
cell cycle to obtain a uniform population, and then stimulated with growth factors to release them from arrest. The conclusion would be hard to draw from the cell cycle profile of an asynchronized population since the distribution of the different stages of the cell cycle would be variable at any given time point.

MCF7 is a tumorigenic breast cancer cell line that is ER positive, and its proliferation is dependent on estrogen. Because of this, removal of estrogen can synchronize these cells effectively. MCF7 is a popular model in breast cancer research due to its extensively characterized features, as well as its estrogen responsiveness. Estrogen controls the cell cycle and proliferation of MCF7 cells by regulating the activation of cyclin dependent kinases and the phosphorylation of Rb [108]. The status of Rb phosphorylation is a better prognostic indicator than total Rb levels for breast adenocarcinoma cells [109]. In addition, the effect of interaction between p55γ and Rb has been demonstrated by expression of a dominant-negative module, the NH2 terminal 24 amino acids of p55γ, in MCF7 cells and achieved cell cycle arrest and decreased DNA synthesis [48]. Although shRNA transduction to reduce p55γ in MCF7 cells did not induce significant growth inhibitory effect, it was hypothesized that MCF7 cells were less dependent on p55γ for proliferation because they express ER, so the estrogen response is the major signaling for their survival. However, the protein depletion experiments were conducted in the presence of estrogen in the cell growth medium, so that the influence of estrogen can not be avoided. Thus, removal of estrogen from the growth medium could potentially sensitize MCF7 cells to the proliferation signal from
other pathways, such as p55γ. It was hypothesized that overexpression of p55γ might substitute for the estrogen requirement for growth in MCF7 cells.

The subcellular localization of p55γ and the co-localization with other proteins before and after growth factor stimulation should provide a clue for revelation of its function. Since there are no commercially available antibodies, a project to generate rabbit anti-serum against a synthetic peptide of N-terminal specific amino acids of p55γ was initiated. Because of the uniqueness of this antigen, the resulting serum should recognize only the p55γ and none of the other regulatory subunits. Unfortunately, although the anti-serum could recognize the peptide antigen with high affinity and specificity, it failed to detect the native folded protein (data not shown). Expression of a GFP-tagged p55γ construct in breast cancer cells then became the next best choice. Recently, a GFP-tagged p85α has been utilized as a convenient tool for the visualization of this protein and to demonstrate that the p85α co-localizes with IRS-1 in the distinct cytosolic foci [17]. It was expected that GFP-tagged p55γ could mimic the behavior of the endogenous protein and provide valuable information on its signaling. The interaction between p55γ and α and β tubulins has been reported [43]. In this dissertation work, cell cycle profile analysis and immunohistochemistry assays proved that both the cytoskeletal structure and the mitosis machinery were disorganized after p55γ knock down (see Chapter 2). F-actin is the microfilament assembled by the globular actin bundles, and can be labeled by Texas red-X phalloidin. Overall, it seemed logical to hypothesize that p55γ formed complexes with the cytoskeleton proteins such
as microfilaments and regulated their functions. Here, results demonstrated that the p55γ co-localized with F-actin after EGF stimulation, and a portion of the p55γ seemed to have a nuclear localization. It also relocated to the same subcellular compartments with Akt, suggesting at least a part of the p55γ biological functions resembles that of p85α.

3.3 Material and methods

Cell line

MCF7 cells were maintained in IMEM supplemented with 10% FBS. HEK 293 cells are maintained in DMEM supplemented with 10% FBS.

Generation of recombinant constructs

All constructs were generated with cDNAs amplified from a Mammalian Gene Collection (MGC) of cDNA clones (Invitrogen, Carlsbad, CA) using Finnzymes Phusion™ High-Fidelity DNA Polymerase (Espoo, Finland). The pcDNA™ 3.1 Directional TOPO Expression vectors (Invitrogen) were used for all other constructs except pEGFPC1/N2 vectors. Restriction enzymes XhoI and EcoRI were from New England Biolabs (Ipswich, MA). The mRFP-AktPH plasmid was a generous gift from Dr. Tamas Balla from NICHD at the National Institutes of Health.

Antibodies and reagents
Mouse monoclonal antibody against p85α was from Upstate (Lake Placid, NY). Mouse monoclonal against total Rb was from Cell Signaling. Goat anti-V5 antibody was from Bethyl (Montgomery, TX). Mouse monoclonal anti-human Rb antibody for detection of phosphorylated forms of Rb was from BD Pharmingen. Agarose immobilized goat anti-V5 affinity purified was from Bethyl. Thymidine was purchased from Sigma-Aldrich (St. Louis, MO).

**Generation of MCF7 stable cell lines**

For proliferation assays, MCF7 cells were transiently transfected with p55V5, pFlagp55, or pCDNA3.1 using Lipofectamine 2000 (Invitrogen) according to manufacturers’ specifications. For localization assays, MCF7 cells were transfected with pEGFPC1-p55 or pEGFPC1 vectors. Briefly, cells were split the night before transfection and reached 80-90% confluent in a 6-well plate, 250 μl of OptiMEM I® per well was combined with 10 μl of Lipofectamine 2000 and allowed to sit at room temperature for 5 min. Then, 250 μl of OptiMEM I® per well was combined with 5 μg of DNA. These two solutions were then combined and complexed for 20 min at room temperature. Forty-eight hours after transfection, cells were trypsinized and plated on 10 cm dishes. The next day, G418 was added to the cells at a final concentration of 600 μg/ml in complete media, and refreshed every three days. After two weeks of selection, the G418 resistant clones were pooled for analysis.
Synchronization by double thymidine block

MCF7 cells were grown to 40% confluency, and then thymidine was added at a final concentration of 2.5 mM and incubated for 16 h. Then, the medium was removed, the cells were washed three times with PBS, and fresh growth medium without thymidine was added. Cells were cultured in the thymidine-free medium for 8 h before fresh thymidine was added, and the cells were cultured for 16 h before analysis.

Estrogen deprivation with charcoal-stripped serum

MCF7 cells were stripped from estrogen following our protocol described previously [110]. Briefly, MCF7 cells were grown in normal growth medium until 70% confluency was reached. Then, the cells were changed to estrogen deprivation medium, which consisted of Phenol red-free IMEM (Invitrogen) supplemented with 5% Charcoal-Stripped Calf Serum (Gemini) (CCS). Every hour for the following five hours, the cells were washed with Phenol red-free IMEM, and maintained in estrogen deprivation medium between washes. Cells were cultured overnight, and then trypsinized with a phenol red-free trypsin. One thousand cells in 100 μl of estrogen deprivation medium were plated in each well of a 96-well plate. Cells were allowed to attach for 5 h, then 100μl of phenol red-free IMEM containing increasing concentration of estrogen from 0 to 1 nM was added to each well. Cells were stained with crystal violet solution on Day 3, 5, and 7 after estrogen addition. These experiments were carried out in quadruplicates.
**Immunoprecipitation**

After treatment, the cells were trypsinized, washed with ice-cold PBS, and lysed with a lysis buffer (10 mM Tris-base, pH 7.4, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of pepstatin, leupeptin, and aprotinin), and cleared by centrifugation. The lysates (500 μg) were mixed with 50 μl of goat anti-V5 conjugated beads or 5 μg of control IgG, at 4°C, overnight, with rotation. The next day, 50 μl of Gammabind G sepharose (Amersham Biosciences) were added to the antigen-antibody complex and incubated for 2 h at 4°C, with rotation. Beads were recovered by centrifugation at 12,000 rpm for 1 minute, and washed four times with the lysis buffer. The pellet was resuspended in 2x laemmli buffer (BIO RAD) and boiled for 5 min, centrifuged, and subjected to SDS-PAGE.

**Immunohistochemistry**

SKBr3 cells were transfected on glass coverslips with pEGFPC1-p55, pEGFPN2-p55 or control empty vector with a nucleofection method as described in Chapter 2, using solution V and program V-23. Cells were serum-starved for 24 h after overnight incubation, and then stimulated or not with 100 ng/ml of EGF for 10 min. The cells were fixed with 4% paraformaldehyde (Sigma) in PBS for 10 min at room temperature, then permeabilized with 0.5% Triton X-100 for 5 min at room temperature, and then
washed with PBS 3 times. Texas Red-X phalloidin was diluted 1/200 with 0.1% gelatin/PBS containing 10% normal goat serum to incubate with the cells for 20 min at room temperature. Stock DAPI (40 μg/ml) was added to the incubation at a dilution of 1/500. The coverslips were washed four times with PBS, and mounted with Aqueous Mounting Medium with Anti-Fading Agents (Biomed, Foster city, CA) on microslides (VWR, Media, PA), then sealed with nail polish. Cells were observed under a Zeiss 510LSM/ META/ NLO live imaging, multiphoton microscope, and the images were analyzed with a Zeiss LSM Image Browser Version 4.

**Total Internal Reflection Fluorescent Microscope Imaging**

SKBr3 cells were transfected on modified glass-bottom 8-well chamber slides (Nalge Nunc, Naperville, IL) with pEGFPC1-p55 plasmid. Twenty-four hours later, the cells were washed three times with PBS, and incubated in serum-free medium for 24 h for starvation. The cells were stimulated with 100 ng/ml of EGF, and GFP signaling was monitored live with an Olympus IX81 Total Internal Reflection Microscope connected with an incubator supplied with a humidifier and a CO₂ supplier to maintain the cells under the normal growth conditions of 37°C and 5% CO₂.

3.4 **Results**

3.4.1 *Generation and characterization of overexpression constructs*
The p55γ protein is potentially oncogenic for breast cancer since its mRNA is upregulated in primary tumor samples in contrast to normal tissues as shown by array analysis (www.oncomine.org). In ovarian tumor samples evaluated by immunohistochemistry, the p55γ protein was found overexpressed in malignant ovarian cancer tissues compared to normal tissues [44]. In order to investigate the effect of p55γ on breast cancer cells, several expression constructs were generated. The V5/His and the Flag-tagged constructs were generated for two reasons. The first reason was to overcome antibody problems, since the commercially available antibody could not co-precipitate p110α in the immunoprecipitation assay. Attempts were also made to obtain anti-p55γ rabbit serum through injection of the animals with a synthetic N-terminal peptide. Although the serum collected recognized the antigenic peptide, it could not detect the naturally folded protein, nor was it successful in recognizing denatured protein in fixed cells. The second reason was that compared to the non-tagged pCDNA3.1-p55γ construct, tagged constructs seemed to be more stable and to generate more protein when equal amounts of DNA was transfected; the reason for this finding will be discussed later.

Expression constructs were generated by PCR and molecular cloning using the SPORT plasmid containing the human cDNA encoding p55γ as the template. Both non-tagged and tagged constructs were generated in pCDNA3.1, pCDNA3.1/Myc-His, pEGFPC1 or pEGFPN2 plasmids. A V5 tag sequence was introduced to the C-terminus of p55γ during PCR for a V5-tagged construct in a pCDNA3.1 plasmid. The primers
for each PCR reaction are listed in Table 2. A brief cartoon representing tags for each construct is shown in Figure 3.1 A. Sequencing reactions for each construct were performed to confirm the correct insertion. HEK293 cells were transfected with these constructs and cell were lysed to detect expression of p55γ. The proteins expressed from each construct were found migrating in PAGE analysis at the expected rate for their respective molecular mass (Figure 3.1 B).

**Table 2.**

<table>
<thead>
<tr>
<th>Cloning Method</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flag p55 TOPO cloning</td>
<td>CAC CAT GGA CTA</td>
<td>TAA CGG CCG TTA</td>
</tr>
<tr>
<td></td>
<td>CAA GGA CGA CGA</td>
<td>TCT GCA AAG CGA</td>
</tr>
<tr>
<td></td>
<td>TGA CAA AAT GTA</td>
<td>GGG</td>
</tr>
<tr>
<td></td>
<td>CAA TAC GGT GTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAG TAT G</td>
<td></td>
</tr>
<tr>
<td>pCDNAp55 TOPO cloning</td>
<td>CAC CAT GTA CAA</td>
<td>TTA TCT GCA AAG</td>
</tr>
<tr>
<td></td>
<td>TAC GGT GTG GAG</td>
<td>CGA GGG CAT CTG</td>
</tr>
<tr>
<td>pCDNA/His/V5 TOPO cloning</td>
<td>CAC CAT GTA CAA</td>
<td>TCT GCA AAG CGA</td>
</tr>
<tr>
<td></td>
<td>TAC GGT GTG GAG</td>
<td>GGG CAT CTG</td>
</tr>
<tr>
<td>pEGFP cloning</td>
<td>AGA TCT CGA GCT</td>
<td>GCA GAA TTC GAT</td>
</tr>
<tr>
<td>(Digestion enzymes:</td>
<td>ATG TAC AAT ACG</td>
<td>TAT CTG CAA AGC</td>
</tr>
<tr>
<td>Xhol/EcoRI)</td>
<td>GTG TGG</td>
<td>GAG GG</td>
</tr>
</tbody>
</table>

Table 2. Primers used for the generation of the eukaryotic p55γ expression constructs.
Figure 3.1 Characterization of p55γ overexpression constructs. (A) Cartoon representation of the different p55γ expression constructs. (B) Transient overexpression of constructs in HEK293 cells; lysates were resolved by SDS-PAGE, transferred to PVDF membranes which were probed with a specific anti-p55γ antibody. (C) Co-immunoprecipitation of Rb and p55γ in HEK293 cells. Lysates from HEK293 cells transiently expressing pCDNA3-p55-His/V5 plasmid was incubated with goat anti-V5 conjugated beads or control IgG as indicated, and the immunoprecipitants were probed for total Rb, and the V5 tag.
3.4.2 Confirmation of binding between p55γ and Rb

HEK293 cells were transfected with the pCDNA3.1-p55V5/His plasmid, and immunoprecipitation assays showed Rb in the immunoprecipitate obtained with a V5 antibody, but not in the control IgG immunoprecipitate, indicating that Rb associated with p55γ (Figure 3.1 C). Since it is proposed to use the established constructs in this study to investigate the effect of p55γ expression on cell cycle and cell proliferation, it become important to determine if the constructs maintain the ability to bind Rb protein and modulate cell cycle when expressed in mammalian cells.

3.4.3 Effect of p55γ on proliferation and estrogen sensitivity in MCF7 cells

The effect of p55γ on estrogen sensitivity and proliferation was determined in breast cancer MCF7 cells. First, MCF7 cell lines stably expressing p55γ were generated from cells transfected with p55γ constructs by G418 selection. Then, protein expression was verified by western blot for p55γ with the equal amount of protein blotted onto a PVDF membrane (Figure 3.2 A). MCF7 can be synchronized at G0/G1 by estrogen deprivation, since MCF7 cell expresses estrogen receptor and its growth is dependent on estrogen. Estrogen can be effectively stripped from cells by washing in charcoal-stripped serum [110]. The phenol red pH indicator used in most commercial available growth media is a weak estrogen mimic that promotes the proliferation of estrogen receptor positive MCF7 cells [111]; hence we used the phenol red-free growth media and trypsin in the estrogen deprivation method. MCF7 cells stably expressing p55γ were
stripped of estrogen to achieve a G0/G1 arrest, and then increasing concentrations of estrogen were added to the cells to release them from the resting phase. No significant difference was found between the proliferation of p55γ expressing and control cells on day 3, 5 and 7 after estrogen deprivation, indicating that p55γ could not help MCF7 to overcome the growth inhibiting effect of estrogen removal. Furthermore, no significant differences were found between p55γ expressing cells and control cells in their growth response to increasing amounts of estrogen, which indicates that p55γ did not noticeably enhance the estrogen sensitivity of the MCF7 cells (Figure 3.2 B).
(A) MCF7-pC, MCF7-pFlag55, MCF7-p55V5
Endogenous p55γ

(B) Absorbance A_{550nm} vs. time (D3, D5, D7)

pC vs. Flag55
- pC
- Flag55

pC vs. p55V5
- pC
- p55V5

estradiol (M)
Figure 3.2 Effect of p55γ expression on estrogen sensitivity of MCF7 cells. (A) Western blot analysis shows the detection of p55γ in MCF7 cells stably expressing the Flag and V5 tagged proteins. (B) Expression of p55γ did not change the estrogen sensitivity of MCF7 stable cell lines expressing pFlag-p55, or p55-His/V5 compared with pCDNA3.1 empty vector. The cells were estrogen-starved and then re-stimulated with increasing concentrations of estrogen; cell proliferation was monitored at 3, 5, 7 days of estrogen incubation. The growth curve was drawn from the absorbance of crystal violet staining of the cells at different time points (3, 5, 7 days) after estrogen addition. Upper panels: Growth assay of cells stimulated with 1nM of estrogen. Lower panels: Growth response to increasing concentration of estrogen on Day 7.
3.4.4  Down-regulation of p85α by overexpression of p55γ

Information from genetic studies revealed that tissues from p85α knockout mice showed altered expression of PI3K subunits, including up-regulated p55γ [18]. Based on data from p55α, p50α knockout mice and p85β knockout mice, which did not result in significant changes in p85α level in the overall tissue expression, I proposed that p55γ modulation may not significantly alter the expression of other regulatory subunits. To detect the p85α levels in response to p55γ expression, protein were prepared from A1N4 cells transiently transfected with constructs expressing p55γ on either end of GFP. Surprisingly, western blot analysis revealed that p85α was down-regulated in cells overexpressing p55γ (Figure 3.3). This result is interesting since it shows that regulatory subunit such as p85α may be regulated by p55γ overexpression, potentially compensating some of the effects caused by p55γ alteration.
Figure 3.3 Expression of p55γ was associated with down-regulation of p85α. P55γ constructs with GFP tag attach either to the N-terminal (pEC55) or the C-terminal (pEN55) end were transiently transfected into A1N4 cells, and the cell lysates were harvested 72 h after transfection. Proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, the membrane was probed with anti-p55γ, anti-p85α, and re-blotted with β-actin as the loading control.
3.4.5 Validation of the GFP-p55γ and p55γ-GFP constructs using immunohistochemistry and live cell imaging

Information on the intracellular trafficking of the GFP-p55γ fusion protein and its co-localization with other regulators or structural proteins may provide clues on its function. Our findings demonstrated that the p85α protein is modulated by the overexpression of p55γ, suggesting a balance of the proteins based on functional similarity. Our findings along with the domain homology of these two proteins suggested that GFP-p55γ may have overlapping functions with p85α in cell growth signaling. GFP-p85α was demonstrated to associate with IRS-1 in the cytosol, in the form of a sequestration complex, and to negatively regulate insulin signaling [17].

GFP-fusion p55γ constructs was used to investigate the co-localization with F-actin and Akt, and to confirm its membrane localization. To investigate the localization of p55γ using the GFP-p55γ construct, the ability of this construct to interact with p110α was first determined by immunoprecipitation assays. The purpose was to confirm that the GFP tagged protein maintained the ability to interact with p110α and of forming kinase complex similar to the endogenous protein. Results showed that p55γ can be detected in immunoprecipitates of p110α antibody (Figure 3.4 A), and conversely the p110α was detected in the immunoprecipitates of GFP antibody (Figure 3.4 B), thus strongly suggested that the GFP-p55γ associates with p110α. Next, as a preliminary experiment, the localization of GFP-p55γ was evaluated in breast cancer SKBr3 cells
and Chinese Hamster Ovary cells using a Nikon E600 upright Epi-fluorescence microscope. After 24 h of incubation in serum-free medium, the GFP-p55γ remained diffusely in the cytosol, with what appeared like big patches along the cell periphery. After EGF stimulation, the GFP fusion protein condensed to form round vesicle-like complexes in the cytosol (data not shown). This observation resembles what was documented previously for p85α [17]. However, the limited resolution of Epi-fluorescent microscopy led us to use laser confocal microscopy to search for additional evidence on the co-localization of p55γ and its interacting proteins.
Figure 3.4 Validation of the GFP-p55γ constructs by co-immunoprecipitation.

Cells were transfected with GFP-p55γ or control GFP constructs and then immunoprecipitated with anti-p110α or control antibody as indicated in (A) A fraction (1/50) of the whole cell lysates (WCL) was loaded as control to verify the protein expression. The membrane was blotted with anti-GFP, anti-p85α, and anti-p110α antibodies as indicated. (B) Lysates were immunoprecipitated with anti-GFP or control antibody, and then blotted for p110α, using 1/50 of the total lysates as control for protein expression.
To investigate the association between p55γ and cytoskeleton protein actin filaments (F-actin), SKBr3 cells were transfected with either N-terminal GFP-tagged p55γ (pEGFPC1-p55) or C-terminal GFP-tagged p55γ (pEGFPN2-p55) expression constructs. Cells were treated with 100 ng/ml of EGF after serum starvation, and then counter-stained with Texas red-X phalloidin for F-actin. Results showed three major findings: the first was that before EGF stimulation the GFP-p55γ is diffusely located in the cytosol with several patches along the cell periphery, close to where the cells were attached to the substrates (Figure 3.5 A and C); after EGF stimulation the GFP-p55γ signal accumulates and co-localize with the signal from F-actin (Figure 3.5 B and D). The second finding was that, regardless of the location of the GFP tag, either at the N- or the C-terminus of p55γ, the localization of GFP fusion proteins is similar, demonstrating that the GFP fusions did not dramatically interfere with the functional localization of p55γ. The control GFP vector expressed protein localized uniformly in the cytosol, and the signal was unaffected by stimulation, which also confirmed the specificity of the GFP tagged p55γ constructs. The third finding was that in addition to the co-localization with F-actin, the GFP tagged p55γ proteins also trafficked to the nuclear vicinity in the cells, suggesting that this protein might be also involved in the DNA replication process.
Figure 3.5 Both N-terminal and C-terminal GFP fusion p55γ proteins re-localized to membrane and co-localized with F-actin upon EGF stimulation. (A) SKBr3 cells transfected with N-terminal GFP-p55γ were serum starved for 24 h and stained with Texa red-X Phalloidin for F-actin (upper left), and Topro3 for nucleus (lower left). (B) After serum starvation, GFP-p55γ expressing cells were incubated with 100 ng/ml of EGF for 10 min and stained as in (A). The localization of C-terminal p55γ-GFP construct transfected SKBr3 cells before (C) and after (D) EGF stimulation is also shown. The images were taken with a LSM 510 confocal microscopic system, and the data were analyzed using the LSM image processing software. Bar: 10μm.
It seemed possible that the localization of p55\(\gamma\) protein would change after it was stably expressed in cells, since the incorporation of the plasmid into the chromosome adds stress to the cells, which may affect the protein itself, compared to ectopic expression. To test such possibility, MCF7 cells stably expressing GFP-p55\(\gamma\) were generated and tested for the response of GFP-p55\(\gamma\) to EGF stimulation. The images were taken with a regular fluorescent microscopy system, and the result showed that the GFP-p55\(\gamma\) signal moved to the cell periphery upon EGF stimulation, merging with the F-actin signal (Figure 3.6 B), comparing to the absence of co-localization before EGF stimulation (Figure 3.6 A). The similar observations in both transient and stable cells supported the localization findings of GFP-p55\(\gamma\), showing that the protein moved to the cell periphery and co-localized with F-actin upon EGF stimulation.
Figure 3.6 **GFP-p55γ stably expressed in MCF7 cells re-localized to plasma membrane and co-localized with F-actin upon EGF stimulation.** MCF-7 cells stable expressing GFP-p55γ were serum starved and EGF stimulated as described for SKBr3 cells. The pictures shown were taken (A) before and (B) after EGF stimulation. Cells were stained with Texas red-X Phalloidin for F-actin (upper left); the phase contrast image (upper right) shows the shape and flatness of the cells. GFP-p55γ was shown in green fluorescence channel (lower left). The images were taken with a Nikon E600 epi-fluorescent microscopic system, and the data were analyzed using the LSM image processing software. Bar: 20μm. The expression of GFP-p55γ was confirmed by western blot with an antibody against p55γ, as shown in the picture inset.
Tests on whether a protein relocates to the cell membrane from the cytosol can be performed by several standard methods. One is to label an established membrane-bound protein and test whether it co-localizes with the target protein; another method is to use a special Total Internal Reflection Fluorescent Microscopy (TIRFM) to identify and detect the proteins once they localize at the membrane. From epifluorescence microscopy images it is difficult to determine the membrane attachment of a certain protein, and impossible to capture the movement of fluorescently labeled proteins in live cells. TIRF microscopy is very reliable to determine the membrane localization of proteins, and can be used also to observe live cells in time lapse. TIRFM images showed that before EGF stimulation, only very small amount of GFP-p55γ localized at the cell membrane, upon EGF addition the signal at the membrane rapidly increased, and reaching a peak around 10 min (Figure 3.7), and afterwards the signal remained detectable but decreased gradually within one hour. The TIRFM technique was performed following the procedure described by Xu [112]. The TIRFM data supported the finding that p55γ migrates to the membrane upon the EGF stimulation.
Figure 3.7 Membrane localization of GFP-p55γ increased with time after EGF stimulation. SKBr3 cells were transiently transfected with GFP-p55γ on glass-bottom chamber slides and serum starved for 24 h before incubation with EGF. Pictures shown are representative images from 0 min to 12 min after EGF addition.
As the major target for PI3K pathway, the function and localization of Akt has been extensively studied. It was proven that Akt is recruited to the membrane by generation of phosphatidylinositol-3,4,5-triphosphate (PIP$_3$) through phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP$_2$) by PI3K [4], where Akt is further phosphorylated and activated by the PDK1 [68] and PDK2 [67]. Akt localization was tracked using an mRFP-AktPH expression construct, a generous gift from Dr. Tamas Balla (NICHD, NIH). Co-expression of GFP-p55$_\gamma$ and mRFP-AktPH in SKBr3 cells revealed that GFP-p55$_\gamma$ co-localizes with Akt after EGF stimulation (Figure 3.8). This data showed that GFP-p55$_\gamma$ in SKBr3 cells serves as a component of PI3K responding to EGF stimulation, and trafficks with the Akt protein to the same subcellular compartment in the membrane vicinity.
Figure 3.8 Co-localization of mRFP-AktPH with GFP-p55γ. SKBr3 cells were co-transfected with the mRFP-AktPH and GFP-p55γ constructs. The cells were serum starved for 24 h and stimulated with 100 ng/ml of EGF for 10 min before being fixed. Nuclei were stained with DAPI (blue). Pictures were taken with a LSM510 confocal microscopic system, and the data were analyzed using the LSM image processing software. Bar: 10μm.
3.5 Discussion and conclusion

Overexpression of the full-length p55γ protein was expected to stimulate cell growth and promote tumorigenesis in mammary epithelial cells. Especially since the dominant-negative module of p55γ inhibited tumor growth [49] and cell cycle progression in a variety of tumor cell lines [48;55;56]. In this study, silencing of p55γ significantly reduced cell growth and induced apoptotic cell death, thus, breast cancer cells are less capable of forming colonies in vitro (Chapter 2). However, in the present studies the use of different constructs to overexpress p55γ in mammary epithelial cells (A1N4 and MCF10A) did not reproducibly show the expected effect (data not shown). Lack of efficient expression and other technical issues with proper controls have been obstacles for further study in mammary epithelial cells.

The expression of p55γ did not substitute for the estrogen requirement for proliferation in MCF7 cells. Since the cDNA inserts were validated by DNA sequencing prior to expression experiments, it seems possible that either the expression did not induce a detectable proliferation effect because expression levels were insufficient, or the ectopic expression produced dysfunctional proteins, or the expressed proteins were rapidly degraded, or the cell lines used already contain sufficient p55γ for proliferation so that the overexpression delivers minimal effect. Other breast cancer cell line models with different endogenous p55γ expression were also evaluated by overexpression of p55γ constructs, such as MDA-MB231 and MDA-MB-436 cells. Unfortunately, none
of these cell lines tested showed reproducible results for cell cycle and proliferation experiments, probably because of the above mentioned reasons. Besides, another reason for lack of growth effects was that the down-regulation of p85α protein potentially compensated the up-regulation of p55γ.

Interestingly, p85α, the most abundant regulatory subunit, was found significantly down-regulated after p55γ overexpression. Gene expression array databases comparing gene expression profiles of pik3r1 (p85α coding gene) and pik3r3 (p55γ coding gene) in several breast cancer cell lines showed their inverse relationship, in which high pik3r1 expression was associated with low expression of pik3r3. Likewise, high pik3r3 expression is correlated with relatively low expression of pik3r1 (www.oncomine.org). In the present study, my experiments evaluating the p85α and p55γ by western blot also showed that p55γ level was high in different cell lines while the p85α level was low. In addition, the knockout mice provide convincing evidence for the existence of a molecular balance of PI3K subunits. As reviewed by Vanhaesebroeck [18], mice with p85α, p55α and p50α (pan-p85α) homozygous deletion is embryonic lethal, and the heterozygous mice are viable. In both these homozygous and heterozygous pan- p85α deletion mice, p85β is up-regulated in the insulin-sensitive cells. In contrast, knockout mice with only p85α depletion contain up-regulated expression of p55α and p50α in muscle and fat cells. It was also reported that when p55α and p50α units were targeted for knockout, the p85α was also decreased in muscle, and unchanged in adipocytes and
liver cells. Interestingly, the p85β knockout did not seem to affect the expression of any other regulatory subunits such as p85α, p55α, p50α and p55γ. These observations indicate that alteration of one subunit may sometimes influence the expression of other subunits of PI3K. The alterations might be dependent on the importance and function of the altered protein in different tissues.

There has been no report on the generation of p55γ knockout mice, but based on observations in the current study, p55γ down-regulation may have some effect on the expression of other regulatory subunits. In this study, overexpression of p55γ was found to lead to down-regulation of p85α protein, which potentially counteracts the effect of p55γ over expression. P55γ and p85α are both regulatory subunits with about 80% similarity in their protein sequences. Overlapping functions by p55γ and p85α in PI3K and other signaling pathways remain to be identified. Nevertheless, the effect of p55γ overexpression on p85α suggested mechanisms underlying the molecular balance among proteins sharing one survival pathway in order to maintain the stability of cellular signaling.

In this chapter, the functionality of GFP-p55γ construct was validated by confirming its membrane localization in response to EGF stimulation in different cell lines, and by using different detection methods, including confocal microscopy and TIRFM. The punctuate localization patches produced by GFP-p55γ is very interesting, in that it resembles the localization of a GFP-p85α construct in a previous study [113].
membrane localization of the PI3K is enough to recruit the downstream target proteins and activate multiple signal transduction kinase pathways [114]. Indeed, our previous research demonstrated the trans-localization of both calmodulin and Akt to the plasma membrane upon EGF stimulation, and the co-localization of these two proteins to the same sub-cellular compartment [115]. F-actins are enriched in some growth factor-inducible cell peripheral structures such as lamellipodia and membrane ruffles. The co-localization of p55γ with F-actin upon EGF in breast cancer cells strongly suggests a role of p55γ in actin network formation. The GFP-p55γ construct is therefore suitable for use in future experiments to explore the function of p55γ through its interacting proteins using immunohistochemistry methods or live cell imaging.

By using the GFP-p55γ fusion protein and confocal microscopy, the localization and translocation of p55γ upon EGF stimulation were determined. Upon activation of PI3K through epidermal growth factor stimulation, the localization of p55γ was shown by a GFP-p55γ fusion protein, shifted from a diffuse cytoplasmic pattern to the cellular membrane and co-localized with F-actin and Akt, and also the nuclear region. These results support the findings in chapter 2 that p55γ may exert some of its roles through a PI3K/Akt independent pathway and further suggest that a novel role of p55γ may be exerted by interaction with nuclear proteins. Both N-terminal and C-terminal tagged p55γ constructs generated similar results, further validating that the p55γ translocation observed in these cells is specific and revealing the functional localization of p55γ.
Based on the above results, it was concluded that balance among the regulatory subunits is important to maintain a consistent biological signaling for cell growth. The observation on up-regulated p55γ in breast cancer cells requires further examination of other regulatory subunits such as p85α, p85β, p55α, and 50α. Furthermore, as the location of p55γ expression not only matches that of PIP3 at the membrane upon stimulation, but also to the nuclear region, its functions related with nuclear proteins require further investigation.
CHAPTER 4

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

4.1 Summary of results and conclusions

PI3K pathway components are highly implicated in breast tumorigenesis. In contrast to its catalytic subunits, roles of the regulatory subunits of PI3K in breast cancer are largely unknown. Our aim was to determine the roles of PI3K regulatory subunit p55γ on regulating survival signaling of breast cancer cells. The data presented in this thesis show the roles of p55γ in breast cancer cells.

In chapter 2, we presented evidence showing that in some breast cancer cell lines, reduction of p55γ significantly reduced cell growth, and induced mitotic catastrophe. The mitotic catastrophic cell death was evidenced by multipolar mitosis observed using confocal microscopy, and supported alteration of the corresponding G2/M checkpoint proteins, such as down-regulation of Chk-1 protein, the up-regulation of cyclin B1 and phosphorylated Cdc2, as well as the down-regulation of mitotic catastrophe marker protein Plk1. Reduction of p55γ also caused G2/M cell cycle arrest in some cells, an increased proportion of cells lingering in mitosis suggested that these cells might die from prolonged mitosis retention. Moreover, reduction of p55γ resulted in down-regulation of the survival signaling protein survivin, further supporting our hypothesis that p55γ is involved in survival signaling. These data indicate that expression of p55γ is necessary for cell growth and survival. Interestingly, p55γ may not regulate survival signaling through the conventional PI3K-Akt pathway because not all cells with p55γ
silencing show reduced activation of Akt. This suggests that in addition to its role as a regulatory subunit of PI3K, p55γ may exert some functions through a PI3K/Akt independent pathway to regulate cell survival signaling.

In chapter 3, overexpression of full-length p55γ did not significantly alter the rate of cell growth, cell cycle progression and apoptosis of “normal” mammary epithelial cells, p55γ expression also did not affect the estrogen response of MCF7 cells. Besides the technical issues which caused insufficient expression, some cell lines tested might already contain sufficient amount of p55γ protein, so that the overexpression did not influence their proliferation and survival. Interestingly, p85α, the most abundant regulatory subunit in the cells, was found significantly down-regulated after p55γ overexpression. This infers that the lack of effect for p55γ on cell growth is possibly a result of the balance adjustment among the regulatory subunits, in order to maintain consistent biological signaling pathways for cell growth. The molecular balance between the PI3K regulatory subunits has been reported in knockout mouse models. It has been shown that pik3r1 knockout leads to up-regulation of pik3r3 gene product. However, the effect of pik3r3 knockout on the level of pik3r1 has not been reported. It was hypothesized that pik3r3 knockout does not have dramatic effect on pik3r1 expression, since pik3r1 is more ubiquitously expressed in the cells, in contrast, pik3r3 only have localized higher expression, which presumably indicates less function. However, our results suggested that after expression in A1N4 mammary epithelial cells,
p55γ could down-regulate the p85α protein, which probably nullified part of the overlapping effect with p85α by p55γ overexpression.

On the other hand, interestingly, we observed the similar molecular balance in the p55γ knock down cell line models, in SKBr3 cells, reduction of p55γ resulted in up-regulation of p85α. These findings emphasized the molecular balance among the signaling proteins sharing a common pathway; modulation of one signal target may lead to the compensational alteration of the other, thus counteracting the effect mediated by the modulation. In spite of the molecular balance, the overexpression experiment did not generate reproducible results but the knockdown experiments did. It is speculated that the reasons why the proliferation and survival effect was detected in shRNA mediated p55γ knockdown model in spite of the compensation from p85α was probably two fold. First, the overexpression was not as effective as the lentiviral mediated knockdown, which was more complete and efficient. Second, the effective p55γ silencing probably triggered a signaling pathway that was not completely covered by p85α, which might be the Akt-independent pathway.

Also in chapter 3, by using a GFP-p55γ fusion protein in immunoprecipitation and confocal microscopy as well as TIRFM, the localization and translocation of p55γ upon EGF stimulation was determined. Upon activation of PI3K through epidermal growth factor stimulation, the localization of p55γ shifts from a diffuse cytoplasmic location to the cellular membrane and co-localizes with F-actin and Akt, as well as to the nuclear region. This result supports the findings in chapter 2 that p55γ may exert some of its
role through a PI3K/Akt-independent pathway and further suggests that a novel role of p55γ may be exerted by its interaction with the nuclear proteins.

In addition, in the Appendix we also investigated the interaction of p55γ, calmodulin and Rb. We demonstrated the formation of a protein complex consisting of calmodulin, p55γ and Rb; with p55γ potentially facilitating the interaction between calmodulin and Rb. We also showed the changes in binding between these proteins in the presence of calcium. Results suggested that p55γ might play a role in modulating the interaction between calmodulin and Rb, thus regulating the signaling transduction between calcium signaling and cell cycle progression. However, we can not determine the exact binding ratio or stoichiometry of the three protein partners, except that they co-exist in the same subcellular complex. The quantitative study of protein-protein interaction such as BIACORE is suggested to verify this modulation.

In conclusion, our study revealed for the first time that p55γ plays a significant role in survival signaling in breast cancer cells. Based on the observations in this thesis, we conclude that p55γ plays a pivotal role in breast cancer cells to regulate cell growth and cell survival. In addition, regulation of cell proliferation, and especially the cytoskeleton apparatus organization may be also important in other cells or organs with limited endogenous expression of p55γ. Although the underlying biological mechanisms warrant further investigations, p55γ may play an important role in anti-cancer drug development.
PI3K have been considered promising targets for cancer treatment. However, existing inhibitors for PI3K so far need to be combined with other cytotoxic drugs or radiation to enhance their effectiveness. Instead of inhibition of the catalytic subunit, this thesis shows a new strategy involving the inhibition of regulatory p55\(\gamma\) subunit signaling that results in blocking cellular proliferation and survival. Importantly, this strategy did not cause changes in Akt activation in all the cell lines. Thus, development of p55\(\gamma\) specific inhibitors may lead to effective therapies for breast cancer. It is also possible that p55\(\gamma\) inhibitors could be used in combination with catalytic subunits inhibitors to create more effective and safer regimens for inhibiting PI3K signaling pathways.

4.2 Future directions

The data generated from the present study on the role of p55\(\gamma\) in breast cancer cells are exciting and very useful to provide the big picture on how p55\(\gamma\) regulates the cell survival and proliferation. A better understanding of the molecular mechanism(s) underlying the ability of p55\(\gamma\) regulation in breast cancer cells will enhance our understanding on the role of PI3K in breast cancer. On the other hand, the present study may also aid in the development of clinical drugs targeting PI3K for breast cancer prevention and treatment. Further investigation of the role of p55\(\gamma\) is warranted. Some future directions were suggested to extend the investigation on the function of p55\(\gamma\).
In the future we need to figure out that besides p85α, if any other regulatory subunits of PI3K are being altered by p55γ modulation, which could potentially improve the understanding of its biological functions. The p55γ expression level with the existing constructs did not meet our expectations, and heterogeneity of the expression might be one of the reasons for the absence of a reproducible effect. A more uniform expression level of p55γ could be obtained through other techniques such as lentiviral mediated expression, or Tet regulated inducible expression system. In addition, in order to evaluate the protein expression, it is necessary to improve the detection of non-tagged p55γ construct, so a sensitive antibody is urgently required to be developed. Since the synthetic peptide antigen did not produce effective antibody, a full-length human p55γ may be generated in large amounts in prokaryotic cells and concentrated for antibody production.

Since the p55γ reduction leads to mitotic defect and increased mitotic spindle asters, it would be useful to detect whether other cellular structure proteins and the mitotic regulatory proteins can be modulated by p55γ reduction. Proteins such as γ-tubulin, centrin, cyclin B1, phospho-Cdc2, and Plk-1 can be evaluated microscopically by immunostaining with the respective antibodies. The other G2/M regulating and stress response proteins potentially activated, such as Cdc25, Wee1, 14-3-3σ, p21, and Gadd45, can be evaluated by western blot.

The ER positive cell line MCF7 was not affected by p55γ reduction. Whether the MCF7 cells were insensitive because they were growth dependent on estrogen, and less
reliable on the proliferation signaling from p55γ, this question could be answered by using a modified MCF7 cell lines that is not dependent on estrogen for growth. Alternatively, other ER positive cell lines (T47D, ZR-75-1) can be used for shRNA transduction and tested for growth resistance.

Although extensive evidence showed the role of p55γ in present study, we do not totally exclude that the phenotypes observed might be influenced by other genetic factors. Therefore, the phenotypes need to be confirmed in other systems. One approach is to overexpress a mutant form of p55γ that avoid the shRNA targeting, in the p55γ shRNA transduced cells, and determine if overexpression can rescue the phenotypes caused by p55γ silencing.

Furthermore, the GFP-p55γ construct generated by present study provided a very useful tool for in vitro functional studies by immunofluorescent microscopy and live imaging analysis. Some known/potential p55γ binding proteins can be examined for co-localization with this GFP-p55γ, such as p110α, IRS-1, IRS-2, centrin. In addition, to explore the nature of the condensed patches that GFP-p55γ is localized in the cell, further co-localization studies are required. For example, to test whether the structure is lamellipodia, cortactin could be co-stained; for focal adhesion complexes composition study, the cell could be co-stained for vinculin.

It is tempting to hypothesize that p55γ specific inhibitors could be used in combination with catalytic subunit inhibitors to create more effective therapeutic regimens for inhibiting PI3K signaling pathways in breast cancer. However, although
the gene expression profile of primary tumors from patients showed that p55γ was
overexpressed in breast cancer compared to normal tissues (www.oncomine.org), more
work should be done to confirm the tumor specific expression of p55γ in order to use it
as a potential cancer target. In addition, targeted delivery by conjugating the p55γ
inhibitor to a tumor cell surface specific antibody could help to avoid damage to the
adjacent normal cells.
APPENDIX

INTERACTION OF P55γ WITH CALMODULIN

A.1 Abstract

Calmodulin functions as a sensor of calcium signaling and a possible mediator for PI3K pathway in cancer cells. To elucidate the possible role of calmodulin in mediating PI3K pathway through association with p55γ, the interactions among p55γ, calmodulin, and Rb were investigated. Association of calmodulin with p55γ was demonstrated by using calmodulin sepharose beads in pull-down assays from 293T cells. The p55γ facilitated the interaction between calmodulin and Rb. The novel observations on the interaction between p55γ and calmodulin suggested that p55γ exert some biological functions through association with calmodulin and Rb.

A.2 Introduction

Calmodulin is a calcium responsive protein that regulates many cellular functions including cell proliferation, cell cycle progression, chemotaxis, and so on. Earlier research in our lab used calmodulin bound to sepharose beads to identify potential calmodulin binding proteins under in vitro conditions. From the murine mammary carcinoma cell line Myc83, a calmodulin binding specific 64KDa protein was identified and was found to be reactive with pan-p85 antibody (Deb T, unpublished data). Because this protein could potentially be a longer murine form of p55γ [42], results strongly suggested the association between p55γ and calmodulin in murine cells. In this study
the association between calmodulin and p55γ was investigated in human cell lines. It was hypothesized that p55γ might serve as an adaptor protein for the crosstalk between the calcium/calmodulin signaling and PI3K/cell cycle pathway. Some preliminary data were generated to test this hypothesis, and the results might be useful in exploring the biological functions of p55γ.

First, attempts were made to concentrate and analyze the protein from the 64KDa protein band in SDS-PAGE gel. I excised the protein from gel which migrated at the same speed with the protein shown to be reactive with calmodulin in a calcium dependent manner. With the MALDI-TOF mass spectrometry method, however, the p55γ protein was not detected. Instead, the most abundant protein in the retrieved protein band was identified to be vimentin, a known calcium-dependent calmodulin interacting cell structure protein. Since the pan-p85 antibody should not be reactive with vimentin, and we could not confirm that the protein band being excised and analyzed was the same band shown in western blot, we decided that this experiment was not very informative.

Additional problems derived from the fact that when the project was initiated, no anti-p55γ antibody was commercially available. Next, a p55γ specific antibody was generated for protein detection. Unable to obtain any anti-serum generated by other labs, attempts were made to produce rabbit anti-serum using a synthetic N-terminal specific peptide. Unfortunately, the anti-serum recognized the antigenic peptide with high affinity and specificity, but did not recognize the endogenous or expressed p55γ
protein in western blot. We also tried the anti-serum for immuno cellular chemistry in AU565 cells, a cell line that has been shown to have detectable p55γ [48]; however the anti-serum generated high background. To date, commercially available anti-p55γ antibodies still have poor sensitivity for protein detection.

In a murine mammary carcinoma cell line overexpressing c-Myc we demonstrated that calmodulin activation successfully phosphorylated and activated Akt [116], which suggested that the PI3K/Akt pathway is associated with calmodulin activity. The hypothesis of this study was that p55γ and calmodulin associate with each other, and calmodulin, p55γ, and Rb co-exist in the same protein complex. The complex formed by these proteins potentially facilitates the crosstalk of cellular signal transduction including those initiated by calcium surges and growth factors.

A.3 Material and Methods

Antibodies and reagents

Mouse monoclonal anti-GFP was obtained from Roche (Indianapolis, IN). Rabbit anti-p110 for immunoprecipitation was obtained from Upstate (Millipore). Mouse monoclonal anti-p110 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-p85α was from Upstate. Monoclonal anti-Flag M1 were from Sigma. Epidermal growth factor was purchased from BD Biosciences.

Calmodulin sepharose pull-down assay
HEK293 cells were transiently transfected with Fugene 6. Briefly, cells were plated the day prior to transfection at a density of 1.5x10^6 cells per 10 cm² dish. Fugene 6 (15 μl) was diluted in 600μl of OptiMEM reduced serum medium and incubated at room temperature for 5 min; then 10 μg of pFlag-p55 plasmid or control pFlag-Sox9 were added to the tube and incubated for another 20 min, before the mixture was added dropwise to the cells. Cells were lysated 24 h later in an EDTA-free lysis buffer (10 mM Tris-base, pH 7.4, 1% Triton X-100, 50 mM NaCl, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of pepstatin, leupeptin, and aprotinin). Lysates were vortexed and centrifuged at 15,000 g for 15 min at 4°C. Lysate protein concentrations were measured using the BCA protein assay kit. For each treatment, 500 μg lysates were precleared by incubation with control sepharose beads with rotation at 4°C for 1h, and then incubated with 50 μl of Calmodulin sepharose 4B (BIOMOL, Plymouth Meeting, PA) or control sepharose in the presence of EDTA or CaCl₂ for 2 h. The pellets were washed four times with the lysis buffer in the presence of EDTA or CaCl₂ according to the specifications, and then the proteins were resolved by SDS-PAGE and transferred to PVDF membranes, and blots were probed for the Flag tag and Rb.

A.4 Results

Identification of calmodulin-p55γ-Rb protein complex
First, three putative calmodulin binding domains were identified on the p55γ sequence using the search engine in the calmodulin target database website (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html), the binding domains are homologous with those found in the regulatory subunits p85α and p85β (Figure A. 1). Second, the association between endogenous human p55γ and calmodulin was confirmed in vitro. The AU565 human breast cancer cell line was the model system used previously by Dr. Hamburger [42;48], and was found to express moderate amounts of p55γ (Chapter 2). Calmodulin conjugated sepharose beads pulled down a 55 KDa protein which could be detected by a pan-p85 antibody in lysates of AU565 cells. The association between calmodulin and p55γ was also found to be calcium-dependent, as the amount of p55γ co-precipitated with calmodulin increased in the presence of calcium compared to that detected in similar experiments done in the presence of a calcium chelator EGTA (Figure A. 2). Next, calmodulin-sepharose pull-down assays performed on lysates from HEK293 cells transfected with Flag-p55 or control Flag-Sox9 showed that more Rb bound to the calmodulin beads in the presence of Flag-p55γ compared to control Flag-tagged protein; the bound Rb decreased in the presence of calcium, meanwhile, more Flag-p55γ was found associated with calmodulin. Control sepharose beads bound to neither Flag-p55γ nor Rb protein. The control unrelated Flag-tagged protein (Flag-Sox9) did not bind the calmodulin beads (Figure A. 3). These results indicated that the binding among calmodulin, p55γ, and Rb is very specific, that
p55γ might facilitate the binding between Rb and calmodulin, and that calcium might decrease the association between calmodulin and Rb.
Figure A.1 Calmodulin binding sites predicted for regulatory subunits including p55γ. Homology analysis on three predicted putative CaM binding sites of human p85α, p55γ, and p85β protein sequences identified using calmodulin target database (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html). The potential CaM binding sites are conserved among the regulatory subunits.
Figure A.2 Calmodulin specifically binds to endogenous p55γ in a calcium-dependent manner. Subconfluent AU565 cells were serum-starved for 24 h and cell lysates were collected. The lysates were incubated with 50 ml of calmodulin-sepharose beads in the absence (Lane 2), or in the presence (lane 3) of 0.1 mM CaCl2, or with 1mM EGTA plus 0.1mM CaCl2 (lane 4) for 2 h. Lysates incubated with non-conjugated sepharose were used as a negative control (lane 1). Proteins released from washed beads were resolved by SDS-PAGE and transferred to PVDF membranes. Blots were probed with anti-p85pan pAb, which recognizes the p85α, p85β, and p55γ subunits. Data are representative of two independent experimental determinations.
Calmodulin is a ubiquitous calcium sensing protein that is upstream of calmodulin kinases as well as a regulator of many cellular functions such as fatty-acid oxidation, autophagy, neuronal growth and migration, cell survival, etc [117].

![Figure A.3 Calmodulin is associated with overexpressed p55γ in a calcium-dependent manner.](image)

<table>
<thead>
<tr>
<th>CaM sepharose</th>
<th>Control sepharose</th>
<th>FlagSOX9</th>
<th>Flag55</th>
<th>1/50 input</th>
</tr>
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<tr>
<td>CaCl2</td>
<td>+</td>
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<td>EGTA</td>
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Bound proteins were detected with anti-Flag or anti-total Rb antibody. 1/50 of the input for pull-down assay was loaded indicating the specific size of the designated proteins.
A.5 Discussion and Conclusions

Calmodulin is a ubiquitous calcium sensing protein that is upstream of calmodulin kinases as well as a regulator of many cellular functions such as fatty-acid oxidation, autophagy, neuronal growth and migration, cell survival, etc [117]. Upon binding to calcium, a conformational change in calmodulin facilitates its binding to other proteins that do not have the ability to associate with calcium directly and utilize calmodulin as a sensor and signal transducer. The calmodulin bound sepharose beads are a convenient tool for in vitro screening of calmodulin binding partners.

Our data showed that Flag-p55γ associated with calmodulin in a calcium dependent manner, and the expression of Flag-p55γ seemed to enhance the binding between calmodulin and Rb. However, in the presence of calcium, when calmodulin and p55γ association was augmented, the association between calmodulin and Rb seemed slightly decreased. This result was interesting not only because it showed that the three proteins co-existed in the same protein complex, but also because it showed that the binding of the three proteins changed in presence of calcium, which indicates this protein complex might involve in signal transduction. Meanwhile, the sepharose pull-down assays and the western blot analysis do not pursuit a quantitative analysis of protein-protein interactions, more accurate quantitative analysis such as BIACORE is suggested to study the relative binding of these proteins in response to calcium signaling.

It is speculated that, when Rb is bound to calmodulin and p55γ, less phosphorylation sites are exposed because of spatial limitations, and Rb becomes less phosphorylated
and more actively bound with the E2F transcription factor, thereby inhibiting the cell cycle progression by regulating the gene expression for S phase entry [118]. Experiments in which physiological amounts of calcium were used to test the cells, resulted in a positive signal for cell cycle progression [119]. Possibly there was less Rb bound to calmodulin and p55γ, making more sites available to be phosphorylated, and less active in inhibiting the cell cycle, thus allowing the cell cycle to proceed to S phase. A role of p55γ as a binding platform for calmodulin and Rb interaction that may regulate their functions in response to calcium signaling becomes an attractive hypothesis, as it provides a potential regulatory mechanism of how the calcium signal is transduced to Rb and regulates cell cycle progression. However, additional experiments are required to identify the potential calmodulin binding site for p55γ.

Calmodulin and Ca^{2+}/calmodulin-binding proteins typically contain either basic amphiphilic alpha-helices [120] or IQ motifs. IQ motifs includes about 25 amino acid residues with the sequence IQXXXRGXXXR (X represents any amino acid) [121]. Rb contains an IQ motif starting from amino acid 731, which suggests a Ca^{2+}-independent calmodulin binding, and p55γ contains alpha-helices, according to the motif prediction based on the information from the calmodulin target database. Our results showed that p55γ, calmodulin and Rb form a protein complex in both transformed human cells and human breast cancer cells. Both the experimental data and the motif prediction indicated that the interaction between p55γ and calmodulin was calcium-dependent, while the Rb and calmodulin interaction was calcium-independent. Overexpression of
p55γ enhanced the interaction between Rb and calmodulin. This finding formed a link among three of the most intensively investigated processes in cell cycle and cell proliferation: Rb/E2F transcription regulation, PI3K/Akt pathway, and calcium/calmodulin signaling. The function of this protein complex might be promoting cell proliferation and cell cycle progression through transferring the signal between calmodulin and Rb, thus facilitating Rb phosphorylation and inactivation by calmodulin.

P55γ selectively binds hypophosphorylated Rb in MCF-7 cells. Stronger binding of active, hypophosphorylated Rb with p55γ was detected when MCF-7 cells were treated with Heregulin, a differentiation factor, compared to serum-starved, quiescent cells [48]. Addition of mitogens disrupts the interaction, and the time course of complete dissociation of the p55γ-Rb complex is consistent with the time course of Rb phosphorylation, suggesting that there is a mechanistic connection between the two events [48]. HEK293 contains hyperphosphorylated Rb, as predicted by viral E1A/E1B expression. However, this study also detected the calmodulin/p55γ/Rb complex in HEK293 cells. Based on our data, we suggest that the Rb phosphorylation status is not relevant for predicting the Rb/p55γ association, although the activated (hypophosphorylated) Rb may bind p55γ with higher affinity.

Discovery of a functional p55γ-calmodulin interaction that leads to Rb phosphorylation and cell cycle progression will identify p55γ as a protein that plays an important role in cell proliferation. The biological mechanisms underlying how p55γ
binds to calmodulin are important. Identification of specific binding domains may allow
the development of novel therapeutics that inhibits tumor cell progression by blocking
the interaction of p55γ and calmodulin for treating human breast cancer.
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