INSULIN RECEPTOR SIGNALING IS NECESSARY FOR THE MAINTENANCE OF EPITHELIAL PHENOTYPE IN MCF10A CELLS

By

Vanessa Carmen Di Palma

A Thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Medical Biophysics
University of Toronto

©Copyright by Vanessa Carmen Di Palma (2013)
Insulin Receptor Signaling is Necessary for the Maintenance of Epithelial Phenotype in MCF10A Cells.
Master of Science, June 2013
Vanessa Carmen Di Palma
Graduate Department of Medical Biophysics, University of Toronto

ABSTRACT

Obesity is an adverse factor in the development and severity of breast cancer. Obesity is often accompanied by an increase in circulating insulin, which is also associated with poor BC prognosis. Although not expressed in normal breast tissue, the insulin receptor (IR) is highly expressed in BC, therefore insulin signaling in BC cells may be responsible for the negative prognostic effects associated with hyperinsulinemia.

This thesis describes the development of a cell-based system to study how insulin affects BC. My work shows that MCF10A, untransformed human breast epithelial cells that express the IR, require insulin for normal proliferation and morphology. Interestingly, I discovered hyperactivation of ERK1/2 in MCF10A cells in response to insulin withdrawal, resulting in a loss of epithelial phenotype. Unexpectedly, while losing epithelial phenotype, MCF10A cells depleted of insulin failed to migrate. In conclusion, breast cells that express IR require insulin for migration and maintenance of epithelial characteristics.
ACKNOWLEDGEMENTS

My graduate experience was shaped by the committed academic support and kind personal guidance from my supervisor Vuk, without whom I could not have completed this work. Thank you for continually challenging me to strive to be a better scientific mind and for giving me the freedom to make mistakes, then helping me learn from them. Thank you to my committee members, Dr. Jane McGlade and Dr. Senthil Muthuswamy, for your valuable advice, support, and guidance throughout my degree. Infinite thank yous to the Stambolic lab, for your friendship, laughter, and for making me look forward to coming to the lab every day. You’ve all provided a positive influence, helped me navigate through the challenges of graduate school, and have become my friends in and outside of the lab. Importantly, I am so grateful to my family and friends for unremittingly supporting me during my years of study and enduring hours of talk about breast cells and insulin, and to Alex for sticking with and laughing with me always. Last and not least, my love and thanks go to Adam for putting everything in perspective.
TABLE OF CONTENTS

i Abstract
ii Acknowledgements
iii Table of Contents
iv List of Figures
v List of Tables
vii Abbreviations

CHAPTER 1: INTRODUCTION

1.1 Circulating Insulin, Obesity and Breast Cancer
  1.1.1 Adipokines, Estrogen and Cancer
  1.1.2 Metformin

1.2 The Insulin Receptor
  1.2.1 The Insulin Receptor Gene and Protein
  1.2.2 Insulin Receptor Expression
  1.2.3 Insulin Receptor Isoforms
  -Ligand binding
  -Receptor Dimerization and Hybrid Receptors
  -IR Isoform Heterodimers and Hybrid Receptor Physiology
  1.2.4 The Insulin Receptor and Cancer

1.3 Insulin Receptor Signaling Pathways
  1.3.1 The PI3K Pathway
  -The Roles of PKB Isoforms
  -The PI3K Pathway and Cancer
  1.3.2 The Ras Pathway

1.4 Epithelial and Mesenchymal Phenotypes and ERK1/2

CHAPTER 2: HYPOTHESIS AND RATIONALE

2.1 Anticipated Impact/Relevance to Breast Cancer

2.2 Hypothesis

CHAPTER 3: MATERIALS AND METHODS

CHAPTER 4: RESULTS

4.1 The Insulin Receptor Pathway is Intact in MCF10A Cells

4.2 Growth Response and Change in Morphology in MCF10A Cells After EGF Withdrawal or Insulin Withdrawal
4.3 Insulin Withdrawal Causes Changes in Cell Migration
4.4 Changes in Morphology are Due to Inactivation of the Insulin Receptor Pathway
4.5 Insulin Withdrawal and Inhibition of the Insulin Receptor Pathway Cause ERK1/2 Activation
4.6 Insulin Receptor Knockdown Causes the Same Changes as Insulin Withdrawal
4.7 Insulin Withdrawal and IR Knockdown Causes Loss of Epithelial Phenotype
4.8 ERK2 Activation is Responsible for the Loss of Epithelial Phenotype.

CHAPTER 5: DISCUSSION

5.1 MCF10A Cells as a Model for Breast Cells that Express the Insulin Receptor in a Hyperinsulinemic, Hyperglycemic Environment
5.2 MCF10A Cells Require Insulin Signaling Through the Insulin Receptor for Proliferation and Migration
5.3 The Role of ERK2 in Migration and Maintenance of Epithelial Phenotype
5.4 Communication Between The IR, PKB, and ERK2
5.5 The Potential Role of IGF-IR in Maintenance of Epithelial Phenotype
5.6 Insulin Withdrawal/Insulin Receptor Knockdown and Possible Senescence

CHAPTER 6: FUTURE DIRECTIONS

6.1 Exploring the Role of the IR and Insulin Signaling in Cell Cycle
6.2 Determining the Involvement of Each Insulin Receptor Isoform in the Response to Insulin Withdrawal, and IGF-IR and IGFI/II in the Insulin Response
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3</td>
<td>The Role of PKB and its Isoforms in ERK2 Activation, and PKB localization</td>
<td>70</td>
</tr>
<tr>
<td>6.4</td>
<td>Further 3D Studies to Determine the Effects of Insulin Withdrawal on Events of Acinar Formation</td>
<td>70</td>
</tr>
<tr>
<td>6.5</td>
<td>Knockdown of PTEN and/or Hyperactivation of PI3K to Determine the Effects of Insulin Withdrawal in a Model of Cancer Initiation</td>
<td>72</td>
</tr>
<tr>
<td>6.6</td>
<td>Determining the Role of the Insulin Receptor in Other Breast Cells Lines</td>
<td>73</td>
</tr>
<tr>
<td>6.7</td>
<td>Determining the Effect of Insulin Withdrawal and ERK2 in Breast Cancer Models <em>In Vivo</em></td>
<td>74</td>
</tr>
<tr>
<td>6.8</td>
<td>Final Summary</td>
<td>75</td>
</tr>
</tbody>
</table>

BIBLIOGRAPHY | 77
# LIST OF FIGURES

## CHAPTER 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Obesity, cancer risk and progression, and circulating insulin levels</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>The effect of an increase in adipose tissue on circulating insulin levels, and tissues expressing the insulin receptor</td>
<td>6</td>
</tr>
<tr>
<td>1.3</td>
<td>The insulin receptor isoforms</td>
<td>11</td>
</tr>
<tr>
<td>1.4</td>
<td>Dimerization of the insulin receptor isoform monomers</td>
<td>15</td>
</tr>
<tr>
<td>1.5</td>
<td>Insulin receptor and IGF-IR dimerization and hybrid receptors</td>
<td>17</td>
</tr>
<tr>
<td>1.6</td>
<td>Signaling through PI3K as part of the insulin receptor pathway</td>
<td>20</td>
</tr>
<tr>
<td>1.7</td>
<td>EMT and the contribution of EMT to cancer progression</td>
<td>27</td>
</tr>
</tbody>
</table>

## CHAPTER 4

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>The insulin receptor pathway is intact in MCF10A cells</td>
<td>39</td>
</tr>
<tr>
<td>4.2</td>
<td>Growth response and change in morphology of MCF10A cells after EGF withdrawal or insulin withdrawal in 2D and 3D</td>
<td>42</td>
</tr>
<tr>
<td>4.3</td>
<td>Insulin withdrawal causes changes in cell migration</td>
<td>44</td>
</tr>
<tr>
<td>4.4</td>
<td>Changes in morphology are due to inactivation of the insulin receptor pathway</td>
<td>47</td>
</tr>
<tr>
<td>4.5</td>
<td>Insulin withdrawal and inhibition of the insulin receptor pathway cause ERK1/2 activation</td>
<td>50</td>
</tr>
<tr>
<td>4.6</td>
<td>Insulin receptor knockdown causes the same changes as insulin withdrawal</td>
<td>56</td>
</tr>
<tr>
<td>4.7</td>
<td>Insulin withdrawal and insulin receptor knockdown cause loss of the epithelial phenotype</td>
<td>54</td>
</tr>
<tr>
<td>4.8</td>
<td>ERK2 activation is responsible for the loss of epithelial phenotype in MCF10A cells</td>
<td>56</td>
</tr>
</tbody>
</table>

## CHAPTER 6

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>Timeline of acinar formation of MCF10A cells in matrigel</td>
<td>71</td>
</tr>
</tbody>
</table>
LIST OF TABLES

CHAPTER 5

Table 5.1 PKB isoforms have distinct functions in IGF-IR signaling in MCF10A cells
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4E-BP1</td>
<td>eIF4E-binding protein 1</td>
</tr>
<tr>
<td>Akt</td>
<td>AKT8 virus oncogene cellular homolog</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternate Reading Frame</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2 associated death promoter</td>
</tr>
<tr>
<td>BC</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>B-Raf</td>
<td>B-Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Cip1</td>
<td>Cell-cycle inhibitory protein</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>CS</td>
<td>Cowden syndrome</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DKO</td>
<td>Double knock out</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Dioxynucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ED50</td>
<td>Effective dose</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ErbB2</td>
<td>Erythroblastosis oncogene B</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracelluar signal regulated kinase1/2</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor α</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box protein O</td>
</tr>
<tr>
<td>Fra</td>
<td>Fos-related antigen</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>Gab1</td>
<td>Grb2-associated binding protein 1</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating proteins</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GLUT1-4</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor bound protein 2</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine -5’- triphosphate</td>
</tr>
<tr>
<td>HDLC</td>
<td>High density lipoprotein cholesterol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney 293T</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HMGA1</td>
<td>High-mobility group protein A1</td>
</tr>
<tr>
<td>HNMPA</td>
<td>Hydroxy-2-napthalenylmethy phosphonic acid</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>HR-A</td>
<td>Hybrid receptor (IR-A:IGF-IR)</td>
</tr>
<tr>
<td>HR-B</td>
<td>Hybrid receptor (IR-B:IGF-IR)</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HT-FIR</td>
<td>Hepatocyte-specific Transcription Factor of the IR gene</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>IEG</td>
<td>Immediate-early gene</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>IGFI/II</td>
<td>Insulin-like growth factor 1/2</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IR-A</td>
<td>Insulin receptor isoform A</td>
</tr>
<tr>
<td>IR-B</td>
<td>Insulin receptor isoform B</td>
</tr>
<tr>
<td>IRNF-I/II</td>
<td>Insulin receptors nuclear factors I/II</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin resistance syndrome</td>
</tr>
<tr>
<td>KD</td>
<td>Knock down</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Kip1</td>
<td>Kinesin-related protein</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LDLC</td>
<td>Low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver kinase B1</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney Cells</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>MEK1</td>
<td>MAPK/ERK kinase 1</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumour virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mSos</td>
<td>mammalian son of sevenless</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1/2</td>
<td>mammalian target of rapamycin complex 1/2</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonly phenoxy polyethoxylethanol</td>
</tr>
<tr>
<td>OIS</td>
<td>Oncogene induced senescence</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PAX2</td>
<td>Paired box gene 2</td>
</tr>
</tbody>
</table>
PBS  Phosphobuffered saline
PCR  Polymerase chain reaction
PDK1 Phosphoinositol-dependent kinase 1
PH  Plekstrin homology domain
PI3K Phosphoinositide 3-kinase
PIK3CA Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit
PIP2 Phosphatidylinositol 4,5-bisphosphate
PIP3 Phosphatidylinositol (3, 4, 5)-triphosphate
PKB Protein kinase B
PR  Progesterone Receptor
PTEN Phosphatase and tensin homolog
qRT-PCR Real-time quantitative reverse transcriptase
Ras  Rat sarcoma
RB  Retinoblastoma
Rheb  Ras homolog enriched in brain
RNA Ribonucleic acid
RNAi interference RNA
RSK p90 ribosomal s6 kinase
RT-PCR Reverse transcription polymerase chain reaction
RTK Receptor tyrosine kinase
S6K S6 kinase
SA-β-GAL Senescence-associated beta-galactosidase
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser Serine
SH2 SRC homology domain 2
SH3 SRC homology domain 3
SHBG Sex hormone-binding globulin
Shc SRC homology 2 domain containing
shRNA short hairpin RNA
Sp1 Specificity protein 1
SRC Sarcoma
T1D Type I Diabetes
T2D Type II Diabetes
TBST Tris-buffered saline with tween
TFIID Transcription factor IID
TGFβ Transforming growth factor beta
Thr Threonine
TNF-α Tumour necrosis factor alpha
TSC2 Tumour suppressor complex 2
WT Wild type
µg microgram
µm micrometer
CHAPTER 1: INTRODUCTION
Obesity is an epidemic in Canada and the United States, with almost 40% of the adult population of Canada the overweight or obese[1]. An established risk factor for a number of diseases such as heart disease, diabetes, and stroke, there is emerging evidence that obesity increases the risk and mortality of a number of human cancers, such as endometrial, colon, ovarian, and breast cancer (BC)[1, 2]. For example, overweight women with BC have a higher risk of recurrence and death compared to women of normal weight[2]. Death incidence in BC patients gradually increases with their body mass index (BMI), a measure of body adiposity[3]. Additionally, breast cancer patients have been found to have 45% more visceral adipose tissue compared with control subjects[4]. The mechanisms by which obesity can increase cancer incidence are not fully understood and are likely to be varied, complex and non-exclusionary. Associated factors include circulating sex hormones, hormones that regulate metabolism, and adipokines. The focus of this thesis is on the role that the circulating hormone insulin may play in promoting breast cancer.

Obesity is often accompanied by an increase in circulating insulin levels and a decrease in insulin sensitivity in insulin-target tissues[3]. Increasing plasma insulin concentrations correlate with a number of markers of obesity, including high waist-to-hip ratio[5], waist circumference and visceral adipose tissue levels[6]. These changes in circulating insulin also affect cancer risk; high fasting insulin levels are associated with poor BC prognosis while insulin resistance syndrome triples BC recurrence[7]. Additionally, high fasting plasma glucose concentrations and hyperinsulinemia have been associated with increased risk of BC in both pre and postmenopausal women[8-10], and an elevated serum concentration of C-peptide, a clinically useful correlate of insulin concentration, has been associated with increased BC risk [8, 11]. High plasma insulin concentrations also increase the severity of cancers, as they correlate with high tumour grade and axillary lymph node involvement[9]. It appears, therefore, that the link between obesity and cancer may in part due to the increase in levels of circulating insulin.
Figure 1.1 Obesity, cancer risk and progression, and circulating insulin levels
(A) The graph indicates the importance of obesity as an adverse factor in breast cancer[7]. Body mass index, a marker of obesity is along the x-axis, and is plotted against the estimated relative risk of the adverse events of recurrence and death of patients with operable breast cancer on the y-axis. Estimated risk of these adverse events increases exponentially as BMI increases[2]. A BMI of 25 is considered overweight, while a BMI of 30 is obese[1]. A major consequence of obesity is an increase in circulating insulin levels. (B) Body mass index is plotted along the x-axis, while fasting insulin level is along the y-axis[7]. Fasting insulin levels increase as body mass index increases[3]. The relationship between obesity and fasting insulin levels is causal[20,29]. Graphs taken from Goodwin, P.J., et al., Fasting insulin and outcome in early-stage breast cancer: results of a prospective cohort study. J Clin Oncol, 2002. 20(1): p. 42-51.
Insulin is a circulating peptide hormone produced by the islet (β) cells of the pancreas. In response to elevated plasma glucose levels, insulin is released into the bloodstream and promotes glucose uptake and processing by its primary target tissues, liver, adipose tissue, and skeletal muscle [12, 13]. In healthy mammals, elevated plasma glucose levels, usually a result of food intake, lead to an increase in circulating insulin, resulting in a removal of glucose from the bloodstream and into peripheral tissues[13]. In addition to its main role in glucose homeostasis, insulin also promotes a number of other cellular events that relate to metabolism, such as amino acid transport, lipid metabolism, glycogen synthesis, and protein synthesis[13].

Given its central role in metabolism, defects in insulin signaling lead to various pathologies. A major outcome of deregulated insulin signaling is diabetes, which is characterized by failure to clear blood glucose and the inability of insulin to communicate with its target tissues [14]. There are two types of diabetes, each caused by a different mechanism. Type I Diabetes (T1D) arises from failure of the pancreas to produce insulin[15] while Type II Diabetes (T2D) is a true metabolic disease, characterized by the development of peripheral insulin resistance, hyperglycemia and hyperinsulinemia[16]. Insulin resistance is the inability of target tissues to respond adequately to physiological levels of insulin in the bloodstream[14]. As the body attempts to compensate for lowered insulin sensitivity in its target tissues, there is a counterintuitive increase in circulating insulin in the blood. This occurs because under normal conditions, insulin suppresses glucose release from the liver, but in the context of insulin resistance, glucose is inappropriately released into the blood, resulting in hyperglycemia [17]. As the insulin receptors (IRs) expressed on target tissue become less sensitive to insulin, β cells in the pancreas secrete increasing amounts of insulin in response to the increasing blood glucose levels in an attempt to compensate for lower insulin sensitivity and elevated blood glucose levels[13, 18]. The resultant hyperinsulinemia can also contribute to further insulin resistance [19]. The progression of long-term insulin resistance, hyperglycemia and hypersinsulinemia to T2D is caused by interplay of genetic and environmental factors, including age, ethnicity, and obesity[20]. As obesity rates rise, so do rates of T2D[1].
In addition to the relationship between cancer, hyperinsulinemia and hyperglycemia, a number of epidemiological studies have shown a relationship between T2D, breast cancer risk and prognosis[21-23]. Two studies performed by Lipscombe et al demonstrate this connection. First, a population-based study[24] showed a significant increase in BC risk in postmenopausal women with T2D. The second, a cross sectional study of 55-79yo women[25], was designed to determine the risk of BC during the prediagnostic phase of T2D. During this prediagnostic phase, serum insulin levels are the highest as the patient is not yet being treated. The likelihood of having a prior BC diagnosis was found to be significantly increased among new/prediabetics than in those participants without diabetes. Both studies demonstrated that T2D is a risk factor for BC. Further evidence for the potential role of insulin in tumorigenesis came from a retrospective study of patients treated for diabetes with various insulin analogues. Namely, increased cancer (including BC) susceptibility was uncovered in patients taking glargine, a long acting insulin analog[26]. In contrast, the T2D drug metformin, which effectively lowers circulating insulin levels instead of increasing the concentration of circulating insulin, reduces risk and severity of cancer in diabetics[27]. Given this evidence, it can be postulated that obesity and the accompanying increase in circulating insulin levels, as well as possible T2D may contribute to cancer.

There are several possible mechanisms by which obesity and hyperinsulinemia may lead to oncogenesis. Paradoxically, breast cancers express the IR at much higher levels than normal breast tissue[28, 29]; >85% of BC have been found to express high levels of the IR, whereas normal breast epithelial cells normally do not express the IR at significant levels[30]. Breast cancers may therefore be sensitive to increases in circulating insulin, which raises the possibility that the effects of obesity on BC may be in part mediated by insulin signaling in cancer cells. In support of this mechanism, insulin has been shown to exert a direct mitogenic impact on BC cells in vitro in ER-positive cell lines[31]. Further, the possibility that insulin promotes cancer progression is raised by the fact that high IR expression in primary BC from lymph node-negative patients is associated with a reduced 5-year disease-free survival[32]. Insulin may also have a synergistic effect on oncogenesis mediated by other hormones. For example, insulin stimulation of the breast
Figure 1.2 The effect of an increase in adipose tissue on circulating insulin levels, and tissues expressing the insulin receptor

(A) In normal cells, circulating insulin acts on target tissues—muscle, liver, adipose tissue, to increase glucose transport into tissues. Insulin also leads to the conversion of glucose to glycogen for storage in the liver and skeletal muscle[12,13]. In the obese state, fat tissue communicates with other insulin target tissue to decrease peripheral insulin sensitivity, leading to a somewhat paradoxical compensatory increase in circulating insulin to ensure clearance of glucose[13,17,18]. This hyperinsulinemia has an effect on cells that express the insulin receptor. (B) Breast cancers express the insulin receptor, rendering them sensitive to increases in circulating insulin. Tumors from 178 patients with early stage breast cancer were tested for expression of several receptors using immunohistochemistry. Interestingly, almost 90% of breast cancers express high levels of the insulin receptor[30].

<table>
<thead>
<tr>
<th>n=178 (Mount Sinai Hospital)</th>
<th><strong>IR</strong></th>
<th><strong>IGFI-R</strong></th>
<th><strong>ER</strong></th>
<th><strong>Pgr</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IHC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• negative (0-2)</td>
<td>1.1%</td>
<td>25.3%</td>
<td>27.5%</td>
<td>44.2%</td>
</tr>
<tr>
<td>• weak (3-5)</td>
<td>11.2%</td>
<td>63.5%</td>
<td>25.2%</td>
<td>23.8%</td>
</tr>
<tr>
<td>• strong (6-8)</td>
<td><strong>87.6%</strong></td>
<td><strong>47.2%</strong></td>
<td><strong>55.0%</strong></td>
<td></td>
</tr>
</tbody>
</table>

* * Allred score 0-8; r≥0.15 , p<0.05  (Mulligan AM, O’Malley F, Goodwin PJ  Br Ca Res Treat 2007)
cancer cell line MCF-7 increases estrogen receptor (ER) and progesterone receptor (PR)
expression and cooperates with estradiol in stimulating proliferation in these cells[33].

**Adipokines, Estrogen and Cancer**

In addition to its contribution to hyperinsulinemia, adipose tissue produces hormones
called adipokines that can act in endocrine, paracrine, and autocrine mechanisms to
contribute to cancer risk. Other mitogenic systemic hormones, like estrogens and
progesterone, are also influenced by hyperinsulinemia and obesity. For example,
progesterone, a sex hormone important in the menstrual cycle and embryogenesis, is
stored in adipose tissue[34]. Adipose tissue can therefore contribute to the bioavailability
of progesterone. The two main adipokines produced by adipose tissue are leptin and
adiponectin, which generally act in metabolic and biological opposition to each other;
leptin is thought to enhance BC risk and progression while adiponectin is thought to be
protective against BC[35]. It is important to consider the complex relationships between
estrogens, progesterone and adipokines when investigating the impact of obesity,
hyperinsulinemia, T2D etc on cancer, as these hormones act in concert to maintain
homeostasis in the body. None can change without influencing the others.

Leptin is produced by adipocytes and preadipocytes, and the plasma concentrations of
leptin increase with adiposity[31, 36], and are therefore high in obese individuals.
Elevated plasma leptin levels are also correlated with T2D, and degree of insulin
resistance, in a relationship that is independent of body fat mass and BMI[37, 38]. This
implies that even after weight loss, insulin resistance and high circulating insulin levels
can cause the same negative health effects as obesity; effects which may have been the
result of insulin all along. In some cases, leptin has been found to act as a mitogen in
normal and cancerous human breast tissue[31, 39, 40]. A study in MCF7 cells showed
that leptin stimulates aromatase (estrogen synthase) activity in these cells[41], a
representation of how adipokines can influence circulating levels of estrogens and
therefore alter other facets of metabolism and homeostasis.
Like leptin, adiponectin is synthesized and secreted by adipocytes, yet its plasma concentration is inversely correlated with BMI and degree of insulin resistance, and is decreased in T2D[42]. Many large-scale studies have demonstrated a relationship between low levels of adiponectin and increased breast cancer risk[43-45]. Tumours in patients with low concentrations of plasma adiponectin have a high histologic grade, and are larger and more invasive [44]. Growth suppression is seen when two ER-negative breast cancer cell lines are exposed to adiponectin[46], and furthermore adiponectin completely blocks estrogen-induced growth of the ER expressing MCF-7 cell line[47]. Both adipokines, therefore, can facilitate the progression of cancers directly, and indirectly through their effects on estrogen signaling.

Before menopause, estrogens are produced mainly by the ovaries, but after menopause, circulating estrogen is synthesized almost exclusively in stromal cells of the adipose tissue[48, 49]. Estrogens have a causal role in BC, and a stimulatory effect on growth and metastasis of ER expressing tumours[48, 49]. Therefore, obese, postmenopausal women produce greater amounts of estrogens and have a higher risk of breast cancer[50]. Communication between the insulin pathway and estrogens may explain the synergistic effects of the high circulating insulin that often accompanies insulin resistance, and estrogen in promoting BC growth and possibly initiation.

**Metformin**

Further evidence for the possible impact of hyperinsulinemia and T2D in cancer, and a possible effective anticancer therapy in IR overexpressing cancers, come from retrospective observational epidemiologic studies of cancer incidence in patients with T2D undergoing various treatments. Significantly, patients taking metformin, a drug that reduces circulating insulin levels, had a reduced risk of cancer diagnosis (all cancer types, including BC). This effect is associated with greater metformin exposure[51]. Furthermore, a separate study reported lower cancer mortality in diabetics being treated with metformin than other treatments that do not work by lowering circulating insulin (sulfonylureas or insulin)[52]. Finally, a recent observational epidemiological study
among 2,529 BC patients reported higher response rates to standard of care neoadjuvant systemic therapy in BC patients who were diabetic receiving metformin, compared to those who were diabetic but not receiving metformin or non-diabetic and not receiving metformin[53].

Metformin functions by causing AMP-dependent protein kinase (AMPK) activation and inhibiting transcription of key gluconeogenesis genes in the liver, and by increasing glucose uptake in skeletal muscle. This results in a very effective reduction in levels of circulating glucose, which in turn increases insulin sensitivity and reduces hyperinsulinemia[54, 55]. Even in healthy non diabetic individuals, administration of metformin leads to a reduction in circulating insulin levels[56]. In addition to the (indirect) metabolic effects, activation of AMPK by metformin and its other agonists, like AICAR, has been found to have a strong direct growth-suppressive effect on many cancer cells. In BC cell lines, this effect was independent of ER and HER2 status and was identified in a variety of cell lines having characteristics of a spectrum of molecular subtypes of breast cancer[55, 57, 58]. The direct anti-proliferative effects of activated AMPK are mediated by its ability to potently inhibit cellular protein synthesis and growth. Mechanistically, AMPK achieves this by phosphorylation and stabilization of the protein product of the tuberous sclerosis complex 2 (TSC2) tumour suppressor gene[59]. TSC2 integrates various regulatory inputs implicated in cell growth, then transmits them to mTOR, which is a key regulator of cellular protein translation, mTOR reviewed in[60].

The ability of metformin to both indirectly effect the elevated circulating insulin levels, as well as directly effect signaling throughput to mTOR in cancer cells has led to the development of several clinical trials evaluating this drug in therapy of breast and other cancers[61]. The outcome of these clinical trials will establish the utility of metformin/insulin-lowering drugs as anti-cancer agents in combination with standard BC care, and inform the development of diagnostic, predictive and molecular mechanism of action biomarkers for their possible use in BC treatment.
THE INSULIN RECEPTOR

Although not expressed in normal breast tissue, the IR is ubiquitously expressed in BC. This raises the possibility that insulin signaling in BC cells, via activation of proliferative and survival pathways, may be responsible for the negative prognostic effects of obesity and high circulating insulin levels.

The Insulin Receptor Gene and Protein

The human IR is encoded by a 22-exon gene spanning 120 kB on chromosome 19[62]. The approximately 5kb cDNA was isolated and characterized by two groups, who found that their sequences predicted IRs of two different lengths- 1343 aa[63] and 1355 aa[64]. The size discrepancy is due to the inclusion or exclusion of the 36 bp exon 11 encoding a 12 aa segment in the C-terminus of the IR α-subunit; this alternative splicing generates two IR isoforms[65]. Expression of IR-A and B are developmentally regulated. The shorter “fetal” IR-A, lacking exon-11, is typically expressed embryonically, while the longer IR-B “adult” isoform is thought to mediate the metabolic functions of insulin during post-natal life. Able to dimerize with the IR, the Insulin-like Growth Factor Receptor I (IGF-IR) also contributes to signaling through the IR. The IGF-IR has significant homology with the IR as the both receptors evolved from the same ancestral gene[66], and their ligand-binding domains and tyrosine kinase domains have over 50% homology[67]. Together, IR and IGF-IR respond to nutrient availability and environmental conditions to coordinate metabolism, growth and differentiation [68].

The IR is a dimeric transmembrane receptor that belongs to the family of receptor tyrosine kinases. Each monomer is composed of an α and β subunit covalently linked by disulfide bonds. Upon dimerization, further disulfide bonds are formed between the monomers. All forms of the mRNA transcript appear to encode for the same complete proreceptor[16, 69-71] Each monomer is synthesized as a single chain 180kD proreceptor, and is extensively glycosylated. The α-subunit is 723aa and 130kDa the β-subunit is 620aa and 95kDa[72, 73], with the difference in predicted mass and actual
Figure 1.3 The Insulin Receptor Isoforms
(A) Diagram of the modular structure of IR. The IR is comprised of the α and β subunit held together by disulfide bonds. On left side of the figure the α and β subunit is show how they are encoded by the 22 exon sequence[82]. The red fragment at the C terminus of the α-subunit is encoded by exon 11 and is present in IR-B, but not in IR-A[80]. The predicted protein modules are indicated in colour on the right. JM indicates the juxtamembrane domain, ID is the insert domain, encoded by exon 10 (and 11)[82]. (B) Side view of the 3D structure of the IR α subunit. The gray loop indicates the insert domain (ID) with and without the red fragment on top of ID impeding ligand binding. (C) Schematic top view of the IR heterotetramer extracellular portion. Insulin (in black) binds to one site of IR-B. The 12aa encoded by exon 11 does not allow for binding of IGF-II (gray molecule) to IR-B. Due to the absence of exon 11 both insulin and IGF-II may bind to IR-A[82].

Figure adapted from Belfiore, A., et al., Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocr Rev. 2009. 30(6): p. 586-623.
mass due to glycosylation[74]. Following glycosylation, intra and inter-subunit disulfide bonds are formed in the rough endoplasmic reticulum. The prereceptor then moves to the Golgi apparatus where proteolytic cleavage by furin occurs at a specific tetrabasic sequence to separate the α and β-subunits, at which point the isoforms are differentiated[74, 75]. Terminal glycosylation and fatty acid acylation also occur in the Golgi post-cleavage before the receptor is transported to the cell surface[74, 75].

Binding of ligand to the IR α-subunit results in dimerization and tyrosine kinase activity in the β-domain by causing a conformational change that brings the two β-subunits into close proximity. After ligand binding, autophosphorylation of residues on the β-subunits induces a series of conformational changes in the receptor that allow ATP-binding, further β-subunit phosphorylation, and recruitment of membrane and cytosolic protein substrates[16, 71]. The α-subunit contains both a low (site 1) and a high affinity ligand binding site (site 2)[16, 71, 72]. Ligand first binds the low affinity site of one α-subunit, and then the high affinity site of the opposing α-subunit. A second ligand molecule (insulin, IGFI/II) bridging the leftover sites 1 and 2 speeds dissociation of the first molecule of ligand[73, 76]. In this way the two kinase domains are brought together to allow trans-autophosphorylation. Interestingly, computational analyses have shown that the length of time that ligand spends bound to the IR may determine which IR-mediated signaling pathway is activated downstream [16, 71, 77], and therefore differences between IR ligands that influence their binding may dictate differences in signaling caused by their association with the IR. A slow ligand dissociation rate also causes an increase in IR pathway signaling, demonstrated by the use of insulin analogs with slower dissociation rates which result in disproportionately high signaling through the IR[73].

**Insulin Receptor Expression**

Though the major targets of insulin action are adipose tissue, liver, and skeletal muscle, IR is also expressed at lower levels in the pancreatic acini, kidney, heart, brain, lungs, placenta, erythrocytes and other cells[78], indicating that IR also has non-metabolic functions in the body. In metabolic tissues IR-B is the predominant isoform accounting
for between 60-80% of total IR expression in the liver, muscle and adipose tissue[79]. IR-A is the predominant isoform in fetal cells, however in adults it is ubiquitously expressed at very low levels and interestingly is the major isoform expressed in many human cancers[80]. In general, the action of insulin is metabolic in tissues that predominantly express IR-B and non-metabolic in those that express IR-A as the major isoform[81].

IR expression is regulated in a tissue and development-specific manner. The 2000bp IR promoter[65] is subject to complex regulation in a tissue and development-specific manner[82, 83], suggesting that IR expression is highly regulated and can respond to changing cellular conditions.

**Insulin Receptor Isoforms**

*Ligand binding*

Studies of the two IR isoforms and their interactions with ligand have been very useful in determining IR function and its role in both normal and pathological conditions like T2D and cancer. The presence of the additional 12aa encoded by exon 11 at the C-terminus of the α-subunit of IR-B, immediately downstream of the C-terminal peptide essential for ligand binding, result in IR-A and IR-B having different ligand binding affinities [76]. Compared to IR-B, IR-A has a 1.7-fold higher affinity for insulin[84], and a higher rate of insulin association and dissociation[85]. Additionally, IR-A is internalized and recycled more quickly than IR-B[86]. Importantly, the presence of the 12aa exon-11 does not allow for binding of the larger IGF-I and II to IR-B[87], and may contribute the slower binding and turnover of IR-B bound insulin[76]. IGF-I, though it binds the IR with lower affinity than insulin does, binds the IR 10-times more frequently in cells with high IR-A expression than in cells expressing only IR-B[88]. Clearly, the inclusion or exclusion of these 12aa affects how each isoform interacts with ligand. The presence of exon 11, therefore, influences the result of IR activation, as binding of specific ligands and the length of time ligand spends bound to the receptor determines downstream signaling.
The functional consequence of these differences in ligand binding has been explored in a number of studies. The isoforms have discrete roles in specific cell types. In murine hematopoetic cells, IR-A induces mitogenic and antiapoptotic signals, while stimulation of IR-B primarily induces cell differentiation[89]. Which ligand binds the IR also dictates the result of IR activation. When stimulated with IGF-II, cells expressing IR-A preferentially undergo proliferation, whereas insulin stimulation results in activation of glucose uptake[90].

*Receptor Dimerization and Hybrid Receptors*

IR hetero and homodimerization adds an additional layer of control and complexity to insulin/IGF signaling. Most tissues express both IR isoforms, though one is usually expressed at higher levels than the other. When dimerization occurs upon the binding of ligand to the IR extracellular α subunit, the result is both homodimers (IR-B/IR-B or IR-A/IR-A dimers) and heterodimers (IR-A/IR-B dimers). Studies using bioluminescence resonance energy transfer (BRET)[91] have shown that these heterodimers form randomly, and their frequency depends on the proportion of each isoform within the membrane[92]. Interestingly, though homodimers of IR-B are unable to bind IGFs, heterodimers are able to recruit intracellular partners upon insulin and IGF-II stimulation with the same affinity as IR-A homodimers[91]. Additionally, IGF-I can activate heterodimers, but with less affinity than it can activate IR-A homodimers[91]. IR-A homodimers have high affinity for IGFI and IGFII, and also bind insulin well[93, 94]. The outcome for signaling, therefore, is that when IR-A is the predominant isoform IR-B is mostly incorporated into heterodimers with the result that the majority of insulin binding sites can also act as high affinity IGF-I/II binding sites[81].

The existence of both IR and IGF-IR suggest that though the pathways of metabolic signaling (insulin/IR) and growth regulation (IGFs/IGF-IR) have been separated through evolution, crosstalk between the pathways remains in the form of IGF-II and IR-A[95]. Cells that express both IGF-IR and IR can form dimers, called hybrid receptors (HR) of an IR isoform hemireceptor and an IGF-IR hemireceptor[85, 96, 97]. These HRs form
Figure 1.4 Dimerization of insulin receptor isoform monomers.
Insulin receptor subunits of each isoform (comprised of one alpha and one beta monomer) can dimerize with other subunits of the same or other isoform. Dimers of the same isoform are called homodimers, and made of both isoforms are called heterodimers [82]. The alpha monomers of each isoform are shown in different colours as they differ by the presence or exclusion of exon-11. The isoform dimers bind ligand with different affinities. The ligands each dimer bind are shown above the receptor dimer. The ligand which binds the receptor with the least affinity is shown outlined with a red box. IR-B homodimers bind insulin with high affinity to exert the metabolic effects of insulin. Heterodimers of IR-A:IR-B bind insulin and IGF-II with high affinity, which leads mainly to mitogenic signaling downstream. IF-A homodimers bind insulin, IGF-I/II with high affinity which results in mitogenic signaling downstream [82, 101, 103].
with the same efficiency as IR isoform homo and heterodimers, so the proportion of HRs
is dependent only on the amount of each hemireceptor present in the membrane[98]. In
fact, in most tissues, such as fat, skeletal muscle and the breast, HRs represent about one
half of the total amount of receptors that bind IGF and insulin in a cell[98]. HRs have
ligand binding properties that differ from those of whichever IR isoform they include. For
example, unlike IR-B homodimers, HR-B (the IGF-IR:IR-B hybrid receptor) have a high
affinity for IGF-I[99], and bind insulin very poorly. Further study of HR binding has
demonstrated that HR-A is better able to bind high affinity ligands like the IGFs, than the
low affinity ligand insulin[100]. HR-A, therefore, is considered a low specificity receptor
that is usually activated by IGF-I or II, except under conditions of high insulin
availability (hyperinsulinemia)[100]. As a result, HR-As represent another way by which
increasing IR-A expression amplifies growth signaling through the IR and IGF-IR
pathways.

IR Isoforms and Hybrid Receptor Physiology

Both insulin and IGFs are required for normal vertebrate and invertebrate development.
These ligands act through various receptors, which are expressed early in fetal
development[78]. During embryogenesis, growth is promoted mainly by IGF-II signaling
through IR-A, whether through HR-A or IR-A homodimers[95]. Thus, through its ability
to bind both insulin and IGFs, IR-A may allow for the cross talk with the IGF-IR
signaling pathway that is necessary for embryonic growth. As fetal development
progresses, binding studies have shown that the proportion of homodimeric IR to HR
gradually increases[101], shifting the IR/IGF-IR expression profile to accommodate for
the physiological needs of an organism in a different stage of life.

Postnatally, IR isoforms continue to exert different roles in mammalian physiology. IR
isoform switching to predominantly express IR-B is linked with cell differentiation in
several cell types. For example, in early hepatocytes induction of differentiation causes a
shift from high expression of IR-A to high IR-B expression[89, 102]. Further,
differentiation of adipose tissue, another major insulin metabolic target, requires an
Figure 1.5 Insulin Receptor and IGF-IR dimerization and hybrid receptors
The insulin receptor and IGF-IR hemireceptors can dimerize to create hybrid receptors (HR)[82]. Hybrid receptors can occur between IGF-IR and IR-A and IR-B hemireceptors. The ligand which binds the receptor with the least affinity is shown outlined with a red box. Homodimers of IR-B bind insulin to exert a metabolic effect[82]. Hybrid receptors of IR-B:IGF-IR (HR-B) bind IGFII well but bind insulin with low affinity[109] and ligand binding therefore mainly exerts a mitogenic effect. HR-A bind all ligands to exert a mitogenic effect, though it only binds insulin in situations of high insulin availability[110]. IGF-IR homodimers bind IGFII and lead to mitogenic signaling downstream[104].
isoform switch from primarily IR-A to IR-B[103]. Differentiation of as preadipocytes into mature adipocytes is associated with a switch in their response to insulin and IGF largely based on differential IR isoform expression[102, 104, 105].

The Insulin Receptor and Cancer

Expression of the IR has been studied in many human cancers. Interestingly, compared to normal tissues, colon, lung, ovarian, thyroid and smooth muscle tumours have all been found to express high levels of the IR[106-108]. In BC, high levels of IR have been found in >85% of tumours tested[109]. Moreover, average IR expression in BC is more than 6 times higher in cancer specimens compared to normal breast tissue[110]. Further, functional studies of breast cancer tissue compared to normal breast tissue indicate higher IR responsiveness to insulin in cancer cells[111].

There is an imbalance in cancer cell IR isoform expression. IR-A is the predominant isoform expressed in breast carcinomas, as well as cancers of the colon, smooth muscle, thyroid, ovary and lung[89, 108, 112]. In contrast with normal breast tissue, IR-A is also the prevalent isoform expressed in BC cell lines and tissue samples[113]. Interestingly, BC cells have been shown to secrete IGF-II, which may act in an autocrine manner[113]. Thyroid carcinomas and ovarian cancer cells[108], which also overexpress the IR, have elevated IR-A:IR-B and produce autocrine IGF-II[112]. Significantly, blocking IR-A, as well as IGF-II, inhibits growth in these cell types[112].

Several studies have examined if IR overexpression or increasing the IR-A:IR-B ratio can initiate cell transformation and tumorigenesis. Expression of IR-A in 184B5 cells, immortalized human breast epithelial cells, at levels similar to those found in human breast cancers does induce a ligand-dependant transformed phenotype that can be inhibited by an IR-blocking antibody[114]. Significantly, these cells fail to induce tumours in nude mice[114], suggesting that full oncogenic transformation requires more than just elevated IR-A expression[114].
INSULIN RECEPTOR SIGNALING PATHWAYS

The activated IR tyrosine kinase leads to activation of a series of cellular signaling pathways responsible for glucose transport across the cell membrane and glycogen synthesis, as well as increases in cell growth, proliferation, migration and protein synthesis. Proximal to the IR are the Insulin Receptor Substrate (IRS) proteins, which are directly phosphorylated by the IR[115]. When phosphorylated by IR, IRS 1-6[73, 116] provide docking sites for Src homology 2 (SH2) domains in adaptor and effector proteins which are responsible for further propagation of the signal[117]. The PI3K and Ras signaling pathways represent the major targets of IR activation and are briefly introduced below.

The PI3K Pathway

The Phosphoinositol 3-Kinase (PI3K) pathway is largely responsible for the metabolic and anabolic signaling downstream of the IR[118]. The class IA PI3Ks, transduce signals from receptor tyrosine kinases (RTKs) by phosphorylating the 3’ hydroxyl group of phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphotidylinositol-3,4,5-trisphosphate (PIP3), a plasma membrane second messenger which regulates glucose homeostasis, proliferation, survival, and growth[118]. Class IA PI3Ks are composed of two subunits: the regulatory subunit p85, and p110, the catalytic subunit. In response to ligand binding, IRS is recruited and phosphorylated by the activated IR, which in turn recruits PI3K by binding its regulatory subunit. The regulatory subunit undergoes a conformational change to allow the catalytic subunit to phosphorylate PIP2 to PIP3[119]. The negative regulator of PI3K signaling is the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which acts in direct opposition to the action of PI3K; PTEN dephosphorylates the D3 position of PIP3 into PIP2[120]. Once formed, PIP3 transduces the IR signal by binding the plekstrin homology (PH) domains of proteins to recruit them to the plasma membrane. One such PH domain containing protein is protein kinase B (PKB)/Akt, which, when recruited by PIP3, is phosphorylated by 3-phosphoinositide-dependent kinase (PDK-1)[119] at threonine 308 (T308). Full activation of PKB is
Figure 1.6 Signaling Through PI3K as Part of the Insulin Receptor Pathway
Upon ligand binding, the insulin receptor is activated, and signals through numerous downstream effectors, including the Phosphatidylinositol 3-kinase pathway. PI3K pathway activation results in cell growth and proliferation, glucose transport, protein synthesis via the mTOR1 complex, inhibition of apoptosis, and effects cell glycogen metabolism through inhibition of GSK3[144]. The tumor suppressor PTEN is an important negative regulator of the PI3K pathway[144]. PI3K and PTEN are major oncogenes[146]. Activating mutations in the PI3K are also common in cancer, and confer growth factor independence to the cell; rendering this pathway ligand independant[146,147].
achieved by its further phosphorylation at serine 473 (S473) by mTORC2[121]. As PKB has over 80 downstream targets, its activation has many consequences, namely decreased apoptosis, and increased cell growth and protein synthesis.

Pro-survival functions of PKB/Akt are carried out in part by direct phosphorylation of forkhead box “Other” (FOXO) 1,3 and 4 transcripts[122]. When phosphorylated, FOXO proteins are exported from the nucleus and degraded in the cytoplasm[123]. Nuclear export of FOXOs reduces the transcription of several proapoptotic factors and cell cycle inhibitory genes, including p27\(^\text{Kip1}\) [118], p21\(^\text{Cip1}\) [124], and Fas-ligand[125]. PKB also phosphorylates and inactivates the mitochondrial proapoptotic protein BAD[126]. Finally, phosphorylation by PKB enhances the activity of both MDM2, which negatively regulates the tumour suppressor p53, and the transcription factor NF-κB, leading to apoptosis inhibition[127].

The PI3K pathway is also a major regulator of cellular protein synthesis and growth, mainly via control of tuberous sclerosis complex 2 (TSC2). Activated TSC2 acts as a GTPase activating protein (GAP) towards the small GTPase Rheb[128]. Inactivation of TSC2 by PKB phosphorylation relieves its inhibition of Rheb[129] with the result that the amount of GTP bound Rheb increases. Rheb is then able to bind and activate mTORC1, the master regulator of mRNA translation[130]. mTORC1 activity phosphorylates and regulates 4E-BP1 and S6K. When phosphorylated by mTORC1, the serine/threonine protein kinase S6K regulates factors involved in protein synthesis, like ribosomal S6 protein (S6) and other translational regulators like eIF4B[131] and PDCD4[132]. Moreover, phosphorylated 4E-BP1 releases eIF-4E, allowing it to interact with eIF-4G and activate cap-dependent mRNA translation[131].

PKB activation regulates metabolism via regulation of the metabolic enzymes 6-phosphofructo-2-kinase and glycogen synthase kinase (GSK3β), through which it stimulates glycogen synthesis. It also induces translocation of the glucose transporter GLUT-4 from the intracellular storage compartment to the plasma membrane[116]. Additionally, PKB can promote aerobic glycolysis, a metabolic process of many
cancers[133]. Which group(s) of PKB substrates are targeted in response to PI3K activation are somewhat unclear, but they may be determined by different expression in particular cell types, localization or other factors that depend on environmental conditions/circumstances, or through cross talk between pathways[134].

The Roles of PKB Isoforms

Considering the variety of cellular targets mediating the effects of PKB it is reasonable to assume further degrees of regulation of substrates by the activated PKB. The more setting-specific expression of PKB isoforms, of which there are three, and PKB substrates may play a part in determining the PKB response to a specific stimulus.

Several studies have investigated isoform specific functions of PKB. Though the three isoforms- PKBα, PKBβ and PKBγ- are structurally homologous and share a common mechanism of activation, they display different physiological functions[135]. While, PKBα and β are expressed ubiquitously, PKBγ is mainly found in tissues of the brain[136]. The viability of knockout mice for each of the isoforms suggests functional redundancy. Nevertheless, mice lacking PKBα display significant growth and developmental defects[137], PKBβ knockout mice fail to maintain glucose homeostasis[138] and PKBγ deficient mice show defects in brain development[139]. Based on this data, it is thought that PKBβ is most responsible for carrying out the metabolic role of insulin, while PKBα seems responsible for mitogenic signaling, and signaling during development.

Double knockouts (DKO) have been more useful in determining non-redundant isoform functions. Knockout mice lacking both PKBα and PKBβ die shortly after birth, are very small, with many deficits in skin and bone, skeletal muscle atrophy and adipose tissue development[140]. Interestingly, adipocyte differentiation can be restored through ectopic expression of PKBα but not PKBβ[141], indicating that in the regulation of this specific process, there is no redundancy in PKB isoform function. Compound knockouts of PKBα and PKBγ reveal a dosage-dependent effect of the PKB isoforms on
development and survival. PKBα/- PKBγ+/- mice die several days after birth and have multiple defects in the heart, thymus and skin, whereas PKBα/- PKBγ/- genotype is embryonic lethal. Remarkably, PKBα+/- PKBγ-/- mice survive[142], indicating that only one copy of PKBα is required for survival. Furthermore, PKBβ-/- PKBγ-/- and even PKBα+/- PKBβ-/- PKBγ-/- mice develop normally and survive, but with severe glucose intolerance and low body and brain mass[143] indicating that PKBα gene is essential for development and survival, while both PKBβ and PKBγ play a role in animal and individual organ size. It is not yet known, however, how signaling through a specific isoform is selected for within the IR/IGF-IR axis.

The PI3K Pathway and Cancer

The link between PI3K and cancer was first noted in the 1980s, when the protein’s lipid kinase activity was associated with two viral oncoproteins[134]. Binding of p85 PI3K, and recruitment of p110α PI3K to phosphotyrosines of the viral oncoproteins lead to activation of the PI3K pathway[144]. Later, PI3K was found to be activated by many oncogenic RTKs[145], and therefore implicated in transmitting cancer-relevant signals. More recently, somatic mutations in p110α and PTEN, leading to increased activity of the PI3K pathway, have been found in many cancers[134].

Of the four genes encoding the PI3K catalytic subunit, PIK3CAis the only one frequently mutated in human cancer. Point mutations of the gene are found in many cancer types, including cancers of the breast (27%), endometrium (22%), and prostate (29%), where somatic mutations of PIK3CA are more frequent than any other genetic change[146]. Around 80% of PIK3CA mutations occur in three “hotspot” locations: E542K and E545K in the helical domain, and H1047 in the kinase domain[147]. These hot spot mutations are sufficient to induce transformation[148], and result in increased PI3K activity in vitro as well as growth factor independent pathway activation and growth[149, 150].

Oncogenic mutations of any of the three PKB isoforms are not frequent in cancer[151, 152]. A transforming point mutation (E17K) within the PH domain of PKBα leading to
its membrane recruitment independent of PIP3 [153] has been found in a subset of ovarian(2%), colorectal(6%), and breast cancers(8%)[153]. More prevalent, PKBβ amplification was identified in a subset of head and neck squamous cell carcinoma (30%), pancreatic (20%), ovarian (12%) and breast (3%) cancers [154-156]. No mutations in PKBγ have been identified thus far.

The PTEN gene is one of the most commonly mutated tumor suppressor genes in human cancer[134]. Found frequently in glioblastoma, breast, lung, prostate, and endometrial cancers[134, 157-159], genetic inactivation or reduced expression of PTEN leads to increased cell proliferation, cell growth, and decreased apoptosis, therefore enhancing tumour development[160, 161]. Despite the fact that PTEN also acts as a serine, threonine, and tyrosine phosphatase, maintenance of its activity as a lipid phosphatase is shown to be the driving force of tumour suppression[162]. Mutations in PTEN that impair lipid phosphatase function lead to accumulation of PIP3 and unfettered activation of proteins downstream of it[120, 134, 163]. The high frequency of PTEN mutations could be explained by its lack of redundancy in this function.

Both inherited and somatic mutations in PTEN cause an increased incidence of cancer. PTEN mutations follow the two-hit hypothesis of tumour suppression; inherited or sporadic mutation of PTEN predisposes an individual to cancer, as a second hit in the other allele causes oncogenesis. “Two hits” in PTEN are seen in sporadic tumours as well as tumours from patients with germline mutations[164]. Many disorders, called collectively PTEN hamartoma tumour syndromes[160], like Cowden’s Syndrome (CS), are caused by inherited PTEN mutations. These disorders are characterized mainly by the formation of benign tumours. In the case of the autosomal dominant CS, patients have a much higher risk for breast, thyroid, and endometrial cancers, as well as renal cell carcinomas, melanoma, and some brain tumours[163].

**The Ras Pathway**
Activation of the Ras pathway is mainly responsible for the mitogenic effects of IR ligand binding. Ras signaling is initiated by binding of Grb2 to a phosphorylated IRS via its SH2 domain[165]. Through its SH3 domain, Grb2 then binds and activates the Ras guanine nucleotide exchange factor (GEF) mSos (son of sevenless)[165]. Activated mSos recruits Ras and promotes its exchange of GDP for GTP[166]. Ras-GTP binds the serine/threonine kinase Raf, which, when active, phosphorylates the dual specificity kinase MAPK kinase (MEK1)[167].

The target of MEK1 is ERK1/2, another MAPK family kinase. In its inactive form in the cytoplasm, ERK1/2 forms a heterodimer with MEK1[168]. Phosphorylation by MEK1 allows ERK1/2 to dissociate from the complex and translocate to the nucleus, where it phosphorylates substrates involved in transcriptional programs that induce cell proliferation, growth, and migration[168, 169]. Activated ERK1/2 also acts in the cytoplasm to phosphorylate numerous targets and initiates protein translation and cell growth. Because ERK1/2 has targets in both the nucleus and cytoplasm, its localization is very important in determining the biological effects of its activation[169].

The 90kD ribosomal S6 kinases (RSKs) are a family of Ser/Thr kinases downstream of the Ras-MAPK cascade activated directly by ERK1/2. Implicated in the regulation of diverse cellular processes, such as cell proliferation, survival, growth and motility, RSKs phosphorylate both cytosolic and nuclear targets[170]. RSKs are just one of the many proteins downstream of Ras/ERK1/2 that have a variety of targets in and outside of the nucleus. Crosstalk with the PI3K pathway also occurs, further complicating untangling the effects of signaling through the IR. Understanding how these pathways affect cell function alone and in tandem will help decipher how to regulate specific axes and their effects without disrupting and potentially accelerating cancer progression.

EPITHELIAL AND MESENCHYMYAL PHENOTYPES AND ERK1/2

Normal epithelial tissue is comprised of regular cells that maintain tight junctions, gap junctions, and cell-cell adherence junctions mediated by interactions between E-cadherin
and glycoproteins on adjacent cells[171]. A prototypical cadherin, calcium-dependent E-cadherin is an intercellular adhesion molecule that couples to the actin cytoskeleton of adjacent cells through the binding of β-catenin to its cytoplasmic tail[172]. Gap junctions allow direct chemical communication between adjacent cells, and tight junctions provide epithelial tissues with its barrier functions[173]. Additionally, through interactions mediated by integrins and other molecules, cells are connected to the extracellular matrix (ECM)[174]. Along with cell-cell contacts, contact with the ECM helps define cell polarity, a property that allows separate functions for the basal and apical cell surface[175]. The structural integrity and polarity provided by these many contacts are important properties of epithelial tissues. Internally, these properties allow epithelia to create defined subdomains with specific physiological roles[174]. An example where maintenance of epithelial characteristics is important to tissue function is in normal breast tissue, where epithelial cells form highly organized tubuloductal structures necessary for lactation[176].

Certain circumstances require dissolution of the rigidly structured epithelial phenotype. During development, for example, embryonic epithelium gives rise to the mesoderm, which is comprised of highly motile cells that migrate to become many tissues[177, 178]. During this program, epithelial cells transform into another cell type, mesenchymal cells, in a process called epithelial to mesenchymal transition (EMT)[178]. Mesenchymal cells are highly motile, non-polarized cells with few cell-cell contacts and an increased ability to differentiate [179]. In adults, mesenchymal cells are usually found in loose connective tissue or in reticular fibers, where they can migrate to aid in wound healing[180] or form new smooth muscle, arteries[181], and bone tissue[182].

Inappropriate induction of EMT in normal tissue can disrupt tissue integrity and therefore function, and result in pathological conditions like chronic inflammation and fibrosis[183, 184]. In cancer cells, EMT is a key process in the metastatic cascade[185, 186]. Mesenchymal cells can detach from neighbouring cells and penetrate to or through surrounding tissue, remodeling the ECM as they go to further disturb any epithelium they pass through[186]; mesenchymal cells are able to synthesize ECM components and
Figure 1.7 EMT and the contribution of EMT to cancer progression.

(A) The transition of an epithelial to a mesenchymal cell (EMT) involves a functional transition, and includes changes in expression of several proteins that mediate the function of each cell type. Epithelial cells are polarized and attached to the cells adjacent. They express the proteins listed in red, which can used to identify them as epithelial cells. Following a stimulus for EMT, such as a change in environmental conditions or during embryogenesis, cells pass through a variety of phenotypes. They express a mixture of epithelial and mesenchymal markers and gradually attain a mesenchymal morphology. Cells are no longer attached to the basement membrane, excrete ECM, and are more motile. They express proteins, listed in green, which are used to identify them as mesenchymal cells[174].

(B) The progression from normal epithelium to metastatic and invasive carcinoma involves several steps. Structured epithelial tissue acquires an oncogenic mutation and an epithelial tumour forms that disrupts tissue architecture but is contained. EMT occurs and cells loose polarity and detach from the basement membrane. Mesenchymal cells secrete factors which alter the composition of the basement membrane and change signaling networks in surrounding cells. These mesenchymal cells enter the blood stream and exit the circulation at a remote site. These remote metastases may revert back to epithelial cells through MET[174].
produce metalloproteinases (MMP) that degrade the matrix[187], as well as secrete signaling molecules like EGF that can act on epithelial cells[188]. Further aiding tumor progression, mesenchymal cells are more resistant to apoptosis and therefore apoptotic agents[189, 190]. In these ways, EMT is especially dangerous in the context of cancer.

Once EMT is induced, cell-cell adhesions are dissembled, and cell polarity is lost. Cells are more invasive and migratory, and instead of appearing regular and cuboidal, are spread and have spindle like protrusions[178]. The molecular hallmarks of EMT are loss of proteins required for cell-cell junctions like cytokeratin and E-Cadherin, as well as de novo expression of mesenchymal markers like vimentin, and N-cadherin[191, 192]. Expression of transcription factors that regulate E-cadherin, like Twist[185], ZEB1 and ZEB2[193, 194], and Snai1 are increased[195]. Deregulation of the pathways controlling these transcription factors [196], like the IR/IGF-IR[197] pathway, can cause EMT and promote cancer progression. In light of this, E-cadherin can be considered a suppressor of tumor invasion, and a key caretaker of the epithelial state[198]. In fact, markers of E-cadherin suppression are seen in many cancer contexts. The most prevalent human malignancies are carcinomas, which originate from epithelial cells. At the leading edge of carcinomas, expression of Snai1 is high, E-cadherin is low, and cells show many more mesenchymal characteristics[199].

Another protein that has been more recently linked to loss of epithelial phenotype is ERK2, an important effector of the MAPK signaling cascade. Activity of the Ras-ERK1/2 axis in this pathway affects E-cadherin function. Inhibition of Ras can restore E-cadherin expression in many human cancer cells[200]. Further, ERK1/2 inhibition downstream of Ras blocks EGF from disrupting E-cadherin facilitated adherens junctions[201]. Finally, expression of activated Ras in intestinal epithelial cells is sufficient to disrupt E-cadherin in an ERK1/2 dependent manner[176]. Shin et al further clarify the role of ERK1/2 in maintenance of the epithelial phenotype and EMT in a study that found ERK2, but not ERK1, is responsible for induction of EMT[202]. Ectopic expression of ERK2 in MCF10A cells results in the dramatic change in cell morphology characteristic of EMT. E-cadherin expression is lost and there is increased expression of
mesenchymal markers like N-cadherin and vimentin in a manner similar to induction of EMT in these cells with oncogenic Ras-V12. Moreover, specific knockdown of endogenous ERK2 prevents these changes in MCF10A cells where EMT has been induced by transfection of activating Ras. Significantly, both expression and activation of Fra1, a transcription factor necessary for EMT, depends on a functional ERK2. If ERK2 is disrupted[203], Fra1 is not activated and EMT does not occur; active ERK2/Fra1 regulates the transcription factor ZEB1/2[203], increasing expression of ZEB1/2 to decrease E-cadherin expression and promote EMT.
CHAPTER 2: HYPOTHESIS AND RATIONALE
Although not expressed in normal breast tissue, the insulin receptor (IR) is ubiquitously expressed in BC, raising the possibility that insulin signaling in BC cells, via activation of proliferative and survival pathways, may be responsible for the negative prognostic effects of obesity and high circulating insulin levels. Considering that insulin has essential metabolic functions, modeling the relationship between obesity, high insulin levels and their impact on BC represents a major challenge. Further adding to the complexity of the challenge are the frequent genetic alterations in the components of the insulin-stimulated pathways in BC.

**Hypothesis**

I hypothesize that insulin mediates the impact of obesity in BC and that genetic alterations impacting signaling pathways regulated by insulin play a key role in sensitizing BCs to the action of this hormone.

**Objective**

To develop a cell-based system to explore the impact of insulin on BC cell properties.
CHAPTER 3: MATERIALS AND METHODS
**Materials and Antibodies**

All materials were from Sigma (St. Louis, MO) unless otherwise stated. α-P-PKB (S473) α-PKB, α-Active (cleaved) caspase 3, α –P-RSK, α –P-ERK1/2 (T202/Y204), α-ERK1/2, α-P-IGF-IR, α-IGF-IR, α-p21, α-P-p53(S15) from Cell Signaling (Danvers, MA), α- β-catenin and α-p27Kip1 from BD Transduction Laboratories (Franklin Lakes, NJ), α –IR from Millipore (Temecula, CA), and α- α tubulin from Calbiochem (Darmstadt, Germany)

**Cell Culture**

The MCF7, and 293FT cell lines were grown in Dulbecco’s Modified Eagle Medium, High Glucose (DMEM), containing 1% penicillin streptomycin and containing 10% (v/v) heat-inactivated Fetal Bovine Serum (DMEM and FBS; GIBCO, Grand Island, New York). The MCF10A cell line was grown in 2D in DMEM/F12 media containing 1% penicillin streptomycin and containing 5% (v/v) heat-inactivated Horse Serum from Invitrogen. Additionally the media contains 20ng/mL EGF, 10µg/mL Insulin, 0.5mg/mL Hydrocortisone, and 100ng/mL cholera toxin. MCF10A cells were grown in 3D in growth factor reduced matrigel (BD Biosciences, Missisauga ON), in DMEM/F12 supplemented with 2% growth factor reduced matrigel, 2% Horse Serum, 5ng/mL EGF, 10µg/mL Insulin, 100ng/mL cholera toxin and 0.5mg/mL hydrocortisone. MCF10A cells in 2D and 3D maintained as described by Debnath et al. {Debnath, 2003 #601}

**Immunofluorescence**

MCF10A cells grown in 3D in matrigel were fixed, stained and visualized as described by Debnath et al. {Debnath, 2003 #601}.

**Immunoblotting**

MCF10A cells washed twice with cold PBS and lysed in cold NP-40 lysis buffer (1%v/v NP-40, 50mM Tris-Cl, 150mM NaCl) plus protease inhibitors (10 mM sodium pyrophosphate (Na₂P₂O₇), 10 mM sodium fluoride (NaF), 10 mM EDTA and 50 mM sodium orthovanadate (Na₃VO₄)). After a 20 minute incubation period on ice, cell membranes were isolated by centrifugation of lysates at 10,000 g for 10 minutes at 4°C.
Lysate protein concentrations were determined by Bradford Protein Assay (Bio-Rad, Hercules, CA) with spectroscopic analysis at 595 nm. Cell lysates were mixed with 5X sample buffer (69 mM Tris- HCl (pH 6.8), 11% (v/v) glycerol, 2.2% (w/v) sodium dodecyl sulfate (SDS), 0.02% (w/v) bromophenol blue and 0.5M dithiothreitol (DTT)) boiled for 5 minutes at 95°C to denature the protein, and resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes for immunoblotting.

Membranes were blocked for one hour with blocking buffer (2.5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) in Tris-buffered saline supplemented with 0.1% (v/v) Tween-20 (TBST; 50mM Tris-HCl (pH 8.0), 150 mM NaCl) and sodium azide). Membranes were incubated for overnight with primary antibody diluted 1:1000 in TBST, after which they were washed three times for 10 minutes with TBST. HRP-conjugated secondary antibodies corresponding to the primary used were then applied at a dilution of 1:10 000 in 5% skim milk diluted in TBST for at least 1.5h. Membranes were then washed again 3 times for 10 minutes each with TBST, after which proteins were visualized using Western Lightning™ Chemiluminescence Reagent Plus (ECL; Perkin Elmer, Waltham, MA) and autoradiographic film (Kodak; Rochester, NY). Membranes were stripped for 30 minutes at 50°C in 63.5 mM Tris-HCl (pH 6.8), 2% (v/v) SDS and 0.1 M %-%ME and washed four times with TBST prior to reprobing for other antibodies.

**Migration Assays**

Transwell migration assays were performed as described by Mandelboim et al[204] with the following changes. 24 well plates and transwell filters from BD Biosciences Cell Culture (San Jose, CA) were coated with poly-D-lysine by adding 500µL of 10% poly-D-lysine in sterile PBS to each well for 2h, removing the poly-D-lysine mixture then allowing the filters to dry for 2h before washing them twice with PBS. MCF10A cells were trypsinized and counted using a Coulter Counter and plated 5000 cells/well. Cells were allowed to migrate for 12h before being fixed with 10% formalin and stained with 10% crystal violet. The lower part of the transwell filter was mounted and visualized under a light microscope. Pictures were taken of three fields of view per filter, and
migrated cells were counted using Image-J.

Wound healing assays were performed as described previously [205], with the following differences- 12 well plates were coated with poly-D-lysine in the same way as during a transwell migration assay. MCF10A cells growing in full media (MCF10A media containing all growth factors and serum) were trypsinized, counted with a Coulter Counter, and plated at $2.5 \times 10^5$/well. They were allowed to grow to confluence in serum free MCF10A media, before the media was replaced with either serum-free full MCF10A media, or serum-free MCF10A media without insulin for 24h before a scratch was introduced with a pipette tip. Cells were allowed to migrate for 12 hours before being fixed and stained with formalin and crystal violet, as before. Pictures of three fields of view of each scratch were taken and the average width of the scratch per field of view determined using image J.

**RT-PCR**

RNA was isolated from MCF10A cells grown in full serum using Qiagen RNEasy Midi Kit. Using RT-PCR, cDNA was made from this isolated RNA (Invitrogen). Primers were designed to a 620bp stretch of the IR that included exon 11 (Forward 5’-ATC TGC AGA ACC AGA GTGA-3’, Reverse 5’-CCG AAT TCG TGG GCA CGC TGG TCGA-3’), and PCR was run on the cDNA sample using Taq and buffer from GenScript (Piscataway, NJ). The PCR product was resolved in a 3.5% agarose gel and visualized under a UV light.

**Generation of Lentiviral Stocks and Infection**

Human embryonic kidney 293FT cells (hereafter referred to as ‘293FT cells’) were thawed 5-7 days prior to transfection for expansion and grown in DMEM media supplemented with 10% FBS. Cells were harvested via trypsinization, counted using a Coulter Counter, and plated at $2 \times 10^6$ cells per 10 cm culture plate (100 plates). Calcium phosphate transient co-transfections (CalPhos Mammalian Transfection Kit, Clontech, Mountain View, CA) of 293FT cells were performed with 6µg of lentivirus containing the shRNA targeting construct, 750ng of PAX2 env packaging construct and 250ng of
MDZ-G envelope vector per plate. Cells were washed with PBS and medium was changed 18 hours post-transfection, followed by supernatant collection at 48 and 72h. Harvested viral supernatant was immediately filtered (0.4 µM filter) and pooled to ensure even distribution of viral particles. The viral stock was then distributed at either 100 or 200 µL into cryovials and frozen on dry ice before transferring the stocks to storage at -80°C.

MCF10A cells were plated at a 2.5x10^5 for 24h before being washed with PBS before addition of lentivirus containing media and MCF10A media at a ratio of 1:2. After 24h of infection, virus-containing media was removed and cells were washed in PBS. Media was replaced with MCF10A media containing 1µg/mL puromycin for 4 days before being used in subsequent experiments.

**Statistical Analysis**

For all data, statistical significance was determined by Student’s t test. For all tests a p-value < 0.05 was considered statistically significant.
CHAPTER 4: RESULTS
4.1 The insulin receptor pathway is intact in MCF10A cells

To study and model the insulin response in breast tissue we used MCF10A cells, a spontaneously immortalized, non-transformed mammary epithelial cell line established from a breast with fibrocystic changes[206]. These cells are capable of growth in 2D, but form spherical acini when grown in 3D, recapitulating the steps of mammary gland development including polarization and apoptosis to form and maintain a clear lumen[207]. Expression of oncogenes in the MCF10A model system can lead to EMT and transformed 2D and 3D phenotypes[207]. To determine if MCF10A cells express key components of the IR pathway, Western blotting was performed to detect the presence of the IR, as well as IRS1, a major insulin signaling adaptor, and PTEN, a negative regulator of PI3K signaling. As seen in figures 4.1A and B, MCF10A cells express these proteins, indicating their potential to respond to insulin stimulation. In order to assess whether the IR pathway is functional in MCF10A cells, they were starved of insulin and serum, and stimulated with 10µg/mL insulin. Immunoblot analysis with phospho-specific antibodies against PKB (S473), p70S6K (T389), and ribosomal protein S6 (S240/244) revealed insulin-mediated activation of the PI3K signaling pathway (Fig 4.1B). Next we tested the response of the IGF-IR to stimulation with insulin, but as all activation specific antibodies against phosphorylated IGF-IR cross react with IR phospho-sites, we can confirm that the pathway is activated, but cannot distinguish whether one or both receptors are involved (Fig. 4.1C)

Alternative splicing generates two IR isoforms[65] which differ in size by the inclusion or exclusion of the 36 bp exon 11 encoding a 12 aa segment in the C-terminus of the IR α-subunit. Expression of IR-A and B is developmentally regulated. The shorter “fetal” IR-A, lacking exon-11, is typically expressed embryonically, while the longer IR-B “adult” isoform is thought to mediate the metabolic functions of insulin during post-natal life. To determine which IR isoform is expressed in MCF10A, we devised a RT-PCR strategy exploiting the difference in transcript lengths. By resolving the RT-PCR product on a 3.5% agarose gel, we found that MCF10A cells express both the short isoform A (584bp) and the long isoform B (620bp) of the IR (Fig 4.1A)
Figure 4.1 The insulin receptor pathway is intact in MCF10A cells.

(A) Lysates from MCF10A cells were immunoblotted (IB) with antibody against the insulin receptor. HEK293 cells were included as a positive control. RNA was isolated from MCF10A cells and RT-PCR was used to make cDNA. Primers were designed to a 620bp section of the insulin receptor that included exon 11. The PCR product was resolved on a high percentage agarose gel. (B) Presence of the insulin receptor pathway was determined using IB of MCF10A lysates for IRS1 and PTEN, using HEK293 cells as a negative control. MCF10A cells were starved (-) and then stimulated with insulin (+) for 1 minute. Lysates of starved and stimulated cells were IB for phosphorylated PKB, S6K and S6. (C) MCF10A cells were starved and then stimulated with various concentrations of insulin for 15 minutes. Lysates were IB for IGF-IR and P-IGF-IR, as well as tubulin as a loading control.
4.2 Growth response and change in morphology of MCF10A cells after EGF or insulin withdrawal.

To investigate the role of insulin in MCF10A proliferation, we plated cells at low density and grew them in media containing no serum, the full media concentration of EGF, and various concentrations of insulin for 3 days. Proliferation was monitored using the Incucyte, which recorded cell density every 4h over the monitoring period. While there was a slight decrease in proliferation at low insulin concentrations, the cells still attained full or near-full confluence after 72 hours, even at 1/10th the full insulin concentration (Fig 4.2A). However, complete insulin withdrawal resulted in a significant impairment of proliferation (Fig 4.2A). Interestingly, even limited EGF withdrawal led to a significant reduction in proliferation (Fig 4.2A).

We next asked how insulin withdrawal affects cell morphology. Cells were plated at low density in no serum and without either insulin or EGF. Cell morphology was monitored using the Incucyte, which recorded cell images at 10X magnification at 4h intervals during cell growth for 3 days. MCF10A cells grown under optimal conditions in 2D are cuboidal and have a regular, cobblestone appearance. After 3 days, the cells grown in full media (without serum but with EGF and insulin) maintained a cobblestone appearance (Fig 4.2B). Cells grown in serum-free media supplemented with EGF but no insulin displayed spindle shaped protrusions, fewer cell-cell contacts, and a spread appearance beginning at 24h, a morphology they maintained after 3 days. In contrast, cells grown in full insulin but no EGF retained the cuboidal, cobblestone appearance observed in cells grown in full media, even at low cell densities, after 3 days. This difference in morphology reflected distinct signaling in response to insulin and EGF in MCF10A cells.

To probe the impact of insulin on cell growth in 3D, MCF10A cells were grown in matrigel, where they form multicellular acinar structures[207], with or without insulin. Acini were visualized after 14 days, which is the length of time necessary for full acinar formation. Cells grown in full media formed regular spheres (Fig 4.2C). In comparison, MCF10A cells grown in no insulin also formed regular spheres, though consistent with
Figure 4.2 Growth response and change in morphology of MCF10A cells after EGF withdrawal or insulin withdrawal in 2D and 3D.

(A) MCF10A cells were plated in 6 well plates at low confluence, with 3 wells of each of the following conditions- serum free media was supplemented with the full amount of EGF and 0, 1, 5, 8, or 10μg/mL of insulin, or the full amount of insulin and 0, 5, 10, 15, 20ng/mL of EGF. Full media denotes the presence of serum, and full amounts of EGF(20ng/mL) and insulin(10 μg/mL). The Incucyte was used to measure cell confluence in each condition every 2 hours for 3 days. Average confluence in each of the 3 wells was plotted. Three biological replicates were performed of each experiment. (B) MCF10A cells were grown in serum free media with full EGF and insulin, full EGF and no insulin, or full insulin and no EGF. Pictures were taken of cells grown in each condition using the Incucyte after 1 and 3 days.
Figure 4.2 Growth response and change in morphology of MCF10A cells after EGF withdrawal or insulin withdrawal in 2D and 3D.

(C) Cells were grown with (10 μg/mL) or without insulin in matrigel for 14 days to allow complete formation of acinar structures. Pictures were taken of acini at 10X magnification with a light microscope. Acini were fixed and stained with primary antibodies against β-catenin and cleaved caspase 3, fluorescent secondary antibodies, and DAPI, and visualized by immunofluorescence. β-catenin marks the baso-lateral cell margins, and cleaved caspase marks apoptosis.
the negative impact of insulin withdrawal on proliferation, they were much smaller. The acinar structures were fixed and stained with antibodies against markers of apoptosis (Cleaved Caspase 3) and polarization (β-catenin), and visualized by immunofluorescence (Fig 4.2C). MCF10A cells grown in full media displayed apicobasal polarization of the cells making up each acini, as well as evidence of the creation and maintenance of hollow lumen at the center of each acini through apoptosis. In contrast, the morphology of acini grown without insulin was disrupted, exhibiting neither cell polarization nor apoptosis at the center of the acini, congruent with the proliferative and morphological effects associated with insulin withdrawal.

4.3 Insulin withdrawal causes changes in cell migration

The influence of insulin for MCF10A migration was tested by transwell migration assays (Fig 4.3A). Cells were grown with or without insulin for 24h and then seeded in the upper chamber of a transwell filter. The bottom transwell chamber contained media with insulin as a stimulant to migrate, or no insulin, as a control (Fig 4.3C). Cells were left for 12h to allow for migration towards the lower chamber. Cells grown in insulin migrated regardless of whether or not there was insulin in the bottom chamber. However, cells grown in no insulin required the presence of insulin in the bottom chamber to migrate.

Wound-healing assays were also performed to further investigate the dependence of MCF10A migration on insulin. The surface of confluent cells grown in media with or without insulin for 24h was scratched with a pipette tip. The wound was allowed to “heal” overnight in media containing insulin, or no insulin. Cells grown without insulin failed to fully close the scratch gap (Fig 4.3B). However, when the healing media contained insulin, the cells migrated and wound width decreased significantly overnight. Taken together, these results suggest that MCF10A cells require insulin signaling for optimal migration.
Figure 4.3 Insulin withdrawal causes changes in cell migration

(A) MCF10A were grown to confluence in 24 well plates. Media was then exchanged for serum free media containing the full concentration of EGF and insulin, or full EGF and no insulin for 24h called “growth media”. The surface of the confluent cells was then scratched with a pipette tip, and the “cell growth media was replaced with serum free media with or without insulin added (“heal media”). Wounds were allowed to heal overnight. P = 1.97E-04

(B) 5000 cells grown with or without insulin in the media were transferred into the upper well of a transwell chamber. The lower chamber contained media with or without insulin. Cells were allowed to migrate overnight. The upper filter was then scraped, and the cells on the lower filter were fixed, stained and counted. The average number of cells migrated in each condition were plotted.
Figure 4.3 Insulin withdrawal causes changes in cell migration

C) Diagram of the transwell migration assay set-up. A known number of MCF10A cells grown with or without insulin were transferred to the upper chamber of a transwell chamber containing media with the same amount of insulin that they were grown in—“growth” media. The bottom well of the transwell chamber was filled with media with or without insulin—“stimulation” media. Cells were allowed to migrate towards the bottom chamber overnight. Cell migration is indicated by a green arrow, no migration by a red cross. D) Representative pictures of one field of view (FOV) taken at 10X magnification of the bottom of a transwell filter (facing the stimulation media) after cell migration. Cells are fixed and stained with crystal violet, and therefore appear purple. Filter pores appear black.
4.4 Changes in morphology are due to inactivation of the insulin receptor pathway.

To determine if lack of insulin signaling through the IR was responsible for the observed changes in MCF10A morphology, cells were treated overnight with various inhibitors of the IR signaling pathway. Inhibition with HNMPA, an IR inhibitor, resulted in the same changes in morphology seen after insulin withdrawal (Fig 4.4A, left panels, top and bottom). Treatment of MCF10A cells with the PI3K inhibitor LY294002 (Fig 4.4A, right panels), or the mTOR inhibitor rapamycin (Fig 4.4A, middle panels) did not lead to changes in morphology, although LY294002 reduced cell viability precluding the full assessment of the morphological changes. The effects of these inhibitors on proliferation were not determined, as LY294002 was found to kill all plated MCF10A cells after approximately 4h of treatment. Importantly, insulin withdrawal did not result in activation of p53, indicating that the differences in proliferation are likely not a result of pro-apoptotic or growth-arrest signaling.

4.5 Insulin withdrawal and inhibition of the insulin receptor pathway cause ERK1/2 activation.

To investigate the changes in signaling that were responsible for the phenotypic, migrational and proliferative changes upon insulin withdrawal, lysates from MCF10A cells grown with or without insulin were immunoblotted with activation-specific antibodies against several downstream effectors of the IR (Fig 4.5A). After 3 days of insulin withdrawal, phosphorylation of PKB at S473 was predictably decreased, while ERK1/2 ((T202/Y204) phosphorylation was surprisingly elevated (Fig 4.5A). A time course of the ERK1/2 response to insulin withdrawal revealed that increased phosphorylation of ERK1/2 occurred 2h following insulin removal. Importantly, phosphorylation of both PKB and ERK1/2 decreased as the concentration of EGF in the media decreased (Fig. 4.5B), indicating that ERK1/2 activation is specific to insulin withdrawal.
Figure 4.4 Changes in morphology are due to inactivation of the insulin receptor pathway. (A) Various inhibitors of the insulin receptor pathway, HNMPA, Rapamycin and LY294002 were added to the media of subconfluent MCF10A cells overnight. Pictures were taken of the cells after the 24h incubation period. Pictures of MCF10A cells grown for 3 days with or without insulin or EGF, or in full media are shown to compare morphology. (B) Lysates of MCF10A cells grown in serum free media with (+) or without (-) insulin, as well as MCF10A cells treated with Cisplatin overnight were IB with antibodies against P-p53, P-PKB and Tubulin.
Figure 4.5 Insulin withdrawal and inhibition of the insulin receptor pathway cause ERK1/2 activation. 
(A) After being grown in serum free media with (+, 10μg/mL) or without(-) insulin, and the full concentration of EGF (20ng/mL) for 3 days, MCF10A cells were lysed and IB for P-PKB, PKB, P-ERK1/2, ERK1/2, and epithelial marker E-Cadherin. To determine timing of ERK1/2 activation, the media of MCF10A cells grown in serum free media with full EGF and insulin was replaced with serum free media without insulin for 1h, 2h and 5h. Lysates were IB for P-ERK1/2, ERK1/2, P-PKB and PKB. (B) To compare the effects of insulin and EGF withdrawal, MCF10A cells were grown for 3 days in serum free media with the full (10μg/mL), and 20ng/mL, 1ng/mL or 0ng/mL . Lysates were IB for effectors of the insulin receptor pathway. (C) MCF10A cells grown in serum free media were treated with P3K inhibitor LY294002 (50nM), or the IR inhibitor HNMPA (50μM) for 2.5h. Cells were also treated with HNMPA overnight (O/N). Insulin was also withdrawn (-) for 2.5h or overnight. Lysates were IB for P-ERK1/2, ERK1/2, and P-PKB. MCF10A were also treated with mTOR inhibitor rapamycin (50nM) for 2.5h (+) and compared against cell lysates were insulin was removed from the media for 2.5h. Lysates were immunoblotted for P-ERK1/2, ERK1/2, and P-PKB to confirm that rapamycin was effective in shutting down mTOR. (D) To determine the level in the IR pathway at which inhibition causes ERK1/2 activation, MCF10A cells were treated with MK-2206 (200nM) (+) for 2.5h, with (+) or without(-) insulin in the serum free media. Lysates were IB for P-ERK1/2, ERK1/2, P-PKB, IR, PKB and P-RSK.
In order to pinpoint where in the IR pathway cross-talk with ERK1/2 occurs in response to insulin withdrawal, we treated MCF10A cells with either HNMPA (IR), LY294002 (PI3K), MK-2206 (a pan-PKB inhibitor), or rapamycin (an mTOR inhibitor). Immunoblotting with antibodies against P-ERK1/2 and P-PKB showed that IR and PI3K inhibition caused an increase in P-ERK1/2 and decrease in P-PKB (Fig 4.5C) whereas mTOR inhibition did not. Thus the observed increase in P-ERK1/2 was IR specific, mediated via PI3K pathway component(s), acting upstream of mTOR.

The ERK1/2 effect was dependent on PKB activity as treatment of cells with the pan-PKB inhibitor MK-2206 also led to ERK1/2 activation, as well as phosphorylation of RSK(S380), a downstream target of ERK1/2 (Fig 4.5D). Interestingly, we observed an increase in IR protein expression after 2.5h of insulin withdrawal and after 2.5h of PKB inhibition (Fig 4.5D).

4.6 Insulin receptor knockdown causes the same changes as insulin withdrawal.

As an alternative means of interfering with insulin signaling, and to further understand the role of the IR in BC, we developed a lentiviral shRNA system to knock down the IR in human cells. Western blotting using an antibody against the IR showed that the use of the lentiviral shRNAs led to an effective IR knockdown in MCF10A (Fig 4.6A), HEK293, and MCF7 cells (not shown). Similar to insulin withdrawal, knockdown of IR in MCF10A resulted in an increase in ERK1/2 phosphorylation (Fig 4.6B).

To investigate how IR knockdown affected MCF10A proliferation in comparison to insulin withdrawal, we plated control MCF10A cells and MCF10A IR-KD cells in serum free full media, as well as MCF10A cells grown in serum free full media without insulin, at low density and let them grow for 8 days. While cells grown in full media attained full or near-full confluence after 72h (Fig 4.6C), both full insulin withdrawal and IR-KD led to a significant reduction in proliferation (Fig 4.6C).
Figure 4.6 Insulin receptor knockdown causes the same changes as insulin withdrawal
(A) Lentivirus containing one of three (sh1,sh2 or sh3) short hairpin RNAs (shRNA) against the insulin receptor were used to infect MCF10A cells. 5 days post infection, after 4 days of selection with 1µg/mL of puromycin, infected cells were lysed. IR-KD lysates and lysates of MCF10A cells grown in serum free media containing EGF, and with 10µg/mL(+) or without(-) insulin (control) were immunoblotted (IB) for IR and B) P-ERK1/2 and ERK1/2 expression (C) MCF10A cells were plated in 6 well plates at low confluence, with 3 wells of each of the following conditions- serum free media was supplemented with the full amount of EGF and 0 or 10µg/mL of insulin, and IR knockdown cells in serum free media containing the full amount of insulin(10µg/mL) and EGF(20ng/mL). The Incucyte was used to measure cell confluence in each condition every 2 hours for 10 days. Average confluence in each of the 3 wells was plotted. 3 biological replicates were performed. (D) MCF10A in which the IR had been knocked down were imaged using a light microscope. (E) The transwell migration experiment described in figure 4.3 was performed with IR-KD MCF0A cells grown only serum free media containing both growth factors, and cells grown in serum free media with or without insulin. Cells were allowed to migrate for 12h towards media with or without insulin, fixed, and the number of cells that migrated were counted. 3 biological replicates were performed. (F) Lysates from MCF7 cells untreated or treated with 300nM doxorubicin overnight, as well as MCF10A cells grown for 24h with and without insulin in serum free MCF10A media, and MCF10A cells in which the IR had been knocked down were IB for p27, p21, IR, P-P53(Ser15).
**D**

Empty Vector  IR(sh1)-KD  IR(sh2)-KD  IR(sh3)-KD

**E**

![Graph showing cell migration](image)

- **Average cells migrated through filter**
  - **Insulin in media of lower chamber**
  - **Control**, **IR(sh2)-KD**, **IR(sh3)-KD**
  - **Legend**: 0ug, 10ug

**F**

<table>
<thead>
<tr>
<th>MCF7</th>
<th>MCF10A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>+</td>
</tr>
<tr>
<td>p27</td>
<td>+</td>
</tr>
<tr>
<td>p21</td>
<td>+</td>
</tr>
<tr>
<td>Insulin Receptor</td>
<td>+</td>
</tr>
<tr>
<td>P-p53 (S15)</td>
<td>+</td>
</tr>
<tr>
<td>Tubulin</td>
<td>+</td>
</tr>
</tbody>
</table>
We next assessed cell morphology upon IR knockdown. Normal MCF10A cells grown in 2D are cuboidal and have a regular, cobblestone appearance. After 3 days of selection with puromycin, 4 days total after lentiviral infection, MCF10A IR-KD cells were imaged under a light microscope. After three days of growth in serum free media, infected cells displayed spindle-shaped protrusions, fewer cell-cell contacts, and a spread appearance (Fig 4.6D), similar to the phenotype observed upon insulin withdrawal (Fig. 4.2B, right panels).

The impact of IR knockdown on MCF10A migration was tested in transwell migration experiments (Fig 4.6E). Equal number of MCF10A IR-KD cells, as well as empty vector infected MCF10A cells were seeded into the upper chamber of a transwell filter. The upper chamber contained full media, whereas the bottom chamber contained media with insulin as a stimulant to migrate, or no insulin, as control. 12h later, vector control cells migrated regardless of whether or not there was insulin in the bottom chamber (Fig 4.7E). Consistent with our previous observations that MCF10A cells grown without insulin did not migrate when there was no insulin in the bottom chamber, IR-KD failed to migrate regardless of the presence of insulin in the bottom chamber (Fig 4.7E).

A possible explanation for the observed changes in morphology accompanied by lack of migration is that the MCF10A cells become senescent upon insulin withdrawal. To test this possibility, we immunoblotted lysates of MCF10A cells grown with and without insulin, as well as IR-KD MCF10A cells, using antibodies against the markers of senescence p21, p27, and P-p53 (S15). MCF7 cells treated with doxorubicin were used as a positive control. Compared to untreated MCF7 cells, MCF7 cells treated with doxorubicin displayed very high expression of senescence markers p21, p27 and P-p53 (S15) (Fig 4.6G). In contrast, MCF10A cells, regardless of growth conditions, did not show increased expression of p27 or P-p53 (Fig 4.6G). While there was a slight increase in p21 in IR-KD and insulin withdrawal cells compared to normal MCF10A (not shown), the increase p21 seen upon induction of senescence in MCF7 cells was much more robust. Therefore MCF10A cells likely do not become senescent upon withdrawal of insulin or IR knockdown.
4.7 Insulin withdrawal and IR knockdown cause loss of epithelial phenotype.

Upon insulin withdrawal and IR knockdown, we found that MCF10A cells lost the regular, cuboidal, phenotype characteristic of epithelial cells[191], and instead resembled mesenchymal cells, with fewer cell-cell contacts and a spread, fibroblastic morphology. We wanted to determine if the apparent loss of epithelial phenotype was indeed indicative of EMT, and whether the morphological changes were accompanied by a loss of expression of epithelial proteins and an increase in mesenchymal proteins. We used MCF10A cells depleted of insulin for 3 days, and IR-KD MCF10A cells, immunoblotting their lysates with antibodies against protein markers of both the epithelial (E-Cadherin) and mesenchymal phenotypes (N-cadherin and Snai1), respectively.

Three days following insulin withdrawal, we observed reduced expression E-Cadherin (Fig 4.7A), a cell adhesion protein an epithelial marker[171], accompanied by an increase in expression of the mesenchymal markers Snai1 (Fig 4.7A) and N-Cadherin (Fig 4.7B), consistent with a loss of the epithelial phenotype. Similarly, E-Cadherin decreased in IR-KD MCF20A cells, further indicating that insulin signaling is necessary for the maintenance of the epithelial phenotype (Fig. 4.7C).

4.8 ERK2 activation is responsible for the loss of epithelial phenotype.

Our data reveals a relationship between insulin withdrawal and activation of ERK1/2 signaling. It has been shown that ERK2 specifically regulates maintenance of the epithelial phenotype in MCF10A cells[202]. In this study, where the introduction of constitutively active ERK2 into MCF10A cells caused increases in N-cadherin and vimentin, a decrease in E-cadherin, and changes in morphology indicative of a switch to a mesenchymal lineage[202]. Significantly these changes did not occur upon the addition of constitutively active ERK1. To determine if ERK2 activation was responsible for the loss of epithelial phenotype in MCF10A cells upon insulin withdrawal and IR knockdown, we performed lentiviral knockdown of ERK1 and ERK2 in MCF10A cells using shRNAs obtained from Dr. John Blenis (Harvard University)(Fig. 4.8A). Cells
Figure 4.7 Insulin withdrawal and Insulin receptor knockdown cause loss of the epithelial phenotype.

(A) After 24h of growth in serum free media supplemented with full EGF, and with (+) or without (-) insulin, MCF10A lysates were IB for IR, E-cadherin, Snail, and (B) N-Cadherin. Additionally, IB with the same antibodies using lysates from MCF10A cells in which the IR had been knocked down with shRNA against the IR in a lentiviral vector. Two different shRNA constructs were used (sh1 and sh2). (C) To determine timing of P-ERK1/2 activation and loss of E-cadherin in MCF10A after lentiviral infection with shRNA (sh1 and sh2) against the IR, lysates were collected 1 day after infection, and after 1 and 2 days of selection with 1µg/mL of puromycin. Lysates were IB with antibodies against E-Cadherin and P-ERK1/2.
were grown in serum-free media with or without insulin. Immunoblotting of cell lysates revealed that ERK1 knockdown cells grown without insulin exhibited the same increase in IR and N-Cadherin expression seen upon IR knockdown and insulin withdrawal in mock knockdown cells (Fig 4.8A). Notably, these changes in expression were not seen in ERK2 knockdown cells after insulin withdrawal (Fig 4.8A), suggestive of a partial reversal of the EMT phenotype upon ERK2 knockdown. Interestingly, Western blotting with an activation specific antibody against RSK (S380) showed an increase in RSK phosphorylation in MCF10A upon insulin withdrawal in both ERK1 and ERK2 knockdown cells, suggesting that this effect is not ERK1/2 dependent.

The importance of ERK1 and ERK2 for insulin-directed MCF10A migration was tested in transwell migration experiments (Fig 4.8B). ERK1 or ERK2 knockdown cells grown with or without insulin for 24h were seeded into the upper chamber of a transwell filter. The upper chamber contained the amount of insulin that the cells were grown in, whereas the bottom chamber contained media with insulin as a stimulant to migrate, or no insulin, as a control. Cells were left for 24h to allow migration towards the lower chamber. Confirming the importance of ERK2 in the IR-KD/insulin withdrawal phenotype, ERK2 KD cells did not migrate regardless of the presence of insulin in the bottom chamber or the media they were grown in (Fig. 4.8B). In comparison, MCF10A cells in which ERK1 had been knocked down (ERK1-KD cells) grown in insulin migrated regardless of the presence of insulin in the bottom chamber (Fig 4.8B, left panel). ERK1 KD grown without insulin required insulin in the bottom chamber to migrate (Fig 4.8B, right panel). These results highlight the importance of ERK2 in regulating EMT and cell migration in the context of insulin withdrawal.
Figure 4.8 ERK2 activation is responsible for the loss of epithelial phenotype in MCF10A cells.

(A) ERK1 or ERK2 were knocked down in MCF10A cells by infection with lentivirus we created containing shRNA against ERK1 (A4 and A5) or ERK2 (G6 and G7). These cells, as well as empty vector control MCF10A cells, were grown in serum free media containing EGF, with or without insulin for 24h. Lysates from these cells, as well as MCF10A cells in which the IR had been knocked down, were IB for the insulin receptor, ERK1/2, P-ERK1/2, P-RSK and N-Cadherin. (B) MCF10A cells in which ERK1 and ERK2 had been knocked down were grown for 24h in serum free media with EGF, with or without insulin, were transferred in known quantity to the upper well of a transwell filter containing the same media the cells were grown in. Cells were allowed to migrate for 12 h towards media with or without insulin added as a stimulant for cells to migrate. Cells infected with an empty vector lentivirus grown with or without insulin for 24h were also transferred in the same manner to the upper well of the transwell filter and allowed to migrate. As before, cells were fixed, stained, and the cells on the bottom of the transwell filter were counted. In each of the 3 biological replicates, there were 3 technical replicates of each condition.
CHAPTER 5: DISCUSSION
There have been many epidemiological studies linking hyperinsulinemia, increased cancer risk, and IR expression in human cancers[106, 108, 110-112, 208, 209]. The goal of my study was to explore the relationship between BC and insulin at the cellular level by investigating the effects of insulin on untransformed breast cells in vitro. These cell-based studies revealed a relationship between insulin withdrawal and cell morphology that involved loss of the epithelial phenotype through specific activation of the MAPK pathway effector ERK2.

**MCF10A cells as a model for breast cells that express the IR in a hyperinsulinemic, hyperglycemic environment.**

Breast cancer cells have been shown to overexpress the IR and to exhibit higher insulin responsiveness compared to normal breast tissue[106, 111]. We initially determined that MCF10A cells exhibited these characteristics and could serve as a valid model system in which to study the role of insulin in BC. High IR expression has been found in as many as in 90% of breast tumours[109], with the mean IR content 6-fold higher in cancer specimens compared to normal breast tissue[110]. MCF10A cells express both isoforms of the IR (Fig. 4.1A), as well as key members of the IR pathway(Fig. 4.1B), and were able to mount an IR specific response to insulin stimulation. Importantly, MCF10A cells do not have any of the three hotspot mutations in PIK3CA (data not show) that confer growth factor-independent pathway activation[150], so their response to insulin was ligand induced.

MCF10A cells express both IR-A and IR-B. IR-A is the isoform most frequently expressed in cancers and is responsible for the mitogenic rather than the metabolic effects of insulin[106, 210, 211]. Interestingly, in breast cancer cell lines and breast cancer tissue samples, the IR-A:IR-B ratio is high- with IR-A constituting 64-100% and 40-80% of total IR in cell lines and tissue samples, respectively[113].

Most BC cells in culture, including MCF10A cells, are grown in media supplemented with an excess of insulin, which, at 10µg/mL is about 40 times the concentration found in
human blood under normal conditions. Most BC tissue culture media also contains 3-5 times the normal blood concentration of glucose. This combination of factors made MCF10A a good model system for studying the effects of insulin on breast cells that express the IR in a hyperglycemic, hyperinsulinemic environment, such as in an obese individual or a T2D patient.

**MCF10A cells require insulin signaling through the IR for proliferation and migration.**

Functional studies of BC tissue compared to normal breast tissue indicate higher IR responsiveness to insulin in transformed cells with high IR expression[111]. My proliferation analysis confirmed that expression of the IR confers high IR responsiveness to insulin in MCF10A cells. These studies demonstrated that even at insulin concentrations of 1/10th the amount of insulin in the normal media, MCF10A were able to normally proliferate (Fig. 4.2A). Only upon complete insulin withdrawal or IR knockdown (KD) did MCF10A cells undergo proliferative arrest. In comparison, any restriction of EGF resulted in a decrease in proliferation, and upon EGF withdrawal cells died within 24h (Fig. 4.2A).

In 3D, MCF10A cells formed smaller acini, which failed to establish proper internal architecture when insulin was removed from the media(Fig. 4.2C). Whether this was due to a lack of growth and proliferation due to the removal of insulin from the media, or whether it was a result of the fact that insulin is crucial to stages of normal acinar development could not be discerned. However, in light of the proliferative defects we observed in 2D growth, it is likely that the size difference can be attributed to the former.

Following insulin withdrawal, MCF10A cells underwent a change in cell morphology that could be seen as early as 24h after removal of insulin from the media (Fig. 4.2B). Normal MCF10A cells, like most epithelial cells, have a regular, cuboidal shape and maintain many cell-cell contacts[207] (Fig 4.2B). When confluent, they have a cobblestone-like appearance. When grown in the full amount, or even limited amounts of
insulin, they retained this normal appearance, but upon insulin withdrawal their morphology changed. Cells became irregular, more spread, and developed spindle-shaped protrusions and fewer cell-cell contacts reminiscent of mesenchymal cell morphology. Restriction of EGF, though it resulted in proliferative defects, did not cause changes in cell morphology (Fig. 4.2B). Changes in morphology consistent with mesenchymal cell fate were also observed in MCF10A IR-KD cells. Thus insulin signaling likely contributes to the function of MCF10A cells as epithelial cells.

My data also strongly supports the role for insulin in cell migration. The fact that inhibition of proliferation with Mitomycin C (data not shown) did not inhibit insulin-induced migration, suggests that the migration and proliferation are uncoupled in insulin deprived cells. Knockdown of the IR also arrested migration, regardless of the presence of insulin in the growth or stimulation media (Fig. 4.6E). As IR-B is not normally responsible for cell motility, it is likely that the migration response is mediated through IR-A hetero and homodimers, as well as HR, which are more responsive to insulin [81, 100]. Future work will be needed to formally test this hypothesis.

Normal breast epithelial cells migrate minimally, however MCF10A cells exhibit a migratory phenotype in culture. This could be due to changes acquired during immortalization, such as the deletion of p16INK4a, or because they express the IR at high levels where normal breast epithelial cells do not. Despite the induction of EMT-like morphological and protein expression changes seen following the removal of insulin from the media, the increase in migration that usually accompanies EMT was not observed. Immortalization may have consequences on aspects of signaling responsible for the observed migratory arrest following insulin removal. Determining if IR expression or immortalization causes this uncharacteristic increase in MCF10A baseline motility (compared to other epithelial cells) and lack of migration following insulin withdrawal/IR-KD would be an important step in determining why we are not observing full EMT. To this end, insulin withdrawal studies using untransformed primary mammary epithelial cells taken from normal mice, and mice genetically altered to overexpress the IR in their mammary glands would be useful.
In light of the finding that both insulin removal and IR-KD in MCF10A cells resulted in a decrease in the epithelial marker E-Cadherin and an increase in the mesenchymal markers N-Cadherin and Snai1, as well as a transition to a more mesenchymal morphology, the decrease in migration after insulin withdrawal or IR-KD is unexpected. Loss of epithelial phenotype and expression of mesenchymal markers usually correlates with a greater propensity to migrate [191, 195]. It is therefore reasonable to assume that we are seeing a loss of epithelial phenotype and but not full EMT, which requires that cells gain motility along with the other changes associated with the phenomenon[178].

**The role of ERK2 in migration and maintenance of epithelial phenotype**

Compared to MCF10A cells grown in insulin, cells grown without insulin showed an expected decrease in PKB/Akt phosphorylation, as well as an unexpected increase in ERK1/2 phosphorylation (Fig. 4.5A) which occurred after just 2h of insulin removal. This increase in P-ERK1/2 was also seen upon IR-KD (Fig. 4.7B). Additionally, E-cadherin levels decreased after about 3 days of insulin removal or IR-KD (Fig. 4.7C), while levels of the mesenchymal markers N-cadherin and Snail, which repress E-cadherin and Snail transcription [169, 187] increased after 24h (Fig. 4.7A, B). These protein expression changes are characteristic of the loss of the epithelial phenotype. There are several studies in MCF10A cells that suggest that ERK2 is responsible for mediating the signaling changes that result in the loss of the epithelial phenotype, including an increase in mesenchymal markers and migration [202, 203, 212], and our data largely confirm these studies (Fig 4.8). After knockdown of ERK2, but not of ERK1, insulin removal and IR-KD failed to cause increases in the mesenchymal markers N-cadherin and Snail (Fig 4.8A). ERK2 may therefore contribute to regulating the loss of expression of epithelial markers and epithelial phenotype.

Further supporting the work of Shin et al on the role of ERK2 in the epithelial phenotype[202], ERK2 specifically was found to play a pro-migratory role in MCF10A cells. Knock down of ERK2, but not ERK1, under conditions of insulin withdrawal repressed migration (Fig 4.8B). Remarkably, knockdown of IR also inhibited migration
despite ERK2 activation (Fig 4.6E). This suggests that though ERK2 is present and hyperphosphorylated in these cells, the signal to migrate is most likely mediated through the IR. It is reasonable to assume, therefore, that breast cells that express the IR require insulin and ERK2 for migration.

The loss of epithelial phenotype in response to insulin withdrawal or IR-KD and the dependency of MCF10A on the insulin for migration raise possible concerns associated with insulin restriction as a treatment for BC. While MCF10A cells are untransformed, and fail to undergo full EMT in response to insulin withdrawal, it is possible that transformed BC cells may develop a pro-migratory phenotype upon insulin restriction.

Activation of RSK, an important effector of ERK1/2 in global transcription regulation[213], was also enhanced upon insulin withdrawal and IR KD (Fig. 4.5D). Preliminary studies of RSK inhibition in the Blenis laboratory have demonstrated that phosphorylated RSK can inhibit the mesenchymal protein Snail. This may account for the maintenance of some epithelial characteristics upon insulin withdrawal, such as the lack of a more migratory phenotype. This observation is supported by the fact that RSK represses some of the ERK1/2 regulated transcription factors that contribute to the EMT immediate-early gene (IEG) response, or are themselves IEG products [214, 215], such as Snail. Additionally, a genome wide RNAi screen revealed RSK as a common effector for multiple migratory stimuli [216], though the effect of its activation on migration is unknown.

Another putative role for RSKs in the context of insulin signaling is participation in a negative feedback loop that restricts activation of the Ras/ERK signaling cascade. Increased ERK1/2 phosphorylation has been seen in skeletal muscle tissue from RSK2 deficient mice [217], and inhibition of RSK using the RSK-specific inhibitor BI-D1870 was shown to increase ERK1/2 phosphorylation in several cell types [218]. These studies both suggest that a normal function of RSK may be to prevent MAPK pathway hyperactivation. Perhaps inhibition of RSK in the condition of IR-KD or insulin withdrawal would enhance ERK2 phosphorylation and result in complete EMT. To avoid
the potential off-target effects of using an RSK inhibitor, and obtain more accurate results as to the function of RSK in our system, knock down of each RSK isoform would be most effective, as well as pulldowns of RSK effectors to establish if Snail is directly acted upon and inhibited by RSK.

All observed signaling and expression changes occurred within 24h of insulin withdrawal except the decrease in E-cadherin expression (Fig. 4.7). Studies by Shin et al exhibit that ERK2-mediated loss of epithelial phenotype is caused by changes Zeb and Fra1[203] activity. Both expression and activation of Fra1, a transcription factor necessary for EMT, depend on the activity of ERK2[202, 203]. Disruption of ERK2 precludes activation of FRA1 and eventual EMT. Important to its role in EMT, ERK2/Fra1 stimulates expression of the transcription factor ZEB1/2[203], which reduces E-cadherin expression. Western blotting to examine Fra1 and Zeb1/2 in the context of insulin withdrawal would determine if this system is intact in MCF10A cells. Determining the timing of Fra1 activation after insulin withdrawal would be informative because our results show that E-Cadherin downregulation requires 3d of withdrawal withdrawal this timing is consistent with other reports that demonstrate that Fra1 is not activated immediately upon ERK2 phosphorylation, and requires 24h to be induced[203].

**Communication between the IR, PKB and ERK2.**

Chemical inhibition of critical effectors of the IR pathway was implemented to characterize the crosstalk between insulin removal/IR-KD and ERK2. Inhibition of IR, PI3K, and PKB all caused ERK2 activation (Fig 4.5C, D), whereas inhibition of mTOR did not (Fig. 4.5C). Thus PKB appears to be the key node of communication between the IR and ERK2. Inhibition of PKB using MK-2206 resulted in blockade of all PKB isoforms, but each phenotypical and signaling change observed upon insulin removal and IR-KD could be due to interplay between the effects of inactivating two of the three PKB isoforms. Distinct roles of PKBα and PKBβ have been seen in regulation of migration and EMT. In a study by Irie et al. hyperproliferation and antiapoptotic activities were induced in normal breast cells (MCF10A) by overexpressing IGF-IR [219]. Subsequent
ligand-induced hyperstimulation of the IGF-IR pathway disrupted epithelial cell morphology and resulted in a more mesenchymal cell phenotype. Knockdown of PKBβ in this context reverted the cells to epithelial morphology, while knockdown of PKBα exaggerated the spindle-shaped, fibroblastic morphology and protrusive extensions (Table 5.1). Activation of PKBβ was found to be necessary for the spindle shaped phenotype induced by PKBα knockdown. During ligand stimulation of IGF-IR overexpressing cells, knockdown of PKBα, but not of PKBβ, was found to cause increased cell migration and invasion, and was accompanied by changes in protein expression seen during EMT. Among other conclusions, this study suggests that the PKBα/ PKBβ balance in cells is crucial to cell phenotype. Especially applicable to our study was the observed increase in phosphorylated ERK1/2 accompanying the EMT-like changes induced by PKBα knockdown. This ERK1/2 activation was determined to be necessary for migration induced by down-regulation of PKBα. It is possible that since insulin removal, MK-2206 and IR-KD all caused non-specific inhibition all PKB isoforms, the somewhat opposing roles of PKBα and PKBβ inactivation suggested by this study occurred, causing the modified EMT phenotype we observed.

Considering that the anti-IR antibodies cannot distinguish between the two IR isoforms, determining which isoform is responsible for the morphological changes and the effects of insulin withdrawal on migration in response to insulin is difficult. Creating a strategy to knockdown each of the isoforms individually is complicated by the fact that the IR is translated as a high molecular weight proreceptor (180kDa) and that all forms of the mRNA transcript appear to encode for the complete proreceptor[16, 69-71]. This makes targeting a specific isoform with shRNA impossible. Perhaps overexpression of one specific isoform at a time in breast cells from mammary-specific IR knockout mice, would be useful in discerning the relationship between each IR isoform, their function in breast cancers, contribution to the regulation of PKB and ERK2 activity, and perhaps differences in how each IR isoform signals to the isoforms of PKB.
Table 5.1 PKB isoforms have distinct functions in IGF-IR signaling in MCF10A cells[219]

MCF10A cells in which the IGF-IR was overexpressed displayed phenotypic and signaling changes. Knockdown of PKB isoforms had distinct effects on cell morphology, migration, proliferation and signaling in the context of IGF-IR overexpression.

<table>
<thead>
<tr>
<th>PKB Isoform Knocked down</th>
<th>PKBα</th>
<th>PKBβ</th>
<th>PKBα/β</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effect in 2D</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Enhanced GF stimulated migration.</td>
<td>• Reverted IGF-IR++ spindly morphology.</td>
<td>• Parental phenotype restored.</td>
<td></td>
</tr>
<tr>
<td>• Decreased proliferation.</td>
<td>• Decreased proliferation.</td>
<td>• PKBα KD migration impaired.</td>
<td></td>
</tr>
<tr>
<td>• Phenotype and markers of EMT.</td>
<td>• Decreased EGF stimulated migration.</td>
<td>• Erk activation</td>
<td></td>
</tr>
<tr>
<td>• Erk activation.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Overexpression suppresses EGF dependent migration and Erk activation.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Effect in 3D</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Disrupted lumen formation, protrusive extensions (EMT)</td>
<td>• Restored lumen formation and apoptosis.</td>
<td>• Small, irregular structures.</td>
<td></td>
</tr>
</tbody>
</table>

Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. 2005. Irie Brugge
IR pathway inhibition with HNMPA and MK-2206 resulted in a significant increase in IR expression. It could be that upon insulin withdrawal, there is a compensatory upregulation of IR to better utilize any insulin or IGF1/II available in the media.

Phospho-specific antibodies against the residues of IGF-IR that are phosphorylated upon insulin stimulation also bind activation-specific residues of the IR, so it was not possible to determine when IGF-IR was activated specifically. Ascertaining relative expression levels of IGF-IR before and after insulin removal or IR KD, as well as immunoprecipitation (IP) of IGF-IR to determine its activation under these conditions would help to resolve whether epithelial phenotype was being partially maintained through insulin/IGF1/II signaling through this receptor.

Previous studies of the link between ERK2 and PKB in MCF10A involved altering MCF10A using extra-physiological levels of IGF-IR[219] or transforming Ras[202, 203]. These changes may have allowed MCF10A to achieve full EMT once other alterations to their signaling were made, such as knocking down specific isoforms of PKB or the addition of constitutively active ERK2.

**Insulin withdrawal/IR knockdown and possible senescence**

Two main senescence pathways cause cell cycle arrest through different methods, and therefore there are discrete markers that indicate activation of each one. Robust elevation of the cyclin-dependent kinase inhibitor (CKI ) and p53 target p21<sup>Cip1</sup>, as well as p27<sup>Kip1</sup>, are characteristic of p53 induced senescence [220, 221], while the second senescence pathway involves activation of the CKI p16<sup>INK4A</sup> and subsequent maintenance of Rb in its unphosphorylated, growth suppressive state. An accumulation of Rb in this form is a marker of senescence initiated via the second pathway[222, 223], and Western blotting for p16<sup>INK4A</sup>, CDK4-6 or E2F levels also help confirm senescence through the Rb pathway[224]. Validating the use of these cell-cycle related proteins as biomarkers of senescence, overexpression of p53, p16<sup>INK4A</sup> or p21<sup>Cip1</sup> have been shown to cause premature senescence-related cell cycle arrest[225, 226]. Rb induced senescence cannot
occur in MCF10A cells, as they do not express p16\textsuperscript{INK4A}, so a model of p53 induced senescence was used as a positive control in this experiment. When comparing IR-KD cells and MCF10A cells undergoing insulin withdrawal, and doxorubicin-treated MCF7 cells for expression of cell cycle proteins and the senescence markers p27\textsuperscript{Kip1}, p21\textsuperscript{Cip1} and P-p53 (S15), it was obvious from the marked increases in each of these senescence markers that the doxorubicin-treated MCF7 were undergoing senescence, while MCF10A cells were not (Fig 4.6G). The IR-KD and MCF10A cells grown without insulin were mostly likely undergoing cell cycle arrest, associated with p21\textsuperscript{Cip1} induction; the three members of the Cip/Kip family, p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip1}, are very effective inhibitors of the CDK2/E-A complexes, through which they cause cell cycle arrest\cite{227}. Nuclear export of FOXOs reduces the transcription of cell cycle inhibitory genes, including p27\textsuperscript{Kip1} \cite{118} and p21\textsuperscript{Cip1}\cite{124}. Usually PKB activation maintains the FOXO proteins in a phosphorylated state, which results in their removal from the nucleus and degradation in the cytoplasm\cite{123}. During insulin withdrawal and IR KD, PKB activity was low and perhaps a portion of FOXO proteins remained unphosphorylated in the nucleus where they performed their function of promoting transcription of p21\textsuperscript{Cip1}\cite{124}. This could account for the slight p21\textsuperscript{Cip1} induction observed.
CHAPTER 6: FUTURE DIRECTIONS
Exploring the role of the IR and insulin signaling in cell cycle.

To further understand our results and to more fully characterize the effects of insulin withdrawal on MCF10A cell cycle, several more assays should be performed following removal of insulin from the media. Cell cycle analysis using flow cytometry and PI or BRDU staining will be important in confirming that decreased proliferation is not due to apoptosis, and in understanding the cause of the moderate induction of \( p21^{Cip1} \) in cells undergoing insulin withdrawal and IR KD. It would be interesting to see what, if any, effects insulin removal has on cell cycle in the context of ERK2 knockdown. Further exploration of this question would assist in understanding the effects of ERK2 phosphorylation in the IR signal cascade. Activation of additional downstream targets of ERK2, such as S6, and the transcription factor Myc, should be also be assessed after insulin withdrawal/IR knockdown, as the MAPK pathway influences the transcription of genes that are important in cell cycle[223], and cell morphology[169].

Determining the involvement of each IR isoform in the response to insulin withdrawal, and IGF-IR and IGF/II in the insulin response.

In humans, two types of the insulin receptors exist [80, 84]. We found that MCF10A cells express both IR isoforms (Fig 4.1 A). Though starvation of MCF10A cells largely eliminated signaling throughput downstream of the IR, it would be interesting to explore the possibility that IGF-II, which can be produced by breast cancer cells, may act in an autocrine manner to stimulate IR signaling. Using a neutralizing anti-IGF-II antibody (Abcam#9574) we could determine the relative, if any, contribution of IGFI present in the media may also activate the IR. The levels of IGFI/II could also be determined using ELISA. Growth factor free MCF10A media alone, or supplemented with a known quantity of IGFI could be used to determine the role of IGFI mediated IR activation in the absence of insulin. Comparing the response of MCF10A cells to the dual IR/IGF-IR inhibitor OSI-906 and their response to insulin withdrawal could assist in differentiating the effects of IGF-IR and IR signaling. Knocking down the IGF-IR in MCF10A would also further this goal.
The Role of PKB and its isoforms in ERK2 activation, and PKB localization.

Treatment of MCF10A cells with a pan-PKB inhibitor caused the same ERK2 activation seen upon removal of insulin from the media (Fig. 4.5D). Each PKB isoform has been shown to have different effects on MCF10A cell growth, motility, and morphology in response to hyperactive IGF-IR[219]. Knock out mice of each PKB isoform have different phenotypes depending on which isoform they are lacking[136-139, 142]. Knockdown of each isoform individually and in combination would determine which isoform was responsible for the effects seen upon insulin withdrawal. Additionally, little is known about the localization of each isoform, and the effects of isoform localization on signaling events and cell phenotype. Knocking down each of the three PKB isoforms, followed by study of the cell response to insulin signaling, and immunofluorescence to determine where the isoforms are localized would help determine the importance of subcellular localization on PKB isoform function. Utilization of the location specific PKB inhibitors available in our lab[228] would further aid in understanding the complicated crosstalk between the PKB and ERK2 pathways in response to insulin signaling and withdrawal.

Further 3D studies to determine effects of insulin withdrawal on acinar formation.

After approximately 15 days of growth in Matrigel, MCF10A cells form three-dimensional acinar structures that resemble in vivo mammary glandular architecture[207, 229, 230]. Mature MCF10A acini proliferate minimally and maintain a constant cell number and size[207]. A tightly controlled apoptotic program accomplishes the formation
Figure 6.1 Timeline of acinar formation of MCF10A cells in matrigel
The sequence of biological events that occur during MCF10A acinar formation in matrigel. Cells are seeded in matrigel on Day 0. Proliferation occurs until Day 14. Formation of acinar structures is evident even during early days of development, and can be seen using Lamenin 5 staining to detect the outer surface of the acinus where it attaches to the matrix[230][231]. Distinct polarization occurs around Day 6, when two distinct subpopulations of cells are evident. The group contacting the matrix have apical and basal surfaces with respect to the inside of the acinus, as indicated with apical Golgi protein staining (GM130) of these cells. At this point, signaling dichotomies between the inner cell population and the outer, polarized cell population can be seen; high phosphorylated PKB (P-PKB) survival signaling can be seen in the cells of the outer cell population[230][231]. Beginning on Day 8, the inner cell population starts to undergo programmed cell death to form and maintain a hollow lumen, as indicated by expression of active caspase 3. The photomicrographs are taken from [231], and the figure is adapted from [231] as well.
of the acinar lumen[231]. As in the development of a mammary duct, cell polarity is established within the external epithelial layer[207, 231]. These events follow a known timeline (Fig. 6.1). To circumvent the impact of insulin restriction on proliferation necessary for the formation of MCF10A acini, restriction of insulin following the initial proliferative phase (after day 6) could be used to evaluate its impact on the later stages of acinar development, such as apoptosis. Cleaved/activated caspase 3 staining could be used to monitor for apoptosis, while antibodies against E-Cadherin and β-catenin could be used to delineate the basement membrane. Cellular apical structures, normally facing the lumen, could be identified using antibodies against the Golgi marker GM130. Acinar structures could be monitored for morphological changes using IF staining.

Compared to acini in full insulin, those grown in the absence of insulin were much smaller (Fig 4.2C). To further determine the effect of insulin on acinar proliferation and maintenance, insulin can be removed from the media at other key points in the process of acinar formation- proliferation (days 0-8), polarization (starting day 4), apoptosis to form a clear lumen (starting day 8), and after 20 days in 3D culture. Removal of insulin after the 20 day time point may determine whether insulin is necessary to maintain acinar architecture. To determine if proliferation occurs after acinar formation, antibodies against phospho-ERM (ezrin/radixin/moesin) can be used to immunostain the entire plasma membrane of the acini from which insulin has been removed after 20 days in 3D culture. The goal would be to delineate a timeline of acinar formation that highlights the function of insulin at key points in their development. MCF10A cells grown under optimal conditions would be used as controls.

**Knockdown of PTEN and/or hyperactivation of PI3K to determine the effects of insulin withdrawal in a model of cancer initiation.**

Activating PI3K mutations and loss of function of PTEN, and subsequent activation of PI3K/PKB signaling are some of the most commonly found oncogenic changes in human breast cancer [232]. To mimic activation of PI3K/PKB signaling and to sensitize MCF10A cells to the changes in insulin signaling, knockdown of PTEN using shRNA
(Sigma Mission TRC-1 shRNAs shPTEN-45 and shPTEN-48) would be useful. By the use of stable PTEN knockdown, and by transfection of constitutively active PI3K, common oncogenic changes would be introduced into MCF10A cells. These cells could be subjected to insulin restriction, as well as pharmacological inhibition of multiple downstream components of the insulin-signaling pathway, such as PI3K, PKB, ERK2 and mTOR, to determine the relative importance of each pathway constituent in the interpretation of the insulin signal.

PTEN knockdown or PI3K hyperactivation are potentially oncogenic events[134, 233], and to determine their tumorigenicity in the presence of insulin or the IR would be an important step in understanding tumour initiation in the context of hyperinsulinemia. Assessing soft agar colony formation and anoikis in these potentially transformed cells with or without the expression of the IR and/or ERK2, would help determine the importance of insulin signaling to tumor initiation potential in vitro. If these cells were able to form anchorage independent colonies in soft agar, injection of these cells (with and without with IR, ERK2, etc) into nude mice would establish a model system to study the involvement of the IR pathway in tumour initiation.

Overexpression of ErbB2 in MCF10A cells leads to hyperproliferation, formation of multiacinar structures, failure to form lumens, as well as uncontrolled and anchorage independent cell growth[231]. Therefore, ErbB2-overexpressing variants and normal MCF10A cells could be utilized for the evaluation of the impact of insulin on BC cell growth. Detailed growth and morphology assays of ErbB2-expressing, activated PI3K, and PTEN-knockdown MCF10A cells in both in monolayer and 3D would also be performed.

**Determining the role of the IR in other breast cell lines and primary breast cells.**

We primarily used MCF10A cells in our study. MCF7 cells, another breast cancer cell line, were used as positive controls for some experiments. These cells express the IR, but carry a mutation in PIK3CA that renders the IR pathway growth factor independent. It
would be very important to determine if other normal breast cells that express the IR at high levels require insulin for proliferation and migration as MCF10A do, and if ERK2 hyperactivation occurs in response to insulin withdrawal and IR knockdown. Breast cancer cell lines that express the IR could also be used for comparison. It would be necessary to first determine if each cell line used had any IR pathway-relevant mutations, such as inactivating mutations in PTEN or activating changes to PIK3CA, which would affect their insulin response. The studies in these BC cell lines would help understand the relevance of studies in MCF10A with various oncogenic changes detailed in the previous aim.

**Determining the effect of insulin withdrawal and ERK2 in breast cancer models in vivo.**

As an extension of the *in vitro* work, it would be informative to explore the dependence of BC on insulin in various mouse models, including the use of orthotopic xenografts in the hyperinsulinemic, but not obese, MKR\(^{+/+}\) mouse[234, 235], and the highly cancer-prone breast-specific PTEN knockout mouse[236]. Work on the biology of PTEN, a major tumor suppressor whose expression is lost in many human BCs has led to the development of several mouse model systems that reproduce the biology of breast cancer development in the absence of PTEN. Female PTEN\(^{+/+}\) mice develop breast tumors with 50% penetrance within one year [237], and all animals with conditional deletion of PTEN in the mammary gland develop mammary tumors [236]. Considering the relatively slow onset of mammary tumors in PTEN\(^{+/+}\) mice and the fact that they develop a broad spectrum of BCs[237], we believe that these mice are an excellent model system to explore the impact of obesity and hyperinsulinemia on BC. Importantly, metabolic functions in PTEN\(^{+/+}\) mice are normal (Stambolic V, unpublished observations).

To study the impact of obesity on mammary tumorigenesis, PTEN\(^{+/+}\) mice on a high fat diet (HFD) could be used. Previous studies, including our own, indicate that within 10 weeks of HFD, mice become obese, lose peripheral insulin sensitivity, and display impaired glucose clearance. These changes are accompanied by an increase in circulating
insulin levels [238-240]. As in humans, an increase in circulating insulin levels in obese animals precedes the development of diabetes[241]. HFD-fed PTEN\(^{+/−}\) mice and their normal chow-fed PTEN\(^{+/−}\) controls would be monitored for the appearance of tumors. Their tumors would be isolated, weighed and analyzed using immunohistochemistry and Western blotting for various markers of insulin signaling activation, and IR and IGF-IR expression. Common sites of metastatic spread of breast tumors in mice, such as the lungs and liver, would also be inspected for the presence of metastasis in all mice presenting with primary breast tumours. Generation of a PTEN\(^{+/−}\) and mammary specific IR overexpressing mouse would be useful, as comparing the BC incidence in these mice that of the PTEN\(^{+/−}\) mouse would help interpret the contribution of IR expression to cancer initiation. Using these mice in the aforementioned experiments involving HFD-induced obesity and hyperinsulinemia would add another layer of understanding to the question of how insulin and IR expression contribute to BC in the context of obesity.

The MKR\(^{+/−}\) mouse expresses a dominant negative form of the IGF-IR in its skeletal muscle, and is therefore diabetic with insulin resistance in muscle, liver, and adipose tissue. These mice exhibit high circulating levels of adiponectin, adiponectin resistance and insulin resistance, despite the fact that MKR\(^{+/−}\) mice show normal expression of adiponectin receptors and normal AMPK phosphorylation in the muscle and liver[235]. Additionally, they show accelerated mammary gland development and increased incidence of breast cancer, despite the fact that they are not obese. Creating a mammary specific IR knockout mouse with an MKR\(^{+/−}\) background, as well as determining the relative expression of each IR isoform in normal, and cancerous breast cells in the normal MKR\(^{+/−}\) mouse would aid in elucidating the link between IR expression and breast cancer initiation. Knocking out ERK2 in the mammary gland in coordination with and without IR knockdown would perhaps clarify the role of ERK2 and loss of the epithelial phenotype relates to metastasis in the presence of hyperinsulinemia.

**FINAL SUMMARY**

Insulin appears to have a profound effect on proliferation, morphology, migration, and signaling in an *in vitro* model of mammary epithelial cells that express the IR in a
hyperinsulinemic environment. Our study establishes a new link between the IR pathway and ERK2 signaling, and provides further evidence that ERK2 activation is responsible for loss of the epithelial phenotype in MCF10A cells. In addition, it brings to light the potential unanticipated effects of energy stress on cancer cell phenotypes. Further investigations of the role of insulin and its receptor in the breast will be key to providing insight into the development and progression of breast cancer and will result in the identification of new, more effective therapeutic targets.
1. Canada, S., Table 105-0501-Health indicator profile, annual estimates, by age group and sex, Canada, provinces, territories, health regions (2011 boundaries) and peer groups, occasional, 2011; CANSIM.
92. Benyoucef, S., et al., Characterization of insulin/IGF hybrid receptors: contributions of the insulin receptor L2 and Fn1 domains and the alternatively


197. Kim, H.J., et al., Constitutively active type I insulin-like growth factor receptor causes transformation and xenograft growth of immortalized mammary


