Mechanistic Analysis of Differential Signal Transduction Mediated by the Insulin Receptor Substrate Proteins IRS-1 and IRS-2: A Dissertation

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MECHANISTIC ANALYSIS OF DIFFERENTIAL SIGNAL TRANSDUCTION MEDIATED BY THE INSULIN RECEPTOR SUBSTRATE PROTEINS IRS-1 AND IRS-2

A Dissertation Presented

By

JUSTINE MARIE LANDIS

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

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AUGUST 11, 2014

CANCER BIOLOGY
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MEDIATED BY THE INSULIN RECEPTOR SUBSTRATE PROTEINS IRS-1 AND IRS-2

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Cancer Biology
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This work is dedicated to my parents. You have always whole-heartedly supported me and have been my biggest cheerleaders through it all.
I would first like to thank my mentor Dr. Leslie Shaw. She has pushed me to grow during my graduate career and I will forever be grateful for all that she has done for me. Through both good experiments and failed experiments, she always encouraged me to keep pushing the science forward and has always believed in me. I couldn’t have asked for a better mentor.

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Finally, to my family, I couldn’t have done it without you! Thank you Mom, Dad and Ryan for your love and constant support. You have always encouraged my veracious love of reading, learning and science. Life hasn’t always made it easy on us, but you always keep me focused on the silver lining.
The Insulin Receptor Substrate (IRS) proteins IRS-1 and IRS-2 are cytoplasmic adaptor proteins that organize and propagate intracellular signaling downstream of specific growth factor receptors, including the Insulin and Insulin-Like Growth Factor-1 Receptors (IR and IGF-1R, respectively). Despite sharing a high level of homology and the ability to stimulate Phosphotidylinositol-3-Kinase (PI3K) and Mitogen-Activated Protein Kinase (MAPK) signaling, IRS-1 and IRS-2 play distinct roles in mammary tumor progression. Specifically, IRS-1 promotes growth and proliferation, whereas IRS-2 promotes motility, invasion, survival, aerobic glycolysis, and metastasis. To further understand the differences between IRS-1 and IRS-2, I investigated the mechanistic basis of IRS-2-mediated PI3K activation. I identified tyrosines in IRS-2 that mediate its recruitment and activation of PI3K in response to insulin and IGF-1 stimulation. Using a PI3K-binding deficient IRS-2 mutant, I demonstrated that IRS-2-dependent PI3K signaling promotes aerobic glycolysis through its ability to selectively regulate the phosphorylation of the Akt effector Glycogen Synthase Kinase-3β (Gsk-3β). I also performed a rigorous comparison of IRS-1 and IRS-2 signal transduction and their ability to regulate functions associated with tumor progression. These studies required the generation of a novel model system where IRS-1 and IRS-2 function could be compared in a genetically identical background. Using this model, I confirmed a role for IRS-1 in growth regulation and IRS-2 in tumor cell invasion, as well as expanded the understanding of differential IRS protein function by showing that IRS-2 more
effectively promotes Akt activation. The model system I have established can be used for further characterization of IRS-1 and IRS-2-specific functions.
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LIST OF ABBREVIATIONS

APE - Akt-phosphorylation enhancer
APPL1 - Adaptor protein, Phosphotyrosine Interaction, PH domain and Leucine Zipper Containing 1
AS160 - Akt substrate of 160 kDa
ATP – Adenosine triphosphate
BSA – Bovine Serum Albumin
CMV – Cytomegalovirus
DMEM - Dulbecco's modified Eagle's medium
EGF – Epidermal Growth Factor
EGFR – Epidermal Growth Factor Receptor
EMT – Epithelial to Mesenchamal Transition
ER – Estrogen Receptor
ERK – Extracellular Signaling-Regulated Kinase
FACS – Fluorescence-Activated Cell Sorting
FBS – Fetal Bovine Serum
FOXO - Forkhead box O
GAP – GTPase Activating Protein
GEF – Guanine Nucleotide Exchange Factor
GFP – Green Fluorescent Protein
Glu – Glutamine
Glut- Glucose transporter
Grb2 – Growth factor receptor-bound protein 2
Grb10 - Growth factor receptor-bound protein 10
GSK-3 – Glyocogen Synthase Kinase
HA - Hemagglutinin
HER2 – Human Epidermal Growth Factor Receptor 2
His – Histidinol
HRP - Horseradish Peroxidase
Hsp90 – Heat Shock Protein 90
IACUC – Institutional Animal Care and Use Committee
IGF-1 – Insulin-Like Growth Factor-1
IGF-IR – Insulin-Like Growth Factor-1 Receptor
IGFBP – Insulin-Like Growth Factor Binding Protein
IL-4 – Interleukin-4
IR – Insulin Receptor
IRS – Insulin Receptor Substrate
iSH2 – inter-SH2
JIP1 – JNK-Interacting Protein-1
kDa – Kilodalton
KRLB – Kinase Regulatory Loop Binding
Lys - Lysine
MAPK – Mitogen Activated Protein Kinase
MEFs – Mouse Embryonic Fibroblasts
MMP – Matrix Metalloproteinase
MMTV – Mouse Mammary Tumor Virus
MOI – Multiplicity of Infection
mTORC1 – Mechanistic Target of Rapamycin Complex 1
mTORC2 – Mechanistic Target of Rapamycin Complex 2
NaCl – Sodium Chloride
NADH - Nicotinamide Adenine Dinucleotide (reduced form)
NCK – Non-Catalytic Region of Tyrosine Kinase Adaptor Protein
Neo - Neomycin
PBS – Phosphate Buffered Saline
PDK-1 – Phosphoinositide-Dependent Kinase
PDGFR – Platelet Derived Growth Factor Receptor
Pen/Strep – Penicillin/Streptomycin
PH – Plextrin Homology
PI3K – Phosphatidylinositol-3-Kinase
PKB – Protein Kinase B
PR – Progesterone Receptor
PtdIns – Phosphoinositide
PTB – Phosphotyrosine Binding
PyMT – Polyoma Middle T
PTEN – Phosphatase and Tensin Homolog
Puro - Puromycin
RPM – Revolutions Per Minute
RTK – Receptor Tyrosine Kinase
S6K – Ribosomal Protein S6 Kinase
Ser – Serine
SH2 – Src Homology-2
SHP-2 – Src-Homology 2 Domain Containing Protein Tyrosine Phosphatase 2
TCA - Tricarboxylic Acid Cycle
Thr – Threonine
TNBC – Triple Negative Breast Cancer
TSC – Tuberous Sclerosis
Tyr – Tyrosine
UMMS – University of Massachusetts Medical School
WT – Wild Type
PREFACE

The majority of the work presented in this dissertation represents my own work with the following exceptions:

In Chapter II, Anuradha Seshdri performed the western blots in Figure 2.1b (Irs-1^{fl/fl} only). Additionally, Yung Wang performed the cell growth, invasion and glycolysis assays and western blots in Figure 2.3.

In Chapter III, Jenny Janusis performed the western blots in Figure 3.6B

In Chapter IV, Jenny Janusis performed western blots that contributed to the quantification depicted in Figure 4.3. However, the depicted blots, quantification and statistical analysis are my own work. The 3D growth assays from Figure 4.5 were performed and photographed by Jenny Janusis. Measurement of colony size, colony number, statistical analysis, and interpretation of the data are my own work. Invasion assays from Figure 4.6 were also performed and quantified by Jenny Janusis, while I performed all statistical analysis and interpretation of the data.
CHAPTER I

Introduction
Breast Cancer: Progression to Metastatic Disease

In the United States, breast cancer is the second leading cause of cancer related death among women (1). In the past two decades, new treatment strategies for localized and regional disease have significantly improved 5-year survival rates for women to 99% and 84%, respectively (2). The same cannot be said for the treatment of metastatic breast cancer, where limited successful treatment options are available and the 5-year survival rate for women with metastatic disease is only 24%. The mechanisms that promote metastasis are still not fully defined and understanding them is key to the development of effective treatment strategies for metastatic breast cancer.

Tumor progression from initiation to dissemination and metastasis is a complicated, multistep process that requires adaptation of tumor cells (3). Many of these changes, the so-called “Hallmarks of Cancer,” are essential for cells to successfully disseminate (4). Following tumor initiation, transformed cells begin to rapidly grow and divide, evading the growth suppressors that would normally cause senescence or cell death. As cells proliferate, they begin to grow into the luminal regions of the ducts and lobules of the breast epithelium, forming carcinoma in situ (3). These cells are subjected to many different stresses including intermittent hypoxia and low nutrient supply due to the insufficient and disorganized vasculature network supporting them (5). These stresses select for those cells able to withstand such harsh growing conditions; a subset of these aggressive cells can eventually acquire the ability to invade through the basement membrane and intravasate into the vasculature (3). Cells then travel through the blood stream, where they extravasate from the capillary bed into the site of metastasis. To
successfully colonize a new organ, cells must be able to survive and grow in an environment very different from where they originated. Adaption to a changing microenvironment causes cells to become more aggressive and resistant to conventional therapies. Understanding how and why these changes occur are essential to effectively targeting metastatic breast cancer.

**IGF-1R Signaling in Breast Cancer**

Aberrant activation of growth factor signaling is a common event in breast cancer. Increased expression and activation of several growth factor receptors have been observed in primary tumors, including Human Epidermal Growth Factor Receptor 2 (HER2), Vascular Endothelial Growth Factor Receptor (VEGFR), Epidermal Growth Factor Receptor (EGFR) and Insulin Like Growth Factor-1 Receptor (IGF-1R) (6). Targeted therapies for each have been developed, with the drug Herceptin that targets HER2 being the most successful to date (7).

IGF-1R expression in breast tumors was first demonstrated over 25 years ago, and several subsequent studies since have implicated IGF-1 signaling in breast cancer (8,9). The IGF-1R is commonly overexpressed in breast cancer and IGF-1R tyrosine kinase activity is increased 40-fold in malignant cells relative to normal breast cells (9,10). High plasma IGF-1 levels in premenopausal women positively correlate with increased risk for developing breast cancer (11). Additionally, high levels of IGF-1R expression correlate with disease relapse following chemotherapy and radiation (12). Despite a clear
correlation between patient prognosis and elevated IGF-1 signaling, IGF-1R targeted therapies have had limited clinical success, presumably due to constitutive activation of signaling pathways downstream of the receptor (13). Fully understanding the mechanism of signal transduction downstream of the IGF-1R is important to finding novel ways to target this signaling pathway in cancer.

**IGF-1R Activation and Signal Transduction**

The IGF-1R is a ubiquitous growth factor receptor tyrosine kinase (RTK) that transmits intercellular signals in response to ligand binding to its extracellular domain (9). The IGF-1R is expressed in nearly all cell types, with the exception of T-lymphocytes and hepatocytes and \textit{Igf-1r} \text{−/−} mice have severe growth defects and die shortly after birth due to respiratory failure (9,14). In addition to its regulation of global organismal growth and development, the IGF-1R also promotes cellular proliferation, migration and survival (15).

Several factors determine IGF-1R activity and bioavailability. First, to generate the functionally mature IGF-1R, the immature IGF-1R pro-receptor polypeptide is proteolytically cleaved into 135-kDa $\alpha$ and 90-kDa $\beta$ subunits (9). Next, the $\alpha$ and $\beta$ subunits are joined by disulfide bonds to form the mature, dimeric IGF-1R. Insulin Like Growth Factor (IGF) -1 and IGF-2 bind with high affinity to the IGF-1R, while insulin can stimulate the receptor only at very high concentrations (16). During the post-translational processing of the IGF-1R in the endoplasmic reticulum, subunits of the
Insulin Receptor (IR), can be co-incorporated with IGF-1R subunits to form hybrid receptors that have high affinity for the IGFs as well as insulin (17,18). Additionally, IGF-IR activation is limited by Insulin Like Growth Factor Binding Proteins (IGFBP), which function to bind IGFs and sequester them from receptors, limiting their bioactivity (19). Therefore, many factors influence the activity of the IGF-1R, including hybrid receptor formation and the availability and concentration of ligands.

Transmission of signals downstream of the IGF-1R is a multi-step process that requires the kinase activity of the receptor as well as recruitment and activation of intracellular adaptor proteins and kinases. Following ligand binding to the extracellular α subunits, the signal is stereologically transmitted to the largely intracellular β subunits, activating the receptor’s tyrosine kinase activity (20,21). This leads to autophosphorylation in trans of tyrosines (Tyr) 1131, 1135 and 1136 in the kinase domain of the IGF-1R (20-22). Phosphorylation of all three of these tyrosines is required for signal transduction as they mediate the association of the IGF-1R with the Insulin Receptor Substrate (IRS) proteins (23). Mutation of these three tyrosines to phenylalanine (Phe) blocks their phosphorylation and abolishes IRS association. Additionally, mutation of Tyr950, present in an NPEY motif in the juxtamembrane region of the IGF-1R receptor, abolishes association of the IRS proteins and the adaptor Shc (23,24). Following their recruitment to the IGF-1R, the IRS proteins are subsequently phosphorylated by the receptor and recruit factors required for activation of Phosphotidylinositol-3-Kinase (PI3K) and Mitogen-Activated Protein Kinase (MAPK)
signaling. The recruitment and phosphorylation of these adaptor proteins following IGF-1R autophosphorylation is a key requirement for activation of downstream signaling.

IGF-1 Signaling and Breast Cancer Progression

The positive correlation between amplified IGF-1 signaling and breast cancer spurred studies of the specific outcomes of increased signaling downstream of the IGF-1R. Early work using mouse embryonic fibroblasts (MEFs) and NIH-3T3 cells done by Renato Beserga’s group clearly demonstrated that loss of IGF-1R expression blocks transformation of these cell types (25,26). Mutation of Tyr950 to Phe also inhibits the transforming ability of the IGF-1R through loss of IRS and Shc binding, highlighting the importance of adaptor proteins in mediating IGF-1R signaling and transformation (27). Similar studies using human breast cancer cell lines have confirmed the role of IGF-1R in transformation as well as implicating it in promoting tumor progression. Expression of a dominant negative IGF-1R in MDA-MB-435 breast carcinoma cells decreased adhesion, invasion, anchorage independent growth, and metastasis to the lungs, liver and lymph nodes following mammary fat pad orthotopic injection (28,29). Expression of a constitutively active IGF-1R in normal, immortalized breast epithelial MCF10A cells disrupts acini formation in matrigel, increases growth in soft agar and invasion and promotes tumor initiation in nude mice (30). These studies show that the IGF-1R is not only sufficient, but required to induce transformation, tumorigenesis and promote
metastasis. Furthermore, the recruitment of adaptor proteins is required for the transforming ability of the IGF-1R in breast cancer.

Of note, the functional outcomes of IGF-1 signaling are dependent on Estrogen Receptor (ER) status. IGF-1R stimulated mitogenesis requires ER expression, as MCF7 cells (ER+) respond to IGF-1 with growth, while MDA-MB-231 (ER-) do not (31). However, non-mitogenic functions, such as adhesion, motility, and invasion are increased in both ER+ and ER- breast cancer cell lines in response to IGF-1 stimulation (28,31,32). Therefore, hormone receptor status is an important factor in determining the functional outcome of IGF-1R activation and could affect the outcome of IGF-1R targeted therapies in specific breast cancer patients.

The IRS Proteins: Mediators of IGF-1 Signaling

The IRS proteins are cytoplasmic adaptor proteins that serve as a key link between the IGF-1R and activation of downstream signaling pathways. The IRS family of proteins has four members, IRS 1-4. IRS-1 and IRS-2 are ubiquitously expressed in both humans and rodents and are considered the primary regulators of mitogenesis and glucose metabolism in response to insulin (33). Irs-3 is expressed only in rodents and IRS-4 has a more limited expression pattern found only in the brain, kidney, thymus and liver in humans and rodents (34-36). The expression of IRS-1 and IRS-2 are hormonally regulated at the transcriptional level, where IRS-1 is an ER responsive gene and IRS-2 is a progesterone receptor (PR) responsive gene (37-39). These data have been confirmed
in breast carcinoma cells lines, as it has been shown that well differentiated, ER+ cell lines predominantly express IRS-1 but less differentiated, ER- cell lines predominantly express IRS-2 (40). In addition to hormonal regulation, IRS-2 has also been shown to be a hypoxia responsive gene as its mRNA and protein expression are both increased under hypoxic conditions (41). Importantly, loss of ER and/or PR expression, as well as increased hypoxia, are common events during breast cancer progression. Therefore, it is important to consider their effects on IRS expression and how this may serve to limit or promote tumor progression.

The IRS proteins were discovered in studies of the events following insulin stimulation. Using phosphotyrosine-specific antibodies, a 185-kDa protein was shown to be phosphorylated in hepatoma cells within seconds of insulin stimulation and termed IRS-1 (42,43). Irs-2 was subsequently discovered as an alternative substrate of the IR in the liver and muscle of Irs-1−/− mice in response to insulin stimulation (44-46). Subsequent studies determined that IRS-1 and IRS-2 also serve as substrates for the similar IGF-1R as well as the interleukin-4 (IL-4) receptor (46-50). Specific integrin adhesion receptors have also been shown to use the IRS proteins as intermediates to regulate intracellular signal transduction (51-53). Limited studies have also implicated the IRS proteins downstream of the VEGF, EGF, growth hormone, and prolactin receptors (54-58). These discoveries show that the IRS proteins serve as adaptors for multiple classes of cell surface receptors, many of which are dysregulated in cancer.

Unlike many proteins involved in signal transduction, the IRS proteins do not contain any intrinsic enzymatic activity and instead function as adaptor proteins
following their phosphorylation by upstream receptor and non-receptor kinases (43,59). Recruitment and receptor association are mediated through interactions of the plextrin homology (PH) and phosphotyrosine binding (PTB) domains of the IRS proteins with phosphorylated tyrosine residues in the cytoplasmic regions of the IR and IGF-1R (24,49,60,61) (Figure 1.1A). IRS-2 contains a unique region encoded by amino acids 591-786 termed the Kinase Regulatory Loop Binding (KRLB) domain that regulates its association with the IR (62) (Figure 1.1A). Binding of this region to the IR is thought to decrease the receptor’s kinase activity and limit tyrosine phosphorylation of IRS-2, as mutation Tyr628 in the KRLB region of IRS-2 increases kinase activity of the receptor (63,64). Once recruited to the receptor, phosphorylation of the C-terminal region of the IRS proteins creates docking sites for downstream effectors including PI3K, Grb-2, SHP-2, Fyn, c-Crk, CrkII and Nck (65-70). IGF-1R signaling mediated by the IRS protein is diagramed in Figure 1.1B. In regard to PI3K activation by the IRS proteins, phosphorylation of tyrosines in YxxM motifs leads to PI3K recruitment through association of src homology 2 (SH2) domains of the regulatory subunit, p85, leading to activation of PI3K signaling (71-73). Using a proteomics phosphopeptide screen, additional proteins were shown to be capable of interacting with IRS-1 and IRS-2 in a phosphorylation dependent manner (74). Many of these proteins were similar between IRS-1 and IRS-2; however, there were several additional proteins that were mutually exclusive to either IRS-1 or IRS-2 (74). Additional studies are required to determine if interaction of these proteins and IRS-1 or IRS-2 occurs in intact cells.
Figure 1.1. Insulin Receptor Substrate-Mediated Signal Transduction.

A.

**IRS-1**

PH  PTB  PI3K  Grb2  SHP2

**IRS-2**

PH  PTB  KRLB  PI3K  Grb2  SHP2

B.
Figure 1.1. Insulin Receptor Substrate-Mediated Signal Transduction. (A) Schematic of IRS-1 and IRS-2 domains (PH, PTB and KRLB) and effector binding regions (PI3K, Grb2, and SHP2). (B) Schematic of IGF-1 stimulated signal transduction mediated by the IRS proteins.
IRS-mediated signal transduction is also controlled by serine phosphorylation in addition to the regulation by tyrosine phosphorylation described above (75). Serine phosphorylation of IRS-1 has been extensively studied, while less information is currently available pertaining to IRS-2 serine phosphorylation (75,76). Phosphorylation of IRS-1 on Ser302 and Ser307 disrupts the interaction between the PTB domain and upstream receptors, limiting both phosphorylation of IRS-1 as well as activation of downstream signaling pathways (77-79). Additionally phosphorylation of IRS-1 on Ser662 and Ser731 limits the association of IRS-1 with PI3K and decreases Akt activation (80). Ser662 and Ser731 are adjacent to YxxM motifs, and it is thought that their phosphorylation functions to limit tyrosine phosphorylation of IRS-1 (80).

Many, but not all, tyrosine and serine residues are conserved between IRS-1 and IRS-2 and the differences may confer different functional outcomes (81). Using quantitative mass spectrometry, phosphorylation of tyrosine residues in YxxM motifs of both IRS-1 and IRS-2 were observed over a time course of insulin stimulation (82). The role of Tyr612 and Tyr632 in IRS-1 were further investigated and were shown to be required for PI3K activation in response to insulin (83). However, the role of specific of tyrosine residues in response to IGF-1 stimulation has yet to be addressed. Additionally, no studies have assessed the contribution of tyrosine residues in YxxM motifs of IRS-2 and are required to truly be able to compare the functions of IRS-1 or IRS-2-mediated PI3K activation.
IRS-1 and IRS-2 Specific Functions

Despite similar structure and ability to activate many of the same signaling pathways, IRS-1 and IRS-2 do not function interchangeably. Irs-1\(^{-/-}\) mice are born runted and display insulin resistance but do not develop diabetes (84,85). Irs-2\(^{-/-}\) mice are infertile, have impaired brain development and ultimately develop diabetes due to β-cell failure combined with insulin resistance (86,87). These studies implicate both Irs-1 and Irs-2 in regulation of whole body glucose homeostasis as loss of either results in insulin resistance. However, Irs-1 plays an important role during developmental growth, while Irs-2 is involved in brain development and survival of the endocrine compartment of the pancreas. These data imply there are some similar functions of the Irs proteins, however Irs-1 and Irs-2 are not redundant.

In vitro studies using cell lines derived from Irs-1\(^{-/-}\) and Irs-2\(^{-/-}\) mice confirm the non-redundant roles of the Irs proteins and additionally indicate that Irs functions are often cell context dependent. In Irs-1\(^{-/-}\) MEFs, IGF-1 dependent mitogenesis is impaired but Irs-2 expression is sufficient to maintain wild type (WT) levels of PI3K activation (88). In response to insulin stimulation, knockdown of Irs-1 expression in L6 myotubes decreases Akt1 activation, actin remodeling and Glucose Transporter-4 (Glut4) translocation to the plasma membrane, while knockdown of Irs-2 expression decreases Akt2 and Erk activation (89). In response to either insulin or IGF-1 stimulation, Irs-1, but not Irs-2, associates with Grb2 and activates MAPK-dependent proliferation in fetal brown adipocytes, while both Irs-1 and Irs-2 can promote PI3K-dependent differentiation.
in this cell type (90). Irs-2 is the primary mediator of insulin-stimulated glucose uptake in adipocytes by increasing Glut4 translocation to the plasma membrane and Irs-1 overexpression cannot rescue this function (91). These data suggest that cell context can mediate some of the differential functions attributed to either Irs-1 or Irs-2 activation.

Some of the differences between Irs-1 and Irs-2 have also been attributed to the relative differences in PI3K activation mediated by the IRS proteins. For example, in keratinocytes, Irs-1 displays higher PI3K association relative to Irs-2 and stimulates PI3K-dependent glucose uptake following insulin stimulation (92). In contrast, Irs-2 serves this function in fibroblasts. The mechanisms that underlie the differences of PI3K activation by either Irs-1 or Irs-2 in a cell context dependent manner remain to be determined.

**The Role of the IRS Proteins in Breast Cancer**

The established role of the IGF-1R in breast cancer and the importance of the IRS proteins in mediating IGF-1R signaling prompted studies on the function of the IRS proteins in tumor initiation and metastasis. Like the IGF-1R, IRS-1 is commonly overexpressed and constitutively tyrosine phosphorylated in many types of cancer, including of the breast (93,94). Localized breast tumors express high levels of IRS-1, whereas more invasive tumors have high IRS-2 expression (95). More recent studies of the IRS proteins have suggested that the subcellular localization of the IRS proteins may be a more predictive marker for patient prognosis versus expression status alone. In
human breast carcinoma cells, both IRS-1 and IRS-2 localize to the cytoplasm, but only IRS-1 localizes to the nucleus and IRS-2 localizes to the plasma membrane (96,97). Nuclear localization of IRS-1 in human tumors correlates with increased survival in response to tamoxifen treatment, while IRS-2 membrane localization correlates with decreased overall survival (96,98). Therefore, in addition to expression status, subcellular localization of the IRS proteins is a key determinant in the functional outcome of their expression in breast cancer.

*In vitro* studies of the IRS proteins have also defined their differential roles in tumor initiation and progression. Overexpression of IRS-1 or IRS-2 in non-transformed MCF10A human mammary epithelial cells leads to increased IGF-1-dependent Akt activation and disrupts normal acinus formation in 3D matrigel (99). In response to IGF-1 stimulation, IRS-1 is required for PI3K- and MAPK-mediated mitogenesis in ER+ breast cancer cell lines (100-102). However, overexpression of IRS-1 in two ER- breast cancer cell lines was not sufficient to stimulate IGF-1 dependent mitogenesis (40). In contrast to IRS-1, IGF-1 signaling mediated by IRS-2 promotes adhesion and motility regardless of ER status of the cell line used (102,103). These data implicate differential functions of the IRS proteins in discrete stages of tumor initiation and progression, and some of these functional outcomes are dependent on hormone receptor expression status.

Irs mouse models have also contributed to the understanding of the different roles that the Irs proteins play in breast cancer. Transgenic overexpression of IRS-1 or IRS-2 in the mammary glands of mice induces mammary hyperplasia, tumorigenesis and pulmonary metastasis (99). Both IRS-1 and IRS-2 overexpressing tumors display higher
levels of PI3K and Extracellular Signaling-Regulated Kinase (Erk) activity relative to matched normal mammary gland tissue. Another model to study breast cancer drives Polyoma Middle T expression with the mouse mammary tumor virus promoter (MMTV-PyMT), which induces tumorigeneis specifically in the mammary glands of mice and these tumors are robustly metastatic (104). Despite no differences in overall tumor growth in PyMT:Irs-1−/− or PyMT:Irs-2−/− mice relative to PyMT:WT mice, decreased pulmonary metastasis is observed in Irs-2−/− mice (105,106). In contrast, Irs-1−/− tumors express elevated levels of Irs-2, are significantly more metastatic and display increased angiogenesis relative to their WT counterparts (105,107). Upon further investigation of the Irs-1−/− tumors, an Irs-2-dependent increase in PI3K/Akt signaling was observed. Cell lines derived from these tumors are more invasive and have increased survival and aerobic glycolysis relative to WT tumor derived cells (107,108). The opposite is true of Irs-2−/− tumor derived cells, which are less invasive, more sensitive to apoptotic stimuli and have decreased aerobic glycolysis (106,108). Using these tumor derived cell lines, it was determined that Irs-2 positively regulates aerobic glycolysis through a mechanistic target of rapamycin complex-1 (mTorc1)-dependent increase in Glut1 surface expression and glucose uptake (108). Together, these studies highlight the differences between the IRS proteins and implicate IRS-2-dependent PI3K signaling in multiple functions associated with metastasis in breast cancer.
PI3K Signaling

The link between IRS2, PI3K signaling, and breast cancer is not surprising, given that the PI3K signaling pathway is one of the major pathways activated in response to RTK signaling. The lipid kinase activity of PI3K was first described in the early 1950’s when it was shown that radiolabeled phosphates could be incorporated into phospholipids following acetylcholine stimulation (109). Phospholipids were further linked to signal transduction in studies showing that the break down of the phospholipid phosphoinositide (PtdIns) was linked to membrane-associated receptors known to regulate intracellular calcium trafficking (110). Pioneering studies in the 1980’s lead to the discovery of a new form of lipid kinase activity directed at the 3-OH position of the inositol head ring. In transformed cells, this lipid kinase activity was discovered following immunoprecipitation of pp60v-src and PyMT (111,112). It was initially thought that the product of the reaction was PtdIns-4-P, however thin layer chromatography experiments in Lewis Cantley’s lab clearly demonstrated that the lipid product was actually PtdIns-3-P, ushering in the field of PI3K signaling (113). In addition to its association with viral oncogenes, PI3K activity was shown to co-immunoprecipitate with the platelet derived growth factor receptor (PDGFR) and the IR following ligand stimulation (112,114-116). Further study determined that PtdIns-3-P levels remain constant in cells, while there is a rapid accumulation of PtdIns-3,4,5-P3 following stimulation with growth factors such as platelet derived growth factor (PDGF) or G-protein coupled receptor (GPCR) agonists (117,118). Transfection of the IR into Chinese hamster ovary cells also results in
increased PtdIns-3,4,5-P$_3$ production (116). From these studies, it was concluded that PtdIns-3,4,5-P$_3$ is the true second messenger generated by PI3K that leads to amplification of intracellular signaling.

While there are three distinct classes of PI3K (Classes I-III), all with kinase activity directed at the 3-OH position of PtdIns, their structure and substrate specificity set them apart. Class I PI3K is the only class that can generate the active second messenger PtdIns-3,4,5-P$_3$ from PtdIns-4,5-P$_2$ (113,117,119). Class I PI3K is further divided into two classes; Class IA activated in response to RTKs and Class IB activated by GPCRs (120). Because of the role of RTKs in breast cancer formation and metastasis, further discussion of PI3K in this dissertation will focus on Class IA PI3K and its roles in amplification of RTK signaling.

Class IA PI3K is a heterodimer comprised of a regulatory subunit and a catalytic subunit (121). The regulatory subunit exists as 5 isoforms (p85$\alpha$, p55$\alpha$, p50$\alpha$, p85$\beta$, and p55$\gamma$) where p85$\alpha$, p55$\alpha$ and p50$\alpha$ are encoded by $PIK3R1$ while p85$\beta$ and p55$\gamma$ are encoded by $PI3KR2$ and $PI3KR3$, respectively (120). Structurally, p85 contains two SH2 domains in the N and C-termini connected by an inter-SH (i-SH2) domain region that binds to the catalytic subunit. The catalytic subunit also exists as three isoforms (p110$\alpha$, p110$\beta$ and p110$\delta$) each of which are encoded by distinct genes $PIK3CA$, $PIK3CB$ and $PIK3CD$, respectively. The p110 isoforms are structurally similar, each containing an N-terminal p85-binding domain, a Ras binding domain, a PIK (Phosphoinositide-3-kinase family) domain, a C2 domain and a C-terminal catalytic domain. Under basal conditions, the association of p85 with p110 stabilizes p110 and inhibits its lipid kinase activity
Following RTK activation, the SH2 domains of the p85 regulatory subunit bind to phosphorylated tyrosine residues within YxxM motifs of upstream receptors and adaptor proteins, inducing a conformational change that is transmitted to the p110 subunit, releasing inhibition of p110 and rendering the kinase active (122-126). This inhibition of p110 is mediated by contacts of its N-terminal p85 binding domain with the i-SH2 of p85 (124). For maximal PI3K activity, both SH2 domains of p85 must be associated with phosphotyrosine residues as mutation of one SH2 domain impairs PI3K activity by 50% and mutation of both abolishes binding to IRS-1 and activation of PI3K following insulin stimulation (125,126). This mechanism of activation, the disinhibition of the catalytic subunit through the binding of SH2 domains of the regulatory subunit to pYxxM motifs, is similar across all PI3K Class 1A isoforms.

While binding of the SH2 domains of PI3K to p-YxxM motifs is required for activation, different classes of proteins serve as binding partners for p85. PI3K is activated in response to ligand stimulation of many RTKs, including the IR and IGF-1R (116,127,128). Downstream of the IR and IGF-1R, activation of PI3K is mediated by pYxxM motifs in the IRS proteins and loss of IRS expression abolishes PI3K activation following insulin or IGF-1 stimulation (129,130). However, downstream of other receptors, like the PDGFR, PI3K is directly activated by association with pYxxM motifs within the receptor and does not require an adaptor protein (72,123). Therefore, depending on the RTK, the mechanism of PI3K activation may require recruitment of the IRS proteins or other adaptor proteins.
The mechanism underlying the intracellular functional responses following generation of PtdInsP-3,4,5-P_3 by PI3K remained in question for many years. However, the discovery that PH domains associate with PtdInsP-3,4,5-P_3 illuminated the mechanism of PI3K mediated signal transduction (131). The first PI3K effector identified was Akt (also called Protein Kinase B (PKB)), which is recruited to PtdIns-3,4,5-P_3 through its PH domain (132-134). PI3K activation of Akt leads to increased survival, growth, proliferation, glucose metabolism, transcription, and protein translation (135). In addition to Akt, the generation of PtdIns-3,4,5-P_3 by PI3K also leads to the activation of Rac signaling through activation of Rac guanine-nucleotide exchange factors (Rac-GEFs) (136,137). Activation of Rac-GEFs increases the rate of exchange of GDP to GTP in Rac's nucleotide binding site increasing Rac activation (138). Rac signaling regulates cell-to-cell contacts, actin cytoskeleton rearrangement, cell adhesion, transcription, translation, survival and entry into the cell cycle (139,140). Through Akt and Rac-GEF activity, PI3K signaling is able to regulate many cellular functions including cell growth and division, survival, protein synthesis, motility, and cell polarity (120).

One of the specific metabolic pathways dependent on PI3K is the regulation of glucose metabolism, and type-2-diabetes is associated with perturbations in this signaling pathway (120). In response to insulin, PI3K stimulates glucose uptake and Glut4 translocation to the plasma membrane, as these effects are lost when PI3K is inhibited using LY294002 or wortmannin (141). Loss of all three isoforms of p85 in insulin-responsive tissues such as the liver and muscle results in an impaired insulin response
Specifically in the liver, loss of p85 results in hyperinsulinaemia and hyperglycemia, while in the muscle it results in decreased glucose uptake as well as whole-body glucose intolerance. Conversely, loss of Phosphatase and Tensin Homolog (PTEN), the phosphatase that antagonizes the actions of PI3K, results in increased insulin sensitivity in the liver, fat and muscle (144). As with other signaling molecules, the effects of PI3K can vary between tissue and cell types. However, perturbed glucose responses are one of the key results of impaired PI3K signaling activity, which occurs in several forms of cancer and promotes tumor growth.

**PI3K Signaling and Cancer**

In addition to its crucial role in normal cell biology, PI3K is considered an oncogene and is mutated in many types of cancer including colon, lung, brain, gastric, and breast (145). Activating mutations in the p110\(\alpha\) catalytic subunit of PI3K (PIK3CA) occur in roughly 25% of all breast cancers and are localized to two “hot spots” in exon 9 and exon 20 which encode the helical and catalytic domains respectively (146). These gain-of-function mutations, E542K, E545K and H1047R, lead to increased PI3K activity and constitutive activation of downstream targets such as Akt, Ribosomal Protein S6 Kinase (S6K), Glycogen Synthase Kinase-3 (GSK-3) and Eukaryotic translation initiation factor 4E-Binding Protein (4EBP) (147,148). E545K and H1047R are considered oncogenic as they induce anchorage-independent growth, growth factor-independent growth and protect against anoikis in MCF-10A cells; they also induce transformation of
both chicken embryo fibroblasts, mammary epithelial cells and human colon cancer cell lines (147-149). The catalytic antagonist of PI3K is the phosphatase PTEN, which is frequently lost in tumors that do not harbor outright PIK3CA mutations (150). The phosphatase activity of PTEN dephosphorylates the 3-OH phosphorylation of PtdIns-3,4,5-P₃, reverting it back to PtdIns-4,5-P₂. Loss of PTEN leads to accumulation of PtdIns 3,4,5-P₃ and aberrant activation of PI3K/Akt signaling (151). Germline mutations of PTEN result in Cowden’s Syndrome and predispose patients to developing breast and thyroid cancers (152). Interestingly, mutations in PIK3CA and PTEN are not mutually exclusive and can coexist in the same tumor suggesting that their alteration may not lead to the same functional outcomes (153,154). In addition, there are other mechanisms that alter PI3K signaling in tumors exclusive of PI3K and PTEN genetic mutations.

PI3K signaling can also be amplified indirectly by constitutive RTK activity. IGF-1R signaling is commonly amplified in breast cancer and leads to increased PI3K activity (9). Another common event leading to increased PI3K signaling is HER2 amplification (155). HER2 is a receptor tyrosine kinase in the EGFR receptor family that can activate PI3K signaling and is overexpressed or amplified in ~25% of all breast cancers (156,157). Interestingly, PIK3CA mutation and PTEN loss are observed following development of resistance to HER2 targeted therapies, including trastuzumab and lapatinib (158,159). Thus, it appears that tumors resulting from aberrant PI3K activation will evolve new mechanisms to sustain pathway activation in response to targeted inhibition of the pathway, suggesting that active PI3K signaling is required for tumor maintenance.
Multiple groups have reported the occurrence of mutations in the PI3K pathway and their association with breast cancer subtype, however some of the results are contradictory. PIK3CA mutations have been shown to be associated with ER/PR+ tumors, while PTEN loss is associated with ER/PR- tumors (160,161). The relationship between PIK3CA mutations and HER2+ breast cancer remains controversial, with some reports of a correlation of PIK3CA mutation and HER2 amplification and others with no correlation (160-162). However, triple negative breast cancers (TNBC, ER-/PR-/HER-) infrequently show PIKCA and PTEN perturbation suggesting that these tumors do not rely on PIKCA or PTEN perturbations for amplification of PI3K signaling (159). Due to the overlapping nature of PIK3CA, PTEN and HER2 perturbations in breast cancer, it is clear that despite functioning in the same signal transduction pathway, these nodes of PI3K signaling are unique and should be viewed as such.

**PI3K Signaling: Akt**

Akt was the first PI3K effector to be described and it is a central player in PI3K signaling downstream of RTKs (133,134). Akt shares considerable homology with protein kinases A, G and C and therefore was classified as a member of the AGC kinase family (132,135). Akt is rapidly activated in response to growth factor stimulation and this activation is dependent on PI3K, as either PI3K inhibitors or overexpression of dominant negative PI3K block Akt activation (134). Additionally, expression of a constitutively active, membrane-targeted version of PI3K promotes Akt activation
Studies using $^{32}$P-labelled phosphates determined that Akt phosphorylation in response to insulin or IGF-1 stimulation occurs on residues Ser473 and Thr308 (165). Phosphorylation of both Ser473 and Thr308 synergize and are required for maximal activity. However, mutation of one site does not affect phosphorylation of the other site. Together these data indicate that Akt phosphorylation is a key step in its activation in response to insulin or IGF-1 stimulation.

The PH domain of Akt plays a critical role in its activation in response to PI3K signaling. Following growth factor stimulation, Akt is recruited to the plasma membrane through association of its PH domain with PtdIns 3,4,5-P$_3$ and treatment with PI3K inhibitors blocks its translocation (166). Plasma membrane targeting of Akt by tagging its N-terminus with the myristoylation/palmitylation motif of Lck tyrosine kinase leads to constitutive phosphorylation and activation of Akt, which cannot be inhibited by expression of a dominant negative PI3K. This result suggests that the targeting of Akt to the membrane is sufficient for its activation. However, phosphorylation of Akt is the primary mechanism leading to activation of this kinase, as deletion of the PH domain does not impact kinase activity, providing that Akt is constitutively targeted to the plasma membrane (167). Membrane localization of Akt leads to Phosphoinositide-Dependent Kinase-1 (PDK-1) phosphorylation of Thr308, thus activating the kinase (168). Akt is also phosphorylated on Ser473 by mechanistic target of rapamycin complex 2 (mTORC2), leading to maximal kinase activation (169). Following these phosphorylation events, Akt is fully functional to promote signal transduction.
Following its activation at the plasma membrane, Akt localizes to the cytoplasm and nucleus where it phosphorylates many downstream effectors, amplifying RTK signaling (170). GSK-3 was the first identified, bona-fide Akt substrate (171). Peptides generated around the Akt phosphorylation site in GSK-3 ultimately lead to the discovery of the minimal recognition motif of Akt’s kinase activity (R-X-R-X-X-S/T-B) (172). This motif is distinct from other AGC kinases such S6K1, which can tolerate K substitutions at the -5 and -3 positions, distinguishing them from Akt. Since the discovery of GSK-3 as an Akt effector, many more Akt substrates have been identified and Akt signaling has been shown to regulate many cellular functions including cell growth, proliferation, survival, metabolism, motility, invasion, and angiogenesis (135,173). These functions are often regulated by Akt-dependent phosphorylation of more than one downstream effector; glucose metabolism, for example, requires phosphorylation of the Akt substrates GSK-3, FOXO, TSC2, PRAS40, and AS160 (135). In addition, phosphorylation of GSK-3 and FOXO by Akt also influence proliferation and survival. Thus, Akt signaling does not occur in a discrete pathway and instead branches at many places, eliciting numerous effects.

The substrate specificity of Akt can be modulated depending on the phosphorylation state of Akt and through its protein-protein interactions. For example, Ser473 phosphorylation is required for the N-terminal phosphorylation of FOXO proteins but it is not required for Akt phosphorylation of other FOXO sites, GSK-3 or TSC2 (169,174). Interestingly, Ser473 phosphorylation is not required for response to growth factor stimulation but does increase Akt kinase activity 5-10 fold (165). In addition to
the phosphorylation state of Akt, protein-binding partners may also direct its substrate specificity. Proteins that have been shown to interact with Akt, some of which have been shown to regulate its activity, include Hsp90, JIP1, Grb10, and APE (175-178). Therefore, it is important to keep cell context in mind, as Akt interacting proteins may be differentially expressed and likely influence the outcome of Akt activation. One particular Akt interacting protein is Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing-1 (APPL1), which promotes phosphorylation of GSK-3β, but not TSC2 (179-183). The proposed mechanism for this APPL1-mediated substrate specificity is the partitioning of Akt activity to specific endosomes containing GSK-3β, but not TSC2. Thus, subcellular localization is an additional factor that influences Akt activation.

It is well known that Akt translocates to both the cytoplasm and nucleus following its activation at the plasma membrane and many studies have confirmed that Akt is active in the nucleus (184). The use of fluorescent Akt reporters has shown that Akt activity in the nucleus occurs more slowly but is sustained relative to its activity in the cytoplasm (170). The larger question of whether nuclear Akt is phosphorylated before import into the nucleus still remains in question, however the factors required for Akt activation (PI3K, PtdIns 4,5-P2, PtdIns 3,4,5-P3, PDK-1, and mTORC2) have been shown to be present in the nucleus (185-190). Based on these studies, subcellular localization of Akt may regulate the effectors that Akt has access to, but further studies are required to fully understand this regulation of Akt substrate specificity.
Akt Isoforms

Akt is present as three isoforms, Akt1-3, which share 80% sequence and structural homology despite being encoded by distinct genes (191). Each isoform contains a C-terminal PH domain, a catalytic domain and a N-terminal hydrophobic motif (192,193). Initial studies of the Akt isoforms suggested they functioned redundantly, however it is now clear they have non-overlapping functions. Akt1−/− mice are born runted, Akt2−/− mice develop diabetes and Akt3−/− mice have developmental defects of the brain (194-197). Interestingly, the Akt isoforms have distinct subcellular localization patterns. Akt1 is present in the cytoplasm, Akt2 in the mitochondria and Akt3 in the nucleus; importantly, knockdown of one isoform does not impact localization of the other two isoforms (198). These data suggest that each Akt isoform has specific functions, which may in part be mediated by subcellular localization.

The most well defined Akt isoform-specific function is the regulation of glucose homeostasis by Akt2. Loss of Akt2 expression, but not Akt1 or Akt3, results in glucose intolerance and decreased glucose uptake by the muscle and fat in response to insulin (196,199). This is due to Akt2-dependent regulation of Glut4 translocation to the plasma membrane in response to insulin and cannot be rescued by overexpression of Akt1 (200-202). Glut4 translocation to the plasma membrane is facilitated by the phosphorylation and inhibition of the Rab-GTPase Activating Protein (GAP) AS160 by Akt2 (203-205). Currently, there are a limited number of Akt isoform specific reagents available and development of superior reagents is a requirement to rigorously understand the specific
roles of each Akt isoform. New conditional and tissue specific knockout mouse models of individual Akt isoforms will also be helpful in determining isoform specific contributions.

**Akt and Breast Cancer**

Just as PI3K activity is amplified in human cancer, Akt signaling has been shown to be hyperactivated in brain, breast, prostate, and ovarian tumors (206). Unlike PI3K, few somatic mutations have been described for Akt. The best characterized is a gain of function mutation of Glu17Lys in the PH domain of Akt1, leading to constitutive activity of Akt1 through its targeting to the plasma membrane (207). This particular mutant occurs in roughly 4-8% of breast cancer patients. Amplification of Akt2 expression has been shown in only 3% of breast cancer patients and currently no studies have shown amplification of Akt1 expression in breast cancer (208). However, increased Akt kinase activity is commonly observed in many different types of cancer and often predicts poor prognosis in patients. In one study, Akt1 kinase activity was increased in 40% of breast tumors (206). Akt3 kinase activity has also been shown to be upregulated in ER- breast cancer (209). In addition, patients with tumors that scored positive for Akt phosphorylation were more likely to relapse and have distant metastases (210). Together these studies demonstrate the importance of increased Akt kinase activity in breast cancer, but suggest that Akt depends on upstream factors to promote its hyperactivity.
Studies of Akt isoform-specific effects in breast cancer have focused mainly on Akt1 and Akt2. Initial studies of the isoforms used overexpression of constitutively active Akt isoforms, leading to some controversy over the specific roles of Akt1 and Akt2 (173). However, more recent knockdown studies have more consistently described the roles of these isoforms. In general, in vitro studies show that Akt1 inhibits migration, invasion, and the epithelial-to-mesenchymal transition (EMT), while Akt2 promotes these functions (211-214). The mechanism of Akt1-mediated inhibition of migration has been well characterized by Alex Toker’s group, who has shown that the actin bundling protein palladin is specifically inhibited by Akt1 leading to decreased invasion of human breast cancer cell lines (215). Studies using invasive variants of human breast cancer cell lines determined that Akt2 expression was upregulated in a Twist-dependent manner and that knockdown of Akt2 reduced Twist-regulated migration and invasion (216). Together these studies demonstrate that Akt1 and Akt2 do not function interchangeably to promote breast cancer progression.

The use of mouse models has confirmed results from in vitro studies on the isoform-specific roles of Akt in tumor formation and growth. In HER2-driven mouse mammary tumors, mammary specific expression of a constitutively active Akt1 confirmed a negative regulatory role of Akt1 in migration and metastasis in breast cancer (217). Other in vivo studies have also suggested that Akt1 functions to limit invasion and metastasis, while Akt2 promotes these functions (214,218). Additionally, Akt1 expression has been shown to accelerate tumor formation and growth, while Akt2 expression inhibits these functions (218). However, one group has reported that Akt1
can promote invasion and metastasis (219). The studies of Akt1 loss were done on different genetic backgrounds with total body knockout of Akt1, which may explain the different observations between studies. Little is known about Akt3 specific functions in breast cancer and further study of all three isoforms is required to fully understand their specific functions in breast cancer progression. However, studies do clearly implicate Akt signaling in many functions associated with tumor progression, including the regulation of tumor cell metabolism.

**Tumor Metabolism: Effects on the Microenvironment and Tumor Progression**

In the early 1920’s, Otto Warburg first noted that cancer cells consume glucose at a higher rate relative to normal cells (220,221). He observed that cancer cells preferentially metabolize glucose using glycolysis, not oxidative phosphorylation, even in the presence of oxygen. This phenomenon as been termed the Warburg Effect. Warburg proposed that this metabolic switch was due to defective mitochondrial metabolism in cancer cells, which has since been shown to be false (5,222). However, the principles of the Warburg effect have been rigorously confirmed and are now considered one of the “Hallmarks of Cancer” (4).

Under normoxic conditions, in normal, non-proliferating cells, glucose is converted to pyruvate and then shuttled into the mitochondria (223). There it is metabolized through the Tricarboxylic Acid Cycle (TCA) cycle, generating NADH that can be used to generate ATP during oxidative phosphorylation. However, under
conditions where oxygen is limiting, cells switch to anaerobic glycolysis and convert pyruvate into lactate to generate ATP. In comparison to oxidative phosphorylation, aerobic glycolysis is an inefficient way to generate ATP, generating 36 versus 2 molecules of ATP respectively (5). Interestingly, following Warburg’s discovery in cancer cells, others noted that normal, rapidly proliferating cells such as lymphocytes and thymocytes also preferentially use aerobic glycolysis (224-227). It was clear that both normal rapidly proliferating cells and cancer cells use aerobic glycolysis but the reasons behind this metabolic shift were still unclear.

Why rapidly proliferating normal and cancer cells preferentially use glycolysis to metabolize glucose remained a biological conundrum for many years. Studies have since shown that glycolysis proceeds faster than oxidative phosphorylation, rapidly producing ATP when glucose is not limiting (228,229). Aerobic glycolysis not only supports increased cell growth and proliferation through ATP generation, but also supplies cells with biomass required for their proliferation (223). Cell division is a huge metabolic challenge for a cell and requires the synthesis of nucleotides, amino acids and lipids. Aerobic glycolysis meets these metabolic requirements by shuttling metabolic intermediates into biosynthetic pathways. To maintain high glycolytic flux to sustain ATP and biomass generation, cells must convert pyruvate into lactate using lactate dehydrogenase, which is then secreted (230). This explains why tumor cells secrete high levels of lactate, which at first glance seems wasteful, but is actually required to sustain flux through the pathway.
To maintain high glycolytic flux, tumor cells must also sustain high levels of glucose uptake from the blood supply. In breast cancer, the expression of glucose transporters Glut1 and Glut3 are increased, along with their targeting to the plasma membrane (231). Of the family of five transmembrane glucose transporters, Glut1 and Glut3 have the lowest $K_m$ values and can facilitate glucose transport at a nearly constant rate (230). In response to growth factor stimulation of Akt, Glut1 expression and surface localization are increased leading to increased glucose uptake and aerobic glycolysis (232,233). The expression and plasma membrane localization of Glut1 are just two mechanisms by which PI3K/AKT positively regulate aerobic glycolysis.

Aerobic glycolysis is a fundamental response to the constantly changing tumor microenvironment. As a tumor grows, cells eventually reach the oxygen diffusion limit and become hypoxic (5). However, the diffusion limit of glucose is greater than that of oxygen and therefore the supply of glucose is not limited. It has been well established that fluctuations in oxygen levels occur frequently in tumors and ultimately favor the survival of cells with increased glycolytic ability (5). Consequently, tumors use glycolysis even in the presence of oxygen due to constitutively upregulated glycolytic pathways. Hypoxia triggers the expression of hypoxia-inducible factor-1α (HIF-1α), a transcription factor, which up-regulates the expression of glucose transporters as well as glycolytic enzymes, promoting glycolysis. In addition to hypoxia, PI3K/Akt signaling has been shown to positively regulate HIF-1α activation and promote glycolysis (234-236). Akt has also been demonstrated to regulate the activity of enzymes that participate in glycolysis. Specifically, Akt has been shown to stimulate phosphofructokinase activity.
and increase hexokinase association with the mitochondria, both of which increase
glycolysis (237,238). Studies have also shown that tumor cells will undergo cell death in
response to glucose withdrawal and treatment of tumors with PI3K or mTOR inhibitors
decreases glucose uptake and induces regression (239,240). These data implicate both
the microenvironment and PI3K/Akt signaling as central players in the regulation of
aerobic glycolysis in cancer.

Aerobic glycolysis also supports important functions that promote tumor
progression. *In vitro* studies using breast cancer cell lines have shown that more invasive
cells such as MDA-MB-231 cells consume higher levels of glucose relative to the less
invasive MCF-7 cells (5). Persistent glycolysis leads to acidification of the tumor
microenvironment, promoting ECM and basement membrane degradation through
increased matrix metalloproteinase (MMP) activity. This allows tumor invasion through
basement membrane, giving tumor cells access to the vasculature, promoting
dissemination and metastasis. Cells likely encounter regions of hypoxia at the site of
metastasis as they proliferate and occlude the blood supply. This once again will favor
the survival of cells with constitutively up-regulated glycolysis. It is clear that aerobic
glycolysis is important in multiple stages of tumor progression and targeting the factors
that contribute to this metabolic reprogramming could lead to more effective treatment
strategies for many types of cancer.
Rationale for Thesis Work

The IRS proteins are required for IGF-1-dependent tumor initiation and progression. Despite their similarity, they do not function interchangeably as IRS-1 promotes growth and proliferation, while IRS-2 promotes survival, invasion, aerobic glycolysis and metastasis (101-103,106-108). The mechanisms that underlie the ability of IRS-2, but not IRS-1, to promote these functions associated with tumor progression to metastasis are unknown. The downstream signaling events following IRS-1 or IRS-2-mediated PI3K activation also remain to be rigorously characterized. Determining the differences in signaling downstream of PI3K activated through IRS-1 or IRS-2 is required to fully understand how the IRS proteins mechanistically differ. Potentially, this could implicate novel drug targets to better treat metastatic disease. The goal of the work presented here was to rigorously establish the mechanistic basis of PI3K activation by IRS-2, as well as the role of IRS-2-dependent PI3K signaling in functions that positively regulate metastatic potential, specifically aerobic glycolysis. Additionally, I sought to generate a novel model system to compare IRS-1 and IRS-2-mediated signaling and functions to better understand how they differentially contribute to mammary tumor progression.
CHAPTER II

Materials and Methods
Rationale and Strategy for Model System Generation

Achieving stable expression of Insulin Receptor Substrate-1 (Irs-1) or Irs-2 in mammary tumor cell lines derived from FVB MMTV-PyMT:Irs-1\(^{-/-}\) or FVB MMTV-PyMT:Irs-2\(^{-/-}\) mice was not possible, with little or no IRS protein expression following antibiotic selection and cell culture passage. Additionally, Wild Type (WT), Irs-1\(^{-/-}\) and Irs-2\(^{-/-}\) cells were derived from discrete tumors and are not genetically matched, thereby limiting the conclusions that can be drawn from their comparison. Therefore, I sought to generate superior cell culture models to address these issues.

My approach was to generate mammary tumor cell lines from mice with conditional alleles of Irs-1, Irs-2 or both Irs proteins. To induce tumorigenesis, I employed the MMTV-PyMT mouse model of tumor progression. Mammary gland specific expression is achieved by driving Polyoma Middle T (PyMT) expression by the mouse mammary tumor virus promoter (MMTV). This model of mammary tumor progression is robustly oncogenic, driving rapid tumorigenesis and metastasis (104). MMTV-PyMT is also a well-characterized, representative mouse model of estrogen receptor negative (ER-) human breast cancer in which the role of the Irs proteins has been previously characterized (106,107,241,242). Following establishment of tumor cell lines in vitro, Irs expression was knocked-out through adenoviral Cre-Recombinase infection. This method allowed for the generation of genetically identical cell lines differing only in
expression of the Irs proteins. Additionally, stable expression of either Irs-1 or Irs-2 was achieved in these cells.

*PyMT:Irs-1<sup>−/−</sup>, PyMT:Irs-2<sup>−/−</sup> and PyMT:Irs-1<sup>−/−</sup>/Irs-2<sup>−/−</sup> Cell Lines*

**Mouse Models.** *Irs-2<sup>−/−</sup>* mouse mammary tumor cell lines were previously isolated from *FVB MMTV-PyMT:Irs-2<sup>−/−</sup>* mice (106). *Irs-1<sup>fl/fl</sup>* and *Irs-2<sup>fl/fl</sup>* mice were a generous gift from the laboratory of Morris White (Children’s Hospital, Boston, MA) (129,243) and were backcrossed through 10 generations onto a *FVB* background. *Irs-1<sup>fl/fl</sup>* and *Irs-2<sup>fl/fl</sup>* mice were crossed with *FVB MMTV-PyMT<sup>−/+</sup>* mice to generate *PyMT:Irs-1<sup>fl/fl</sup>, PyMT:Irs-2<sup>fl/fl</sup>* and *PyMT:Irs-1<sup>fl/fl</sup>/Irs-2<sup>fl/fl</sup>* mice. Females of these genotypes were observed for mammary tumor formation. All mice were housed and bred in accordance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of UMMS.

**Tumor Harvesting and Cell Line Establishment.** At 85 days of age, mammary tumors were dissected from female mice and placed in 3mls high glucose (4g/l) DMEM (Invitrogen) with 2% FBS (Sigma), collagenase (2mg/ml, Sigma) and Pen/Strep (Sigma). Tumors were minced into roughly 1mm<sup>3</sup> pieces, placed back into the same media and incubated at 37°C under constant agitation (220 rpm, 3 hrs). Periodically, tumor digests were pipetted up and down to dissociate any clumps. Following collagenase digestion, cells were pelleted at 1000 RPM for 5 min. Media was aspirated and cells were washed 5 times with 10mls of PBS containing 5% FBS. During the first wash, cell culture plates
were coated with collagen (8mls/10cm plate, 50 mg/ml, Advanced BioMatrix) and allowed to sit in the tissue culture hood during the washes. Before seeding cells, excess collagen was rinsed from the plates with PBS. Cells were resuspended in DMEM/F12 (Invitrogen) containing 2% FBS and Pen/Strep. Approximately 4x10^6 cells were seeded per 10 cm plate.

Cells were maintained in DMEM/F12 containing 2% FBS with Pen/Strep and media was changed every 2-3 days. Once cells reached confluence, they were passaged 1:4 using trypsin and seeded onto collagen-coated plates as described above. Contaminating fibroblasts were periodically removed using differential tyrpsinization. Following addition of trypsin (Sigma), cells were monitored microscopically for detachment of fibroblasts, while the more adherent mammary tumor epithelial cells rounded but maintained their adherence to the plate. Detached fibroblasts were washed away with PBS and fresh media was added. This process was repeated as necessary to remove any contaminating fibroblasts during the crisis period, which lasts approximately 4 weeks. During this crisis period, growth medium was switched to low glucose (1g/l) DMEM (Invitrogen) containing 10% FBS. Following crisis, cells began to actively proliferate and tissue culture plates were no longer pre-coated with collagen prior to cell seeding.

**In Vitro Knock-Out of Irs-1 and Irs-2 expression.** *PyMT:*Irs-1^fl/fl*, *PyMT:*Irs-2^fl/fl* and *PyMT:*Irs-1^fl/fl/Irs-2^fl/fl* cells were infected with adenoviruses containing either GFP or GFP and Cre recombinase (Gene Transfer Vector Core, University of Iowa) at an MOI of
10 in DMEM containing 2% FBS for five hours. Virus containing media was removed and cells were placed in DMEM containing 10% FBS. 24 hours post-infection, GFP positive cells were isolated using fluorescence-activated cell sorting (FACS). Representative FACS profiles for control cells and GFP-Cre cells pre- and post-sort are shown in Figure 2.1A. During and after sorting, cells were kept in 2% FBS/DMEM containing Normocin (100µg/ml, InvivoGen) to prevent contamination. Cells were then screened for Irs-1 and Irs-2 expression by western blot. Irs-1 or Irs-2 expression were successfully knocked-out in PyMT:Irs-1<sup>fl/fl</sup> and PyMT:Irs-2<sup>fl/fl</sup> cells (Figure 2.1B). However, following three rounds of Cre infection with subsequent sorting, PyMT:Irs-1<sup>fl/fl</sup>/Irs-2<sup>fl/fl</sup> cells had residual Irs-1 expression (Figure 2.1C). These cells were subcloned to isolate cell populations with a complete knockout of both Irs-1 and Irs-2 (Figure 2.2). Five subclones with no Irs-1 or Irs-2 expression were selected for further screening and showed no significant difference in cell growth (Figure 2.3A). Additionally, these subclones were also screened for invasion (Figure 2.3B), glucose uptake (Figure 2.3C) and lactate production (Figure 2.3D). In contrast to the cell growth data, the subclones varied in their ability to stimulate invasion and aerobic glycolysis. The level of Igf-1r expression was also determined in each of these subclones, with subclone A having the highest level of IGF-1R expression and subclone C having the lowest (Figure 2.3E).
Figure 2.1. Establishing novel models to study the IRS proteins.

A.

No Infection

Cre

Pre-Sort

Post-Sort

B.

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<tr>
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C.

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| Irs-1           |                 |
| Irs-2           |                 |
| Tubulin         |                 |
Figure 2.1. Establishing novel models to study the IRS proteins. (A) Representative sorting profiles showing the fluorescence of non-infected cells relative to Cre-GFP infected cells pre and post sort. (B) PyMT:Irs-1^fl/fl and PyMT:Irs-2^fl/fl cells were infected with Adenoviral GFP or Adenoviral Cre-GFP and cells were sorted for GFP+ cells 24 hours post infection. Cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize IRS-1, IRS-2, and Tubulin. (C) PyMT:Irs-1^fl/fl/Irs-2^fl/fl cells were infected with Adenoviral Cre-GFP either once or three times and sorted for GFP+ cells 24 hours post infection. Cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize IRS-1, IRS-2, and Tubulin.
Figure 2.2. Evaluating Irs expression in PyMT:Irs-1^{floxed}/Irs-2^{floxed} single cell clones.
Figure 2.2. Evaluating Irs expression in PyMT:Irš-1^{fl/fl}/Irš-2^{fl/fl} single cell clones. PyMT:Irš-1^{fl/fl}/Irš-2^{fl/fl} cells that had been infected and sorted were single cell cloned. Cell extracts from multiple individual clones that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize IRS-1, IRS-2, and Tubulin.
Figure 2.3. Characterizing five PyMT:Irs-1\(+/\)Irs-2\(−/−\) clones.

A. Relative Cell Growth

B. Relative Invasion

C. Glucose Uptake

D. Lactate Production

E. Irs-1\(+/\)Irs-2\(−/−\)

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<td>Tubulin</td>
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Figure 2.3. Characterizing five PyMT:Irs-1\textsuperscript{-/-}/Irs-2\textsuperscript{-/-} clones. (A) PyMT:Irs-1\textsuperscript{-/-}/Irs-2\textsuperscript{-/-} clones (A-E) were assayed for cell growth over three days. The data shown represent one experiment. (B) PyMT:Irs-1\textsuperscript{-/-}/Irs-2\textsuperscript{-/-} clones (A-E) were subjected to a transwell invasion assay for 4hrs. The data shown represent the mean (±SEM) of three experiments. (C,D) PyMT:Irs-1\textsuperscript{-/-}/Irs-2\textsuperscript{-/-} clones (A-E) were grown in 0.1% BSA/DMEM supplemented with IGF-1 (20ng/mL) for 24 hrs. Glucose uptake and lactate production were measured and normalized to total protein concentration. The data shown are expressed as a rate measurement (mM/mg/hr) and represent one experiment with measurements in triplicate. (E) Cell extracts from PyMT:Irs-1\textsuperscript{-/-}/Irs-2\textsuperscript{-/-} clones (A-E) that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Igf-1R, and Tubulin.
Methods and Materials

Cells, Antibodies and Reagents. All cell lines were maintained in DMEM (1g/l, glucose, Invitrogen) containing 10% FBS (Sigma). The murine pCMV-His-Irs-2 construct was kindly provided by Morris White (Children’s Hospital, Boston, MA). Murine Irs-2 was sub-cloned into the pExchange-Puro vector (Stratagene). Tyrosine residues in Irs-2 were mutated to phenylalanine using the Quickchange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. Mutagenesis primers were purchased from Integrated DNA Technologies (Y538F: 5’ GGGCGAACTCTATGGGTTCATGAGCATGC 3’ and 3’ GTCTATCCATGCTCATGAACC CATAGAGT 5’; Y628F: 5’ CCCTTACCCAGAGGACTTTGGAGACATTGAG 3’ and 3’ CTCA ATGTCTCCAAAGTCTCTGGGTAAAGG 5’; Y649F: 5’ GCCAGATGATGGCTCTCATGCC CATCAGGC 3’ and 3’ GGGTCATGGGATGAAGCCATCTGCTGCC 5’; Y671F: 5’ GCAA GAGCGATGACTTCCATGCCCATGCC 3’ and 3’ GGGCTCATGGGATGAAGCAGGTCA TCGCTCTTGCC 5’; Y734F: 5’ CCAGAAGACAGTGGGTTCATGCGAATGTC 3’ and 3’ ACA CCACATTCGCATGAACCACACTGTCTT 5’; Y758F: 5’ CCGGACATGTTGAGGAAGTCCCCTGCTCTCAACA TGTCCCC 3’ and 3’ GGGGACATGTTGAGGAAGTCCCCTGGG 5’; Y814F: 5’ CAG CGGAGACAATGACCAGTTTGTGCTCATGAGC 3’ and 3’ GTCATGAGCACAAACTGG TCATTGTCTCCGCTG 5’). All cell transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. For stable selection, cells were grown in puromycin (100 µg/ml; Fisher).

Human pcDNA-Neo-IRS-1-HA or pcDNA-Neo-IRS-2-HA constructs along with pcDNA-Neo (a generous gift from Adrian Lee, UPMC, Pittsburgh, PA) were transfected
using Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocol. For stable selection, cells were grown in Neomycin (500µg/ml, Gibco).

The following antibodies were used for immunoprecipitation or immunoblotting: Irs-1 (Bethyl Laboratories), Irs-2 (immunoblot, Calbiochem or Cell Signaling Technologies; immunoprecipitation, Bethyl Laboratories), p85 (Millipore), PY99 (Santa Cruz), Glut1 (Abcam) and α6-integrin subunit (a kind gift from Anne Cress, University of Arizona). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Jackson Biolabs. All other antibodies were obtained from Cell Signaling Technologies.

**Immunoprecipitation and Immunoblotting.** Cells were serum starved for 4hrs in 0.1% BSA/DMEM (1g/l glucose) and then stimulated with IGF-1 (100ng/ml) or insulin (100ng/ml) for the times listed in the Figure Legends. If the following inhibitors were used, cells were pretreated for 1hr prior to stimulation: LY294002 (25µM, Cell Signaling Technologies), MK2206 (0.5µM, Selleckchem) and PD98059 (6µM, Selleckchem). Cells were solubilized on ice for 20 minutes in RIPA lysis buffer (50mM Tris, pH 7.4, containing 0.15M NaCl, 1% NP-40, 0.1% SDS, 1% sodium deoxycholate, 1mM sodium orthovanadate, 1mM sodium fluoride, and protease inhibitors (Complete Mini, Roche)). Insoluble material was removed by centrifugation at 14,000 RPM for 10 min at 4°C.

Aliquots of cell extracts containing equivalent amounts of total protein were incubated under constant agitation at 4°C overnight with Irs-1, Irs-2, Akt1, or Akt2 specific antibodies and protein A sepharose beads (GE Healthcare). Immune complexes
were washed with RIPA lysis buffer and incubated with Laemmli sample buffer containing β-mercapatoethanol for 5 minutes at 95°C. Immune complexes, as well as aliquots of cell extracts containing equal amounts of total protein, were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 1X TBST buffer (50mM Tris, pH 7.5, containing 0.15M NaCl, 0.1% Tween-20) containing 5% (w/v) Carnation dry milk. Membranes were then incubated overnight at 4°C in blocking buffer containing primary antibodies. After washing, membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature in blocking buffer and proteins were then detected using enhanced chemiluminescence (Pierce and Bio-Rad). For phospho-antibodies, the blocking buffer contained 5% BSA (w/v) (Sigma).

**HA Pull-Down Experiments.** Cells were serum starved for 4hrs in 0.1% BSA/DMEM (1g/l glucose) and then stimulated with IGF-1 (100ng/ml) for the times listed in the Figure Legends. Cells were solubilized on ice with a 50mM Tris, pH 7.4 buffer containing 0.15M NaCl, 1% NP-40, 10% glycerol, 1mM sodium orthovanadate, 1mM sodium fluoride, and protease inhibitors (Complete Mini, Roche). Insoluble material was removed by centrifugation at 14,000 RPM for 10 min at 4°C.

Aliquots of cell extracts containing equivalent amounts of total protein were incubated under constant agitation at 4°C overnight with HA antibody-conjugated sepharose beads (Thermo Scientific). Pull-down complexes were washed with lysis
buffer and incubated with Laemmli sample buffer containing β-mercaptoethanol for 5 minutes at 95°C. Samples were subjected to western blot analysis as described above.

**Lactate and Glucose Uptake Assays.** Cells were grown in 24 well plates to near confluence, washed with PBS and then incubated with 0.1% BSA/DMEM (1g/l glucose) supplemented with IGF-1 (20ng/ml) for 24 hrs with or without the addition of the Gsk-3β inhibitor SB 216763 (10uM, Sigma). Lactate levels in the conditioned media were measured using a lactate assay kit (Trinity Biotech) and glucose levels were measured using a glucose assay kit (Sigma) according to manufacturers’ instruction. Total cellular protein per well was quantified using a Bradford Assay (BioRad) and lactate production and glucose uptake were expressed as a rate measurement (mM/mg/hr) normalized to protein content.

**Cell Surface Biotinylation.** Cells were grown to near confluence and then incubated in 0.1% BSA/DMEM (1g/l glucose) with IGF-1 (20ng/ml) and with or without SB 216763 (10 uM) for 24 hrs. Following two washes with cold PBS, cells were biotinylated at 4°C for 30 min using EZ-Link™ Sulfo-NHS-SS-Biotin (0.5 mg/mL; Pierce Biotechnology). Biotin was removed and the cells were incubated in a 50mM Tris buffer (pH 7.4) for 5 minutes to quench any residual biotin. Cells were washed with cold PBS and solubilized on ice with a 50mM Tris, pH 7.4 buffer containing 0.15M NaCl, 1% NP-40, 10% glycerol, 1mM sodium orthovanadate, 1mM sodium fluoride, and protease inhibitors
Nuclear contaminants were removed by centrifugation at 14,000 RPM for 10 min at 4°C.

Aliquots of cell extracts containing equal amounts of protein were incubated with Neutra-Avidin Agarose Beads (Pierce Biotechnology) with constant agitation overnight at 4°C. Pull-down complexes were washed lysis buffer and incubated with Laemmli sample buffer containing β-mercapatoethanol for 5 minutes at 95°C.

**2D Growth Assay.** Cells were dissociated using trypsin and resuspended in low glucose DMEM supplemented with 10ng/ml IGF-1. Cells were plated at 5000 cells/well into a total of three wells and this was done for four individual 48-well plates (one for each day for the assay). Media was changed every other day during the course of the assay and one plate was measured for cell growth each day. Media was removed and cells were washed two times with PBS. Cells were fixed with 100% methanol for 15 min at room temperature. Methanol was removed and cells were stained with 0.2% crystal violet in 2% ethanol for 15 min at room temperature. Crystal violet stain was removed, cells were rinsed with H2O and crystal violet was solubilized with 200µl of 1% SDS. A595 readings were measured using a spectrophotometer and cell growth was expressed as relative increase in A595 over day 0.

**3D Growth Assay.** In a 24-well plate, 200µl of Matrigel was added per well per cell line and left to solidify at 37°C for 30 min. During this incubation period, cells were dissociated from their growth plates with trypsin and cells were resuspended at a
concentration of 50,000 cell/ml in DMEM (1g/l glucose) supplemented with 10ng/ml IGF-1. Cells were then mixed with matrigel at a 2:1 ratio and 300µl of this mixture was added to each matrigel-precoated well. The cell/matrigel mix was then allowed to incubate at 37°C for an additional 30 min. Following this incubation, 500µl of low glucose DMEM supplemented with 10ng/ml IGF-1 was carefully added to each well. Cell culture media was changed every three days and pictures were taken on day nine of the assay. Colony size and number were then quantified.

**Transwell Invasion Assays.** 24 hours prior to the assay, transwells (Costar, 8.0um polycarbonate membrane, 6.5mm inserts) were coated with matrigel (5µg/well) and left to dry overnight. The following day, 40µl of 0.1% BSA/ DMEM was added to each well and incubated for an hour at 37°C. Cells (1x10^4/well) resuspended in 0.1% BSA/DMEM were added to the top well of the transwell and 600µl of NIH-3T3 conditioned media was added to the bottom well. After 4hrs of incubation at 37°C, the each transwell was swabbed with a Q-tip twice to remove the media and remaining cells. The transwells were fixed in methanol (10min, room temperature), rinsed with water and left to dry overnight at room temperature. Transwells were stained with DAPI, five representative images were taken of each transwell and invaded cells were counted.

**Statistical Analysis.** In Chapter III, all p-values were calculated using an unpaired Students t test. In Chapter IV, all p-values were calculated using a paired t test. All error bars shown represent standard error of the mean (SEM).
Chapter III

Insulin Receptor Substrate-2 Mediated Phosphatidylinositol 3-kinase Signaling Selectively Inhibits Glycogen Synthase Kinase-3β to Regulate Aerobic Glycolysis

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ABSTRACT

Insulin Receptor Substrate-1 (IRS-1) and IRS-2 are cytoplasmic adaptor proteins that mediate the activation of signaling pathways in response to ligand stimulation of upstream cell surface receptors. Despite sharing a high level of homology and the ability to activate Phosphatidylinositol-3-Kinase (PI3K), only Irs-2 positively regulates aerobic glycolysis in mammary tumor cells. To determine the contribution of Irs-2-dependent PI3K signaling to this selective regulation, we generated an Irs-2 mutant deficient in the recruitment of PI3K. We identified four tyrosine residues (Y649, Y671, Y734 and Y814) that are essential for the association of PI3K with Irs-2 and demonstrate that combined mutation of these tyrosines inhibits glucose uptake and lactate production, two measures of aerobic glycolysis. Irs-2-dependent activation of PI3K regulates the phosphorylation of specific Akt substrates, most notably Glycogen synthase kinase-3β (Gsk-3β). Inhibition of Gsk-3β by Irs-2-dependent PI3K signaling promotes glucose uptake and aerobic glycolysis. The regulation of unique subsets of Akt substrates by Irs-1 and Irs-2 may explain their non-redundant roles in mammary tumor biology. Taken together, our study reveals a novel mechanism by which Irs-2 signaling preferentially regulates tumor cell metabolism and adds to our understanding of how this adaptor protein contributes to breast cancer progression.
INTRODUCTION

The IRS proteins are cytoplasmic adaptor proteins that organize signaling complexes downstream of cell surface receptors (33). Originally discovered as substrates of the insulin receptor, they function as adaptor proteins for additional surface receptors including the closely related insulin like growth factor-1 receptor (IGF-1R) (43,49). Upon ligand stimulation, the IRS proteins are recruited to activated receptors where they are phosphorylated on tyrosine residues within their C-termini, generating binding sites for the recruitment of downstream signaling effectors, including PI3K, Growth factor receptor-bound protein-2 (GRB-2) and Src homology-2 domain-containing protein-tyrosine phosphatase-2 (SHP-2) (93). Following receptor activation, the combinatorial recruitment of these effectors by IRS-1 and IRS-2 is required for amplification of signaling cascades that regulate changes in cellular behavior. IRS-1 and IRS-2 are ubiquitously expressed and are the primary mediators of insulin-dependent mitogenesis and glucose metabolism in most cell types (33). Although they share considerable homology, in vitro cell line studies and distinct phenotypes of the Irs-1 and Irs-2 knockout mice confirm that these proteins do not function in a redundant manner in normal cell biology or development (44,86-88,90,244).

IRS-1 and IRS-2 also play divergent roles in breast cancer. Stimulation of human breast carcinoma cell lines expressing only IRS-1 with IGF-1 increases their proliferation, whereas stimulation of cells expressing only IRS-2 promotes cell migration (102,103). These differences in IRS function have also been demonstrated in vivo using
the Mouse mammary tumor virus – Polyoma virus middle-T antigen (MMTV-PyMT) mouse model of mammary tumor progression. Specifically, in mice expressing PyMT, mammary tumor metastasis is significantly diminished in the absence of Irs-2 expression, and Irs-1 does not compensate for this loss (106). In fact, PyMT:Irs-1⁻/⁻ tumors have elevated expression and tyrosine phosphorylation of Irs-2, and these tumors are more metastatic when compared with their wildtype (WT) counterparts (107). Cells derived from PyMT:Irs-2⁻/⁻ tumors are also significantly less invasive and display decreased aerobic glycolysis relative to PyMT:WT or PyMT:Irs-1⁻/⁻ tumor cells in vitro (106,108).

Distinct functions for IRS-1 and IRS-2 in human breast tumors are also indicated by their unique intracellular localization patterns. Tumor cells express both IRS-1 and IRS-2 in the cytoplasm, while IRS-1 also localizes to the nucleus and IRS-2 to the cell membrane (96,97). Nuclear localization of IRS-1 correlates with increased response to tamoxifen and improved patient survival, whereas IRS-2 cell membrane localization correlates with decreased overall patient survival (96,98). Differential intracellular compartmentalization may contribute to the ability of the IRS proteins to regulate distinct tumor cell functions.

The IRS proteins share their highest degree of homology in their N-terminal Plextrin Homology (PH) and Phosphotyrosine Binding (PTB) domains, which mediate their interactions with upstream receptors (61,62,245,246). The C-termini of the IRS proteins are less conserved, and it is these C-terminal differences that likely confer upon the IRS proteins their divergent functions through specific interactions that impact localization and signaling (93). With regard to signaling, activation of the PI3K and
mechanistic target of rapamycin (mTor) pathway is enhanced in PyMT:Ir$^{-\beta}$ tumors that express only Irs-2, and is significantly lower in tumors that lack Irs-2 expression, indicating that Irs-1 does not compensate fully for the activation of this pathway in vivo (107,108). Similarly in vitro, mammary tumor cells derived from PyMT:Ir$^{-\beta}$ tumors have enhanced Irs-2-dependent PI3K/mTor signaling (107). The PI3K signaling pathway is one of the most commonly mutated pathways in cancer, including breast cancer (160,247). PI3K itself is an oncogene and activating mutations have been observed in many types of cancer (248). In tumors that do not harbor PI3K mutations, other components of this signaling pathway, such as the lipid phosphatase Phosphatase and Tensin Homolog (PTEN) and Akt are often mutated such that PI3K signaling is enhanced (150,248). One of the major contributions of the PI3K/Akt signaling pathway to tumors is the regulation of metabolic pathways that promote aerobic glycolysis, a hallmark of cancer (4,249). The overall goal of our current study was to establish the contribution of Irs-2-mediated PI3K activation to mammary tumor cell metabolism and to determine the mechanism by which Irs-2/PI3K signaling preferentially regulates aerobic glycolysis.
RESULTS

Identification of Tyrosine Residues within Irs-2 that contribute to the recruitment of PI3K.

PI3K is activated by the interaction of src homology 2 (SH2) domains within the p85 regulatory subunit with phosphotyrosine residues in upstream receptors or adaptor proteins (45,46,48,59,126). To understand how Irs-2 activates PI3K signaling, we initially sought to identify specific tyrosine residues that participate in the recruitment of PI3K. Tyrosines located within consensus PI3K binding motifs (YxxM) were mutated to phenylalanine to prevent phosphorylation while maintaining wild type Irs-2 structure (71-73). These residues included Y538, Y649, Y671, Y734 and Y814 (Figure 3.1A). Two additional tyrosine residues that were shown previously in an Irs-2 proteomics phosphopeptide screen to be capable of binding to PI3K (Y628 and Y758) were also mutated to phenylalanine (74). WT Irs-2 and the individual Irs-2 tyrosine mutants were assayed for tyrosine phosphorylation in response to IGF-1 stimulation when expressed transiently in PyMT:Irs-2-/- cells. Mutation of Y649, Y671 or Y814 reduced significantly the overall tyrosine phosphorylation of Irs-2 when compared with the level of WT Irs-2 phosphorylation (Figure 3.1B). In contrast, mutation of Y538, Y628 or Y758 did not significantly reduce Irs-2 tyrosine phosphorylation (Figure 3.1B and C). It
Figure 3.1. Identification of tyrosine residues that mediate Irs-2 recruitment of PI3K.

A.

B.

C.

D.

E.
Figure 3.1. Identification of tyrosine residues that mediate Irs-2 recruitment of PI3K. (A) Schematic of WT Irs-2 and Irs-2 tyrosine mutants. Individual tyrosines that were mutated to phenylalanine are indicated for each mutant construct. (B-E) Irs-2−/− cells were transiently transfected with WT Irs-2 or the individual tyrosine mutant constructs and stimulated with IGF-1 (100ng/mL) (B,C) or insulin (100ng/ml) (D,E) for 10 minutes. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoprecipitated with Irs-2 specific antibodies and the immune complexes were immunoblotted with antibodies that recognize either phosphotyrosine (p-Tyr) or p85α. The p-Tyr blots were stripped and re-probed for Irs-2. The data shown in the graphs represent the mean (±SEM) of four (B), three (C and D) or two (E) independent experiments. NT, mock transfection. *, p<0.05 relative to WT Irs-2.
was not possible to assess the impact of mutating Y734 on phosphorylation due to the protein instability of this Irs-2 mutant.

The ability of the Irs-2 tyrosine mutants to recruit PI3K was assayed by co-immunoprecipitation (co-IP) of the p85 regulatory subunit of PI3K. Only the Y649F, Y671F and Y814F Irs-2 mutants exhibited a significant reduction in their ability to recruit p85 when compared with WT Irs-2 (Figure 3.1B), which correlated with their decreased level of tyrosine phosphorylation. The association of p85 with Irs-2 was reduced by 43%, 57% and 70%, respectively, for each of these Irs-2 mutants. Of note, the tyrosine residues that were implicated in the IGF-1-dependent recruitment of PI3K were also required for the recruitment of PI3K in response to insulin stimulation (Figure 3.1D and E).

We next combined multiple tyrosine mutations to generate an Irs-2 mutant that was impaired in its ability to recruit and activate PI3K. These Irs-2 mutants included: Irs-2 Y3F, which combined mutations in the three tyrosines that individually contributed to the association of Irs-2 with p85 (Y649, Y671, Y814); Irs-2 Y4F, which added an additional mutation at Y734 to Y3F; and Irs-2 Y5F, which added an additional mutation at Y538 to Y4F (Figure 3.1A). The level of tyrosine phosphorylation and association with p85 that was observed for the Irs-2 Y3F mutant was only marginally lower than that observed for the individual Y671F and Y814F Irs-2 mutants (Figure 3.2A and B). Additional mutation of Y734 significantly reduced the level of p85 association when compared with Irs-2 Y3F, indicating that Y734 participates in the interaction of Irs-2 with PI3K (Figure 3.2A and C). The reason that protein expression can be obtained when this
Figure 3.2. Generation of a PI3K-binding deficient Irs-2 mutant.

A. 

B.

C.

D. 

E.

F.
Figure 3.2. Generation of a PI3K-binding deficient Irs-2 mutant. *Irs-2*−/− cells were transiently transfected with WT Irs-2 or Irs-2 multiple-tyrosine mutant constructs (Y3F, Y4F and Y5F) and stimulated with IGF-1 (100ng/mL) (A-C) or insulin (100 ng/mL) (D-F) for 10 minutes. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoprecipitated with Irs-2 specific antibodies and the immune complexes were immunoblotted with antibodies that recognize either phosphotyrosine (p-Tyr) or p85α. The p-Tyr blots were stripped and re-probed for Irs-2. The data shown in the graphs represent the mean (±SEM) of three independent experiments. NT, mock transfection. *, p<0.05 relative to WT Irs-2. **, p<0.05 relative to Irs-2 Y3F.
tyrosine is mutated in conjunction with other tyrosine mutations, while mutation of this tyrosine alone leads to protein instability, remains to be determined. Additional mutation of Y538 (Irs-2 Y5F) did not provide further significant reduction in tyrosine phosphorylation or p85 association, confirming that this tyrosine residue does not contribute to the interaction of Irs-2 with PI3K (Figure 3.2A, B and C). Similar levels of tyrosine phosphorylation and p85 association were observed for each of the combination mutants in response to insulin stimulation (Figure 3.2D, E, and F).

Irs-2-dependent activation of PI3K stimulates aerobic glycolysis.

To establish a model system with which to study Irs-2-dependent PI3K activation and its role in the regulation of aerobic glycolysis, we derived cell lines from PyMT:Irs-2fl/fl mammary tumors and acutely deleted Irs-2 expression in vitro by transient adenoviral infection of Cre-recombinase. These PyMT:Irs-2fl/fl cells were stably transfected to express either empty vector, WT Irs-2 or the Irs-2 Y5F mutant at equivalent protein levels compared to the parental population (Irs2fl/fl) (Figure 3.3A). Similar to the results obtained after transient expression of Irs-2 Y5F, a significant, but not total, reduction in Irs-2 tyrosine phosphorylation and association with PI3K was observed when compared with WT Irs-2 (76% and 86% reduction, respectively) (Figure 3.3B and C). PyMT:Irs-2fl/fl cells expressing empty vector and PyMT:Irs-2+/− cells expressing empty vector, WT Irs-2 or Irs-2 Y5F were assayed for their level of aerobic glycolysis. As shown in Figure 3.3D and E, IGF-1 stimulated glucose uptake and lactate production were reduced by
Figure 3.3. Irs-2-dependent PI3K signaling regulates aerobic glycolysis.

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<td>WT Irs-2</td>
<td>Irs-2&lt;sup&gt;−/−&lt;/sup&gt; Y5F</td>
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<td>Tubulin</td>
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B.

C.

D.

E.

N.S.

Glucose uptake (mM/mg/min)

Leakage resistance (mM/mg/min)

Irs-2<sup>−/−</sup>
Figure 3.3. Irs-2-dependent PI3K signaling regulates aerobic glycolysis. (A) Stable cell lines were generated of PyMT::Irs-2<sup>fl/fl</sup> cells expressing empty vector (EV) and PyMT::Irs-2<sup>−/−</sup> cells expressing EV, WT Irs-2 (WT) or Irs-2 Y5F (Y5F). Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-2 and Tubulin. (B, C) Cells were serum deprived and then stimulated with IGF-1 (100ng/ml) for 10 minutes. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoprecipitated with Irs-2 specific antibodies and the immune complexes were immunoblotted with antibodies that recognize either phosphotyrosine (p-Tyr) or p85α. The p-Tyr blots were stripped and re-probed for Irs-2. The data shown in (C) represent the mean (+SEM) of five independent experiments. *, p<0.0001 relative to WT Irs-2. **, p<0.0001 relative to WT Irs-2. (D,E) Cells were grown in 0.1% BSA/DMEM supplemented with IGF-1 (20ng/mL) for 24 hrs. Glucose uptake (D) and lactate production (E) were measured and normalized to total protein concentration. The data shown are expressed as a rate measurement (mM/mg/hr) and represent the mean (+SEM) of five independent experiments. *, p<0.02 relative to WT Irs-2. **, p<0.02 relative to Irs-2<sup>fl/fl</sup>.
nearly 40% in the absence of Irs-2, and these activities were rescued by restoring WT Irs-2 expression. However, expression of Irs-2 Y5F did not restore glucose uptake or lactate production to the levels observed in WT Irs-2 expressing cells, establishing the requirement for direct PI3K activation by Irs-2 in the regulation of aerobic glycolysis (Figure 3.3D and E).

**Identification of Irs-2/PI3K-dependent Akt effectors.**

Although both Irs-1 and Irs-2 are capable of recruiting PI3K and stimulating the activation of Akt, which plays an important role in regulating metabolism, Irs-2 preferentially regulates aerobic glycolysis in mammary tumor cells (46,73,108,135). To investigate further the mechanism of this metabolic regulation, we sought to identify downstream signaling effectors of Akt that are selectively activated by Irs-2-dependent signaling. Loss of Irs-2 expression did not diminish the overall level of IGF-1 stimulated Akt activation, as assessed by phosphorylation on either Ser473 or Thr308 (Figure 3.4A). Similarly, restoring WT Irs-2 or Irs-2 Y5F expression in the Irs-2-/− cells also did not significantly alter phosphorylation of Akt on either site relative to the level of phosphorylation observed in vector control, Irs-2-/− cells (Figure 3.4B-D). This lack of impact on signaling is likely explained by increased expression of Irs-1 in the Irs-2-/− cells, which compensates for the activation of PI3K and Akt.

To assay for Irs-2-specific Akt activation independent of Irs-1, WT Irs-2 and Irs-2 Y5F were expressed in cells that lack expression of both Irs-1 and Irs-2 (Figure 3.5A).
Figure 3.4. Signaling downstream of a PI3K-binding deficient Irs-2 mutant.
Figure 3.4. Signaling downstream of a PI3K-binding deficient Irs-2 mutant. (A-D) 
PyMT::Irs-2fl/fl cells expressing empty vector (EV) and PyMT::Irs-2−/− cells expressing 
EV, WT Irs-2 or Irs-2 Y5F were stimulated with IGF-1 (100ng/mL) for 2, 10, and 30 
minutes. Aliquots of cell extracts that contained equivalent amounts of total protein were 
immunoblotted with antibodies that recognize Irs-1, Irs-2, pIgf-IR (Tyr1135/1136), Igf-
1R, pAkt (Thr308, Ser473), Akt, pMapk (Thr202/Tyr204), Mapk, and Tubulin. The data 
shown in (C) and (D) represent the mean (+SEM) of four independent experiments.
Figure 3.5. Signaling downstream of a PI3K-binding deficient Irs-2 mutant in *irs-1*/*irs-2* cells.

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| Glucose Utilization (mmol/kg)
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| Lactate Production (nmol/kg)
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<td>Irs-2 YSF</td>
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Figure 3.5. Signaling downstream of a PI3K-binding deficient Irs-2 mutant in Irs-1^{+/+}/Irs-2^{+/+} cells. (A) Stable cell lines were generated of *PyMT::Irs-1^{−/−}/Irs-2^{−/−}* cells expressing empty vector (EV), WT Irs-2 or Irs-2 Y5F. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-1, Irs-2 and Tubulin. (B-C) *PyMT::Irs-1^{−/−}/Irs-2^{−/−}* cells expressing empty vector (EV), WT Irs-2 or Irs-2 Y5F were stimulated with IGF-1 (100ng/mL) for 10 minutes (B) or 2, 10 and 30 minutes (C). (B) Aliquots of cell extracts that contained equivalent amounts of total protein were immunoprecipitated with Irs-2 specific antibodies and the immune complexes were immunoblotted with antibodies that recognize either phosphotyrosine (p-Tyr) or p85α. The p-Tyr blots were stripped and re-probed for Irs-2. The data shown in the graph represent the mean (±SEM) of 3 independent experiments. *, p<0.0002 relative to WT Irs-2. (C) Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-1, Irs-2, pIgf-IR (Tyr 1135/1136), Igf-1R, pS6k (Thr389), S6k, pAkt (Thr308, Ser473), Akt, and Tubulin. (D.E) *PyMT::Irs-1^{−/−}/Irs-2^{−/−}* cells expressing empty vector (EV), WT Irs-2 or Irs-2 Y5F were grown in 0.1% BSA/DMEM supplemented with IGF-1 (20ng/mL) for 24 hrs. Glucose uptake (D) and lactate production (E) were measured and normalized to total protein concentration. The data shown are expressed as a rate measurement (mM/mg/hr) and represent the mean (±SEM) of three independent experiments. *, p<0.003 relative to EV.
This *Irs-1−/−/Irs-2−/−* mammary tumor cell line was generated from *PyMT:Irs-1−/−/Irs-2−/−* mammary tumor cells after transient infection with adenoviral Cre-recombinase. As we had observed using the *PyMT:Irs-2−/−* cells (Figure 3.3B and C), Irs-2 Y5F tyrosine phosphorylation and association with PI3K was significantly, but not completely, reduced in the *Irs-1−/−/Irs-2−/−* cells (Figure 3.5B). Akt was not activated in response to IGF-1 stimulation in the double null cells, keeping with previous reports that the Irs proteins are required for PI3K activation downstream of the IGF-1 and insulin receptors (Figure 3.5C) (129,130). In the absence of background Irs-1 signaling, Akt phosphorylation was diminished in the Irs-2 Y5F expressing cells relative to cells expressing WT Irs-2 (Figure 3.5C). Phosphorylation of p70-S6Kinase, which we previously showed was preferentially activated by Irs-2, was also reduced (Figure 3.5C) (107). Phosphorylation of the Igf-1R was similar in all cells examined, indicating that neither Irs expression nor the ability of Irs-2 to activate PI3K is required for upstream receptor activation (Figure 3.4A and B and Figure 3.5C). Importantly, glucose uptake and lactate production were significantly enhanced upon expression of WT Irs-2, but not Irs-2 Y5F, in the *Irs-1−/−/Irs-2−/−* cells (Figure 3.5D and E).

Akt phosphorylates a large number of effectors, a subset of which have been implicated in the regulation of aerobic glycolysis (135,250). To identify Irs-2-dependent Akt substrates, *Irs-1−/−/Irs-2−/−* cells expressing either empty vector, WT Irs-2 or Irs-2 Y5F were stimulated with IGF-1 and total cell extracts were immunoblotted with a phospho-Akt substrate-specific (RxXP/S/T) antibody (Figure 3.6A) (251). Although many of the detected bands were common between the three cell lines, some IGF-1-dependent
Figure 3.6. Irs-2-mediated PI3K signaling preferentially regulates phosphorylation of Gsk-3β.
Figure 3.6. Irs-2-mediated PI3K signaling preferentially regulates phosphorylation of Gsk-3β. (A,B) PyMT::Irs-1+/Irs-2-/ cells expressing empty vector (EV), WT Irs-2 or Irs-2 Y5F were stimulated with IGF-1 (100ng/mL) for 2, 10 and 30 minutes. (A) Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-2, the p-Akt substrate motif (RXXpS/T) and Tubulin. Molecular weights (kDa) are indicated on the left. (B) Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-2, pGsk3β (Ser9), Gsk-3β, and Tubulin. (C,D) PyMT::Irs-2fl/fl cells expressing empty vector (EV) and PyMT::Irs-2-/ cells expressing EV, WT Irs-2 or Irs-2 Y5F were stimulated with IGF-1 (100ng/mL) for 2, 10 and 30 minutes. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-1, Irs-2, pGsk-3β (Ser9), Gsk-3β, and Tubulin. The data shown in the graph represent the mean (±SEM) of 3 independent experiments from (D). *, p<0.03 relative to WT Irs-2.
differences were observed. In particular, one protein with a molecular weight of approximately 45 kDa showed higher levels of phosphorylation in cells expressing WT Irs-2 relative to vector control cells or cells expressing Irs-2 Y5F (Figure 3.6A). We surmised that this protein could be Gsk-3β based on the molecular weight and its known involvement in the regulation of glucose metabolism (252). Phosphorylation of Gsk-3β Ser 9, the Akt-dependent phosphorylation site in Gsk-3β, increased significantly in Irs-1−/−Irs-2−/− cells expressing WT Irs-2 relative to vector controls and this increase was not observed in the same cells expressing Irs-2 Y5F (Figure 3.6B) (171). Similar results were observed using Irs-2−/− cells expressing either empty vector, WT Irs-2 or Irs-2 Y5F. Although Irs-1 is expressed in these cells, Gsk-3β phosphorylation in response to IGF-1 stimulation is predominantly dependent on Irs-2 expression and its ability to activate PI3K (Figure 3.6C and D) and Irs-1-dependent signaling is unable to compensate for this phosphorylation.

It has been suggested that Mitogen Activated Protein Kinase (MAPK) signaling can also promote GSK-3β phosphorylation in some cell types (253). To determine which pathway was responsible for Gsk-3β phosphorylation in our model system, cells were incubated with PI3K (LY294002), Akt (MK2206) or MAPK (PD98059) inhibitors prior to stimulation with IGF-1. Treatment of Irs-2fl/fl cells with a PI3K or Akt inhibitor prevented phosphorylation of Gsk-3β (Figure 3.7A). In contrast, MAPK inhibition had no effect on Gsk-3β phosphorylation (Figure 3.7B). These data support that PI3K/Akt signaling is the primary pathway that regulates Gsk-3β phosphorylation downstream of the Igf-1r in mammary tumor cells.
Figure 3.7. Gsk-3β inhibition is mediated by PI3K/Akt signaling.
Figure 3.7. Gsk-3β inhibition is mediated by PI3K/Akt signaling. (A,B) PyMT::Irs-2<sup>−/−</sup> cells were treated with or without a PI3K (LY294002), Akt (MK2206) or MAPK (PD9805) inhibitor prior to IGF-1 stimulation (100µg/ml, 10min). Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-1, Irs-2, pIgf-IR (Tyr1135/1136), Igf-1R, pAkt (Thr308, Ser473), Akt, pGsk-3β (Ser9), Gsk-3β, pMapk (Thr202/Tyr204), Mapk, and Tubulin.
Irs-2 regulates aerobic glycolysis through inhibition of Gsk-3β.

To determine if Irs-2 specific activation of PI3K signaling leads to inhibition of Gsk-3β activation, thereby relieving its inhibition of glycolysis, glucose uptake and lactate production were measured in the presence or absence of the GSK-3 inhibitor SB 216763. Inhibition of Gsk-3β rescued glucose uptake and lactate production in the Irs-2−/− vector control and Irs-2 Y5F expressing cells to the level observed in cells expressing WT Irs-2 (Figure 3.8A and B). Previous studies have reported that Gsk-3β regulates aerobic glycolysis at the level of glucose uptake either by regulating Glut1 protein expression, promoting Glut1 retention at the cell membrane or increasing glucose transporter activity (232,254). To address the mechanism by which Irs-2/Gsk-3β regulates glucose uptake, we first assessed total Glut1 expression. Glut1 protein expression levels were equivalent in cells that lack Irs-2 expression, express WT Irs-2 or Irs-2 Y5F (Figure 3.8C and D). Additionally, total Glut1 expression was not altered by Gsk-3β inhibition (Figure 3.8C and D). Irs-2+/− cells expressed significantly less Glut1 on the cell surface compared to Irs2+/+β cells and Irs-2−/− cells with restored WT Irs-2 expression, confirming our previous data that Irs-2 regulates Glut1 surface expression (Figure 3.8C) (108). However, Glut1 surface levels in cells expressing Irs-2 Y5F were equivalent to that observed for WT Irs-2, suggesting that direct activation of PI3K by Irs-2 is not required for regulating Glut1 expression on the cell surface (Figure 3.8D). Moreover, Gsk-3β inhibition did not significantly increase Glut1 surface levels (Figure
Figure 3.8. Irs-2 regulates aerobic glycolysis through Gsk-3β inhibition.

A. Glucose uptake (mM/mg protein/

B. Lactate production (mM/mg protein/

C. Western blot analysis showing the expression levels of Irs-2, Glut1, Tubulin, α6, and Glut1 under different conditions.

D. Western blot analysis showing the effect of SB 216763 on the expression of Irs-2, Glut1, Tubulin, α6, and Glut1.

E. Bar graph showing the relative Glut1 cell surface expression under different conditions.
**Figure 3.8. Irs-2 regulates aerobic glycolysis through Gsk-3β inhibition.** Cells were grown in 0.1% BSA/DMEM supplemented with IGF-1 (20ng/mL) with or without the Gsk-3β inhibitor SB-216763 (10µM) for 24 hrs. Glucose uptake (A) and lactate production (B) were measured and normalized to total protein concentration. The data shown are expressed as a rate measurement (mM/mg/hr) and represent the mean (±SEM) of five independent experiments. (C and D) The cell surface was biotinylated and cell surface proteins were isolated using Neutravidin beads. Avidin interacting proteins were immunoblotted with antibodies that recognize the α6 integrin subunit and Glut1. Total cell lysates from biotinylated cells were immunoblotted for antibodies that recognize Irs-2, Glut1, and Tubulin. (E) The data shown represent the mean (±SEM) of 2 independent experiments from (D).
Therefore, the regulation of Gsk-3β by Irs-2-dependent PI3K signaling likely impacts glucose uptake through a mechanism independent of Glut1 surface expression.
DISCUSSION

In the current study, we investigated the mechanism by which Irs-2 recruits and activates PI3K and demonstrated the requirement of Irs-2-dependent PI3K signaling for breast carcinoma cell metabolism. Our work identifies specific tyrosine residues within Irs-2 that are essential for its association with PI3K and provides a mechanistic basis for the amplification of PI3K signaling downstream of this adaptor protein. Irs-2-dependent PI3K signaling promotes glucose uptake and lactate production, two measures of aerobic glycolysis. The regulation of these functions is specific for Irs-2, as genetically identical mammary tumor cells expressing only Irs-1, which can also activate PI3K, are diminished in their glycolytic capacity. Although global Akt activation was not diminished in the absence of Irs-2-dependent PI3K activation, a reduction in the phosphorylation of specific Akt substrates was observed, indicating that Irs-1 and Irs-2 regulate unique subsets of Akt effectors. Inhibition of one of these Irs-2-specific Akt effectors, Gsk-3β, is required for the regulation of glucose uptake and lactate production.

In summary, our data reveal a novel mechanism by which Irs-2 signaling preferentially regulates tumor cell metabolism and adds to our understanding of how this adaptor protein contributes to breast cancer progression.

Aerobic glycolysis is a hallmark of aggressive tumor cells that supports rapid proliferation, survival and invasion (4,255). The importance of this altered tumor cell metabolism is underscored by the fact that aerobic glycolysis is regulated by many oncogenic and tumor suppressor pathways that promote tumor progression. In previous
work, we reported that signaling through Irs-2 regulates mammary tumor cell glycolysis through the control of Glut1 surface expression, which was dependent upon PI3K and mTor pathway activation (108). We now provide evidence that the direct activation of PI3K by Irs-2 is required for regulating aerobic glycolysis and demonstrate that specific downstream effectors of this pathway control glucose uptake, the rate-limiting step in glycolysis. Glut1 surface expression is decreased in cells lacking Irs-2, but not in cells expressing an Irs-2 mutant with a significantly reduced ability to stimulate PI3K signaling, indicating that Irs-2-dependent PI3K activation is not required for Glut1 surface localization. However it is possible that the low level of Irs-2/PI3K/Akt signaling retained by the Irs-2 Y5F mutant (~15%) is sufficient to regulate Glut1 surface localization. Generation of an Irs-2 mutant that is completely deficient in PI3K recruitment is required to address this potential mechanism of regulation. Of note, inhibition of Gsk-3β activity rescues glucose uptake, but does not increase Glut1 expression on the cell surface. Taken together with our previous study that implicated mTorc1 in the regulation of Glut1 surface expression, our data support that Irs-2 coordinates the activation of signaling pathways to promote Glut1 expression on the cell surface and stimulate optimal uptake of glucose and aerobic glycolysis (108). Given that Irs-2 is a hypoxia-regulated gene and its expression is required to sustain Akt activation in hypoxic environments, Irs-2-dependent PI3K signaling likely plays a key role in controlling glucose uptake and glycolytic metabolism in both normoxic and hypoxic tumor microenvironments (41).
We identified four individual tyrosine residues within Irs-2 that are required for the recruitment and activation of PI3K in response to both IGF-1 and insulin stimulation. These tyrosines had previously been reported to be phosphorylated in response to insulin stimulation, but their involvement in recruiting PI3K had not been assessed (82). Likewise, each of these tyrosines had been shown to have the potential to bind to PI3K in a phosphopeptide pull-down proteomics screen (74). However, this screen also implicated Y628 and Y758, which do not contribute to PI3K association with Irs-2 upon stimulation of mammary tumor cells with either IGF-1 or insulin. A previous study had reported that Y628 inhibits Irs-2 tyrosine phosphorylation in response to insulin, but not IGF-1 stimulation (63). We did not observe any difference in the phosphorylation of Irs-2 in response to either insulin or IGF-1 stimulation when this site was mutated, suggesting a cell context dependent role for this regulation. Combined mutation of all of the tyrosines located within canonical PI3K binding motifs did not completely inhibit PI3K association with Irs-2 or downstream activation of Akt signaling. Although the tyrosines we identified play a dominant role in recruiting PI3K (>90%), additional tyrosine residues within Irs-2 may recruit PI3K through a non-canonical interaction. Additionally, PI3K recruitment may occur through an indirect interaction with another binding protein. A rigorous study of Irs-2 interacting proteins will be necessary to identify these alternative mechanisms of PI3K recruitment.

The regulatory subunit of PI3K, p85, contains two SH2 domains that must be engaged by phosphorylated tyrosines to induce a conformational change, leading to disinhibition and activation of the p110 catalytic subunit (126). Although binding to one
SH2 domain can partially activate PI3K, binding of both SH2 domains promotes maximal kinase activity. We implicate four tyrosines in the association of Irs-2 with PI3K and based on primary sequence alone, Y649/Y671 and Y734/Y814 could be predicted to form docking sites for two tandem p85 SH2 domains. However, Irs-2 is an intrinsically disordered protein with minimal structural information available to guide predictions of physical proximity (256). Therefore, additional potential pairings are possible both in cis and trans, given that the IGF-1 and insulin receptors are dimeric and two Irs proteins can be recruited simultaneously (257). When comparing Irs-1 with Irs-2, only two of the four tyrosines implicated in Irs-2-dependent PI3K activation have corresponding residues within Irs-1. Y612 and Y628 (human numbering) in Irs-1, which correspond to Y649 and Y671 in Irs-2, mediate full PI3K recruitment, suggesting that only a single PI3K heterodimer is recruited to Irs-1 (83). Y734 and Y814 do not share homologous sites within Irs-1 and individual mutation of Y814 had the most significant impact on the association of Irs-2 with PI3K. Therefore, Y734 and Y814 may be determining factors in the enhanced activation of PI3K by Irs-2.

Our study reveals a novel mechanism for how Irs-1 and Irs-2 differentially regulate tumor cell metabolism and contribute to breast cancer progression. Both Irs-1 and Irs-2 can recruit and activate PI3K to promote Akt signaling when stimulated with IGF-1, yet the functional outcomes that result from this activation are different. Irs-1 and Irs-2 have distinct subcellular localization patterns in human tumors, with Irs-1 localized to both the cytoplasm and nucleus, and Irs-2 to the cytoplasm and at the cell membrane (96,97). Irs-2 membrane localization is a predictor of reduced breast cancer patient
overall survival, while Irs-1 nuclear localization is associated with tumors that are more differentiated and non-metastatic, and more sensitive to tamoxifen response (96,98). These studies reveal that differences in the localization of Irs-1 and Irs-2 impact outcomes in human cancer and point toward localization as being a key determinant of the function of these adaptor proteins. Localizing to distinct intracellular compartments would not only determine access to unique subsets of downstream effectors, but also to the substrates of these effectors to impact cell function. With regard to Irs-2, we hypothesize that activation of Akt at the plasma membrane would target inhibition of Gsk-3β where it could regulate glucose uptake. We identify Gsk-3β as one Akt target that is preferentially regulated by Irs-2-dependent PI3K signaling. Additional Akt effectors that are selectively regulated by this signaling pathway may also play a role in metabolism and other functions regulated by Irs-2. In this regard, Foxo1 is preferentially regulated by Irs-2 because PI3K is not sufficiently activated by Irs-1 to support this regulation (258). The PI3K kinase signal is likely enhanced when activated through Irs-2 because it has a greater potential for PI3K recruitment. The enhanced activation of mTorc1, Gsk-3β and Foxo1, key regulators of metabolism, confirms the role of Irs-2 in metabolic regulation and highlights the importance of this signaling pathway for tumor progression.
ACKNOWLEDGEMENTS

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CHAPTER IV

A Novel Model System to Study the IRS Proteins: Differential Activation of Signaling and Regulation of Cell Functions by IRS-1 and IRS-2

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ABSTRACT

The Insulin Receptor Substrate (IRS) proteins, IRS-1 and IRS-2, are the primary mediators of insulin-stimulated growth and glucose homeostasis. Studies of IRS-1 and IRS-2 have shown that they do not function interchangeably in both normal cellular biology and in tumor progression. Specifically in studies of breast cancer, IRS-1 promotes growth and proliferation, while IRS-2 promotes motility, invasion, survival, aerobic glycolysis and metastasis. However, many of these studies have been performed in model systems that limit the conclusions that can be drawn about the differences between IRS-1 and IRS-2. We have generated a novel model system that allows for the rigorous comparison of IRS-1- and IRS-2-mediated signaling and functions in mammary tumor cells. We have generated cells from PyMT:Irs-1fl/flIrs-2fl/fl mouse mammary tumors and knocked-out Irs-1 and Irs-2 expression in vitro using Cre-recombinase. This made it possible to express IRS-1 or IRS-2 in a genetically matched background and allowed for the rigorous comparison of their function. Using these cells, we have confirmed a role for IRS-1 in tumor cell growth and a role for IRS-2 in tumor cell invasion. Additionally, our data suggest that IRS-2 can more robustly stimulate Akt phosphorylation on Thr308 and Ser473. This study supports that this model system is suitable for further investigation of IRS-1- and IRS-2-specific functions.
INTRODUCTION

The Insulin Receptor Substrate (IRS) proteins are cytoplasmic adaptors that function to recruit and activate downstream signaling effectors following upstream receptor stimulation (33). The IRS family of proteins has four members, IRS-1-4, and only IRS-1 and IRS-2 are ubiquitously expressed in both humans and rodents (33). In contrast, Irs-3 is expressed only in rodents and IRS-4 is expressed only in the brain, kidney, thymus, and liver (34-36). Mouse models of whole body knockout of each of the Irs proteins suggest that despite having a similar structure they do not function interchangeably. Irs-1-/− mice are born runted, develop insulin resistance, but do not go on to develop diabetes. However, Irs-2-/− mice are infertile, display brain developmental defects and develop insulin resistance, ultimately progressing to diabetes due to β-cell failure (84-87). In contrast to Irs-1 and Irs-2, Irs-3-/− mice are born normal size and do not display any abnormalities in glucose homeostasis, while Irs-4-/− mice display only mild effects on growth, reproduction and glucose homeostasis (259,260). From these studies it has been concluded that Irs-1 and Irs-2 are the primary mediators of growth and insulin-dependent glucose homeostasis.

In vitro experiments have confirmed the distinct functions of the Irs proteins. In Irs-1-/− mouse embryonic fibroblasts (MEFs), cell proliferation is reduced relative to wild type (WT) controls, suggesting that endogenous expression of Irs-2 in Irs-1-/− cells is insufficient to promote proliferation (88). The role of Irs-1 in the regulation of proliferation has been confirmed in L6 myotubes, as knockdown of Irs-1, but not Irs-2,
decreases proliferation. Additionally, the effects of signaling mediated by the Irs proteins on metabolism have been shown to be cell context dependent. For example, in adipocytes Irs-2 promotes Glut4 translocation to the plasma membrane, whereas in L6 myotubes, Irs-1 promotes Glut4 translocation (89,91). Together, these data suggest non-redundant, cell context specific roles of the Irs proteins.

Studies of the IRS proteins in cancer have also suggested that they do not function interchangeably in all steps of tumor initiation and progression, with the most significant data coming from breast cancer. Overexpression of either IRS-1 or IRS-2 leads to disrupted acini formation of MCF10A cells in 3D matrigel assays and in vivo, mammary specific, transgenic overexpression of either IRS-1 or IRS-2 promotes mammary tumor initiation and pulmonary metastasis (99). Together, these experiments suggest that both IRS-1 and IRS-2 are capable of promoting tumor initiation. However, in other stages of tumor progression, the IRS proteins function in a non-redundant manner as shown by in vivo experiments using Irs-1-/ and Irs-2-/ mice crossed with the PyMT mouse model of mammary tumor progression. In this model, tumor growth was not impaired in PyMT:Irs-1- or PyMT:Irs-2- mice relative to their PyMT:WT counterparts (105,106). However, Irs-1- tumors had increased expression and activation of Irs-2 as well as increased angiogenesis and significantly increased pulmonary metastasis (105,107). In contrast, Irs-2- tumors were more apoptotic and had decreased metastasis relative to their WT counterparts (105,106). Additionally, in vitro studies using breast cancer cell lines have suggested that overexpression of IRS-1 promotes IGF-1-dependent growth and proliferation only in estrogen receptor + (ER+) breast cancer cell lines that predominantly
express IRS-1, but not ER- cell lines that express predominantly IRS-2 (40,100-102). In contrast, IGF-1 signaling mediated by IRS-2 promotes adhesion, motility, invasion, and aerobic glycolysis regardless of ER expression status (102,103,108). These data show divergent functional outcomes of either IRS-1 or IRS-2-mediated signaling in breast cancer.

Despite their differential functions in normal biology and cancer, the mechanism by which the IRS proteins regulate these distinct functions remains unknown (93). Both IRS-1 and IRS-2 mediate signal transduction downstream of the Insulin Receptor (IR) and the Insulin-Like Growth Factor-1 Receptor (IGF-1R) (42-50). They have also both been shown to function downstream of additional receptors including the interleukin-4 (IL-4) receptor and certain integrins (50-53). Following either IR or IGF-1R activation, the IRS proteins are recruited via their Plextrin Homology (PH) and Phosphotyrosine Binding Domains (PTB) domains where they share the highest level of homology and are subsequently phosphorylated by the receptor on tyrosine residues in their C-termini (24,43,49,59-61). This creates docking sites for downstream effectors including Phosphatidylinositol-3-Kinase (PI3K), Growth factor receptor-bound protein 2 (GRB2) and Src-Homology 2 Domain Containing Protein Tyrosine Phosphatase 2 (SHP2), leading to activation of the PI3K and Mitogen Activated Protein Kinase (MAPK) signaling pathways (59,65,67,68). In addition to tyrosine residues, the IRS proteins are also phosphorylated on serine residues, some of which inhibit their ability to recruit and activate PI3K signaling (75). The outcome of serine phosphorylation on IRS-1 has been more extensively studied, while less is known about IRS-2 serine phosphorylation. Not
all tyrosine and serine residues are conserved between the IRS proteins and these differences may confer unique protein associations with IRS-1 and IRS-2 (81). In fact, a phosphoproteomic study of all the tyrosine residues in IRS-1 and IRS-2 suggest that they have unique binding partners (74). Together, these differences between IRS-1 and IRS-2 may mediate the differential functional outcomes with which they have been associated. IRS-2 also contains a unique region called the kinase regulatory loop-binding (KRLB) region that associates with the IR and limits the receptor’s tyrosine kinase activity (62-64). IRS-1 does not contain a KRLB region and this difference may also contribute to the differences in functional outcomes of signaling downstream of IRS-1 and IRS-2.

The differential localization of the IRS proteins mediated by unique interacting partners may also play a role in determining some of their discrete functional outcomes. IRS-1 has been shown to localize to both the cytoplasm and nucleus, while IRS-2 strictly localizes to the cytoplasm with some studies reporting localization of IRS-2 at the plasma membrane (96,97). These localization patterns have been shown to correlate with breast cancer patient prognosis. Specifically, IRS-1 nuclear staining predicts increased response to tamoxifen treatment, while IRS-2 plasma membrane staining predicts decreased overall survival (96,98).

The current models that exist to study the IRS proteins are not ideal for rigorous comparison of IRS-1 and IRS-2 specific functions. Cell lines derived from Irs-1−/− and Irs-2−/− mouse tissues and tumors have given great insight into the differential functions of the IRS proteins, but these cell lines are not genetically matched and limit the conclusions that can be made from their comparison (88,89,91,108). The use of breast carcinoma cell
lines has helped to define the roles of the IRS proteins in breast cancer (40,100-103). However, these discoveries have been based on either overexpression or knockdown of either IRS-1 or IRS-2. It has been well established that protein overexpression studies are complicated by the fact that high levels of protein expression may promote functions that are not observed with endogenous levels of expression. Additionally, many of these overexpression studies have been done in such a way that IRS-1 and IRS-2 expression cannot be compared (99,102). Knockdown studies, while useful because either IRS-1 or IRS-2 expression can be knocked down in the same cell line, are limited by the fact that protein levels are only reduced, and not completely eliminated (101,103). In addition, all experiments examining IRS-1 specific functions have been done in a background of IRS-2 expression and vice-versa. Therefore, the effects attributed to either IRS-1 or IRS-2 have not been shown independently of expression of the other IRS protein.

The purpose of the current study was to generate a model system that would allow for the rigorous comparison of IRS-1 and IRS-2 signaling and functions. This model can be used for future identification of novel mechanisms of IRS-1 and IRS-2 action that underlie their unique functions.
RESULTS

**Generation of a model system to compare IRS-1 and IRS-2 function**

To establish a genetically matched model system to investigate IRS-1 and IRS-2-mediated signaling and functions independent of the other, we derived cell lines from PyMT:Irso/Irso mammary tumors and acutely deleted Irs-1 and Irs-2 expression *in vitro* by transient adenoviral infection of Cre-recombinase. The resulting population of PyMT:Irso/Irso cells were single-cell subcloned to generate cell lines with complete loss of Irs-1 and Irs-2 expression. HA-tagged IRS-1 (IRS-1-HA) and IRS-2 (IRS-2-HA) were stably expressed in five distinct PyMT:Irso/Irso subclones. IRS-1, IRS-2 and HA protein expression were assessed by western blot (Figure 4.1). The use of HA-tagged IRS constructs allowed for direct comparison of IRS expression levels. Levels of IRS-1 and IRS-2 expression were equivalent in all five subclones (Figure 4.1). PyMT:Irso/Irso subclones A and D were chosen for further analysis, as they have the highest level of Igf-1r expression (Figure 2.3E).

**IRS-mediated PI3K Activation in Response to IGF-1 stimulation**

In response to IGF-1 stimulation, the IRS proteins are phosphorylated on tyrosine residues in their C-termini to facilitate recruitment and activation of downstream signaling molecules (33). To compare the tyrosine phosphorylation of IRS-1 and IRS-2,
Figure 4.1. Stable expression of IRS-1-HA or IRS-2-HA in PyMT:Irs-1/−/Irs-2/− cells.
Figure 4.1. Stable expression of IRS-1-HA or IRS-2-HA in PyMT:Irs-1⁺/⁻/Irs-2⁺/⁻ cells. Five PyMT:Irs-1⁺/⁻/Irs-2⁺/⁻ clones (A-E) were engineered to stably express empty vector (EV), IRS-1-HA or IRS-2-HA. Following selection, aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize IRS-1, IRS-2, HA, and Tubulin.
*PyMT*: *Irs-1*<sup>-/-</sup>/*Irs-2*<sup>-/-</sup> subclones A and D stably expressing either IRS-1-HA or IRS-2-HA were stimulated with IGF-1 (Figure 4.2A). Each displayed tyrosine phosphorylation within two minutes of IGF-1 stimulation. Phosphorylation levels began to decrease after 10 minutes of stimulation for both IRS-1 and IRS-2. Relative tyrosine phosphorylation of IRS-1 was higher when compared with IRS-2 over the time course of IGF-1 stimulation, however this difference was not statistically significant (Figure 4.2B).

Specific phosphorylated tyrosines within consensus PI3K binding motifs (YxxM) are required for association of the IRS proteins with PI3K following insulin and IGF-1 stimulation (83,261). The interaction of src homology-2 (SH2) domains within the p85 regulatory subunit of PI3K with pYxxM motifs in the IRS proteins leads to disinhibition of the p110 catalytic subunit and renders the kinase active (72,73,122,124-126). This leads to generation of the phosphoinositide-3,4,5-P<sub>3</sub> (PtdIns-3,4,5-P<sub>3</sub>) at the plasma membrane, promoting recruitment and phosphorylation of the PI3K effector Akt (132-134,165). Our previous study identified four tyrosine residues in Irs-2 that participate in its interaction with PI3K, while IRS-1 requires only two tyrosines to mediate full PI3K activity (261). However, a side-by-side comparison of the ability of the IRS proteins to recruit PI3K has yet to be undertaken (83,261). To rigorously compare the ability of the IRS proteins to recruit PI3K, IRS-1 and IRS-2 were assayed for their recruitment of p85, the regulatory subunit of PI3K. p85 co-immunoprecipitated (co-IP) with both IRS-1 and IRS-2 following IGF-1 stimulation (Figure 4.2A). p85 was recruited to IRS-1 and IRS-2 within 2 minutes of IGF-1 stimulation and began to decrease following 10 minutes of stimulation. Consistent with its higher level of tyrosine phosphorylation, IRS-1 also
Figure 4.2. Comparison of the ability of IRS-1 and IRS-2 to associate with PI3K.

A.

<table>
<thead>
<tr>
<th>Irs-1&lt;sup&gt;−/−&lt;/sup&gt;/Irs-2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>IRS-1-HA</th>
<th>IRS-2-HA</th>
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<tr>
<td></td>
<td>0 2 10 30 60 120</td>
<td>0 2 10 30 60 120</td>
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<td>Pull Down: HA</td>
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B.

C.

n.s.
Figure 4.2. Comparison of the ability of IRS-1 and IRS-2 to associate with PI3K.

(A-C) *Irs-1*−/*Irs-2*− D cells stably expressing IRS-1-HA or IRS-HA were serum deprived and then stimulated with IGF-1 (100ng/ml) for 2, 10, 30, 60, and 120 minutes. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoprecipitated with HA-specific antibodies conjugated to agarose beads and the immune complexes were immunoblotted with antibodies that recognize either phosphotyrosine (p-Tyr) or p85α. The p-Tyr blots were stripped and re-probed for Irs-2. The data shown represent the mean (+SEM) of three (B; Tyrosine phosphorylation) or four (C; p85 association) independent experiments.
exhibited higher levels of p85 association relative to IRS-2, but this increase was also not statistically significant (Figure 4.2C).

**Akt activation downstream of IRS-1 or IRS-2**

Previous experiments have demonstrated that Irs-1 can compensate for the loss of Irs-2-mediated PI3K activation of Akt following Cre-mediated knockout of Irs-2 expression, as these cells do not display reduced IGF-1-mediated Akt phosphorylation (261). To compare the ability of IRS-1 and IRS-2 to mediate Akt activation, cells expressing vector control, IRS-1-HA or IRS-2-HA were stimulated with IGF-1 and assayed for their ability to promote Akt activation. Regardless of IRS expression status, Igf-1r phosphorylation was equivalent across the panel of cells lines following IGF-1 stimulation and was sustained during the time course of stimulation (Figure 4.3A and D). Following IGF-1 stimulation, phosphorylation of Akt at Ser473 or Thr308 was not induced in vector control cells (Figure 4.3A and D). This confirms that the Igf-1r requires the IRS proteins to mediate activation of PI3K/Akt signaling (129,130). In contrast, IRS-1-HA and IRS-2-HA expressing cells show an increase in both Ser473 and Thr308 phosphorylation in response to IGF-1 stimulation (Figure 4.3A-F). However, phosphorylation of both sites is higher in both subclones expressing IRS-2-HA relative to cells expressing IRS-1-HA and these data are statistically significant for subclone D (Figure 4.3A-F). In addition to Akt phosphorylation, S6 Kinase (S6k) phosphorylation was increased in cells expressing IRS-2-HA relative to cells expressing
Figure 4.3. Comparison of IRS-1- and IRS-2-mediated signaling.
Figure 4.3. Comparison of IRS-1- and IRS-2-mediated signaling. (A,D) Irs-1<sup>-/-</sup>/Irs-2<sup>-/-</sup> A and D subclones stably expressing pcDNA vector, IRS-1-HA or IRS-HA were serum deprived and then stimulated with IGF-1 (100ng/ml) for 30, 60, and 120 minutes. Aliquots of cell extracts containing equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-1, Irs-2, pIgf-IR (Tyr1135/1136), Igf-1R, pS6k (Thr389), S6k, pAkt (Thr308, Ser473), Akt, pGsk-3β (Ser9), Gsk-3β, and Tubulin. The data shown in (B) and (C) represent the mean (±SEM) of three independent experiments. The data shown in (E) and (F) represent the mean (±SEM) of four independent experiments. *, p<0.035.
IRS-1-HA (Figure 4.3A and D). These data show that the increased phosphorylation of Akt downstream of IRS-2 correlates with its increased kinase activity, as S6k is a downstream effector of Akt and the mechanistic target of rapamycin complex-1 (mTorc1). Additionally, we previously identified Glycogen synthase kinase-3β (Gsk-3β) as an Irs-2-specific Akt substrate (261). While IRS-1 mediated signaling induced a modest phosphorylation of Gsk-3β in response to IGF-1 stimulation, phosphorylation of this Akt effector downstream of IRS-2 was significantly more robust (Figure 4.3A and D).

Akt exists as three isoforms, Akt 1-3, which are highly homologous but, not unlike the IRS proteins, do not function interchangeably in cancer (262). Previous studies of IRS-1 and IRS-2 signaling have suggested that the IRS proteins may differentially regulate Akt1 and Akt2 (89,263). One study suggested that IRS-1 and Akt2 may function together to regulate glucose metabolism, while IRS-2 and Akt1 function to regulate lipid metabolism (263). However, in an independent study, Irs-1 was shown to regulate Akt1, while Irs-2 regulated Akt2 (89). To determine if the IRS proteins differentially activate either Akt1 or Akt2, each of these isoforms were immunoprecipitated following IGF-1 stimulation and the level of phosphorylation of Thr308 and Ser473 was measured. As had been observed for the analysis of total Akt phosphorylation (Figure 4.4), IGF-1-stimulated phosphorylation of Akt requires expression of the IRS proteins. However, phosphorylation of Akt1 on Thr308 is higher downstream of IRS-1 than downstream of IRS-2. These results are preliminary and require further investigation for validation. However, these data do indicate that
Figure 4.4. Akt1 and Akt2 phosphorylation mediated by IRS-1 or IRS-2.
Figure 4.4. Akt1 and Akt2 phosphorylation mediated by IRS-1 or IRS-2. *Irs-1*+/−/*Irs-2*+/− D subclones stably expressing pcDNA vector, IRS-1-HA or IRS-HA were serum deprived and then stimulated with IGF-1 (100ng/ml) for 30, 60, and 120 minutes. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoprecipitated with Akt1 or Akt2 specific antibodies and the immune complexes were immunoblotted with antibodies that recognize either pAkt (Thr308, Ser473), Akt1 or Akt2.
expression of either IRS-1 or IRS-2 is sufficient to promote phosphorylation of either Akt1 or Akt2 in response to IGF-1 stimulation.

IRS-1 promotes growth in 3D Matrigel

Previous studies of the IRS proteins have suggested that IRS-1, but not IRS-2, functions to promote growth and proliferation. Only one of these studies directly compared the ability of IRS-1 and IRS-2 to promote proliferation (102). However, this study used expression of untagged IRS proteins and thus relative IRS expression could not be determined. Therefore, these experiments do not exclude the possibility that the ability of IRS-1 to promote proliferation was dependent on its expression being higher than expression of IRS-2. Using our model system where IRS expression is equivalent, we sought to compare the ability of IRS-1 and IRS-2 to regulate cell growth. Standard 2D assays showed no difference in growth rates when comparing PyMT:Irs-1−/−/Irs-2−/− subclones expressing vector control, IRS-1-HA or IRS-2-HA (data not shown). However, the ability of IRS-1 to stimulate IGF-1 dependent growth is this type of assay has been previously shown to be dependent on ER expression (40). Given that MMTV-PyMT mammary tumors and cell lines derived from these tumors are ER-, our results recapitulate these prior experiments (107,242).

To further assess the role of the IRS proteins in the regulation of cell growth, PyMT:Irs-1+/−/Irs-2+/− cells expressing vector control, IRS-1-HA or IRS-2-HA were subjected to 3D-matrigel growth assays in the presence IGF-1. Following nine days in
culture, the number and size of the colonies formed was determined. In both PyMT:Irs-1–/Irs-2– subclones expressing IRS-1-HA, but not IRS-2-HA, the size of the colonies relative to vector control cells was significantly increased (Figure 4.5A and B). However, the number of colonies formed under IGF-1 stimulated conditions was similar in cells expressing vector control, IRS-1-HA or IRS-2-HA (Figure 4.5A and C). Despite observing no difference in the proliferative ability of these cells in a 2D growth assay, 3D growth assays clearly show that IRS-1 promotes mammary tumor cell growth.

IRS-2 promotes invasion

Multiple studies have suggested that IRS-2, but not IRS-1, positively regulates motility and invasion (102,103,108). Similar to experiments that suggested a role for IRS-1 in proliferation, studies of the regulation of motility and invasion by IRS-2 were done using untagged versions of the IRS proteins (102). Therefore, these experiments do not exclude the possibility that the ability of IRS-2 to promote motility and invasion was dependent on its expression being higher than expression of IRS-1. Other experiments have also shown increased invasion in cells derived from PyMT:Irs-1– tumors relative to those derived from PyMT:Irs-2– tumors (108). However, these cell lines are not genetically matched and it is possible that additional factors could mediate these differences. One additional study using metastatic variants of breast cancer cell lines determined that there was a correlation between increased IRS-2 tyrosine phosphorylation and invasion; however, the ability of IRS-1 to promote invasion was
Figure 4.5. IRS-1 expression promotes cell growth in 3D.
Figure 4.5. IRS-1 expression promotes cell growth in 3D. (A) Equivalent numbers of *Irs-1*−/*Irs-2*− A and D subclones stably expressing pcDNA vector, IRS-1-HA or IRS-HA were grown in a 3D matrix composed of Matrigel in the presence of IGF-1 (10ng/ml). The data shown for colony size (B) and colony number (B) represents the mean (±SEM) of three independent experiments. *, p<0.03, **, p<0.05.
never addressed (103). To assess the ability of the IRS proteins to promote invasion, *PyMT:Ir*rs-1\(^{-/-}\)/Ir*rs-2\(^{-/-}\) cells expressing vector control, IRS-1-HA or IRS-2-HA were assayed using a matrigel transwell invasion assay. Expression of IRS-2, but not IRS-1, significantly increased the invasive ability of cells relative to vector control cells (Figure 4.6A). These data confirm a role for IRS-2 in mediating invasion in mammary tumor cells.

IRS-2 also preferentially increases aerobic glycolysis in mouse mammary tumor cell lines (108). As described above, the original WT, Ir*rs-1\(^{-/-}\) and Ir*rs-2\(^{-/-}\) cell lines used for these studies were not genetically identical. To directly compare the contribution of the IRS proteins to the regulation of aerobic glycolysis, glucose uptake and lactate production were measured in our genetically matched model system. Expression of IRS-2-HA, but not IRS-1-HA, significantly increased glucose uptake, while both IRS-1-HA and IRS-2-HA significantly increased lactate production (Figure 4.6B and C). Of note, in prior studies in which we expressed murine Ir*rs-2 in subclone A, a more significant increase in both glucose uptake and lactate production were observed in response to Ir*rs-2 expression (Chapter 3, Figure 3.5D and E). These data suggest that there may be species differences between the human and murine IRS-2 genes.
Figure 4.6. IRS-2 expression promotes invasion.

A. Number of Invaded Cells

B. Glucose Uptake and Lactate Production

C. Glucose Uptake and Lactate Production
Figure 4.6. IRS-2 expression promotes invasion.  (A) Equivalent numbers of *Irs-1*<sup>−/−</sup> *Irs-2*<sup>−/−</sup> D cells stably expressing pcDNA vector, IRS-1-HA or IRS-2-HA were subjected to a Transwell invasion assay for 4hrs.  The data shown represent the mean (+SEM) of five independent experiments.  *, p<0.0003.  *Irs-1*<sup>−/−</sup>*Irs-2*<sup>−/−</sup> A (B) and D (C) subclones stably expressing pcDNA vector, IRS-1-HA or IRS-HA were grown in 0.1% BSA/DMEM supplemented with IGF-1 (20ng/mL) for 24 hrs.  Glucose uptake and lactate production were measured and normalized to total protein concentration.  The data shown are expressed as a rate measurement (mM/mg/hr) and represent the mean (+SEM) of three (A) or five (D) independent experiments.  *, p<0.04 relative to pcDNA.
In the study presented here, we have performed a rigorous comparative study of IRS-1 and IRS-2-mediated signaling and functions. This study has confirmed that IRS-1 and IRS-2 do not function interchangeably in the activation of PI3K signaling and in the regulation of growth and invasion. Despite IRS-1 sustaining a marginally increased ability to recruit PI3K, Akt phosphorylation and activity are significantly increased downstream of IRS-2. These data suggest that IRS-2 is a more potent activator of Akt and this may contribute to the functional differences associated with the IRS proteins. Both IRS-1 and IRS-2 can mediate activation of Akt, but specific functions regulated by Akt can be attributed either to IRS-1 or IRS-2 in breast cancer. For example, IRS-1 stimulates growth and IRS-2 stimulates invasion and metabolism, but Akt can regulate all of these functions. Interestingly, the Akt effector FOXO1 is preferentially phosphorylated downstream of IRS-2, as the magnitude of Akt activation downstream of IRS-1 is insufficient to promote its phosphorylation (258). This increased level of Akt phosphorylation downstream of IRS-2 is then sufficient to promote glucose uptake. These findings suggest that some of the specific functions associated with IRS-2 may correlate with the magnitude of Akt activation when signals are initiated through this adaptor.

Differential phosphorylation of Akt1 may allow IRS-1 and IRS-2 to promote discrete functions. While both IRS-1 and IRS-2 are capable of facilitating Akt1 and Akt2 phosphorylation, IRS-1 promotes a higher level of Akt1 Thr308 phosphorylation. This
result is exciting as correlations can clearly be made between functions that are associated with IRS-1 and Akt1. Specifically, \( Irs-1^{-/-} \) or \( Akt1{-/-} \) mice are born runted and both Irs-1 and Akt1 have been suggested to suppress metastasis in mouse models of breast cancer \( (85,105,194,212) \). Therefore, it is interesting to consider that both the magnitude of Akt activation as well as the regulation of specific Akt isoforms are mechanisms by which IRS-1 and IRS-2 can regulate differential functions.

Based on our previous work, we hypothesized that IRS-2 would have an increased ability to recruit PI3K. We suggested that Irs-2 may have the ability to associate with two PI3K heterodimers, while studies from another laboratory suggested that Irs-1 has the ability to recruit only one PI3K heterodimer \( (261) \). In the current study IRS-1 displayed a slight increase in the ability to recruit PI3K relative to IRS-2. Despite this increase, Akt signaling is significantly increased downstream of IRS-2 relative to IRS-1. Following ligand binding, the IGF-1R is known to be internalized and traffic through the endosomal compartment, leading to its degradation or recycling back to the cell surface \( (264) \). Recycling of the IGF-1R back to the cell surface promotes sustained Akt activation in response to IGF-1 stimulation. IGF-1R association with IRS-2 may promote its recycling back to the cell surface, facilitating increased Akt phosphorylation. In contrast, IRS-1 may not promote this recycling step and therefore Akt activation may not be sustained. A differential ability of IRS-1 and IRS-2 to regulate the trafficking of the IGF-1R and elevate Akt signaling could be one of the determining factors in the differential outcomes of IRS-mediated signaling.
Recent studies have implicated the differential localization of the IRS proteins in mediating some of their discrete functional outcomes. IRS-1 localizes to both the cytoplasm and nucleus, while IRS-2 strictly localizes to the cytoplasm with some studies reporting localization of IRS-2 at the plasma membrane (96,97). These localization patterns have been shown to correlate with breast cancer patient prognosis. Specifically, IRS-1 nuclear staining predicts increased response to tamoxifen treatment, while IRS-2 plasma membrane staining predicts decreased overall survival (96,98). We have shown that IRS-1, and not IRS-2, promotes growth in 3D and the ability of IRS-1 to regulate growth may be due to its localization to the nucleus. It has been suggested that IRS-1 regulates growth through increased transcription of factors that are involved in cell cycle progression. IRS-1 been shown to associate with ERα in the nucleus and to promote Cyclin D1 transcription (265,266). The specific localization of IRS-1 to the nucleus, and not IRS-2, may explain how IRS-1 promotes growth, as it alone has the ability to promote transcription of cell cycle related genes. Previous studies investigating the role of IRS induced growth have been limited by the model systems that were used to address this question. Specifically, one study addressed the contribution of IRS-1 and Shc, but not IRS-2, in the regulation of human breast carcinoma cell growth (101). Here we have shown that IRS-1 promotes growth in a genetically matched model system in which we can confirm that the level of IRS-1 and IRS-2 expression are equivalent.

A role for IRS-2, but not IRS-1, in the regulation of invasion has been reported previously. Like the studies of the involvement of the IRS proteins in growth regulation, investigations of invasion have been limited by the model systems that were available at
the time the studies were undertaken. Specifically, the same study that used overexpression to show IRS-1 promotes proliferation while IRS-2 is unable to, additionally suggested that IRS-2, and not IRS-1, promotes invasion (102). Here we have confirmed these findings using a system where the ability of IRS-1 and IRS-2 to stimulate invasion could be compared with known equivalent expression levels. It is possible that for invasion to occur, a higher level of Akt phosphorylation is required, thus IRS-2 alone is sufficient to promote this function. As is the case with FOXO1-mediated glucose uptake, invasion may only be stimulated by the high levels of Akt activation that IRS-2, but not IRS-1, can stimulate (258).

We were unable to draw rigorous conclusions about the differential role of IRS-1 and IRS-2 in aerobic glycolysis. Specifically, we observed only a small increase in glucose uptake and lactate production in cells expressing either IRS-1 or IRS-2. Although this effect of the IRS proteins on lactate production was modest, it was statistically significant. However, expression of murine Irs-2 in subclone A increased glucose uptake and lactate production more robustly (Chapter 3). The use of HA-tagged human versions of the IRS proteins in this study could impact their subcellular localization or association with interacting proteins, which may influence their ability to regulate aerobic glycolysis in mammary tumor cells. Additional studies comparing murine Irs-1 and Irs-2 would resolve this question.

Here we have presented a novel model system to compare IRS-1 and IRS-2 specific functions in mammary tumor cells. In this system, IRS-1 and IRS-2 can be studied on a genetically identical background and this is superior to previous models used
to compare the IRS proteins. Using these cells we have confirmed that IRS-1 functions to promote tumor cell growth, while IRS-2 promotes invasion and glucose uptake, suggesting that this model system is suitable for future use in characterizing the distinct roles of the IRS proteins. In this system the specific roles of the IRS proteins in breast cancer can be addressed in a genetically matched background where the other IRS protein is not expressed (i.e. only IRS-1 or only IRS-2 expression). This prevents the contribution of negative feedback loops that could be initiated by either IRS-1 or IRS-2 that could confound results attributing specific functions to the IRS proteins. These cells will be a valuable tool for future comparison of IRS-1 and IRS-2 function and their role in mammary tumor progression.
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CHAPTER V

Discussion
Summary of Findings

In the studies presented in this dissertation, I have investigated the role of Insulin receptor substrate-2 (Irs-2)-dependent Phosphatidylinositol-3-Kinase (PI3K) signaling in breast cancer and more rigorously demonstrated that IRS-1 and IRS-2 do not act interchangeably in cellular functions that are associated with mammary tumor growth and progression. Specifically, I have determined that tyrosines 649, 671, 734, and 814 significantly contribute to the ability of Irs-2 to recruit and activate PI3K signaling in response to Insulin-like growth factor-1 (IGF-1) or insulin stimulation. This information allowed me to generate a PI3K binding-deficient Irs-2 mutant with a significantly impaired ability to recruit and activate PI3K signaling. Using this mutant, I determined that Irs-2-dependent PI3K signaling regulates glucose uptake and lactate production through the inhibition of the Akt effector, Glycogen Synthase Kinase-3β (Gsk-3β). In response to IGF-1 stimulation, Gsk-3β is preferentially phosphorylated downstream of PI3K/Akt signaling mediated by Irs-2, but not Irs-1. Additionally, I determined that the regulation of aerobic glycolysis is not mediated through Gsk-3β-dependent modulation of Glut1 protein expression or cell surface localization.

I also generated and characterized a novel model system that allowed me to perform a controlled comparison of IRS-1 and IRS-2 signaling and function. Using this model, I have demonstrated that in response to IGF-1 stimulation, tyrosine phosphorylation and PI3K association of IRS-1 are marginally higher than they are for IRS-2. However, in response to IGF-1 stimulation, Akt phosphorylation and activation
downstream of IRS-2 are enhanced relative to that observed downstream of IRS-1. Additionally, only expression of IRS-1 is sufficient to mediate increased cell growth, while only IRS-2 expression is sufficient to promote invasive potential. This new model system confirms previous reports regarding IRS-1 and IRS-2 function, but does so in a more rigorous and controlled manner. This model system will allow for future studies to investigate IRS-1 and IRS-2 function in a genetically matched tumor cell background to further characterize and understand the mechanisms of IRS function in tumor progression.

**Differential Akt Activation by IRS-1 and IRS-2**

I have discovered novel ways in which IRS-1 and IRS-2 differ in their function. I have shown that IRS-2 promotes aerobic glycolysis and invasion and has increased ability to activate PI3K/Akt signaling, while IRS-1 promotes growth and stimulates lower levels of Akt activity. In addition, specific Akt effectors are differentially phosphorylated downstream of IRS-1 and IRS-2. Case in point, Gsk-3β, which is predominantly phosphorylated in response to Irs-2-mediated Akt activation.

In early studies of the IRS proteins, IRS-1 was thought to be the predominant substrate phosphorylated in response to insulin and was the primary regulator of insulin functions (267). This was suggested because loss of IRS-1 expression increased the level of IRS-2 tyrosine phosphorylation and IRS-2 phosphorylation was lower in the background of IRS-1 expression. The studies presented here suggest that in mouse
mammary tumor cells IRS-1 only has a slightly increased tyrosine phosphorylation and ability to recruit PI3K when compared directly with IRS-2. Furthermore, increased PI3K recruitment by IRS-1 does not translate into increased phosphorylation of its downstream effector Akt. I hypothesize that one mechanism by which the differential magnitude of Akt phosphorylation is achieved is through differential regulation of Insulin-Like Growth Factor-1 Receptor (IGF-1R) receptor trafficking by IRS-1 and IRS-2. The IGF-1R traffics through the recycling compartment of the endosomal pathway and upon ligand stimulation, it is internalized and eventually recycled back to the cell surface (264). Inhibition of receptor internalization blocks Akt phosphorylation, while inhibition of receptor trafficking blocks receptor recycling and does not promote sustained Akt activation. These results suggest that factors that inhibit the recycling of the IGF-1R through the endosomal compartment could decrease Akt phosphorylation. The IRS proteins could differentially effect this transition and based on the observed increase in Akt phosphorylation, I suggest that IRS-2 could increase recycling of the IGF-1R back to the cell surface.

Following ligand-mediated activation of the IGF-1R, phosphorylation of IRS-2 may preferentially recruit factors involved in trafficking through the recycling compartment of the endosomal pathway that IRS-1 is unable to recruit. Interestingly, the p85 regulatory subunit of PI3K contains a Rab binding domain that associates with the Rab GTPase Rab5 and functions as a Rab GTPase Activating Protein (GAP) (268,269). Rab5-GTP promotes endocytosis and recycling of receptor tyrosine kinases (RTKs) in response to ligand binding (270,271). The GAP activity of p85 would serve to limit
Rab5 activity by promoting the hydrolysis of GTP to GDP; however, Guanine Nucleotide Exchange Factors (GEFs) are also recruited to these vesicles, promoting nucleotide exchange and increased Rab5 activity (269). It would be interesting to investigate the possibility that IRS-2, but not IRS-1, can preferentially recruit GEFs to these compartments and thereby increase trafficking of the IGF-1R through the recycling pathway. In addition to its role as a second messenger, PtdIns-3,4,5-P3 generated by PI3K serves as a substrate for the phosphatases PI4-Pase and PI5-Pase, generating PtdIns-3-P, which serves as a docking site for the early endosome antigen 1 (EEA1) (272). Binding of EEA1 to early endosomes promotes soluble N-ethylmaleimide-sensitive fusion attachment receptor (SNARE)-mediated endosome fusion (273,274). Perhaps IRS-2 promotes trafficking of the IGF-1R through the recruitment of these lipid phosphatases and thus promotes endosome maturation through recruitment of EEA1. Further studies of IRS-1 and IRS-2 interacting proteins would suggest if there is a differential recruitment of such factors. Additionally, it would be interesting to follow the trafficking of the IGF-1R through the early endosomal compartment using total internal reflection fluorescence (TIRF) microscopy in cells expressing either IRS-1 or IRS-2. Potentially, IRS-2 may increase the rate of IGF-1R trafficking, while IRS-1 may not have this ability.

A second possible mechanism for differences in IRS signaling is that Akt activation mediated by IRS-2 is less sensitive to the phosphatases PH domain Leucine-rich repeat Protein Phosphatase (PHLPP)-1 and PHLPP2, which negatively regulate Akt activity through dephosphorylation of Ser473 (275). It is possible Akt activation
downstream of either IRS-1 or IRS-2 occurs in specific subcellular regions in which Akt phosphatases differentially localize. Currently no studies have rigorously studied the localization of these phosphatases in the cell; however, PHLPP1 and PHLPP2 have been shown to co-immunoprecipitate with specific Akt isoforms (275). The data in Chapter IV do not exclude the possibility that downstream of IRS-1, Akt phosphorylation is more sensitive to dephosphorylation than Akt activated downstream of IRS-2. This would lead to increased Akt phosphorylation and activity downstream of IRS-2, which was observed in response to IGF-1 signaling.

In addition to Akt phosphatases, the lipid phosphatase Phosphatase and Tensin Homolog (PTEN) antagonizes the action of PI3K by converting PtdIns-3,4,5-P₃ to PtdIns-4,5-P₂ (276). This decreases the level of PtdIns-3,4,5-P₃ at the plasma membrane and the subsequent recruitment and activation of Akt. It is possible that IRS-1 and IRS-2 may differentially recruit PTEN to the membrane and based on my data, I would suggest that IRS-1 may have an increased ability to recruit PTEN. Interestingly, PTEN also functions as a tyrosine phosphatase for IRS-1 and has been shown to decrease IRS-1-mediated Mitogen Activated Protein Kinase (MAPK) activation by perturbing its ability to associate with Grb-2 and Sos (277,278). However, no studies have addressed the ability of PTEN to act as a phosphatase for IRS-2. PTEN can also directly bind to p85 and in this way, p85 can served to limit the production of PtdIns-3,4,5-P₃ (279). My studies do not exclude the possibility that p85 bound to IRS-1 may recruit a higher level of PTEN relative IRS-2. Co-immunoprecipitation assays could be used to determine if there is a differentially ability of IRS-1 and IRS-2 to associate with PTEN following IGF-
1 stimulation. Additionally, measuring the levels of PtdIns-3,4,5-P\textsubscript{3} and PtdIns-4,5-P\textsubscript{2} following either IRS-1 or IRS-2-mediated PI3K signaling would suggest if there is a differential potential for Akt recruitment and activation.

With regard to the level of PtdIns-3,4,5-P\textsubscript{3} produced, PI3K effectors other than Akt may be differentially regulated by IRS-1 and IRS-2. Specifically, activation of the Rac signaling pathway would promote actin cytoskeleton rearrangement, adhesion and cell-to-cell contacts, all of which affect the motile and invasive ability of cells (139,140). Therefore, if higher levels of PtdIns-3,4,5-P\textsubscript{3} are generated downstream of IRS-2, this could be one mechanism by which IRS-2 promotes invasion.

**Functional Differences Mediated by IRS-1 and IRS-2**

My data demonstrate that IRS-2 is a more potent stimulator of Akt activation and I hypothesize that the differential ability of IRS-1 and IRS-2 to stimulate PI3K/Akt activity underlies the differences in functional outcomes associated with IRS-1 and IRS-2 signal transduction. Based on my studies and what is known about the IRS proteins, I suggest that multiple layers of regulation affect the specificity of IRS-1 and IRS-2 signaling. The first layer of this regulation is the role that the differential localization of the IRS proteins may play in regulating the phosphorylation of Akt effectors, specifically GSK-3β. Studies have shown that both IRS-1 and IRS-2 can localize to the cytoplasm, while IRS-1 also localizes to the nucleus and IRS-2 to the plasma membrane(96-98). These differences in localization patterns have been correlated with patient prognosis.
with IRS-1 nuclear localization correlating with increased patient response to tamoxifen treatment and IRS-2 plasma membrane localization correlating with reduced overall patient survival (96,98). Together, these data suggest that the subcellular localization of the IRS proteins impacts the functional outcomes of the signaling they mediate. In response to IGF-1 or insulin stimulation, Akt can undergo translocation into the mitochondria where it mediates GSK-3β phosphorylation (280). This finding suggests that signaling originating from plasma membrane RTKs facilitates the activation of Akt which is then translocated to specific subcellular locations. Studies in our lab have shown that IRS-2-mediated Akt activation is dependent on an intact microtubule cytoskeleton, as treatment with microtubule disrupting agents inhibits Akt activation downstream of IRS-2 (unpublished data). It is interesting to speculate that activation of Akt mediated by IRS-2 may direct its localization in the cell through a microtubule dependent mechanism. However, the question of whether Akt co-localizes with a complex containing IRS-2 or if IRS-2 merely directs this localization would be important to fully understand the mechanism of this regulation.

The data described above suggest a role for trafficking in the regulation of IRS-2-mediated Akt activation. Interestingly, GSK-3β activity has been shown to correlate with its location within the cell and GSK-3β is more active in the nucleus and mitochondria, while displaying decreased activity in the cytoplasm (281). Putting this in context of what is known about IRS-1 and its localization to the nucleus, it is possible that Akt activation mediated by IRS-1 does not promote GSK-3β phosphorylation, as GSK-3β activity is high in the nucleus where IRS-1, and not IRS-2, has been shown to localize.
Therefore, I would suggest a rigorous study to compare the localization of Akt and GSK-3 phosphorylation in response to either IRS-1 or IRS-2-mediated signaling, as these experiments would suggest if and where the IRS proteins direct localization of Akt activity.

Another mechanism that I hypothesize contributes to the disparate functions associated with the IRS proteins is a differential ability to regulate Akt isoform activation. My data suggest that IRS-1 and IRS-2 are both capable of promoting phosphorylation of Akt1 and Akt2, but IRS-1 promotes increased Thr308 phosphorylation of Akt1 relative to IRS-2. Interestingly, studies of isoform specific functions of Akt1, Akt2 and Akt3 correlate well with the specific functions that have been attributed to either IRS-1 or IRS-2. Studies of either Irs-1−/− or Akt1−/− mice have shown both genotypes are born runted, while Irs-2−/− or Akt2−/− mice develop diabetes (85,86,194-196). Further, Irs-2−/− or Akt3−/− mice both display impaired brain development (87,197). These correlations extend to the role of the IRS proteins or Akt isoforms in tumor initiation and progression. Akt1 has been shown to suppress migration through phosphorylation of the actin bundling protein palladin and inhibits metastasis in vivo (215). Irs-1 has been suggested to negatively regulate metastasis in vivo, as PyMT:Irs-1−/− mice have significantly increased pulmonary metastasis relative to PyMT:wildtype mice, while PyMT:Irs-1+/− mice display an intermediate level of metastasis (105). Additionally, both Akt2 and Irs-2 have been shown to promote metastasis (105,214). Although, Akt isoform-specific regulation downstream of the IRS proteins is not completely discrete, the fact that IRS-1 mediates a higher level of Akt1 phosphorylation in response to IGF-1
stimulation does correspond with the functional comparisons of IRS-1 and Akt1. Additionally, like the IRS proteins, the Akt isoforms have been shown to adopt different subcellular localization patterns. Specifically, Akt1 localizes to the cytoplasm, Akt2 to the mitochondria, and Akt3 to the nucleus (198). It is interesting to speculate that the IRS-specific regulation of Akt isoforms may be dependent on their subcellular localization.

Fluorescent reporters for global Akt activity have been developed and recently an Akt1-specific reporter has been characterized (170,282,283). These reporters function as substrates for Akt and upon phosphorylation, a conformational change is induced that modulates their fluorescence wavelength and the localization of Akt activity can be determined (282). I suggest using these reporters to determine if IRS-1 and IRS-2 mediate Akt activation in a specific region of the cell. Using PyMT:Irs-1⁻/⁻/Irs-2⁻/⁻ cells expressing either IRS-1 or IRS-2, the localization of Akt activation could be assessed with these reporters and co-localization of the IRS proteins to these regions could also be determined. In regard to isoform specificity, the Akt1 fluorescent reporter would be useful in determining if IRS-1 or IRS-2 preferentially mediate Akt1 activation and if this occurs in a specific subcellular compartment. In the future, development of Akt2 and Akt3 fluorescent reporters will also be very useful in characterizing IRS-specific regulation of the Akt isoforms.

I would like to additionally hypothesize that the magnitude and dynamics of Akt amplification downstream of either IRS-1 or IRS-2 act as another level of regulation that contributes to their differences in functional outcomes. Consistent with my findings, the
magnitude of Akt activation downstream of IRS-2, and not IRS-1, has been shown to be sufficient to promote FOXO1 phosphorylation and increase glucose uptake (258). As is the case for FOXO1, it is possible that a greater magnitude of localized Akt activity is required for GSK-3β phosphorylation and IRS-1 cannot sufficiently mediate this level of Akt activation. Additionally, IRS-1 mediates a higher magnitude of Akt1 phosphorylation despite the ability of IRS-2 to mediate a more robust level of global Akt phosphorylation. These data suggest that global Akt activation may not be indicative of a particular isoforms’s activity. I suggest that to fully understand signal transduction downstream of IRS-1 and IRS-2, the magnitude and dynamics of each isoform must be evaluated and considered. In regard to dynamics of Akt activation, it is possible that IRS-1-mediated Akt activation is sustained longer relative to IRS-2 and that specific Akt effectors are phosphorylated only after sustained Akt activation. In response to insulin stimulation, the Akt signaling pathway has been shown to encode temporal patterns of insulin stimulus in the dynamics of Akt phosphorylation (284). These temporal patterns of Akt activation are subsequently decoded into the level of phosphorylation of Akt effectors. However, this study did not correlate these temporal patterns of stimulation with functional responses or look at IRS-1 or IRS-2 involvement. Studies have suggested that IRS-1 mediated growth is dependent on the sustained activation of signaling downstream of IRS-1. Therefore, longer time courses of IGF-1 stimulation should be performed to test this hypothesis.
One interesting mechanism of regulation that my studies do not directly address, but indirectly suggest could impact differential functions, is the potential for IRS-1 or IRS-2 to mediate negative feedback loops that negatively impact their ability to mediate PI3K/Akt signaling. It is well established that downstream of Akt and Mechanistic Target of Rapamycin Complex 1 (mTORC1), Ribosomal Protein S6 Kinase (S6K) will phosphorylate IRS-1 on serine residues that inhibit its ability to mediate PI3K signaling (285). Based on my data, I hypothesize that IRS-2 promotes feedback regulation on IRS-1 and that when both IRS-1 and IRS-2 are expressed, IRS-2 promotes serine phosphorylation of IRS-1, dampening PI3K and Akt activation. Consistent with this idea, despite a higher level of Akt phosphorylation downstream of IRS-2 relative to IRS-1 as shown in Chapter IV, in Chapter III loss of Irs-2 expression did not decrease Akt activation in response to IGF-1 stimulation. These data suggest that Irs-1 compensates for the loss of Irs-2-mediated PI3K activity. In the context of only IRS-1 or IRS-2 expression, it is clear that the level of Akt and S6K activation mediated by IRS-2 is much greater than that downstream of IRS-1. Therefore these data suggest that IRS-2 more effectively activates this negative feedback loop. Studies comparing the sensitivity of IRS-1 and IRS-2 to S6K-mediated serine phosphorylation have yet to be undertaken and will be helpful in determining if IRS-2 is potentially less sensitive to this feedback regulation. These data do not exclude the possibility that IRS-1 may also promote
negative feedback on IRS-2 or that IRS-2 can mediate this feedback on itself, as studies have suggested that IRS-2 can also be phosphorylated by S6K (286, 287).

In addition to the negative feedback loop initiated by S6K, GSK-3β phosphorylation of IRS-1 has also been shown to negatively regulate IRS-1-mediated Akt activation. GSK-3β phosphorylates Irs-1 on Ser332 and phosphorylation of this serine requires an additional priming phosphorylation of Ser336 (288). Mutation of these serines to alanine leads to increased tyrosine phosphorylation of IRS-1 as well as increased phosphorylation of Akt on both Thr308 and Ser473 in response to insulin stimulation. I hypothesize that Irs-2-specific inhibition of Gsk-3β would decrease phosphorylation of Irs-1 on Ser332, increasing the ability of IRS-1 to activate Akt. This contrasts with the potential role of IRS-2 in the S6K feedback loop, where IRS-2 would negatively regulate IRS-1-mediated signaling. A phospho-specific Ser332 IRS-1 antibody is commercially available and will be of significant use in determining if IRS-2-mediated GSK-3β inhibition promotes decreased phosphorylation of this site. Together, these data suggest that IRS-2 can both inhibit IRS-1 activity through S6K activation, as well as promote IRS-1 activity through inhibition of GSK-3β. It is likely the balance of these feedback loops that determines the outcome on IRS mediated functions.

**Intrinsically Disordered Proteins**

Both IRS-1 and IRS-2 are considered intrinsically disordered proteins (IDPs), as only their N-terminal plextarin homology (PH) and phosphotyrosine binding (PTB)
domains have been shown to adopt stable tertiary structure while their central and C-terminal regions do not adopt any discernable structure (256). IDPs are commonly involved in cellular signaling and are often associated with human cancer (289). Structural analysis of IDPs are limited as this class of proteins are poor candidates for X-ray crystallography. However recent advances in nuclear magnetic resonance (NMR) spectroscopy have allowed for more informative study of these proteins (290). These studies have suggested that IDPs may adopt some secondary like structures that are not picked up by structural prediction programs (256). In the case of Gab1, another IDP that is also an adaptor protein, Ser552 directly binds to the C-terminal PH domain (256). From these studies the “N-terminal folding nucleation (NFN) hypothesis” was suggested by Stephen Feller as a mechanism by which IDPs adopt structure and escape aggregation and degradation (256). This hypothesis suggests that during translation, the N-terminal region folds as it leaves the ribosome. As translation continues, regions of IDPs that are relatively unstructured associate with specific regions of the folded N-terminus, increasing the stability of the IDP as well as preventing any non-specific interactions from occurring. It has been suggested that these intramolecular interactions between different regions of the same protein would create loops that may serve as docking sites for downstream effectors. In this case, loops are beneficial as they confine the protein to a more defined region while still allowing for binding of multiple factors. It is interesting to speculate how the intrinsically disordered structure of IRS-1 and IRS-2 contributes to their differences. IRS-1 and IRS-2 share the greatest level of homology in the N-terminal regions, while they are less homologous in their C-termini (81). I
hypothesize that these non-homologous regions of IRS-1 or IRS-2 differentially interact with either their PH or PTB domains, promoting differential formation of looped structures that mediate unique interactions of the IRS proteins. To test this hypothesis, peptides of both IRS-1 and IRS-2 C-terminal regions could be assessed for their ability to associate with constructs of IRS-1 or IRS-2 PH and PTB domains. This would reveal if there are specific C-terminal regions of the IRS proteins that make contacts with the PH and PTB domains. Further, if a specific region is shown to associate with the PH or PTB domain, it would be important to consider the phosphorylation status of serine or tyrosine residues in that region. Mutation of these sites could suggest if these interactions are phosphorylation dependent.

It has been suggested that posttranslational modifications such as tyrosine phosphorylation of IDPs modulate the conformations they adopt. NMR studies of CD79a and CD79b before and after phosphorylation have shown tyrosine phosphorylation will shift their propensity to adopt helical-like secondary structure (291). In the case of CD79a, tyrosine phosphorylation decreases helical propensity, while the opposite is true of CD79b. It is interesting to consider the possibility that IRS-1 and IRS-2 may adopt different conformations depending on their phosphorylation status and this may serve to inhibit or promote association with binding partners.

As suggested from the work done in Chapter III, Irs-2 has four tyrosine residues (Tyr649, Tyr671, Tyr734, Tyr814) that mediate the recruitment and activation of PI3K. In comparing Irs-1 and Irs-2 protein primary sequence, Tyr734 and Tyr814 do not have homologous sites in YxxM motifs in this region of Irs-1. Therefore, tyrosine
phosphorylation in this particular region of the IRS proteins is dissimilar and could
differentially effect the structural conformation the IRS proteins would adopt in response
to phosphorylation. Currently, mass spec studies of differential interacting proteins of
IRS-1 and IRS-2 do suggest that proteins differentially associate with them under basal
and IGF-1 stimulated conditions (unpublished data). It may be possible that these
differential regions of tyrosine phosphorylation in IRS-1 and IRS-2 may induce unique
conformational changes that function as determinants of the protein interactions that IRS
proteins can then mediate. Further along these lines, it has been suggested that protein
interactions can also mediate structural changes within IDPs (292). So it will be
important to consider how unique protein associations of IRS-1 and IRS-2 may impact
their structure, as well as the functional outcomes of these possible conformational
changes. While it is difficult to speculate exactly how IRS-1 and IRS-2 may structurally
differ, I hypothesize that despite overall homology, non-homologous, unstructured
regions of the IRS proteins allow for them to adopt different conformations and
ultimately lead to different functional outcomes through unique protein interactions.

**IRS-2-Mediated Aerobic Glycolysis**

IRS-2 is a key mediator of aerobic glycolysis (108). I have confirmed and
extended the understanding of this regulation to include the requirement for direct PI3K
recruitment and activation by IRS-2 and GSK-3β inactivation. Previous studies of GSK-
3β action have suggested that it inhibits glucose uptake through inhibition of Glut1
protein expression plasma membrane localization (232,254). However, in the studies presented here, I have not found any indication that Gsk-3β regulates Glut1 expression or trafficking to the cell surface. I hypothesize that GSK-3β increases the rate of glucose transport by Glut1, thereby increasing glucose uptake. A mechanism for increased catalytic activity of Glut1 has yet to be described. However, one group has reported that AMPK is similar to GSK-3β in that it does not promote increased Glut1 expression or surface localization and instead increases the catalytic turnover of the transporter (293). To date, no studies have shown that kinase-mediated direct phosphorylation regulates the activity of Glut1, but it remains a possibility. It is also possible that GSK-3β regulates other members of this glucose transporter family, such as Glut3 or Glut4; however expression of these proteins has not been determined in our model systems and should be assessed. Further, the studies here do not exclude the possibility that GSK-3α, an additional isoform of GSK-3, may regulate glucose uptake through a yet to be described mechanism (294). GSK-3 inhibitors are not isoform specific, so both isoforms are blocked following inhibitor treatment. Less is currently known about GSK-3α, but mouse models do not suggest a role for GSK-3α in growth, development, metabolism, or insulin sensitivity (295).

In previous studies from our lab, it was determined that Irs-2 promoted increased glucose uptake through mTorc-1-dependent Glut1 surface expression (108). I have confirmed the role of IRS-2 in the regulation of Glut1 surface expression, but was unable to rigorously tie IRS-2-dependent PI3K signaling to this regulation. The ability of the Irs-2 mutant to recruit PI3K was not completely inhibited and it is possible that the level
of residual PI3K association that the IRS-2 mutant was sufficient to promote Glut1 surface localization. Generating an Irs-2 mutant in which PI3K association is completely abolished will be required to fully address the contribution of Irs-2-dependent PI3K signaling to Glut1 surface expression.

**Aerobic Glycolysis and Metastasis**

The specific contribution of IRS-2-dependent regulation of aerobic glycolysis in promoting metastasis has yet to be determined. I hypothesize that increased aerobic glycolysis downstream of IRS-2 may promote metastasis through cell autonomous effects as well as effects on the tumor microenvironment. With regard to cell autonomous effects, previous studies in erythrocytes, lymphocytes and smooth muscle cells have suggested that specific proteins recruit and associate with multiple glycolytic enzymes at the plasma membrane (296-299). In the case of lymphocytes and smooth muscle cells, Caveolin-1 (Cav-1) functions as a scaffolding protein, recruiting multiple glycolytic enzymes to the plasma membrane (297,298). It is thought that this co-localization of glycolytic enzymes increases pathway flux through channeling of metabolic intermediates and can also localize ATP production at sites of high-energy utilization. It would be interesting to investigate the possibility that IRS-2 functions to recruit these enzymes to regions that require high levels of ATP production, such as the leading edge of an invading cell. Invasive protrusions undergo constant changes in adhesion and rearrangements in their actin cytoskeleton, which require rapid and constant production
of energy (300). Recent, unpublished mass spec studies from our lab suggest that IRS-2, but not IRS-1, associates with metabolic enzymes. Therefore, it is possible that IRS-2 may serve a key function in localization of these enzymes at local sites of invasion. However, further characterization of these IRS-2 interactions as well as localization studies of both IRS-2 and glycolytic enzymes must be undertaken. Interestingly, IRS-2, and not IRS-1, has been shown to localize to the plasma membrane, which further suggests an important role for IRS-2 plasma membrane localization.

In addition to generating energy and biomass for rapidly dividing cells, much of the lactate produced in tumor cells is secreted into the microenvironment (5). This lactate lowers the pH, activating MMPs which function to degrade the ECM, allowing for the invasion of tumor cells through the basement membrane where they gain access to the vasculature, promoting metastasis. It is therefore possible that IRS-2-stimulated lactate production can promote invasion of cells through the basement membrane, increasing the potential for metastasis.

Interesting studies have also suggested that the lactate secreted by tumor cells can promote angiogenesis through multiple mechanisms. Lactate can engage specific cell surface receptors such as Axl and Tie of endothelial cells to promote angiogenesis (301). Additionally, lactate increases the level of VEGF in endothelial cells and stabilizes HIF1α, leading to increased VEGF expression (302-306). Interestingly, \( Irs-2^{-/-} \) tumors display decreased vascularization relative to their WT counterparts, while vascularization is increased in \( Irs-1^{-/-} \) tumors. This difference suggests a role for Irs-2 in promoting angiogenesis, and this may occur in part through its ability to regulate lactate production.
Together these studies suggest that the effects of IRS-2-mediated aerobic glycolysis in tumor cells are more than just cell autonomous and extend into the tumor microenvironment.

**Future Contributions of Model System**

In addition to the insight that my studies have provided into the role of Irs-2-dependent PI3K signaling and the specific functions that are associated with either IRS-1 or IRS-2, a novel model system has been generated to rigorously characterize signaling by, and functions of, the IRS proteins. The studies that were done prior to the work presented here contributed to our understanding of the roles of the IRS proteins in breast cancer. However, many conclusions of these studies were limited by the model systems that were available at the time of the study. In studies done using Irs knockout mice, a true comparison of Irs-1\(^{-/-}\) and Irs-2\(^{-/-}\) tumor derived cells could not be made as these cells came from different tumors from different mice (108). However, here we have generated Irs-1\(^{-/-}\)/Irs-2\(^{-/-}\) mammary tumor derived cells in which a rigorous comparison of IRS-1 and IRS-2 can be performed in a genetically matched background. Going forward, it will be interesting to further investigate IRS-specific functions in this model system. The studies done here comparing IRS-1 and IRS-2 have confirmed previous results and suggest that this model system is suitable for further study of the IRS proteins.

One of the most interesting experiments going forward will be re-introducing IRS-2 expression in Irs-1\(^{-/-}\)/Irs-2\(^{-/-}\) cells that express IRS-1 and vice versa. These types of experiments are key to understanding how IRS-1 and IRS-2 either work together or
impede the other’s function. It has been suggested that IRS-1 may negatively regulate metastasis, as PyMT:Irs-1+/ mice show greater levels of pulmonary metastasis relative to their PyMT:WT counterparts, while PyMT:Irs-1-/- mice display an intermediate level of metastasis (105). Re-introducing IRS-1 expression to cells Irs-1-/-/Irs-2-/- cells that only express IRS-2 and determining the level of invasion compared to cells that just express IRS-2, will determine if IRS-1 can function to inhibit the actions of IRS-2. Additionally, similar in vivo studies using these cell lines could help to address the potential role for IRS-1 in the inhibition of metastasis. This interplay between IRS-1 and IRS-2 could occur through their competition for binding sites at receptors. The study of these IRS/IGF-1R interactions when one or both IRS protein are expressed would further the mechanistic understanding of IRS-mediated signaling.

Additionally, Irs-1-/- and Irs-2-/- cells will be of great use in future studies of the Irs proteins in mouse models of breast cancer. For example, comparing tumor growth and metastasis in mice with mammary fat pad injections of either Irs-2fl/fl or Irs-2-/- cells is the most ideal system to determine the contribution of Irs-2 to tumor progression and metastasis. The same can be said for similar studies using Irs-1fl/fl and Irs-1-/- cells. Using these genetically matched cell lines, the true contribution of Irs-1 and Irs-2 to tumor progression and metastasis can be determined and it will be interesting to see how these studies compare to those done in PyMT:Irs-1+/ and PyMT:Irs-2+/ mice. Further, tumor progression studies following injection of Irs-2-/- cells expressing WT IRS-2 or IRS-2 Y5F will help define the role of Irs-2-dependent PI3K signaling tumor progression and metastasis. Based on my work, I hypothesize that Irs-2 Y5F expressing tumors
would show a significant decrease in their ability to metastasize relative to WT Irs-2 expressing tumors. I further suggest that Irs-2 Y5F tumors would have fewer invasive cells and the tumor would display decreased angiogenesis, due to a reduction in aerobic glycolysis. This type of study is required to fully understand how Irs-2-mediated PI3K signaling and aerobic glycolysis affect tumor progression and metastasis in vivo.

**Overall Significance**

In this dissertation I have focused on the role of IRS-2-dependent PI3K signaling in mammary tumor cell biology. When compared with IRS-1, IRS-2-dependent PI3K signaling preferentially leads to the phosphorylation of the Akt effector GSK-3β. This finding shows that specific downstream effectors are differentially regulated by IRS-1 and IRS-2 and suggests a mechanism that would promote functional differences when signals are initiated downstream of either IRS-1 or IRS-2. Additionally, my studies demonstrate that IRS-1 and IRS-2 do not function interchangeably. IRS-1 promotes growth, while IRS-2 promotes invasion, aerobic glycolysis and a more robust activation of Akt. The studies presented here recapitulate prior studies of the IRS proteins, but do so in a more rigorous and controlled manner.

There is significant evidence to support a role for IRS-2 in promoting metastasis as it regulates many functions associated with metastatic potential such as motility, invasion and aerobic glycolysis (102,103,108). Additionally, studies using mouse models have suggested that IRS-2, but not IRS-1, promotes metastasis (105).
presented here elucidate the underlying mechanisms that specifically allow for IRS-2 to positively regulate metastasis. I would suggest that the ability of IRS-2-dependent PI3K signaling to promote aerobic glycolysis is one of the most important mechanisms by which IRS-2 drives metastasis. This shift in metabolism affects multiple functions that promote metastasis. Specifically, it drives the growth and survival of cells under hypoxic conditions as well as promotes the invasive ability of cells. It additionally primes the microenvironment for invasion and metastasis. Figure 5.1 summarizes my hypothesized role of the contribution Irs-2-dependent PI3K signaling makes to invasion and angiogenesis, both of which would promote metastasis. I believe it is important to consider IRS-2 expression status in breast cancer patients and more specifically, evaluate the phosphorylation status of tyrosines that I have implicated in the recruitment of PI3K. Together with localization and expression, understanding the level of IRS-2 activity may allow for a better determination of which patients have tumors that may be likely to metastasize. My studies suggest that not all PI3K and Akt activation is equal, and it is important to consider the upstream factors that are involved in their activation.
Figure 5.1. Hypothesized Role of IRS-2 in Invasion and Angiogenesis.
Figure 5.1. Hypothesized Role of IRS-2 in Invasion and Angiogenesis. (A) IRS-2 can mediate a higher level of Akt activation through recycling of the IGF-1R back to the cell surface. (B) IRS-2-mediated PI3K signaling preferentially regulates the phosphorylation of the Akt effector GSK-3β. This stimulates increased glucose uptake and supports increased aerobic glycolysis. (C) IRS-2 specifically localizes to regions of localized invasion while subsequently recruiting glycolytic enzymes. This allows for localized aerobic glycolysis and ATP production, promoting actin cytoskeleton rearrangement and cell motility. (D) The lactate produced is secreted into the microenvironment resulting in acidification and the subsequent activation of matrix metalloproteases (MMPs). MMP activation promotes degradation of the extracellular matrix and cell invasion. (E) The secreted lactate engages specific receptors on endothelial cells, stimulating angiogenesis.
Appendix

*Generation of Phospho-specific IRS-2 Antibodies*
Antibody Isolation and Verification

We sought to generate rabbit polyclonal phosphotyrosine-specific IRS-2 antibodies to further investigate IRS-2 function. We chose to generate antibodies directed at the human sequences of IRS-2 for potential use in applications with human tissues such as immunohistochemistry and western blotting. Using sequence alignment tools, the sequence surrounding Tyr734 (Human: Tyr742) and Tyr814 (Human: Tyr823) were similar between mouse and human IRS-2 and could potentially allow for species cross reactivity (Figure A.1A). Additionally, the sequence surrounding these tyrosine residues was determined to have the least amount of sequence homology between IRS-1 and IRS-2 when compared with Tyr649 (Human: Tyr653) and Tyr671 (Human: Tyr675) (Figure A.1B). The sequences of the IRS-2 peptides that were used for immunization are shown in Figure A.1C. The company ProSci (Poway, CA) was selected to generate the peptides and perform the antibody production and purification.

A total of four rabbits (two per peptide) were immunized with KLH-conjugated peptides every two weeks for a total of six weeks. Three different bleeds were performed on each rabbit and serum was purified by a two-step immuno-affinity purification. First, serum was purified through a non-phospho-IRS-2 peptide column to remove antibodies that recognize unphosphorylated IRS-2. The flow through was then purified over a phospho-IRS-2 peptide column to isolate phospho-specific antibodies. For each site, two different phospho-specific antibodies were generated, one for each rabbit (p742-1, p742-
Figure A.1. IRS sequence alignment and peptide sequence.

A.

Query: Human IRS-2
Subject: Mouse IRS-2

B.

Query: IRS-2
Subject: IRS-1

C.

Peptide Sequences
pyY42 - CEDSG(pY)MRMW
pyY823 - CDSDQ(pY)VLMSS
Figure A.1. IRS sequence alignment and peptide sequence. (A) Sequence alignment of human and mouse IRS-2 protein sequence. Red squares indicate tyrosines chosen for phospho-specific antibody generation. (B) Sequence alignment of human IRS-1 and IRS-2 protein sequence. Red squares indicate tyrosines chosen for phospho-specific antibody generation. (C) Phospho-specific peptide sequence used for immunization.
2, p823-1, p823-2). Both non-phospho-specific and phospho-specific antibodies were eluted from the columns and sent to us for subsequent verification and testing.

To determine if the phospho-specific antibodies that recognize Tyr742 and Tyr823 are IRS-2 specific, Irs-1−/−/Irs-2−/− cells expressing either IRS-1-HA or IRS-2-HA were serum starved and stimulated with IGF-1 (Figure A.2a). Little reactivity was observed for p823-1 with either human or mouse IRS-2, suggesting that this rabbit did not have a robust immune response. The other three antibodies showed cross reactivity with IRS-1. However, these antibodies are truly phospho-specific as there is no recognition of IRS-1 or IRS-2 under serum starved conditions. Further optimization of antibody and protein lysate concentrations suggests that p832-1 does recognize the phosphorylated form of IRS-2 (Figure A.2b). Additionally, both Tyr742 and Tyr823 are phosphorylated over a time course of IGF-1 stimulation, with phosphorylation of both sites decreasing after 10 min of stimulation. Further testing is required to verify that these antibodies recognize murine Irs-2.
Figure A.2. Initial screening of pTyr742-IRS-2 and pTyr823-IRS-2 antibodies.

A.

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<th>Irs-1-/-/Irs-2-/-</th>
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<th>IRS-2-HA</th>
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<td>-</td>
<td>+</td>
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<td>pY742-1 IRS-2</td>
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<td>pY742-2 IRS-2</td>
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<td>pY823-2 IRS-2</td>
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B.

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<th>10</th>
<th>30</th>
<th>60</th>
<th>IGF-1</th>
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</table>
Figure A.2. Initial screening of pTyr742-IRS-2 and pTyr823-IRS-2 antibodies. (A) *Irs-1*^-/-/Irs-2*^-/- cells expressing either IRS-1-HA or IRS-2-HA were serum starved and then stimulated with IGF-1 (100ng/ml, 10min). Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with the phospho-specific IRS-2 antibodies pTyr742-1, pTyr742-2 and pTyr823-1. (B) *Irs-1*^-/-/Irs-2*^-/- cells expressing IRS-2-HA were serum starved and then stimulated with IGF-1 (100ng/ml, 2, 10, 30, 60 min). Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with the phospho-specific IRS-2 antibodies pTyr742-1, pTyr742-2 and pTyr823-1 and pTyr823-2.
Bibliography
REFERENCES


52. Shaw, L. M. (2001) Identification of insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the alphaβ4 integrin-dependent activation of phosphoinositide 3-OH kinase and promotion of invasion. Molecular and cellular biology 21, 5082-5093


86. Withers, D. J., Gutierrez, J. S., Towery, H., Burks, D. J., Ren, J. M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G. I., Bonner-Weir, S., and White, M.


110. Lapetina, E. G., and Michell, R. H. (1973) A membrane-bound activity catalysing phosphatidylinositol breakdown to 1,2-diacylglycerol, D-myoinositol 1:2-cyclic


SH2 domains and regulates binding of PI3-kinase to the PDGF beta-receptor. *Cell* **65**, 75-82


mild growth deficiency in mice lacking Akt2/PKB beta. The Journal of clinical investigation 112, 197-208


218. Maroulakou, I. G., Oemler, W., Naber, S. P., and Tsichlis, P. N. (2007) Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV)-


