Snail induction of nuclear erk 1/2 promotes epithelial-mesenchymal transition and chemotherapy resistance in breast cancer cells

Bethany Nicole Smith
Clark Atlanta University

Follow this and additional works at: http://digitalcommons.auctr.edu/dissertations
Part of the Biology Commons

Recommended Citation
ABSTRACT

BIOLOGICAL SCIENCES

BETHANY NICOLE SMITH MS, DELAWARE STATE UNIVERSITY DOVER, DE, USA 2009

SNAIL INDUCTION OF NUCLEAR ERK1/2 PROMOTES EPITHELIAL-MESENCHYMAL TRANSITION AND CHEMOTHERAPY RESISTANCE IN BREAST CANCER CELLS

Committee Chair: Valerie A. Odero-Marah, Ph.D.

Dissertation dated December 2013

Snail is high in several cancers, correlates with poor clinical prognosis, and associated with increased tumor progression via epithelial-to-mesenchymal transition (EMT). EMT is a latent embryonic program that alters epithelial cells to appear more mesenchymal, regulates embryonic development and wound healing. Snail binds to enhancer box sequences on its target genes like E-cadherin, maspin, and estrogen receptor-alpha (ER-α) to increase EMT. MAP kinase (MAPK/ERK1/2) protein signaling regulates the effects of EMT during tumor progression by regulating cell proliferation, growth, migration, adhesion, invasion, and survival. Recent reports suggest that ERK1 and ERK2 isoforms may function differently although they share similar stimulants and substrates. We investigated the mechanism(s) of Snail-mediated EMT that may be regulated by ERK1/2 in breast cancer cells. Snail expression and phosphorylated ERK (p-
ERK) were higher in breast cancer cells compared to normal breast epithelial cells. Snail siRNA in T47-D and MDA-MB-231 breast cancer cells led to p-ERK relocating from the nucleus to the cytoplasm, and MDA-MB-231 had decreased p-ERK expression. Snail overexpression in MCF-7 Snail cells had increased EMT in vitro and in vivo as compared to MCF-7 Neo (control) cells. MCF-7 Snail had less p-ERK than MCF-7 Neo, which was nuclear-localized. ERK2 isoform activity was also higher in the nucleus of MCF-7 Snail compared to MCF-7 Neo. p-ERK and import protein nucleoporin98 (NUP98) were co-localized at the nuclear membrane in MCF-7 Neo suggesting a shift to the nucleus as cancer progresses. MAPK inhibition decreased cell migration and increased cell adhesion in MCF-7 Snail cells, and also re-induced E-cadherin expression, but decreased adhesion and E-cadherin in MCF-7 Neo. ERK2 isoform regulates EMT because ERK2 siRNA decreased Snail in MCF-7 Snail, but decreased E-cadherin in MCF-7 Neo. MCF-7 cells overexpressing Snail decreased ER-α expression. MCF-7 Snail were less responsive to 4-hydroxytamoxifen (4-OHT) chemotherapy using mitochondrial membrane permeability and cytotoxicity assays. MCF-7 Snail were only sensitive after being treated with 4-OHT at 10µM plus UO126 at 10µM. Collectively, our data suggest that nuclear and cytoplasmic ERK1 isoform activity positively regulates cell adhesion and may have a suppressive role in preventing EMT and breast cancer progression. Conversely, nuclear ERK activity which is predominantly ERK2 isoform activity increases EMT, and promotes resistance to chemotherapy and may promote tumor progression.
SNAIL INDUCTION OF NUCLEAR ERK1/2 PROMOTES EPITHELIAL-MESENCHYMAL TRANSITION AND CHEMOTHERAPY RESISTANCE IN BREAST CANCER CELLS

A DISSERTATION

SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

BETHANY NICOLE SMITH

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA

DECEMBER 2013
ACKNOWLEDGEMENTS

This research project would not have been possible without the help of people around me during this journey of discovery and knowledge. First, I would like to express my deepest gratitude to my advisor, Dr. Valerie A. Marah, for her guidance, optimism, patience, vision, honesty and providing me with an excellent atmosphere for doing research at Clark Atlanta University. She has truly been a blessing to me and I will always cherish her inspiring spirit. I would also like to thank my dissertation advisory committee members: Drs. Cimona V. Hinton, LaTonia Taliaferro-Smith, Jaideep Chaudhary and Shafiq A. Khan for their valuable advice, time, and effort on professional and personal levels. Special thanks to the former and current members of Dr. Odero-Marah’s lab for their support. I would also like to thank the financial, academic, and technical support at Clark Atlanta University, Department of Biological Sciences Chairperson, Dr. Marjorie Campbell, the staff, Graduate students and Post-Doctoral students of the Center for Cancer Research and Therapeutic Development. I am thankful to God the creator for continued blessings, my family and friends for their endless support, guidance, patience, kindness, but most of all their LOVE during my matriculation at Clark Atlanta University. This research was supported by the NIH/NCRR/RCMI 2G12RR003062, P201MD002285, Title III program and NIH Research Initiative for Scientific Enhancement (RISE) 2R25GM060414 grants.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter/Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>CHAPTER 1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 2 LITERATURE REVIEW</td>
<td>5</td>
</tr>
<tr>
<td>2.1 The human mammary gland</td>
<td>5</td>
</tr>
<tr>
<td>2.2 Breast cancer development</td>
<td>7</td>
</tr>
<tr>
<td>2.3 Breast cancer subtypes</td>
<td>8</td>
</tr>
<tr>
<td>2.3.1 Ductal hyperplasia</td>
<td>10</td>
</tr>
<tr>
<td>2.3.2 Ductal carcinoma in situ (DCIS)</td>
<td>11</td>
</tr>
<tr>
<td>2.3.3 Lobular carcinoma in situ (LCIS)</td>
<td>12</td>
</tr>
<tr>
<td>2.3.4 Triple-negative breast carcinoma (TNBC)</td>
<td>12</td>
</tr>
<tr>
<td>2.4 Breast cancer metastasis</td>
<td>14</td>
</tr>
<tr>
<td>2.4.1 Sites of breast cancer metastasis</td>
<td>15</td>
</tr>
<tr>
<td>2.5 Epithelial-mesenchymal transition (EMT)</td>
<td>16</td>
</tr>
<tr>
<td>2.6 Snail zinc finger transcription factor</td>
<td>17</td>
</tr>
<tr>
<td>2.6.1 Snail function in normal epithelial cells</td>
<td>17</td>
</tr>
<tr>
<td>2.6.2 The role of Snail in breast cancer</td>
<td>18</td>
</tr>
<tr>
<td>2.6.3 Subcellular localization of Snail</td>
<td>19</td>
</tr>
<tr>
<td>2.7 Estrogen and Estrogen receptor-alpha (ER-α)</td>
<td>21</td>
</tr>
<tr>
<td>2.7.1 Breast cancer therapy</td>
<td>23</td>
</tr>
<tr>
<td>2.8 Mitogen-activated protein kinase/Extracellular-regulated kinase (MAPK/ERK) signaling pathway in breast cancer</td>
<td>25</td>
</tr>
<tr>
<td>2.8.1 MAPK signaling and EMT</td>
<td>26</td>
</tr>
<tr>
<td>2.8.2 Subcellular localization of ERK1/2</td>
<td>27</td>
</tr>
<tr>
<td>2.8.3 Targeting breast cancer by ERK1/2 inhibition</td>
<td>29</td>
</tr>
<tr>
<td>2.9 Snail and MAPK signaling</td>
<td>31</td>
</tr>
<tr>
<td>CHAPTER 3 MATERIALS AND METHODS</td>
<td>32</td>
</tr>
<tr>
<td>3.1 Snail and active nuclear ERK1/2 study</td>
<td>32</td>
</tr>
<tr>
<td>3.1.1 Cell culture, antibodies, and reagents</td>
<td>32</td>
</tr>
<tr>
<td>3.1.2 Animal experiments</td>
<td>33</td>
</tr>
<tr>
<td>3.1.3 Short interfering RNA transfection (siRNA)</td>
<td>33</td>
</tr>
<tr>
<td>3.1.4 Western blot analysis</td>
<td>34</td>
</tr>
<tr>
<td>3.1.5 Immunohistochemistry (IHC)</td>
<td>34</td>
</tr>
<tr>
<td>3.1.6 Immunocytochemistry (ICC)</td>
<td>35</td>
</tr>
<tr>
<td>3.1.7 In vitro cell migration assays</td>
<td>36</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Human body: mammary gland anatomy</td>
<td>(62)</td>
</tr>
<tr>
<td>2. Morphological stages of embryonic development of the mammary gland</td>
<td>(63)</td>
</tr>
<tr>
<td>3. Epithelial-mesenchymal transition (EMT)</td>
<td>(65)</td>
</tr>
<tr>
<td>4. Snail genes occupy a central position in triggering EMT in physiological and pathological situations</td>
<td>(66)</td>
</tr>
<tr>
<td>5. Snail regulates ERK activity in breast cancer cells</td>
<td>(67)</td>
</tr>
<tr>
<td>6. Snail increases EMT and tumorigenicity</td>
<td>(69)</td>
</tr>
<tr>
<td>7. Snail increases tumorigenicity <em>in vivo</em></td>
<td>(71)</td>
</tr>
<tr>
<td>8. Snail promotes nuclear translocation of phospho-ERK</td>
<td>(72)</td>
</tr>
<tr>
<td>9. Phospho-ERK co-localizes with NUP98 nuclear membrane marker in MCF-7 parental cells</td>
<td>(74)</td>
</tr>
<tr>
<td>10. Snail knockdown correlates with nucleo-cytoplasmic translocalization of phospho-Elk-1</td>
<td>(75)</td>
</tr>
<tr>
<td>11. Inhibition of ERK activity decreases Snail and partially reverts EMT independent of proteasomal degradation</td>
<td>(76)</td>
</tr>
</tbody>
</table>
12. Differential role of ERK1 and ERK2 isoforms..............................................................(78)

13. Differential functional roles of ERK activity in MCF-7 Neo vs MCF-7 Snail...........(79)

14. Snail decreases estrogen receptor-α expression *in vitro* and *in vivo*......................(81)

15. Hydroxytamoxifen therapy does not affect MCF-7 Snail morphology or Snail expression...........................................................................................................................................(82)

16. Sustained UO126 and 4-OHT combination treatments effectively reduce MCF-7 Snail viability.............................................................................................................................................(84)

17. Mitochondrial permeability is not responsive to UO126 and/or 4-OHT treatments.(86)

18. Cell apoptosis induced by UO126 or UO126/4-OHT is caspase-independent...........(88)
LIST OF ABBREVIATIONS

AP-1 ................................................................. Activating Protein-1
ARCaP ......................................................... Androgen-repressed Prostate Cancer Cells
ATCC ................................................................. American Type Culture Collection
BPH ................................................................. Benign Prostatic Hyperplasia
BSA ................................................................. Bovine Serum Albumin
BM ................................................................. Basement Membrane
BMP ................................................................. Bone Morphogenetic Protein
β-TRCP .......................................................... Beta-transducin Repeat Containing
cDNA .............................................................. Complementary Deoxyribonucleic Acid
CHiP ................................................................. Chromatin Immunoprecipitation
Cgn ................................................................. Collagen V
CRC ................................................................. Colorectal Cancer
CXCL5 ............................................................. Cysteine (C)-X-C Ligand 5
CXCR4 ............................................................ Cysteine (C)-X-C Receptor 4
DAPI ................................................................. 4'-6-Diamidino-2-Phenylindole
DNA ................................................................. Deoxyribonucleic Acid
ECM ................................................................. Extracellular Matrix
EGF ................................................................. Epidermal Growth Factor
EGFR ............................................................... Epidermal Growth Factor Receptor
EMT ................................................................. Epithelial-mesenchymal Transition
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-α</td>
<td>Estrogen Receptor-α</td>
</tr>
<tr>
<td>R-β</td>
<td>Estrogen Receptor-β</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-regulated Kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen Synthase Kinase-3β</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome Wide Association Study</td>
</tr>
<tr>
<td>HMEPiC</td>
<td>Human Mammary Epithelial Primary Immortalized Cells</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
</tr>
<tr>
<td>IGFR-1</td>
<td>Insulin-like Growth Factor Receptor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Androgen-sensitive Prostate Cancer Cells</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madine Darby Canine Kidney Cells</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metallo-Proteinases</td>
</tr>
<tr>
<td>mNLS</td>
<td>Monopartite Nuclear Localization Signal</td>
</tr>
<tr>
<td>MSK1</td>
<td>Mitogen- and Stress-activated Kinase-1</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Bioinformatics</td>
</tr>
<tr>
<td>NED</td>
<td>Neuroendocrine Differentiation</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutions of Health</td>
</tr>
<tr>
<td>cNLS</td>
<td>Classical Nuclear Localization Signal/Sequence</td>
</tr>
<tr>
<td>PAK1</td>
<td>p21-activating Kinase 1</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3, 4, 5)-Trisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PKD1</td>
<td>Protein Kinase D 1</td>
</tr>
<tr>
<td>PLC</td>
<td>Pregnancy-lactation Cycle</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>PRLR</td>
<td>Prolactin Receptor</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid Hormone-related Protein</td>
</tr>
<tr>
<td>Raf</td>
<td>Rapidly Accelerated Fibrosarcoma</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator of Nuclear kappa-B Ligand</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat Sarcoma</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RSK</td>
<td>Ribosomal s6 Kinase</td>
</tr>
<tr>
<td>SDF1 α</td>
<td>Stromal Cell-Derived Factor 1α</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short Hairpin Ribonucleic Acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interferring Ribonucleic Acid</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted Protein Associated Morphogenesis</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

TrKB..................................................Tyrosine Kinase Receptor, Type 2
CHAPTER 1
INTRODUCTION

Breast cancer is the second most commonly diagnosed cancer, accounting for almost 1 in 3 cancers diagnosed in US women.\(^1\) One of the main causes of mortality from cancer is metastasis, a process facilitated by epithelial-mesenchymal transition (EMT).\(^2\) EMT is a process that regulates normal physiological states like embryonic development and wound healing. EMT also regulates pathological events like tumor development, progression and metastasis.\(^3\) Snail (snail\(^\text{I}\)) transcription factor is a C\(_2\)H\(_2\) zinc finger protein that increases EMT by binding to Enhancer box regions of target promoters. Snail expression corresponds with decreased expression of E-cadherin, VE-cadherin, claudins, occludin, desmoplakin, cytokeratins, and mucin-1 epithelial markers.\(^3\)\(^4\) Snail has also been shown to bind to estrogen receptor-alpha (ER-\(\alpha\)) and maspin promoters and downregulate transcriptional activity in cancer.\(^5\)

Snail genes are convergence points in EMT induction because several signaling pathways such as transforming growth factor beta (TGF-\(\beta\)) and epidermal growth factor (EGF) activate their expression.\(^3\) Snail is involved in several phases of mammary gland development, from birth through puberty, pregnancy, lactation, and continues until menopause.\(^3\)\(^6\) Snail has been shown to increase tumor recurrence and therapy resistance in hepatocytes and Madine Darby Canine Kidney (MDCK) cells.\(^3\)\(^7\) Snail increases tumor resistance to Paclitaxel, suppresses caspase-mediated apoptosis by targeting tumor necrosis factor alpha (TNF-\(\alpha\)), and anoikis by increasing TrKB.\(^7\) Snail is induced by
TGF-β and that upregulates pro-inflammatory interleukins and MMPs which help to degrade the extracellular matrix (ECM).\(^3\) This activity shifts the microenvironment to a more invasive profile.\(^7\) There is direct evidence for EMT in breast and prostate cancer cells in vitro and in vivo.\(^8\) EMT-specific functions of migration and invasion are strongly associated with Snail activity in prostate cancer cells.\(^8\)

Modulation of Snail phosphorylation has been a hallmark of several studies on EMT where Snail was closely involved.\(^8\) Active Snail is localized within the nucleus as a 264 amino acid transcriptional repressor of target promoters, and is phosphorylated by a p21-activated kinase 1 (PAK1) on Ser246.\(^10\) PAK1 phosphorylation of Snail activates Snail protein and promotes Snail accumulation within the nucleus to promote EMT. Snail phosphorylation by glycogen synthase kinase-3β (GSK-3β) at two consensus motifs mediates a negative feedback mechanism.\(^11\) GSK-3β phosphorylation induces β-TRCP-mediated ubiquitination, localization of Snail in the cytoplasm and proteasomal degradation.\(^11\) Furthermore, Snail phosphorylation by protein kinase D1 (PKD1) on Ser11 leads to nuclear export of Snail and EMT inhibition.\(^12\) The zinc finger domain of Snail serves as its nuclear localization sequence and authorizes its movement into the nucleus.\(^13\) Cross-communication between different pathways allows cells to identify and respond appropriately to the extracellular environment.\(^14\) The receptor tyrosine kinases (RTK) like epidermal growth factor receptor (EGFR), Ras/Raf/MEK/ERK, and PI3K/AKT pathways are all involved in cancer development, progression, and metastasis. However, Snail has been strongly implicated in the MAPK/ERK pathway in breast cancer cells.\(^15\)
Mitogen-activated protein kinase (MAPK) and extracellular-regulated kinases 1 and 2 (ERK1/2) are significant signaling proteins which control several processes, including: proliferation, survival, motility, adhesion, and invasion. The ERK cascade has several distinct functions which differ depending on its subcellular localization. Nuclear ERK activity has been associated with malignant mammary tumors and poor prognosis. Breast tumor progression was also associated with increased ERK/MSK1/Elk-1/Snail activity in breast cancer cell lines and in mice. ERK is an attractive target for clinical studies because shifts in its activity and localization are related to tumor progression. ERK1/2 signaling has been portrayed as indistinguishable in vitro. While others have conclusive data which states differential roles of ERK1 and ERK2. Furthermore, the controversial idea that persists concerns the shifts in function for each isoform as epithelial cells transform into malignant tumors. These tumors have a higher potential to become metastatic if not detected or treated properly. ERK1 and ERK2 have been shown to have differential roles during physiological events. Microarray (GenMAPP program) gene expression profiling of ERK1 and ERK2 knockdown was used to analyze stages of early embryogenesis. Specific genes were identified for ERK1 and ERK2, which directly related to separate functions during embryogenesis. Genes involved in dorsal-ventral patterning and embryonic cell migration were identified for ERK1, and genes involved in cell-migration, mesoderm differentiation and patterning were identified for ERK2. There were also genes that were common to ERK1 and ERK2. These findings were identified and confirmed biologically using ERK1 or ERK2 knockdown in zebrafish embryos.
We investigated the role of Snail in regulating MAPK in breast cancer cells and found that Snail and ERK1/2 activity is higher in breast cancer cells as compared to normal mammary epithelial cells. MCF-7 cells overexpressing constitutively active Snail (MCF-7 Snail) displayed EMT, predominantly nuclear active ERK1/2 as well as increased tumorigenicity. The nuclear ERK activity was shown to activate Elk-1 downstream substrate, increase cell migration, decrease cell adhesion, and increase EMT. Conversely, high ERK1/2 activity was observed in MCF-7 Neo cells which was predominantly the ERK1 isoform within the cytoplasm and nucleus, and this ERK1 activity was associated with increased cell adhesion and decreased EMT. These findings begin to uncover nuclear ERK2 regulated by Snail as a specific subcellular target of therapy for metastatic breast cancer cells, whereas ERK1 activity may prevent tumor progression. Therefore, we hypothesized that Snail promotes the translocalization of p-ERK into the nucleus and remains functional in metastatic breast cancer cells. The current studies were designed to test the following objectives:

1. Snail and active ERK are functional within the nucleus of breast cancer cells.
2. Active ERK within the nucleus plays a direct role in breast cancer EMT.
3. Nuclear Snail and ERK increase resistance to hydroxytamoxifen by down-regulating estrogen receptor-alpha.
CHAPTER 2
LITERATURE REVIEW

2.1 The human mammary gland

The mammary gland is unique to mammals, functions to synthesize and secrete milk from specialized lobes and ducts, and nourish the infant. Mammary glands are derived from modified sweat glands that develop during the sixth week of embryogenesis and only function during the pregnancy-lactation cycle (PLC). The proliferating cells from the ectoderm are characterized as the milk line, which is arranged from the buds of the lower limbs to the buds of the upper limbs (Appendix Figure 2). Mammary glands are arranged from layers of specialized epithelial cells, derived from the intercostal muscles, suspensory ligaments, lactiferous sinus and ducts, gland lobules and a layer of adipose tissue. The surrounding stroma is also composed of a basement membrane (BM) and extracellular matrix (ECM) that functions to protect the mammary gland and maintain homeostatic functions within the breast tissue.

Changes in mammary gland morphology influence the composition, architecture, and functions of mammary tissue during specific life stages. The major mammary developmental stages include fetal growth, infancy (pre-pubertal growth), pubertal expansion, and post-lactational and post-menopausal involution.

During embryogenesis, the primary structures of the mammary gland form and lead to a substantial outgrowth of ducts; this is the initiation of ductal branching.
A rudimentary ductal tree forms from the initial sprouting of the mammary buds during embryo growth. The extension of the ductal branches is unique to embryogenesis, although they appear similar to those which occur during puberty. The most obvious and well-documented difference is that embryogenesis occurs in a hormone-independent manner whereas puberty must occur from the initial and continuous production and circulation of hormones throughout the body. This conclusion has been indicated in mice deficient in estrogen receptors alpha (α) and beta (β) (ER-α, ER-β), progesterone receptors (PR), prolactin receptors (PRLR), and growth hormone receptors (i.e., insulin-like growth factor - IGF, epidermal growth factor - EGF). Deficient mice were able to still initiate the growth and branching of morphogenesis during embryonic development. Moreover, mice without EGF receptor (EGFR), a regulator of estrogen activities, still initiated mammary sprouting and duct development. These activities occurred although the neonatal duct system was smaller than wildtype mice. Three signaling pathways have become primary targets to determine the mechanism behind embryonic mammary development: parathyroid hormone-related protein (PTHrP), bone morphogenetic protein (BMP), and Wnt. Disruption or loss of one or a combination of these pathways leads to inhibition of epithelial ductal morphogenesis. In fact, the disruption or loss of several other pathways and the interactions between populations of epithelial and stromal cells can detrimentally affect the development of mammary ducts and other epithelial structures. In a pre-pubescent girl, the mammary gland does not produce high levels of the hormones estrogen and progesterone; this production increases at the onset of puberty from the ovaries. During pregnancy there are strong fluctuations in hormone production, and
comparatively during lactation.\textsuperscript{24a, 29} During normal aging; however, production of these hormones begins to regress and the anatomical structures of the mammary gland begin to involute considerably\textsuperscript{22b}. Menopausal involution is an apparent factor in several pathologies that have been observed in human breast tissues. The hormone-related nonmalignant changes due to aging are neoplastic in nature and were at one time only associated with older women\textsuperscript{22b}.

The PLC has been shown to induce mammary growth but also provides protection against the development of breast cancer in the long term.\textsuperscript{30} PLC can induce a permanent change in the breast architecture which may exert a strong protective effect in women. Still, the mechanism of this process as an effector against breast cancer development or progression is not well understood.

\textbf{2.2 Breast cancer development}

Breast cancer development is a serious pathology of the mammary gland and is currently reported as the most frequent cancer and cancer-related cause of death in women worldwide.\textsuperscript{31} Breast cancer is a hormone-dependent malignancy that leads to uncontrolled growth of mammary cells.\textsuperscript{24, 32} The levels or lack of response to therapy is directly correlated to the subtypes of breast cancer observed both in the clinic and laboratory.\textsuperscript{33} The incidence and survival rates from breast cancer are different from one country to another and among ethnic groups within a single country or region.\textsuperscript{33b} There is a clear disparity among certain ethnic groups and groups that fail to obtain proper preventive or therapeutic benefits. In 2012, 226,740 new cases of breast cancer had been diagnosed and 39,510 deaths had been estimated in the United States.\textsuperscript{34} The cancer statistics among Caucasian (CA) and African-American (AA) women are startling
because the incidence and rate of mortality are consistently the highest among these groups.\textsuperscript{1,35} SEER data collected from 2006-2010 stated that incidence rates among CA women were 127.4 per 100,000 women, and among AA women 121.4 per 100,000 women, indicating that the incidence is slightly higher in CA women.\textsuperscript{36} Mortality rates collected during 2006-2010 showed that AA mortality was 30.8 per 100,000 women, and CA mortality was 22.1 per 100,000 women.\textsuperscript{36} These findings are further supported by studies that implicate that genetic predisposition may play a role in breast cancer development.\textsuperscript{37}

There is currently no clear cause for breast cancer and it is recognized as a multifactorial disease. Several factors contribute to the development of breast cancer. These factors include, but are not limited to, environment and behavior. PLC, the use of birth control, and hormone replacement therapy (HRT) are noted as factors in breast cancer development.\textsuperscript{38}

The relationship between plasma concentrations of estradiol, progesterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and sex hormone-binding globulin (SHBG) were analyzed and the findings indicated that SHBG decreased with increasing body mass index.\textsuperscript{39} Obesity is a risk factor of breast cancer and these molecules have become markers for diagnosis and treatment. Estradiol was higher in current smokers, but lower in two separate populations of postmenopausal women and ex- and non-smokers. FSH decreased with increased alcohol intake in all groups of women in this study.

### 2.3 Breast cancer subtypes

According to the National Cancer Institute, breast cancer is classified into stages:
stages 0, IA, IB, IIA, IIB, IIIA, IIIB, IIIC, and IV. Stage 0 encompasses carcinoma \textit{in situ}, meaning that the tumor remains within the breast. There are three types of carcinoma \textit{in situ}, including: ductal carcinoma \textit{in situ} (DCIS), lobular carcinoma \textit{in situ} (LCIS) and Paget disease of the nipple. DCIS can be characterized into typical and atypical types of breast carcinoma based on close to normal and abnormal cell morphology, respectively.

These tumors have just begun to develop hyperplasia lesions that usually do not develop into invasive tumors. During stage I, carcinoma has formed from proliferating cells and can be divided into stage IA and IB. In stage IA tumors measure about 2 centimeters or smaller. In stage IB tumors are clustered and densely packed into a more organized tumor structure measuring between 0.2 millimeters to 2 millimeters and mostly found in the lymph nodes. For these reasons, it is difficult to detect tumors in the breast during stage IB. When tumors have progressed to stage IIA and IIB their size increases and decreases as they enter nearby lymph nodes. Specifically during stage IIA, tumors are no more than 2 centimeters in length, and tumors can be detected in axillary lymph nodes. During stage IIB the tumor is between 2 and 5 centimeters. Clusters of cancer cells between 0.2 millimeters and 2 millimeters are detected in lymph nodes.

The tumor cannot be detected in the breast or may be detected at any size during stage IIIA. There are a higher number of tumors found in more axillary lymph nodes (4 to 9) or in the lymph nodes near the breastbone. Radiological imaging and physical exams can easily detect these tumors and have become necessary for diagnostic procedures. These tumors have proliferated to larger than 5 centimeters, while smaller clusters near this main tumor are still between 0.2 and 2 millimeters. During stage IIIB, tumors are detected at various sizes and can also spread to the chest cavity and/or skin of the breast.
This breast cancer is termed inflammatory breast cancer because it induces breast swelling and in some cases ulcers form. Stage IIIB tumors also spread to breast bone axillary lymph nodes while continuing to proliferate. Stage IIIC tumors continue to spread to more lymph nodes and usually tumors in the breast are more difficult to detect. Stage IIIC is an advanced form of inflammatory breast cancer and these tumors can develop inoperable clusters of tumor that is treated with non-conventional methods (i.e., combinational therapy, immunotherapy). These cells are primed to become metastatic and leave the original organ site. Stage IV breast cancer has spread to other parts of the body via the circulatory, lymphatic or nervous system pathways. The brain, lungs, liver, and bone are prime locations for breast cancer metastasis.

2.3.1 Ductal hyperplasia

Ductal hyperplasia is a type of non-proliferative lesions, occurring without atypia. The cells have a normal morphology, but grow excessively. These cells develop in the ducts or lobules of the breast tissue and raise the risk of breast cancer by 1.5 to 2 times. Some examples of ductal hyperplasia include fibrosis and cysts. The most common symptoms of ductal hyperplasia cysts or lesions are breast pain and tenderness brought on by rapidly growing nodules. Fibrocystic changes of this nature are challenging to diagnose due to similar morphology such as those observed with atypical hyperplasia. Atypical hyperplasia lesions have transformed morphology, which is less epithelial and more mesenchymal or appear flattened. There are specific cyto-morphological characteristics of hyperplasia, indicated by studies that analyzed various grades of hyperplasia. Cellularity, cell composition, architectural pattern, nuclear diameter and pleomorphism, chromatin pattern and number of single epithelial cells were used to
characterize the cells.

The markers of typical and atypical ductal hyperplasia are estrogen receptor, E-cadherin, cytokeratins 1, 5, 10, and 14, human epidermal growth factor receptor, Ki-67, and progesterone receptor.\(^{48}\) Ductal hyperplasia was identified in association with malignant neoplasia in 56 of 115 cases (48.8\%) and indicated a role that canine mammary atypical hyperplasia plays in malignant neoplastic transformation during lesion development.\(^{48}\) Adjuvant therapy such as hormonal or radiation therapy is useful in treating ductal hyperplasia, usually atypical, which can easily progress to carcinoma.\(^{49}\)

2.3.2 Ductal carcinoma in situ (DCIS)

From its discovery during the early 20\(^{th}\) century until 80 years later, DCIS accounted for fewer than 5\% of newly diagnosed breast cancers, but today accounts for 20\% to 30\% of all breast cancers diagnosed.\(^{50}\) Ductal carcinoma is non-invasive cancer that occurs in the ductal tubes that carry milk to the nipple. DCIS is staged at 0, meaning that the tumors are local and do not move like tumors staged at II, III, or IV.\(^{40}\) However, DCIS is the fastest growing subtype of breast cancer that represents a clonal proliferation of malignant ductal cells that can also become a precursor for invasive breast cancer.\(^{49}\) These neoplastic lesions rapidly progress to become tumors. Patients who have DCIS treated with incomplete biopsy alone develop invasive breast cancer 40\% of the time. Developing an approach to detect and excise DCIS tumors then follow up with therapy is crucial to increase survival rates.

There are several modes of therapy that are useful to treat DCIS, despite the increasing number of new cases every year.\(^{50a}\) Tamoxifen is used to treat DCIS tumors that are ER- and PR-positive, and the growth of these tumors significantly decreases.
More recent investigations are focused on inhibiting DCIS growth factor signaling to prevent increased propensity to become invasive breast tumors. One study used ZD1839 to target EGFR tyrosine kinase signaling in patient tissue samples.\textsuperscript{51} Proliferation decreased after treatment, indicated by Ki67 immunostaining.

### 2.3.3 Lobular carcinoma in situ (LCIS)

Lobular carcinoma in situ (LCIS) is a form of inflammatory breast cancer (IBC), and characterized as the most lethal form of breast cancer.\textsuperscript{52} LCIS/IBC tumors express high levels of estrogen, progesterone, and epidermal growth factor receptors which are significant for diagnostic and prognostic applications. The difference in expression of these growth factors is what defines LCIS/IBC apart from other forms of breast cancer. LCIS/IBC tumors have high levels of HER2 and low levels of ER/PR, which correlates with a high level of malignancy and shorter disease-free survival rates.\textsuperscript{53} Therapy for LCIS/IBC tumors ranges from radiation to adjuvant chemotherapy, but resistance to these is correlated with rapid repopulation of the cells between doses.\textsuperscript{54} Targeting inflammatory mediators and associated signaling pathways in IBC is being used in pre-clinical phases.\textsuperscript{52} Inhibitors for several mediators are being used: RO4929097 targets Notch to downregulate expression of cytokines interleukin-6 (IL-6), and IL-8; CX-4945 targets protein kinase CK2 to downregulate IL-6. Vitamin D calcitriol reduces motility, invasion and tumor spheroid formation in SUM149 IBC tumors.\textsuperscript{55}

### 2.3.4 Triple-negative breast carcinoma (TNBC)

Oncologists, pathologists and geneticists report that approximately 12 to 17% of women with breast cancer have been diagnosed with triple-negative breast cancer.\textsuperscript{56} The loss of estrogen, progesterone, and human epidermal growth factor receptors are major
contributors to the triple negative breast cancer (TNBC) stage. The heterogeneous nature of TNBC and TNBC subtypes has been analyzed for its lack of response or high resistance and recurrence to standard adjuvant therapies. The basal-type epithelial phenotype of TNBC is associated with a poor outcome and cannot be treated with endocrine or HER2-directed therapies.

Although TNBC have similar gene expression profiles and phenotypes to basal-myoepithelial normal breast cells, certain biological markers have been uncovered to determine mechanisms for their development and progression. Immune response genes CIQA, IGLC2, LY9, TNFRSF17, SPP1, XCL2, and HLA-F have been identified as markers for TNBC. Mesenchymal-like (MSL) subtypes of TNBC express markedly high levels of growth factor signaling mediators (TGFβ1L1, BGN SMAD6, SMAD7, NOTCH1, TGFβ1, TGFβ2, TGFβ3, TGFβR1, TGFβR2, TGFβR3, FGF, IGF, PDGF) and EMT genes (MMP2, ACTA2, SNAI2, SPARC, TAGLN, TCF4, TWIST1, ZEB1, COL3A1, COL5A2, GNG11, ZEB2), and Wnt/β-catenin signaling (CTNNB1, DKK2, DKK3, SFRP4, TCF4, TCF7L2, FZD4, CAV1, CAV2, and CCND2). Decreased expression of E-cadherin is also a marker for TNBC as it is decreased during the development and progression. Gene mutations observed in basal-like (BL) and MSL tumors occur in the same genes (i.e., PTEN, TP53, RB1, BRCA1, BRCA2, CDKN2A, and BRAF). Combinational therapies of anthracycline-based regimens and taxanes have become a therapeutic option for TNBC patients, but the combination is met with side effects and high risk of recurrence. EGFR inhibitors, DNA repair inhibitors, anti-angiogenic agents and other chemotherapies are currently being tested in trials that have generated promising results. These therapies are more useful in comination with
traditional chemotherapeutics. Paclitaxel, docetaxel, gemcitabine, and others are being used to directly inhibit TNBC by abrogating their ability to signal to axillary lymph nodes. Kuo et al used microarray data from 157 invasive breast carcinomas to identify a novel 45-gene prognostic signature for predicting risk and recurrence of TNBC. Findings from this gene analysis are clinically significant for studying TNBC in patients, but may also serve as therapeutic targets in vitro.

2.4 Breast cancer metastasis

Breast cancer accounts for 23% of cancer diagnoses (1.38 million women) and 14% of cancer deaths (458,000) each year. Breast cancer has a higher incidence in developed countries, and 50% of new cases and about 60% of deaths are thought to occur in the developing world. The majority of cancer patient deaths are attributed to metastatic spread of the tumor cells throughout the body. In fact, metastasis is one of the main causes of mortality from all cancers.

Understanding the mechanism of metastasis is of great importance to develop more effective therapy and prevention strategies. The mechanisms of metastasis under investigation include the complexity of signaling which is required for its initiation and maintenance. These features of metastatic disease make it more difficult to treat the tumors, and unfortunately there is currently no cure available.

The establishment of metastases at distant sites from the primary tumor is not random and predictable. Breast tumors primarily metastasize to lung and bone marrow tissues, while colorectal tumors metastasize to the liver. In 1989, Paget proposed a mechanism for metastasis with his 'seed and soil' hypothesis. The migrating cells are like seeds and the new environment that they metastasize to is the soil, a nourishing environment for them.
to establish new microvasculature. The different levels of growth and metastasis are based on the idea that tumors are heterogeneous tumors.64b,65

Tumors cells that have metastasized are the same type of cancer cell as the primary tumor. Metastatic tumors must proliferate to generate new colonies and this process involves changes in numerous cell functions.66 Cell-cell and cell-matrix marker expression change to accommodate these shifts and promote epithelial-to-mesenchymal transition. Cell surface adhesion molecules like the cadherins, in particular E-cadherin and N-cadherin help to perpetuate this process.67 During metastasis, there is a loss of E-cadherin and a gain of N-cadherin, a classical shift in expression which plays a critical role in the transformation of malignant or localized tumors. The shift is due to changes in N-linked glycosylation of either molecule. N-acetylglucosaminyltransferase III (GnT-III) structural modifications of E-cadherin increases cell-cell adhesion while N-acetylglucosaminyltransferase V (GnT-V) structural modifications of N-cadherin decreased cell-cell adhesion.65

2.4.1 Sites of breast cancer metastasis

Changes in epithelial cell composition and architecture lead to increased migration and invasive activities. Moreover, the upregulation of O-glycans and galectin-3 adhesion markers favor breast cancer cell metastasis to the brain.68 E-selectin adhesion marker has been implicated in the spread of colorectal and breast cancer because it aids in the adhesion of tumor cells to endothelial cells (blood vessels).69 The cross-talk between the metastatic tumor environment, circulatory system, and immune system is extensive. P-selectin has been reported to facilitate colon, lung, breast, melanoma, gastric, tongue squamous, and neuroblastoma tumors to form complexes in an L-selectin-dependent
manner with platelets and leukocytes. These activities eventually help to facilitate angiogenesis and the progression of metastatic colony formation.

Moreover, there are genes whose function helps to determine a relationship between tissue type and site of metastasis. The human metastatic breast cancer cell line MDA-MB-231 was used to study the site of breast cancer metastasis. MDA-MB-231 were transplanted into the tail vein of immunodeficient mice. Metastatic activity was assayed by bioluminescence imaging of luciferase-transduced cells. Gross examination of lungs at necropsy was also performed. The chemokine receptor cysteine x cysteine receptor 4 (CXCR4), inhibitor of DNA binding 1 (ID1), VCAM1, IL12Rα2, and SPARC are genes which have been indicated in lung metastases. More recently, it has been indicated that bones is also a preferential site for breast tumor metastasis. When breast cancer cells metastasize to the bone tissue, the microenvironment changes and these cells help recruit bone cell osteoblast and osteoclasts to remodel the microvasculature.

During normal bone turnover, osteoblast cells are responsible for bone formation while osteoclast cells degrade the bone resulting in homeostasis between bone formation and bone degradation. These lesions lead to symptoms of bone pain, fractures, cord compression and other physiological complications.

2.5 Epithelial-mesenchymal transition (EMT)

The process of metastasis is complex and requires several steps to occur which initiate movement to a secondary site and development of metastatic colonies. Metastasis is a type of epithelial-mesenchymal transition (EMT) where cells also remodel the extracellular matrix to move and invade the surrounding microenvironment, which has been implicated in several cancers (Figure 3). Malignant cells that are still
localized tumors must escape from the primary tumor surrounded by the basement membrane and extracellular matrix. These cells intravasate into the vascular circulation or lymphatic systems, evade immune surveillance, extravasate from the circulation, and establish metastatic colonies.\textsuperscript{44b, 62, 74, 75b}

The anatomical changes that occur during EMT are characterized by the cells becoming more mesenchymal and neuronal, which is indicative of neuroendocrine differentiation, as indicated in prostate and breast cancer.\textsuperscript{76} EMT has been analyzed \textit{in vitro} and \textit{in vivo}.\textsuperscript{77} Human breast epithelial cells (HBEC) and human breast epithelial cell line (MCF-10F) were used to mimic the initial steps of EMT. MCF-10F cells transformed after 24 hour 17-β-estradiol treatment had increased invasiveness in Matrigel invasion assays, colony formation in agar methocel, and decreased ductulogenesis in collagen assay.\textsuperscript{77b}

2.6 Snail zinc finger transcription factor

Snail is a member of the Snail gene superfamily (consisting of Snail, Slug, Scratch, Smuc) and characterized by a highly conserved carboxyl-terminal region with four to six C2H2-zinc fingers.\textsuperscript{5d, 78} Snail and Slug (Snail2) repress the transcription of their target genes when their zinc domains bind to the enhancer box (E-box) motifs at the target promoter region (5'-CANNTG-3').\textsuperscript{5d} Snail (snail1) transcription factor expression is associated with decreased expression of E-cadherin, VE-cadherin, claudins, occludin, desmoplakin, cytokeratins, and mucin-1.\textsuperscript{79} Snail also suppresses TGF-β-induced apoptosis and its activity is sufficient enough to trigger EMT in hepatocytes.\textsuperscript{7b}

2.6.1 Snail function in normal epithelial cells

Studies have implicated that Snail is required during many important stages of
life, including: embryogenesis, neurogenesis and nerve development, early neonatal and infant development, wound healing, and healing after spinal cord injury. Snail regulates embryogenesis and tissue development, along with other Slug, Twist, Six1, and Cripto. TGF-β and Wnt/β signaling pathways regulate Snail activities during development. Abnormal expression of these pathways and co-regulators is common in breast cancer with poor prognosis. During gastrulation, three primitive germ layers develop and the basement membrane under the epiblast degrades. FGF activity in the primitive streak stimulates Snail upregulation, then represses E-cadherin and destabilizes cell-cell junctions. Snail is expressed in murine extraembryonic tissues and embryonic mesoderm, and in activated fibroblasts in mouse skin wound healing (i.e., fibromatosis).

2.6.2 The role of Snail in breast cancer

High levels of Snail after puberty and well into adulthood have been linked to the induction of hyperplasia and the development of primary tumors in the prostate and breast. The EMT program is switched on as tumor cells become aggressive and Snail begins to regulate migration. Snail and Twist transcription factors were analyzed in a review on signaling in mammary gland development and cancer. Snail is widely observed as a repressor of target gene promoters for adhesion of E-cadherin EMT marker, ectodermal genes observed in Drosophila and zebrafish and tumor suppressors Bim and Bmf. For example, Snail downregulates maspin tumor suppressor in prostate cancer. Snail-mediated downregulation of maspin contributes to an increased potential for the cells to migrate. Analyzing the regulation of Snail on its target genes is critical to developing more effective treatment options for patients with breast cancer. TGF-β induction of Snail upregulates pro-inflammatory interleukins and MMPs which
help to degrade the extracellular matrix (ECM). This activity shifts the microenvironment to a more radical and invasive profile. Stromal- and epithelial-specific cre-transgenic mice were bred to study EMT during cancer development and progression. Through genome-wide association studies (GWAS), investigators suggested that breast cancer invasion seems to be rare, but is initiated after myc activation. Myc has also been noted to activate snail during EMT. EMT-specific functions of migration and invasion are strongly associated with Snail activity in prostate cancer cells. Snail is involved in several phases of mammary gland development, from birth through puberty, pregnancy, lactation, and continues until menopause.

When Snail gene expression is low in node-negative invasive breast (ductal) carcinomas, it is a good prognostic factor. The expression of Snail is observed in normal breast tissue, but is upregulated in breast cancer; a pattern that is inverse for E-cadherin EMT marker expression. Changes in hormone receptor activity and adhesion markers are attributed to Snail upregulation, which is an ever-changing mechanism during breast cancer development. Snail promotes tumor recurrence. A reversible HER-2/neu-induced breast cancer mouse model was used to analyze this mechanism and findings suggest that increased Snail expression correlates with decreased relapse-free survival. This model was intended to improve response to therapy by inactivating HER-2/neu expression and regressing breast tumors. These previous findings have strongly indicated Snail as a target for analyzing therapy recurrence.

2.6.3 Subcellular localization of Snail

Snail localization is mostly due to serine phosphorylation. The C-terminal DNA-binding domain (amino acids 152 to 264) and N-terminal regulatory domain
(amino acids 1 to 151) of Snail have been previously identified. The C-terminal region is composed of four C2H2 zinc finger, and is conserved in vertebrate and invertebrate homologues of Snail. Snail C-terminal domain has high affinity for oligonucleotides with the 5'-CACCTG-3' sequence, as indicated for E-cadherin. The SNAG box sequence of amino acids is necessary for repression of Snail target genes and a subdomain that functions as a nuclear export sequence. Dominguez et al. reported that Snail is localized in the nucleus and cytosol of NIH-3T3, in the nucleus of MDCK cell stably transfected with Snail and MDA-MB-231 TNBC, in the cytoplasm of MCF-7, NIH-3T3, Caco-2, and HCT-116 cells, and undetected in RWP-1 cells by immunofluorescence. These cells also expressed varying levels of Snail mRNA and protein, indicated by RT-PCR and Western blot. Therefore, the localization of Snail may be dependent on the level of transformation for cancer cells.

The increased levels of Snail may be associated with more malignant tumors of different types. Zhou et al. determined that Snail is regulated more specifically by glycogen synthase kinase-3β (GSK-3β) phosphorylation which eventually controls migration and invasion. GSK-3β binds to and phosphorylates Snail at two consensus motifs. The first motif regulates Snail degradation via β-Trcp ubiquitination and the second motif retains Snail in the cytoplasm. Snail-6SA variant abrogates Snail phosphorylation at both sequences to translocate Snail protein in the nucleus but does not decrease its activity there. GSK-3β kinase activity functions as a switch during EMT induction or inhibition by controlling Snail. On the other hand, p21-activated kinase 1 (PAK1) on Ser246 regulates Snail nuclear localization by activating its expression. Protein kinase D 1 (PKD1) inhibits Snail in the nucleus and leads to nuclear export on
Ser\textsuperscript{11}. The activity of Snail as a transcription factor only last about 25 minutes so the tightly controlled localization of Snail in subcellular compartments is highly significant.\textsuperscript{86} Therefore, Snail localization contributes to tumor progression and metastasis via EMT induction.

2.7 Estrogen and Estrogen receptor-alpha (ER-\(\alpha\))

Estrogen hormones play key roles in the development and maintenance of normal sexual and reproductive functions.\textsuperscript{96} In men and women, estrogens exert a vast range of biological effects in the cardiovascular, musculoskeletal, immune, and central nervous systems.\textsuperscript{97} Several cells and tissues have the capacity to synthesize estrogen (17-\(\beta\)-estradiol), including ovarian glandulosa cells, placental synchtiotrophoblasts, adipose and skin fibroblasts, bone, and brain.\textsuperscript{98} The most potent estrogen produced in the body is 17-\(\beta\)-estradiol \textsuperscript{40}; it is a stronger estrogen receptor (ER) agonist than estrone or estriol.\textsuperscript{96} Estrogen synthetase (aromatase) enzyme aids in biosynthesis of estrogen hormones and it is found in a number of human tissues and cells.\textsuperscript{98} Aromatase and estrogen in adipose and skin tissue are associated with increases of body weight and advancing age.\textsuperscript{98} Aromatization of androstenedione to estrone then subsequently estradiol in peripheral tissues is associate with uterine bleeding and endometrial hyperplasia and cancer in obese and anovulatory or post-menopausal women.\textsuperscript{98}

Estrogen signaling is mediated by estrogen receptors (ER), ER-\(\alpha\) (NR3A1) and ER-\(\beta\) (NR3A2), members of the nuclear receptor (NR) family of transcription factors.\textsuperscript{99} ER domains are evolutionarily conserved as indicated by structural and functional analyses of the DNA-binding domain (DBD) and multifunctional ligand-binding domain (LBD).\textsuperscript{99} The N-terminal domain is the most variable of both receptors in sequence and
length based on two distinct activation functions of AF-1 and AF-2 at the C-terminal.\textsuperscript{100} Ligand-dependent estrogen signaling involves estrogen binding to ER then cell-specific transcriptional responses to this activity.\textsuperscript{100-101} ER-\(\alpha\) and ER-\(\beta\) are products of separate genes located on different chromosomes, despite previous hypotheses about the receptors.\textsuperscript{102}

In young adults, estrogen is important for male and female sexual development and behavior, maintenance of the skeleton and cardiovascular system, and normal function of the testis and prostate.\textsuperscript{103} Androgens are the substrates of aromatase and the precursor for estrogen, so it is also important for male and female tissues of ovary, uterus, breast and brain. The mechanisms of both ERs have been under analysis for several years and plays important roles in physiological and pathological events. The consequences of estrogen and ER deficiencies have become more evident in mouse models.\textsuperscript{103}

Estrogen and ER activities regulate Snail and Slug during EMT by participating in the down-regulation of E-cadherin.\textsuperscript{104} The balance between Snail and E-cadherin during normal and tumor development is a step that occurs similarly but which separate consequences. Estrogen stimulation increased the metastatic potential of human epithelial ovarian cancer cell lines while leading to morphological changes characteristic of epithelial-mesenchymal transition (EMT) and increased migration.\textsuperscript{104} E-cadherin promoter and expression were suppressed following estrogen stimulation, and siRNA against Snail attenuated estrogen-mediated E-cadherin. These prometastatic actions were regulated by ER-\(\alpha\), and reversed by ER-\(\beta\). Another study found that ER-\(\beta\) inhibits prostate cancer proliferation in DU-

Estrogen regulates the same growth factor pathways observed during EMT
(increased migration invasion) and MET (increased adhesion, loss of motility) to conduct the branching morphogenesis in the mammary gland and prostate. Estrogen signaling plays a significant role in EMT and the regulation of cell adhesion, migration, and invasion during tumorigenesis and metastasis. Estrogen treatment can elevate Snail expression in the mammary gland. In MCF-7 breast cancer cells, estrogen stimulates morphological changes associated with the rearrangement of actin filaments, a major cytoskeleton component, while also modifying cell-cell and cell-ECM adhesion plaques. Therefore, estrogen treatment is capable of elevating the EMT program in breast cancer cells that are ER-positive, meaning that they express the ER-α subtype of the estrogen receptor.

2.7.1 Breast cancer therapy

Breast cancer is usually diagnosed from a visual, physical or mammogram examination. Sometimes lumps of the breast can be observed by examining the breasts visually or by feeling the breasts for lumps or abnormalities. A mammogram is an X-ray of the breast and used to screen for breast cancer, so the use of this exam is also important for preventive measures. If an abnormality is detected by a mammogram, a diagnostic mammogram is used to evaluate the results of the first exam. Other analysis techniques include ultrasounds, biopsies, and magnetic resonance imaging (MRI). These procedures can use sound waves to produce images of the breast tissue and discern between solid tissue or cysts (ultrasound), removal of breast tissue to test by grading through pathological analysis (biopsy), and magnetic-radio waves to create pictures of the breast interior (MRI). Early detection and patient-directed treatments are more beneficial at antagonizing tumor cells.
The main types of treatment for breast cancer are surgery (partial or complete mastectomy), radiation therapy, chemotherapy, hormone therapy (HRT), targeted therapy, and bone-directed therapy. Unfortunately, these forms of therapy are only effective for localized or regional tumors. Adjuvant and neo adjuvant therapy options are designed to prevent tumor recurrence or systemic therapies (i.e., chemotherapy, hormone therapy) prior to surgery so that surgery is less invasive, respectively. Some therapy options are being improved or reconfigured to determine their efficacy for metastatic disease. Tamoxifen (TAM) is an antiestrogen that has been widely used for decades to treat or prevent low-grade breast cancer occurrence. 4-hydroxytamoxifen (4-OHT) is an active metabolite of TAM and is also used for similar applications. When MCF-7 breast cancer cells were treated with TAM, the maspin promoter activity significantly increased which required increased expression of ER-α. The use of TAM of 4-OHT has proven to be futile when patients are diagnosed with more advanced breast cancer. ER-positive MCF-7 cells treated with estradiol had an induction of PR, but growth and PR were suppressed by tamoxifen treatment, then reversed by 17-β-estradiol. Snail downregulates ER-α in MCF-7 breast cancer cells. This regulation occurred based on the ER-α promoter which was either acetylated (activated) or trimethylated (deactivated). Snail adenovirus reduced acetylation in MCF-7 cells 4-fold, when compared to ER-negative MDA-MB-231 cells (untreated) which are heavily trimethylated without any manipulation.

Breast cancer vaccines are being used in clinical trials to increase the immune system’s potential to defend against cancer cell proliferation. Drug therapy is also being explored which works in different ways: to block the effects of certain growth
factors that help the cancer cells grow (i.e., Iressa ZD1839), chemotherapy drugs that block the activation of signaling pathways and their effectors. The lack of ER-α is also associated with increased signaling pathways like MAPK/ERK and PI3K.

2.8 Mitogen-activated protein kinase/Extracellular-regulated kinase (MAPK/ERK) signaling pathway in breast cancer

Several signaling pathways allow cells to identify and respond appropriately to the extracellular environment. The receptor tyrosine kinases (RTK) like epidermal growth factor receptor (EGFR), Ras/Raf/MEK/ERK, and Phosphoinositol 3 Kinase/AKT (PI3K/AKT) pathways are all involved in cancer development, progression, and metastasis. Two predominant isoforms of ERK (ERK1 and ERK2) were once thought to have similar kinase activities in vitro. However, these kinases have very distinct and separate functions. ERK1 plays a specific role in non-invasive behavior and more epithelial morphology, while ERK2 plays a role in promoting invasion and a phenotype which drives metastasis/EMT in breast cancer cells. Extracellular stimulation of ERK1/2 has been used to study changes that occur during EMT in tumors. Rab17 and liprin-β2 are genes that inhibit invasive properties were upregulated following knockdown of ERK2 and returned to normal levels when ERK2 was re-expressed. Re-expression of ERK1 after knockdown had no effect on the expression of Rab17 or liprin-β2. ERK2-directed suppression of possible tumor suppressors or genes which inhibit metastatic functions is a major function of this kinase. MAPK and Extracellular-regulated kinases 1 and 2 (ERK1/2) are significant signaling proteins that control several processes, including: proliferation, survival, motility, adhesion, invasion and survival. Tracing the steps of MAPK from the external membrane to the nucleus has been the focus of EMT
Although it is well known that several MAPK enzymes regulate cellular functions, determining specific functions during breast cancer development and progression have been more challenging. MAPK functions interact with almost all oncogenes and oncogenic factors. MAPK signaling functions via sequential activation of distinct isoforms that have little or no cross-reactivity, described as MAP3K, MAP2K, MAPK, and MAPKAPK, along with their specific substrates ERK, JNK, and HOG.

### 2.8.1 MAPK signaling and EMT

Breast tumor progression is associated with the ERK/MSK1/Elk-1/Snail signaling pathway in breast cancer cell lines and in mice. This particular investigation focused on the function of chemokine (C-X-C) ligand 5 (CXCL5) on cellular signaling pathways in breast cancer cell lines (MCF-7, MDA-MB-231) and in mice. Treatment with CXCL5 was associated with activation of Raf/MEK/ERK, MSK1, Elk-1, and Snail, while E-cadherin was downregulated.

The components of the ERK1/2 cascade are the main factors responsible for controlling EMT. These include membrane receptors (G-protein coupled receptors (GPCRs); receptor Tyrosine kinases (RTKs); ion channels), adaptor proteins (i.e., Grb2), exchange factors (i.e., SOS), Ras/Raf kinases, proto-oncogenes (i.e., TPL2), and approximately 200 substrates. The activation of MEK1/2 is required on both isoforms in order to phosphorylate both ERK1/2 isoforms. Threonine (ERK1) and Tyrosine (ERK2) residue phosphorylation initiates an activation loop, consistently stimulating the downstream functions of this mechanism. This phosphorylation-dependent loop is the limiting step which accelerates 5-6 times greater than at basal levels (5 mM/min/mg).
Previous studies did not analyze Snail and ERK1/2 activated within the nucleus to determine if there is a mere correlation between the transcriptional control of Snail and phosphorylation activity of the ERK2 isoform in breast cancer cells. Other reports have strongly indicated that active ERK1/2 possesses functionality while within the nucleus or other organelles (i.e., cytoplasm – endosomes, mitochondria, cytoskeletal components like actin and tubulin). Current therapies do not discriminate between both isoforms of ERK and therefore, the dual roles of ERK1 and ERK2 are not considered.

2.8.2 Subcellular localization of ERK1/2

The ERK cascade has several distinct functions which differ depending on its subcellular localization. Several steps tightly controlled by the Ras/MAPK pathway aid in the nuclear import of ERK1/2. One step that occurs post-stimulation is the release of MEK1/2 from adaptor proteins. These proteins function as structural scaffolding which holds MEK1/2 in an inactive conformation. Prior to release of these proteins, MEK1/2 has no catalytic ability on its substrates, therefore impeding direct phosphorylation of ERK1/2.

Whether ERK1/2 translocates into the nucleus, the activation of this kinase can be associated with shifts in other cellular functions due to its localization on the membranes of other organelles (i.e., mitochondria, cytoskeletal components, Golgi apparatus, endosomes). For example, ERK bound to the mitochondrial membrane can directly or indirectly regulate apoptosis, cell survival, and proliferation by activating or inhibiting various pro- or anti-apoptotic factors (i.e., Bcl-2, p21, StAR, PP2A). ERK bound to the cytoskeletal components tubulin or actin can be associated with changes in cellular composition, volume, and potentially promote loss of adhesion and motility. ERK bound
factors that help the cancer cells grow (i.e., Iressa ZD1839), chemotherapy drugs that block the activation of signaling pathways and their effectors.\textsuperscript{110} The lack of ER-\(\alpha\) is also associated with increased signaling pathways like MAPK/ERK and PI3K.

2.8 Mitogen-activated protein kinase/Extracellular-regulated kinase (MAPK/ERK) signaling pathway in breast cancer

Several signaling pathways allow cells to identify and respond appropriately to the extracellular environment.\textsuperscript{14} The receptor tyrosine kinases (RTK) like epidermal growth factor receptor (EGFR), Ras/Raf/MEK/ERK, and Phosphoinositol 3 Kinase/AKT (PI3K/AKT) pathways are all involved in cancer development, progression, and metastasis. Two predominant isoforms of ERK (ERK1 and ERK2) were once thought to have similar kinase activities \textit{in vitro}. However, these kinases have very distinct and separate functions. ERK1 plays a specific role in non-invasive behavior and more epithelial morphology, while ERK2 plays a role in promoting invasion and a phenotype which drives metastasis/EMT in breast cancer cells.\textsuperscript{115} Extracellular stimulation of ERK1/2 has been used to study changes that occur during EMT in tumors.\textsuperscript{116} Rab17 and liprin-\(\beta\)2 are genes that inhibit invasive properties were upregulated following knockdown of ERK2 and returned to normal levels when ERK2 was re-expressed. Re-expression of ERK1 after knockdown had no effect on the expression of Rab17 or liprin-\(\beta\)2. ERK2-directed suppression of possible tumor suppressors or genes which inhibit metastatic functions is a major function of this kinase. MAPK and Extracellular-regulated kinases 1 and 2 (ERK1/2) are significant signaling proteins that control several processes, including: proliferation, survival, motility, adhesion, invasion and survival.\textsuperscript{16} Tracing the steps of MAPK from the external membrane to the nucleus has been the focus of EMT
Although it is well known that several MAPK enzymes regulate cellular functions, determining specific functions during breast cancer development and progression have been more challenging. MAPK functions interact with almost all oncogenes and oncogenic factors. MAPK signaling functions via sequential activation of distinct isoforms that have little or no cross-reactivity, described as MAP3K, MAP2K, MAPK, and MAPKAPK, along with their specific substrates ERK, JNK, and HOG.

2.8.1 MAPK signaling and EMT

Breast tumor progression is associated with the ERK/MSK1/Elk-1/Snail signaling pathway in breast cancer cell lines and in mice. This particular investigation focused on the function of chemokine (C-X-C) ligand 5 (CXCL5) on cellular signaling pathways in breast cancer cell lines (MCF-7, MDA-MB-231) and in mice. Treatment with CXCL5 was associated with activation of Raf/MEK/ERK, MSK1, Elk-1, and Snail, while E-cadherin was downregulated.

The components of the ERK1/2 cascade are the main factors responsible for controlling EMT. These include membrane receptors (G-protein coupled receptors (GPCRs); receptor Tyrosine kinases (RTKs); ion channels), adaptor proteins (i.e., Grb2), exchange factors (i.e., SOS), Ras/Raf kinases, proto-oncogenes (i.e., TPL2), and approximately 200 substrates. The activation of MEK1/2 is required on both isoforms in order to phosphorylate both ERK1/2 isoforms. Threonine (ERK1) and Tyrosine (ERK2) residue phosphorylation initiates an activation loop, consistently stimulating the downstream functions of this mechanism. This phosphorylation-dependent loop is the limiting step which accelerates 5-6 times greater than at basal levels (5 mM/min/mg).
Previous studies did not analyze Snail and ERK1/2 activated within the nucleus to determine if there is a mere correlation between the transcriptional control of Snail and phosphorylation activity of the ERK2 isoform in breast cancer cells. Other reports have strongly indicated that active ERK1/2 possesses functionality while within the nucleus or other organelles (i.e., cytoplasm – endosomes, mitochondria, cytoskeletal components like actin and tubulin). Current therapies do not discriminate between both isoforms of ERK and therefore, the dual roles of ERK1 and ERK2 are not considered.

2.8.2 Subcellular localization of ERK1/2

The ERK cascade has several distinct functions which differ depending on its subcellular localization. Several steps tightly controlled by the Ras/MAPK pathway aid in the nuclear import of ERK1/2. One step that occurs post-stimulation is the release of MEK1/2 from adaptor proteins. These proteins function as structural scaffolding which holds MEK1/2 in an inactive conformation. Prior to release of these proteins, MEK1/2 has no catalytic ability on its substrates, therefore impeding direct phosphorylation of ERK1/2.

Whether ERK1/2 translocates into the nucleus, the activation of this kinase can be associated with shifts in other cellular functions due to its localization on the membranes of other organelles (i.e., mitochondria, cytoskeletal components, Golgi apparatus, endosomes). For example, ERK bound to the mitochondrial membrane can directly or indirectly regulate apoptosis, cell survival, and proliferation by activating or inhibiting various pro- or anti-apoptotic factors (i.e., Bcl-2, p21, StAR, PP2A). ERK bound to the cytoskeletal components tubulin or actin can be associated with changes in cellular composition, volume, and potentially promote loss of adhesion and motility.
to the Golgi may be associated with the packaging of proteins that are activated by ERK stimulation. The tightly regulated phosphorylation steps also relate to nuclear import and export of ERK1/2. Nuclear ERK activity has been associated with malignant mammary tumors and poor prognosis. A lower proportion of phosphorylated ERK1/2 (p-ERK) molecules can be detected in the nucleus of HOSE (<10%) compared to nuclear p-ERK in SKOV3 ovarian carcinoma cells (65%) shortly after extracellular stimulation. These observations suggested that primary an carcinoma cells transform partially based on the nuclear translocalization of activated ERK1/2.

When ERK1/2 has entered the nucleus, the effects are dependent on many substrates, interacting proteins, and interactions that occur directly with DNA. These substrates directly regulate several functions of nuclear activation of Elk-1 and suppression of Erf-1. ERK1/2 also influences chromatin remodeling (i.e., PARP-1 regulation) and nuclear translocation (i.e., nucleoporin50 - NUP50 phosphorylation). Elk-1 is an ERK1/2-activated transcription factor which controls cell proliferation and differentiation by inducing immediate early gene (IEG) c-Fos. ERK1/2 is also involved in the activity of IEGs c-Myc and Fral oncogenes following PDGF stimulation.

When the signal of nuclear ERK1/2 has runs its course and influenced the activation or of partnering transcription factors and other kinases, export out of the nucleus is a necessary activity for several reasons, which are: prolonged nuclear ERK1/2 activation can overload the system with upregulated functions to possibly induce cellular transformation, nuclear ERK1/2 that remains idly within the nucleus can cause global shifts in the signaling mechanisms within the nucleus, and ERK1/2 that remains in the nucleus is able to induce various pathological conditions (i.e., neurogenerative...
diseases, developmental diseases, diabetes, and cancer.

Coupling of ERK1/2 to MEK1/2 in the nucleus stimulates its nuclear export via exportins. Determination of ERK1/2 activation in the cytoplasm is via the expression and/or activation of its cytoplasmic targets. Ribosomal s6 kinases (i.e., RSK1) is a kinase with four protein components (RSK1, RSK2, RSK3, RSK4) and is a direct downstream effector.

2.8.3 Targeting breast cancer by ERK1/2 inhibition

There are several studies whose focus is to develop better ERK1/2 antagonists and a more clear understanding of ERK1/2 signaling in order to target cancer. There are various ways to antagonize the signaling pathways that control cellular functions downstream of ERK1/2 activation. Since the ERK pathway is involved in increasing proliferation and motility, it is also important to note that MAPK/ERK promotes breast cancer resistance to tyrosine kinase inhibitor gefitinib. Gefitinib works by inhibiting the activity of the epidermal growth factor receptor (EGFR), but triple negative (basal-like) MDA-MB-361 and MDA-MB-468 cells indicated intermediate and low sensitivity to gefitinib, respectively. MAPK pathway inhibition has proven to be a promising method of decreasing tumor growth by directly hindering cell survival. However, the use of oral MEK inhibitor CI-1040 (800 mg) to treat non-small-cell lung, breast, colon, and pancreatic cancer has been unsuccessful. Investigators reported a dramatic increase in PI3K/Akt activity after administration of CI-1040, indicating a shift in this pathway and potential tumor resistance. Further studies deem hopeful, especially with a second generation MEK inhibitor, PD 0325901. More importantly, MAPK pathway inhibition has focused on general MAPK inhibition without dissecting out the
role of nuclear vs cytoplasmic ERK. Dual-specificity phosphatases (DUSPs) are also being utilized to inhibit the activity of MAPkinases. These phosphatases can function in a direct or indirect approach to dephosphorylate MAPK. MAPK phosphatases and DUSPs (MKP-DUSPs) remove phosphates at either Thr or Tyr residues of the MAPK catalytic loop, to directly inactivate MAPK. MKP-DUSPs can dephosphorylate kinases upstream of MAPK.

Another method of inhibiting the activity and nuclear import of ERK1/2 is to inhibit its upstream activator, MEK1/2. MEK1/2 phosphorylates ERK1/2 in the Thr/Tyr pattern which induces a conformational change. At this point, ERK1/2 is in an active conformation, which permits its binding to partnering importins to be imported into the nucleus. UO126 (1,4-diamino-2,30dicyano-1,4-bis[2-aminophenylthio] butadiene has a high affinity for MEK1 and MEK2, with a higher potency than PD98059, which binds to MEK1 more effectively than MEK2. UO126 inhibits phosphorylation activities on Ser217 and Ser221 of MEK1/2, which inhibits its ability to phosphorylate MAPK/ERK1/2 at the Thr202 and Tyr204 binding sites.

Inhibition of both ERK1 and ERK2 using UO126 (20 μM for 24 hours) greatly decreased drug resistant tumors. This effect after treatment was indicated by a decrease in the expression of drug resistance genes (Bcl-2, ABCB1, ABCC3) and pro-survival genes [DNA repair – BRCA1, BRCA2; hormone receptor – androgen receptor (AR), estrogen receptor-β (ER-β), PPAR-γ; drug metabolism – CYP3A4]. A study observed that inhibition of ERK1/2 with UO126 could decrease the expression of nuclear-localized Snail and cell migration in MCF-7 Snail breast cancer cells in vitro and in vivo. These
findings may be a way to determine the role of Snail and nuclear-localized ERK1/2 in breast cancer.

2.9 Snail and MAPK signaling

There is a very clear connection between the ERK1/2 signaling pathway and Snail functions in several pathologies. Snail has been shown to regulate tumor associated functions through the MAPK pathway.\(^3\,6\,8\) Snail can increase migration, decrease adhesion, and has been shown to collaborate with other regulators such as growth factors, receptors and respective ligands, and kinases.\(^7\,16\) Hepatocyte growth factor (HGF) induces hepatocyte cell scattering and potentiates cells for migration through MAPK/Egr-1-mediated increases of Snail, while cross-talk between Wnt and fibroblast growth factor (FGF) pathways converge at GSK-3\(\beta\) regulate \(\beta\)-catenin through Snail in mammary carcinomas.\(^13\,5\) Development of a more effective method to treat tumors with Snail- and nuclear MAPK-mediated functions may have promising results future applications to inhibit metastasis.
CHAPTER 3
MATERIALS AND METHODS

3.1 Snail and active nuclear ERK1/2 study

3.1.1 Cell culture, antibodies, and reagents

The human breast cancer cells lines, T47-D, MCF-7, and MDA-MB-231 were obtained from ATCC, Manassas, VA. The MCF-7 cells stably transfected with empty Neo vector (MCF-7 Neo) and or constitutively active Snail (MCF-7 Snail) was established as described previously. Cells were grown in RPMI medium supplemented with 10% fetal bovine serum and 1% P/S (complete RPMI), at 37°C with 5% CO₂ in a humidified incubator. Cells cultured to 60-70% confluence were used for subsequent analyses. RPMI medium and penicillin/streptomycin (P/S) were purchased from VWR Int., West Chester, PA. Fetal bovine serum (FBS) and Charcoal/dextran treated FBS (DCC-FBS) were from Hyclone, South Logan, UT. Anti-human β-actin antibody was from Sigma-Aldrich, Inc., St Louis, MO. Rat monoclonal anti-human Snail antibody, HRP-conjugated goat anti-rat antibody, rabbit monoclonal phosphorylated-ERK antibody, rabbit polyclonal ERK antibody, rabbit polyclonal phospho-p90RSK (9344P), rabbit polyclonal phospho-Elk-1 (9181S), rabbit polyclonal Elk-1 (9182S), and rabbit monoclonal GAPDH (5174S) were from Cell Signaling Technology, Inc., Danvers, MA. HRP-conjugated sheep anti-mouse, sheep anti-rabbit and the Enhanced chemiluminescence prime (ECL prime) detection reagent were purchased from
Amersham Biosciences, Buckinghamshire, UK. Rabbit polyclonal anti-Rsk-1 (SC231), anti-Topoisomerase1 (SC-271285), and anti-β-tubulin (SC-55529) were from Santa Cruz. Anti-human mouse monoclonal E-cadherin (610182) was from BD Biosciences, San Jose, CA. NE-PER Nuclear and Cytoplasmic Extraction Kit (78835) was from Thermo Scientific. Luminata Forte HRP chemiluminescence detection reagent was purchased from EMD Millipore (Billerica, MA). The protease inhibitor cocktail was from Roche Molecular Biochemicals, Indianapolis, IN. UO126 (MEK1/2 inhibitor) and MG132 were purchased from EMD Calbiochem (Billerica, MA).

3.1.2 Animal experiments

All of the animal procedures were approved and performed in accordance with Emory University Institutional IACUC guidelines. Four-week-old female athymic nu/nu mice (National Cancer Institute) were implanted subcutaneously with 17β-estradiol-sustained release pellets and subsequently injected subcutaneously with 2 x 10^6 cells per mouse of Neo or Snail-overexpressing MCF-7 cells mixed 1:1 volume with matrigel (BD Biosciences). The mice were sacrificed after 2 weeks, the tumors excised and tumor volume measured with a caliper (tumor volume was calculated as 3.14 / 6 x largest diameter x smallest diameter squared). The tumors were used for histology studies and immunohistochemistry.

3.1.3 Short interfering RNA transfection (siRNA)

Transient transfections were performed with 200 nM of Snail ON-TARGETplus siRNA (Thermo Scientific - Dharmacon, Lafayette, CO), per the manufacturer’s instructions. T47-D and MDA-MB-231 cells were seeded overnight in 6-well dishes then incubated with ON-TARGETplus complexes in phenol-free RPMI without FBS or
antibiotics for 5 hours; subsequently the media was replaced with 5% DCC phenol-free RPMI for an additional 72 hours. Whole cell lysates were harvested for respective experiments.

### 3.1.4 Western blot analysis

Confluent cells were lysed in a modified RIPA buffer (50 mMTris pH 8.0, 150 mMNaCl, 0.02% NaN₃, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) containing 1.5X protease inhibitor cocktail, 1 mMphenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate. Whole cell lysates were frozen-thawed at -80°C/4°C for three cycles, then centrifuged at 13,500 rpm for 30 min at 4°C. Supernatants were collected and quantified using a micro BCA assay (Promega, Madison, WI). 30 or 50 µg of cell lysate was resolved using 10% SDS PAGE, followed by transblotting onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 5% milk (TBS with 0.05 % Tween-20, 0.05 % BSA containing 5% milk) or 3% milk (TBS-T containing 3% milk), then washed and incubated with primary antibody dilution buffer. After washing, the membranes were incubated in peroxidase-conjugated sheep anti-mouse, sheep anti-rabbit, or goat anti-rat IgG, washed, and visualized using ECL prime reagent (GE Healthcare, Buckinghamshire, UK) or Luminata Forte ECL reagent (Millipore, Billerica, MA). The membranes were stripped using Restore Western blot stripping buffer (Pierce Biotechnology, Inc., Rockford, IL) prior to re-probing with a different antibody.

### 3.1.5 Immunohistochemistry (IHC)

Paraffin-embedded tumor tissues were sliced into 5µm thick sections and mounted on glass. The slides were deparaffinized and rehydrated through a down-graded
alcohol series to deionized water followed by antigen retrieval with Reveal Decloaker RTU antigen unmasking solution (Biocare Medical, Concord, CA). The slides were incubated with Snail primary antibody overnight at 4°C, washed with TBS-T 3x5 min, 1x PBS 3x5 min, and incubated with HRP-conjugated secondary antibody for 30 min at room temp. Slides were washed with TBS-T 3x5 min, 1x PBS 3x5 min, then incubated with an avidin+biotin solution from (Biocare Medical, Concord, CA). Slides were gently and briefly washed with 1x PBS two times; the first wash was for 5 min in a humidified chamber and the second wash was for 15 min with low agitation. Slides were incubated with 3,3-Diaminobenzidine (DAB) for no more than 2 min at room temp. Slides were washed with deionized water 3x5 min, dipped in hematoxylin/eosin for 3 min, rinsed in tap water for 5 min, and dehydrated in an up-graded alcohol series. Slides were dipped in three different containers of xylene for 5 min each. Excess xylene was removed gently and slides were mounted using a xylene-based mounting media. Slides were left to dry overnight at room temp. When completely dry, images of slides were taken using Zeiss Axiovision microscope and camera using AxiovisionRel 4.8 software.

3.1.6 Immunocytochemistry (ICC)

When cells reached 60-80% confluence, 2x10³-5x10³ cells were plated into 16 well chamber slides (Bio-Tek, Nunc, Winooski, VT). For treatments cells were treated with DMSO (control) or MEK inhibitor (UO126). Fixation was performed with methanol/ethanol 1:1 volume for 5 min at room temp followed by 3x5 min washes with 1X PBS, blocking with protein blocking solution without serum (Dako, Camarillo, CA) for 10 min at room temp. Slides were incubated with primary antibody at 1:50 or 1:100 dilutions in Dako antibody diluent solution for 1 hour at room temp. Primary antibodies
include those listed in section 3.1.2, but rabbit polyclonal nucleoporin98 (NUP98) primary antibody was also used (Cat 2598, Cell Signaling, Danvers, MA). Negative control slides were incubated with antibody diluent solution alone instead of primary antibodies. Slides were washed 3x5 min with 1X TBS-T (Dako, Camarillo, CA), then incubated with secondary antibody of Oregon green (rabbit) or Alexa red (mouse) both from Invitrogen in the dark for 1 hour at room temp. Slides were washed 3x5 min with 1X TBS-T and briefly dipped into double deionized water, incubated with DAPI (1μg/ml) for 5 min at room temp in the dark. Slides were washed 3x5 min with double deionized water, then mounted using Fluorogel mounting medium (Electron Microscopy Sciences, Hatfield, PA). Slides were left to dry overnight in the dark then fluorescence microscopy was performed using Axiovision Rel 4.8 software.

3.1.7 In vitro cell migration assay

We utilized Costar 24-well plates containing a polycarbonate filter insert with an 8-μm pore size, to coat with 3.67 μg/μl rat tail collagen V (Cgn) or 2.5 μg/cm² human fibronectin (FN) on the outside for 24 h at 4°C. 5x10⁴ cells were plated in the upper chamber containing RPMI supplemented with 0.1% fetal bovine serum (FBS) while the lower chamber contained complete RPMI. After 5 h, cells that migrated to the bottom of the insert were fixed, stained with 0.05% crystal violet, and either counted to obtain the relative migration or the stain solubilized with Sorenson solution. Optical density (OD) was measured at 490 nm to obtain relative cell migration.

3.1.8 In vitro cell adhesion assay

96 well plates were coated with 3.67 μg/μl of rat-tail collagen I or 2.5 μg/cm² of fibronectin overnight at 4°C. Wells were rinsed with 1X PBS the following day,
preheated to 37°C, for surface neutralization. Remaining binding sites were blocked with 0.1% bovine serum albumin (BSA) in PBS for a period of 1 hr. 3\times10^4 cells were in complete RPMI were added to the plates. After incubation for 20 or 60 minutes, cells were treated with Percoll flotation medium and Percoll fixative for 15 minutes at room temperature, washed with 1X PBS and stained with 0.05% crystal violet, washed again and allowed to dry overnight. A Gen5 automated plate reader was used to quantitate cell attachment on the next day, once each well was solubilized with Sorenson solution, and OD read at 590 nm.

3.1.9 Subcellular fractionation

MCF-7 Neo and MCF-7 Snail were cultured in complete RPMI-1640 containing 10% FBS and 1% P/S until cells reached 80-90% confluence. Subcellular fractionations were performed per the manufacturer’s instructions (Thermo Scientific). Briefly, cells were lysed in a series of buffers containing protease inhibitors (25X) with CERI (100 μl), CERII (5.5 μl), or NER (50μl). Centrifugation steps were performed to obtain a non-nuclear fraction and an intact nuclear pellet, followed by further lysing to isolate the nuclear fraction. Fractionated lysates were stored at -80°C until needed for experiments. 50μg of non-nuclear and nuclear fractions were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. Rat anti-Snail, rabbit anti-p-ERK, rabbit anti-ERK, rabbit anti-p90RSK and rabbit anti-p90RSK, were detected using Luminata forte chemiluminescent substrate. Mouse anti-topoisomerase I (Santa Cruz; 1:5000) and mouse anti-GAPDH (Cell Signaling; 1:1000) antibodies were used to ensure the integrity of nuclear and non-nuclear fractions and as loading controls. LAS-3000 (Fujifilm) imaging software was used to detect expression of the proteins.
3.1.10 Statistical analysis Where appropriate, data were analyzed by a two-tailed student’s T-test or ANOVA (1-way or 2-way) using GraphPad Prism software. P-values less than 0.05 were considered statistically significant.

3.2 Snail and hydroxytamoxifen resistance study

3.2.1 Cell culture, antibodies, and reagents

The MCF-7 cells stably transfected with empty Neo vector (MCF-7 Neo) and or constitutively active Snail (MCF-7 Snail) was established as described previously. Cells were grown in RPMI medium supplemented with 10% fetal bovine serum and 1% P/S (complete RPMI), at 37°C with 5% CO2 in a humidified incubator. Cells cultured to 60-70% confluence were used for subsequent analyses. RPMI medium and penicillin/streptomycin (P/S) were purchased from VWR Int., West Chester, PA. Fetal bovine serum (FBS) and Charcoal/dextran treated FBS (DCC-FBS) were from Hyclone, South Logan, UT. Anti-human β-actin antibody was from Sigma-Aldrich, Inc., St Louis, MO. Mouse monoclonal anti-estrogen receptor alpha antibody was from Santa Cruz biotechnology (Santa Cruz, CA). Luminata Forte HRP chemiluminescence detection reagent was purchased from EMD Millipore (Billerica, MA). The protease inhibitor cocktail was from Roche Molecular Biochemicals, Indianapolis, IN. UO126 (MEK1/2 inhibitor) and 4-hydroxytamoxifen (4-OHT) were from EMD Calbiochem (Billerica, MA).

3.2.2 Animal experiments

All of the animal procedures were approved and performed in accordance with Emory University Institutional IACUC guidelines. Four-week-old female athymic nu/nu mice (National Cancer Institute) were implanted subcutaneously with 17β-estradiol-
sustained release pellets and subsequently injected subcutaneously with 2 x 10^6 cells per mouse of Neo or Snail-overexpressing MCF-7 cells mixed 1:1 volume with matrigel (BD Biosciences). The mice were sacrificed after 2 weeks, the tumors excised and tumor volume measured with a caliper (tumor volume was calculated as \( \frac{3.14}{6} \times \text{largest diameter} \times \text{smallest diameter squared} \)). The tumors were used for histology studies and immunohistochemistry.

3.2.3 Western blot analysis

Confluent cells were lysed in a modified RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.02% NaN₃, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) containing 1.5X protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate. Whole cell lysates were frozen-thawed at -80°C/4°C for three cycles, then centrifuged at 13,500 rpm for 30 min at 4°C. Supernatants were collected and quantified using a micro BCA assay (Promega, Madison, WI). 30 or 50 µg of cell lysate was resolved using 10% SDS PAGE, followed by transblotting onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 5% milk (TBS with 0.05% Tween-20, 0.05% BSA containing 5% milk) or 3% milk (TBS-T containing 3% milk), then washed and incubated with primary antibody dilution buffer. After washing, the membranes were incubated in peroxidase-conjugated sheep anti-mouse, sheep anti-rabbit, or goat anti-rat IgG, washed, and visualized using ECL prime reagent (GE Healthcare, Buckinghamshire, UK) or Luminata Forte ECL reagent (Millipore, Billerica, MA). The membranes were stripped using Restore Western blot stripping buffer (Pierce Biotechnology, Inc., Rockford, IL) prior to re-probing with a different antibody.
3.2.4 Immunohistochemistry (IHC)

Paraffin-embedded tumor tissues were sliced into 5μm thick sections and mounted on glass. The slides were deparaffinized and rehydrated through a down-graded alcohol series to deionized water followed by antigen retrieval with Reveal Decloaker RTU antigen unmasking solution (Biocare Medical, Concord, CA). The slides were incubated with ER-α primary antibody overnight at 4°C, washed with TBS-T 3x5 min, 1x PBS 3x5 min, and incubated with HRP-conjugated secondary antibody for 30 min at room temp. Slides were washed with TBS-T 3x5 min, 1x PBS 3x5 min, then incubated with an avidin+biotin solution from (Biocare Medical, Concord, CA). Slides were gently and briefly washed with 1x PBS two times; the first wash was for 5 min in a humidified chamber and the second wash was for 15 min with low agitation. Slides were incubated with 3,3-Diaminobenzidine (DAB) for no more than 2 min at room temp. Slides were washed with deionized water 3x5 min, dipped in hematoxylin/eosin for 3 min, rinsed in tap water for 5 min, and dehydrated in an up-graded alcohol series. Slides were dipped in three different containers of xylene for 5 min each. Excess xylene was removed gently and slides were mounted using a xylene-based mounting media. Slides were left to dry overnight at room temp. When completely dry, images of slides were taken using Zeiss Axiovision microscope and camera using Axiovision Rel 4.8 software.

3.2.5 Caspase-Glo® 3/7 assay

Equilibrate the buffer and substrate to room temperature before using, then transfer the buffer contents into the amber bottle containing the substrate. Mix by gently inverting the contents to dissolve the substrate. Cells were culture in T-75 flasks until confluent, then 1x10^4 cells were plated in white-walled 96-well plates then allowed to
attach overnight at 37°C with 5% CO₂. Cells were treated with single and combination doses of UO126 and 4-OHT at 1μM and/or 10μM and incubated for 24 hours. Cells were removed from the incubator an equilibrated to room temperature. 100μl of reagent was added to each well and plates were covered with a lid. Plates were gently mixed with a plate shaker at 300 rpm for 30 seconds, incubated at room temperature for 3 hours. Luminescence was measured using a luminometer. Wells were left blank to designate blank reaction, negative control wells were used which contained the reagent, and assay wells contained cells and reagent.

3.2.6 TMRM mitochondrial permeability assay

Cells were cultured up to 1x10⁶ cells per mL of culture media. Cells were either left untreated, treated with CCCP (carbonylcyanide m-chlorophenylhydrazone) as a positive control, or treated with single and combination doses of UO126 and 4-OHT at 1μM and/or 10μM and incubated for 24 hours. Cells were incubated with 10μM of TMRM (tetramethylrhodamine methyl ester) for 20 min at 37°C in the darkness. Cells were washed 1x with wash buffer and analyzed using fluorescence microscopy and by flow cytometry to quantitate results.

3.2.7 Statistical Analysis

Where appropriate, data were analyzed by a two-tailed student’s T-test or ANOVA (1-way or 2-way) using GraphPad Prism software. P values less than 0.05 were considered statistically significant.
CHAPTER 4
RESULTS

4.1 Snail and active nuclear ERK1/2 study

4.1.1 Snail is associated with increased ERK activity in breast cancer cells

We examined the expression of Snail transcription factor in a panel of normal breast epithelial cells and breast cancer cells lines of increasing aggressiveness, by Western blot analysis. The normal breast epithelial cells (HMEPiC) and MCF-7 breast cancer cell line did not express detectable levels of Snail, while Snail was detectable in breast cancer cell lines T47-D and MDA-MB-231 (Figure 5A). ERK1/2 activity (p-ERK) was also increased in breast cancer cell lines as compared to normal breast epithelial cells (Figure 5A). This demonstrates that Snail expression positively correlates with ERK1/2 activity.

To determine the effects of Snail knockdown on ERK1/2 activity in T47-D and MDA-MB-231 breast cancer cells, we transiently transfected these cells with control siRNA or Snail siRNA and analyzed the expression of Snail, p-ERK, total ERK by Western blot and immunofluorescence analyses. Snail knockdown decreased p-ERK in MDA-MB-231 and T47-D (Figure 5B, C). Interestingly, there was also a marked re-localization of p-ERK from the nucleus to the cytoplasm upon Snail knockdown in T47-D cells as seen by immunofluorescence (Figure 5C). Additionally, Snail knockdown increased levels of E-cadherin in T47-D and decreased levels of vimentin in MDA-MB-231 cells, suggesting a
partial reversion of EMT. These data demonstrate that Snail and ERK activity increase with breast cancer progression and that Snail may regulate ERK activity in breast cancer cells.

4.1.2 Snail increases EMT in vitro and tumorigenicity in vivo

Since hormone-dependent MCF-7 cells expressed barely detectable levels of Snail, we utilized MCF-7 cells that have been transfected with either the Neo empty vector or constitutively active Snail cDNA for in vitro and in vivo studies. The MCF-7 Neo/MCF-7 Snail cell model has been used as an EMT progression model for breast cancer.95 We confirmed that MCF-7 Neo maintain a more epithelial and cuboidal morphology which was completely transformed to a more mesenchymal and fibroblast-like morphology in MCF-7 Snail (Figure 6A). We also confirmed by Western blot analysis that this model represents an EMT model by analyzing the expression of EMT markers; MCF-7 Snail demonstrated higher levels of Snail and vimentin and lower levels of E-cadherin as compared to MCF-7 Neo (Figure 6B). We also analyzed the effects of Snail overexpression on cell adhesion and migration using rat tail collagen V and human fibronectin matrices, and found that MCF-7 Snail displayed decreased cell adhesion and increased cell migration on both matrices (Figure 6C, 6D). To determine the effects of Snail overexpression in vivo, we injected MCF-7 Neo and MCF-7 Snail subcutaneously into female nude mice. Significantly larger tumor volumes were observed in MCF-7 Snail tumor xenografts as compared to MCF-7 Neo after 2 weeks (Figure 6E). H&E staining was performed as well as immunohistochemistry to demonstrate higher expression of Snail in MCF-7 Snail tumor xenograft tissues as compared to MCF-7 Neo (Figure 7A). Hematoxylin and eosin (H&E) staining was performed to demonstrate tumor
histology (Figure 7B). Therefore, Snail increases EMT and tumorigenicity in MCF-7 breast cancer cells.

4.1.3 Snail promotes nuclear translocation of p-ERK

Next, we examined ERK1/2 activity upon Snail overexpression and observed surprisingly that MCF-7 Snail had decreased active ERK1/2 as compared to MCF-7 Neo control cells as shown by immunofluorescence and Western blot analyses (Figure 8A, B). Interestingly, though Snail decreased phospho-ERK1/2 levels as shown in Figure 8A, B, it was exclusively localized within the nucleus of MCF-7 Snail (Figure 8A). We further examined ERK1/2 localization utilizing Western blot analysis of nuclear and cytoplasmic fractions. We observed that Snail overexpression led to ERK1/2 activity primarily within the nuclear fraction as compared to the cytoplasmic fraction (Figure 8C). However, in MCF-7 Neo, although there was ERK activity in both the nuclear and cytoplasmic fractions, it was interesting to note that the ERK1 isoform activity was predominantly present within the nucleus, while the ERK2 isoform activity was excluded from the nucleus (Figure 8C). Furthermore, we noted that in MCF-7 parental cells, phospho-ERK staining was similar to MCF-7 Neo cells and co-localized with nuclear import protein nucleoporin98 (NUP98), a nuclear membrane marker, suggesting that the predominant nuclear expression of phospho-ERK within these cells may reside within the nuclear membrane (Figure 9). To determine whether Snail-mediated nuclear translocation of phospho-ERK1/2 may be associated with downstream effectors of phospho-ERK1/2, we reviewed literature about MAPK and its effectors within the subcellular compartments and found that p90 ribosomal s6 kinase (p90RSK) is a cytoplasmic substrate of p-ERK, while Elk-1 transcription factor is a nuclear substrate of p-ERK.\textsuperscript{5b} p90RSK and Elk-1 are
involved in tumor progression and biochemical changes in chromatin structure and indirectly increasing cell proliferation through c-Fos activation, respectively. We analyzed the protein expression of phosphorylated and total forms of p90RSK and Elk-1 in MCF-7 Neo and MCF-7 Snail. We found that the phospho-p90RSK was expressed predominantly within the cytoplasmic fractions of both MCF-7 Neo and MCF-7 Snail; however, phospho-Elk-1 was highly expressed predominantly in the nuclear compartment and at significantly higher levels in MCF-7 Snail as compared to MCF-7 Neo (Figure 8C). Further evidence that Snail may promote activation of ERK1/2 which subsequently activates Elk-1 was supported by the fact that Snail knockdown in MDA-MB-231 cells led to translocation of phospho-Elk-1 from the nucleus into the cytosol (Figure 10).

4.1.4 Inhibition of ERK activity decreases Snail and partially reverts EMT independent of proteasomal degradation

MAPK/ERK signaling is involved in many types of cancer and ERK1/2 is closely involved during cancer development, progression and metastasis. ERK1/2 has been shown to regulate cell migration. We sought to determine if Snail-mediated EMT may be regulated via ERK1/2 activity in our model of breast cancer. To accomplish this task, we inhibited ERK1/2 activity using MEK inhibitor UO126 (20 μM) for 30 min, 2 h, 6 h, and 24 h. MCF-7 Neo and MCF-7 Snail cells treated with UO126 led to phospho-ERK inhibition within 30 min up to 24 h (Figure 11A). Interestingly, Snail expression was decreased in MCF-7 Snail within 24 h after phospho-ERK inhibition (Figure 11A). Also noted was the dramatic changes in MCF-7 Snail morphology after ERK activity inhibition; the cells began to become more epithelial and clumped after 24 h (Figure 11B)
and were still viable as shown by DAPI staining (Figure 11C). We also analyzed effects of inhibition of ERK activity on E-cadherin protein expression. We observed that U0126 treatment of MCF-7 Neo cells altered morphology and led to spindle-shaped cells with extensions (Figure 11C, arrows), which was accompanied by decreased expression of E-cadherin (Figure 11D). Conversely, expression of E-cadherin was increased after U0126 treatment of MCF-7 Snail cells (Figure 11D). To determine whether ERK inhibition was inducing proteasomal-mediated degradation of Snail, we pre-treated MCF-7 Neo and MCF-7 Snail with MG132 proteasomal degradation inhibitor for 2 h prior to treatment with U0126. We observed that inhibition of Snail expression following U0126 treatment was not restored by MG132 (Figure 11E), suggesting that U0126 does not decrease Snail protein expression via the proteasomal pathway. These data suggest that although Snail can regulate p-ERK, there may also be a feedback loop by which ERK activity can also positively regulate Snail expression. Furthermore, it appears that ERK inhibition in MCF-7 Snail cells partially reverted EMT while surprisingly ERK inhibition in MCF-7 Neo cells partially induces EMT.

4.1.5 Differential role of ERK1 and ERK2 isoforms

Since we had observed that MCF-7 Neo expressed a higher level of active ERK1 isoform in both the cytoplasm and nuclear fraction, whereas MCF-7 Snail expressed a higher level of active ERK2 isoform within the nuclear fraction, we dissected the roles of the different isoforms further. We used siRNA against ERK1 or ERK2 to determine the role of the different isoforms. ERK1 and ERK2 siRNA decreased E-cadherin in MCF-7 Neo, but did not induce Snail expression (Figure 12). Conversely, in MCF-7 Snail cells, knockdown of ERK2 but not ERK1 led to decreased Snail expression in MCF-7 Snail
cells, however, there was no detectable induction of E-cadherin expression (Figure 12). Therefore, MAPK regulation of Snail may be specific to ERK2 isoform.

**4.1.6 Differential functional roles of ERK activity in MCF-7 Neo vs MCF-7 Snail**

Since MCF-7 Neo displays predominantly ERK1 activity with no activation of Elk-1 in the nucleus while MCF-7 Snail displays ERK1/2 activity with Elk-1 activation within the nucleus, we examined whether ERK activity may have differential roles in MCF-7 Neo vs MCF-7 Snail cells. We treated MCF-7 Neo and MCF-7 Snail cells with UO126 for 24 h and subsequently conducted migration and adhesion assays on rat tail collagen V or human fibronectin. Antagonizing ERK activity did not significantly affect MCF-7 Neo cell migratory potential, but significantly decreased its adhesive potential (Figure 13A, B). However, in MCF-7 Snail cells, U0126 significantly decreased cell migration and increased cell adhesion (Figure 13A, B). These findings indicated that cells with lower Snail (MCF-7 Neo) may utilize active ERK for increasing cell adhesion to extracellular matrix and those with higher Snail (MCF-7 Snail) may utilize active ERK within the nucleus for increasing cell migration but decreasing cell adhesion to extracellular matrix.

**4.2 Snail and hydroxytamoxifen resistance study**

**4.2.1 Snail decreases estrogen receptor-α expression in vitro and in vivo**

We used Western blot analysis to determine the expression of ER-α in MCF-7 Neo and MCF-7 Snail (Figure 14A). We injected MCF-7 Neo and MCF-7 Snail subcutaneously into female nude mice. H&E staining was performed as well as immunohistochemistry to demonstrate higher expression of ER-α in MCF-7 Neo tumor tissues as compared to MCF-7 Snail (Figure 14B).
4.2.2 Hydroxytamoxifen therapy does not affect MCF-7 Snail morphology or Snail expression

Since MCF-7 Snail repressed ER-α protein and loss of ER-α is associated with resistance to chemotherapy, we next examined whether Snail overexpression is associated with resistance to therapy. MCF-7 Neo and MCF-7 Snail were treated with 5% DCC or ethanol, 17-β-estradiol, 4-OHT, 17-β-estradiol plus 4-OHT. Cell morphology of MCF-7 Neo and MCF-7 Snail treated with 17-β-estradiol, 4-OHT, 17-β-estradiol plus 4-OHT was also analyzed (Figure 15A). MCF-7 Neo and MCF-7 Snail treated with 17-β-estradiol appeared similar to the control groups treated with 5% DCC. Cells were round, tightly packed and appeared to possess more adherent ability than MCF-7 Snail. MCF-7 Neo became more sickly-looking after being treated with 4-OHT and several of the cells were floating in the medium after treatment (indicated by arrows). Many of the cells contained blebs and rough outer membranes following treatment with 4-OHT. MCF-7 Snail treated with 4-OHT appeared similar to those treated with 5% DCC and 17-β-estradiol. These cells remained mesenchymal with fibroblastic extensions and loosely packed colonies, with a few cells that may have died (indicated by arrows). The outer membranes of MCF-7 Snail were mostly smooth following 4-OHT treatment. Protein expression of Snail and ER-α was determined using Western blot analysis (Figure 15A, B). The expression of Snail was not detected in MCF-7 Neo with or without treatment, and was not affected in MCF-7 Snail. Expression of ER-α was highly expressed in MCF-7 Neo, and increased with 4-OHT treatment (Figure 15C). Expression of ER-α was slightly expressed in MCF-7 Snail in complete RPMI, increased after ethanol and 4-OHT
treatment, but undetected after 17-β-estradiol or 17-β-estradiol and 4-OHT treatments (Figure 15C).

### 4.2.3 Sustained UO126 and 4-OHT combination treatments effectively reduce MCF-7 Snail viability

The effects of the treatments on cell proliferation and cytotoxicity were analyzed using resazurin dye assay after 48 hour treatments with UO126 and 4-OHT in single and combination doses (control viability was set at 100%). UO126, 4-OHT (10μM), and UO126 (10μM) plus 4-OHT (10μM) significantly decreased viability in MCF-7 Neo and MCF-7 Snail (Figure 16A). 4-OHT (1μM) did not affect viability in either cell line. The higher the dose, the more dramatic the decrease in cell viability as indicated using 4-OHT at 10μM, UO126 10μM plus 4-OHT 1μM, and UO126 10μM plus 4-OHT 10μM. 4-OHT (10μM) surprisingly had an effect on MCF-7 Snail by decreasing viability that was insignificant compared to the 10μM dose combined with UO126 at 10μM. The number of dead cells following treatments were analyzed (Figure 16B). MCF-7 Snail had a higher number of dead cells as compared to MCF-7 Neo, except when treated with UO126 (10μM) plus 4-OHT (1μM). UO126 (10μM) plus 4-OHT (1μM) resulted in the lowest number of dead cells compared to the other treatments.

### 4.2.4 Snail is associated with reduced cell death following 4-OHT therapy

We analyzed the changes in mitochondrial permeability using a Tetramethylrhodamine, methyl ester (TMRM) assay. The lower the staining indicates less the mitochondrial integrity and higher the cell apoptosis. We found that MCF-7 Neo staining (red) was generally dimmer compared to MCF-7 Snail, suggesting that Snail expressing cells have more membrane integrity and possibly less apoptosis as compared
to Neo control (Figure 17A,B). We also observed that UO126 or 4-OHT treatment decreased staining in MCF-7 Neo as compared to untreated control and comparable to positive control suggesting that the 10µM led to cell apoptosis. This was not seen with MCF-7 Snail suggesting that they are more resistant to UO126. However, co-treatment with both UO126 and 4-OHT now displayed dimmer staining, suggesting that UO126 sensitizes MCF-7 Snail cells to tamoxifen therapy. We also used flow cytometry to quantitate the effects of the treatments on cell death which confirmed the qualitative staining (Figure 17C). Flow cytometry data corresponded with fluorescence data because MCF-7 Neo seemed more sensitive to the treatments used, especially when treated with UO126 plus 4-OHT. There were several peaks for all treatments in MCF-7 Neo and a high reduction in mitochondrial permeability (indicated by green peak at ~35%) with combination treatment. However, MCF-7 Snail did not appear to be as responsive to the treatments and we noticed a smaller peak with combination treatment (~15%).
CHAPTER 5
DISCUSSION

Snail and active nuclear ERK1/2 study

Our research focused on physiological functions that Snail zinc finger transcriptional repressor utilizes to promote breast cancer progression. Snail regulates epithelial-mesenchymal transition (EMT), which involves a loss of epithelial markers like E-cadherin and an increase in mesenchymal markers like vimentin. Snail transcriptionally represses genes by binding to the enhancer sequence (E-box). Such genes include: E-cadherin, occludin, claudins, and mucin-1. Master regulatory pathways like MAPK and PI3K function to activate or inhibit cellular functions like cell motility, adhesion, invasion, and survival. Snail also mediates EMT in breast cancer by repressing estrogen receptor-activity at the promoter level, similar to how it regulates E-cadherin adhesion marker. Snail is a prognostic factor in clinical analyses of human bladder cancer and an early biomarker for malignancy, gastric cancer and non-small cell lung cancer. This communication studied the relationship between Snail and phosphorylated ERK, to discover a new way that ERK1/2 may be altered during breast cancer progression.

ERK1/2 is involved in several functions of breast cancer progression. ERK1/2 can regulate cell proliferation, survival, motility, and differentiation by indirectly shifting normal mammary epithelial cells into a more mesenchymal, less adherent state.
tissue microarray-based immunohistochemical study was conducted on 479 invasive breast carcinomas and 12 carcinosarcomas. The status of 28 different biomarkers was assessed and the findings indicated an up-regulation of vimentin, smooth-muscle-actin, and cadherin-11. There was also an overexpression of proteins that remodel the extracellular matrix (ECM) to promote invasion (SPARC, laminin, and fascin), with an overall reduction of epithelial markers (E-cadherin and cytokeratins). We sought to uncover Snail transcriptional repressor as a major contributor to this program with regards to ERK signaling. Subcellular localization of ERK1/2 and its ability to undergo nucleocytoplasmic translocation within the cell has been the focus of several investigations. Although ERK1/2 is found abundantly throughout the cell in many organelles and cell structures, entry of this protein into the nucleus is highly selective. Nuclear entry of MAPK does not occur in primary ovarian and mammary epithelial cells. Import assays were performed in vitro and primary cells showed lower import activity for ERK1/2 than cancer cells. Non-transformed immortalized ovarian and breast epithelial cells did not possess the import machinery required for active ERK1/2 to enter the nucleus. These findings indicate that targeting nuclear MAPK may be an appropriate method to diagnose and/or treat cancer. Cell lines used for in vitro investigations have shown that activation of the Ras/MAPK pathway regulates activate MAPK nuclear translocation. Nuclear MAPK controls cell proliferation by targeting Elk-1 which induces c-Fos expression. The activation of MAPK/ERK in the nucleus is required for the mitogen-stimulated growth response. In vivo analyses indicate that the nuclear entry of MAPK is more tightly controlled. In developing mouse embryos, p-MAPK is only cytoplasmic, and nuclear MAPK is associated with isolated mitotic cells
or cells that have been mechanically injured to stimulate mitosis.\textsuperscript{132b} In \textit{Drosophila}, the cytoplasmic localization of p-MAPK is so important that when a strong nuclear signal was added to disrupt the cytoplasmic MAPK, differentiation of eye pattern was retarded.\textsuperscript{138} Mouse embryo differentiation increased as the nuclear localization of p-MAPK was reduced. Retention of MAPK also occurs in seneescence of human fibroblasts and during cytoskeleton changes in cultured cells during motility.\textsuperscript{139} Previous studies have indicated an evident link between Snail and other Snail gene members (i.e., Snail, Slug, Twist) and MAPK/ERK signaling.\textsuperscript{3, 5b, 80} But the clinical implications for ERK subcellular localization in malignant breast cancer are unclear.

The current study correlates Snail expression with breast cancer progression, as Snail protein was absent in human mammary epithelial cells isolated from mammoplasty tissue (HMEPiC) and MCF-7 breast cancer cells, while it was expressed in adenocarcinoma breast cancer cells (T47-D) and triple negative breast cancer cells (MDA-MB-231). MDA-MB-231 breast cancer cells are highly aggressive and exhibit high invasion and migratory behavior. MDA-MB-231 expressed the highest level of Snail protein, reiterating its use as a metastatic cell line in this study. Transient Snail knockdown using siRNA decreased the expression levels of p-ERK markedly in MDA-MB-231 cells and led to re-localization of p-ERK from the nucleus to the cytoplasm in T47-D cells. This would suggest that Snail may regulate p-ERK and more specifically regulate its levels and localization in cancer cells.

We utilized the MCF-7 Neo/ MCF-7 Snail EMT model to more closely study the relationship between Snail expression, ERK activity and EMT functions. Indeed, we found that when Snail is overexpressed in less invasive MCF-7 cells, the morphology of
these cells shifted from epithelial to mesenchymal and fibroblastic. Snail overexpression also decreased E-cadherin epithelial marker while vimentin mesenchymal marker was increased which was concomitant with decreased with cell adhesion and increased cell migration. Additionally, Snail overexpression increased tumorigenicity in vivo. This study reports evidence for the first time, that Snail oncogene can negatively regulate adhesion to fibronectin and collagen in breast cancer cells, while also regulating other properties of epithelial cells that have been previously studied (i.e., E-cadherin reduction and increased migration). Our previous studies have shown that similarly in prostate cancer cells, Snail can decrease cell adhesion and increase cell migration by regulating integrin signaling.\textsuperscript{5b}

We found that Snail overexpression in MCF-7 breast cancer cells corresponds to decreased ERK activity which is surprisingly localized in the nucleus. However, this was associated with nuclear localization of active ELK-1, a downstream effector of nuclear p-ERK. Even more surprising was that MCF-7 Neo cells had high levels of p-ERK; however it was predominantly localized within the cytoplasm which corresponded also with cytoplasmic localization of active p90RSK, a downstream effector of cytoplasmic p-ERK. Active p90RSK was localized in the cytoplasm of MCF-7 Neo, MCF-7 Snail, T47-D, and MDA-MB-231; active Elk-1 was localized in the cytoplasm of MCF-7 Neo. Interestingly, active Elk-1 was localized in the nucleus of MCF-7 Snail and T47-D, with both nuclear and cytoplasmic localization in MDA-MB-231, and cytoplasmic localization in MCF-7 Neo. This would suggest that cytoplasmic ERK activity may be associated with less aggressive breast cancer while nuclear ERK activity may be associated more with aggressive breast cancer.
To determine if p-ERK was localized in the nucleus or at the nuclear membrane, we used immunocytochemistry to stain MCF-7 and MCF-7 Neo with p-ERK and/or nucleoporin98 (NUP98). Both cell lines expressed p-ERK and NUP98 at the nuclear membrane, but p-ERK was also localized within the nucleus. These data indicate that p-ERK has dual roles in less aggressive MCF-7 and MCF-7 Neo. We also noticed that the expression of ERK1 was higher in MCF-7 Neo, whereas the expression of ERK2 was higher in MCF-7 Snail. The inhibition of ERK1 or ERK2 has been analyzed in mesothelioma\textsuperscript{19}, and it has been indicated that ERK2 drives tumor migration by modulating the microenvironment.\textsuperscript{117} We found that the expression of ERK1 and ERK2 were differentially dependent on the level of Snail expression. We used siRNA for ERK1 and ERK2 to determine the effects on Snail, p-ERK, ERK, and E-cadherin protein expression. We used MCF-7, MCF-7 Neo, and MCF-7 Snail to demonstrate a progression model of breast cancer coupled with the transient knockdowns.

Another study examined expression of p-ERK1/2 in a total of 886 breast cancer patients and found that surprisingly, ERK1/2 phosphorylation correlated inversely with tumor size.\textsuperscript{140} However, they did not dissect out the localization of these low levels of p-ERK. It is possible that some tumors with low levels of p-ERK may actually have it localized within the nucleus which would still correlate with poor prognosis. These findings suggest that there may be a shift in MAPK localization as breast cancer cells become more metastatic, but the mechanism was not analyzed.

We observed that MAPK inhibition in MCF-7 Snail cells led to decreased expression of Snail and EMT as evidenced by decreased E-cadherin, decreased cell migration but increased cell adhesion. Normally, the molecular half-life of Snail is only
about 25 min, and is highly unstable.\textsuperscript{95} The molecular mechanism that drives tightly-controlled Snail activation can include GSK-3β-directed phosphorylation that can include β-TrCP-directed ubiquitination and eventually proteasomal degradation.\textsuperscript{95} We investigated whether inhibition of proteasomal degradation was the basis behind decreased Snail protein upon inhibition of MAPK activity by pre-treatment with MG132 proteosomal degradation inhibitor. However, we found that UO126 was not leading to decreased Snail protein expression by targeting it for degradation by the proteosomal pathway. It has been reported that AP-1 activation can be induced by cellular stress brought on by UV-irradiation.\textsuperscript{132a} This mode of AP-1 activation induces Snail expression through the MAPK pathway in keratinocytes. Therefore, we propose alternatively that AP-1 activation may be an alternative pathway by which MAPK may regulate Snail and subsequently EMT.

Clinical trials have attempted to treat breast cancer with CI-1040 (PD184352), an orally active, highly potent selective MEK1 and MEK2 inhibitor, but were unsuccessful.\textsuperscript{17a} Breast cancer patients treated with epidermal growth factor receptor (EGFR) inhibitor, Gefitinib suffered relapses which were due to MAPK inhibition that paradoxically lead to PI3K/AKT activation.\textsuperscript{17b} Current clinical trials are testing the use of MEK and PI3K inhibitors in order to generate more effective therapeutic strategies. Our studies attempt to demonstrate that it may not be enough to target whole cell ERK activity. Targeting in this manner would also target cytoplasmic p-ERK which may actually play a role in hindering tumor progression. It may be useful in future studies to focus on targeting nuclear p-ERK to provide more efficacious therapy for breast cancer more difficult to treat with conventional methods.
Snail and hydroxytamoxifen resistance study

Snail induces resistance to cell death, noted in skin tumors induced in mice, biopsies of breast carcinomas from patients, gastric cancer, and hepatocellular carcinomas. Immunofluorescence, chromatin immunoprecipitation (ChIP) analyses indicated that as Snail was high in T47-D and MDA-MB-231 breast cancer cells, the activity of estrogen receptor-α was decreased due to high levels of tri-methylation at the promoter Enhancer box (E-box). Moreover, there are drastic differences between MCF-7 and MDA-MB-231 breast cancer cells in response to the protein kinase C (PKC) activator PMA (TPA). This contrast in how the cells respond directly relates to the differences in cell morphology and tumor behavior; MCF-7 are epithelial and non-invasive, while MDA-MB-231 contrast these cells as highly metastatic. Similar to previous investigation, we found that MCF-7 Snail had undetectable levels of ER-α, a finding that may actually be due to hypermethylation or trimethylation. Tumor volumes taken from nude mouse xenografts were higher in MCF-7 Snail compared to MCF-7 Neo. ER-α primary antibody was analyzed using immunohistochemistry. We found that positive staining only occurred in MCF-7 Neo. We also sought to determine if the inverse correlation between Snail and ER-α expression in MCF-7 Snail was linked to Snail-mediated resistance following 4-OHT chemotherapy. MCF-7 Neo appeared to be more sensitive to 4-OHT in single and combination treatments with 17-β-estradiol compared to MCF-7 Snail which did not seem to respond. Even when the concentration of 4-OHT was increased from 1uM to 10uM, there was still a lack of response from MCF-7 Snail. We also observed these effects by visualizing the cells with light microscopy. Untreated MCF-7 Neo were epithelial and tightly packed, and floating after treatment. MCF-7 Neo
were less tightly packed, cell membranes were rough and blebs started to form after 72 hours. On the contrary, MCF-7 Snail were mesenchymal with fibroblastic extensions prior to and after treatment. The expression of Snail did not change with treatment in either cell line; MCF-7 Neo lacked Snail and MCF-7 Snail expressed Snail. ER-α was decreased in MCF-7 Neo after 4-OHT which is biochemically relevant because it attributes to the function of 4-OHT. Lack of ER-α expression was maintained after 4-OHT treatment in MCF-7 Snail. Using a resazurin dye, we detected the reduction of cell culture medium as the cells grew over a period of 48 hours after being treated. Control cells had higher levels of viability, which was significantly decreased in single and combinational treatments. We noticed that the decreases in cell viability were dose-dependent, but that treating MCF-7 Snail with UO126 and 4-OHT generated a protective effect which restored cell viability. Perhaps inhibiting the MAPK signaling in these cells could be triggering activation of another signaling cascade we have not investigated here (i.e., PI3K/Akt, STAT, JNK). It would be beneficial to analyze other ERK molecules as well (i.e., ERK5, ERK7). Overall, ERK inhibition and 4-OHT treatments in combination were more effective in decreasing cell viability in more metastatic MCF-7 Snail versus single treatments.

We also tested changes in mitochondrial permeability (TMRM mitochondrial dye) and caspase activation to determine if Snail-overexpressing cells would be sensitive to the treatments. We treated MCF-7 Neo and MCF-7 Snail with single and combination doses of UO126 and 4-OHT for 48 hours and found that MCF-7 Snail remained brightly stained following either treatment, and MCF-7 Neo were dimly stained. These findings indicated that cell death is not induced after treatment in cells with high levels of Snail
and Snail that is localized in the nucleus. Flow cytometric analysis of the TMRM staining indicated that MCF-7 Neo responded to several treatments used during these experiments, including the single and combination treatments. There was a sharp peak detected after MCF-7 Neo were treated with UO126 (10uM) and 4-OHT (10uM), which resulted in continued shifts in the mitochondrial potential. These shifts were indicated by several increments and decrements which followed after the initial reading (various peaks and valleys graphically displayed). However, MCF-7 Snail did not seem to respond as quickly to the treatments. We did detect a sharp decrease in mitochondrial membrane potential when MCF-7 Snail were treated with UO126 (10uM) and 4-OHT (10uM), which seemed to affect the cell’s ability to recover from this pharmacological insult (indicated by a shift in the peak).

We used a caspase-3/7 activity assay with a 24 hour treatment and found no significant change in caspase activity in MCF-7 Snail. MCF-7 Neo and MCF-7 Snail had consistent levels of caspase activation for most of the treatments. Caspase activity did increase in MCF-7 Snail following 4-OHT (1uM) treatment, but was lower with a higher dose (10uM). Caspase activity increased in MCF-7 Neo treated with UO126 (10uM) and 4-OHT (10uM), but this increase was not significant. Overall, none of the treatments used to analyze caspase activity induced an increase in caspase activity compared to untreated cells with 100% viability. We also used flow cytometry to stain cells after treatment. The results from this assay confirmed what we observed using qualitative analysis.
CHAPTER 6
CONCLUSION

MAPK/ERK signaling pathway is regarded as a major regulator of signals that promote EMT and metastasis through Snail zinc finger transcription factor. Therapeutics have been designed to target MAPK/ERK signaling throughout subcellular compartments. Therapies have also been designed to target Snail and inhibit metastasis. The data presented here provide the first clear evidence that Snail and phosphorylated-ERK (p-ERK) located in the nucleus of transformed breast cancer cells function to increase EMT. More specifically, we determined that Snail expressed at high levels directly interacts with the ERK2 isoform in these breast cancer cells. Furthermore, this study also provides evidence for the interaction of nuclear Snail and nuclear p-ERK in regulation of EMT. A survival mechanism is present in breast cancer cells that express p-ERK in the nucleus, and have repressed estrogen receptor-α (ER-α). These cells are less responsive to single ERK1/2 and ER-α antagonism, and require combination therapy to induce a therapeutic response. This study is clinically significant for therapeutic and preventive applications; if Snail and p-ERK2 activity are in the nucleus tumors may be more capable to evade chemotherapeutics designed to treat each molecule individually. Therefore, antagonizing the localization of p-ERK and Snail into the nucleus could provide an efficacious approach for the prevention and therapy of breast cancer progression. Nuclear p-ERK may be a mechanism that breast cancer cells utilize to survive and develop therapy recurrence. Most chemotherapeutics function by targeting
ER-α or kinase activities but cannot delineate differences in other cell type-specific molecules. Therefore, these studies on Snail and ERK1/2 translocation into the nucleus contribute to current knowledge of breast cancer cell migration and survival that will improve future therapeutics.
Figure 1. Human body: mammary gland anatomy. Milk-producing gland characteristic of all female mammals and present in a rudimentary and generally nonfunctional form in males. Mammary glands are regulated by the endocrine system and become functional in response to the hormonal changes associated with parturition.
Figure 2. Morphological stages in the embryonic development of the mammary gland. a | Around embryonic day 10 (E10) of mouse development the milk line (orange) is defined by a slight thickening and stratification of the ectoderm (grey) as depicted here in this series of cross sections through the trunk. On E11.5 the milk line breaks up into
individual placodes (orange) and the underlying mammary mesenchyme (blue) starts to condense. Over the following days the placodes sink deeper into the dermis and the mammary mesenchyme becomes organized in concentric layers around the mammary bud (orange). Starting on E15.5, the mammary epithelium (orange) starts to proliferate at the tip and the primary sprout pushes through the mammary mesenchyme towards the fat pad (green). On E18.5 the elongating duct has grown into the fat pad and has branched into a small ductal system. The cells of the mammary mesenchyme have formed the nipple, which is made of specialized epidermal cells (purple). 

The schematic diagrams show the position of the milk line, placodes and mammary buds along the lateral body wall of early mouse embryos. Secreted molecules, receptors and transcription factors that are important at the different stages are listed in the table below. At the mammary bud stage, proteins that are expressed in the epithelium and in mesenchyme are listed separately. BMP, bone morphogenetic protein; ERBB, erythroblastic leukaemia viral oncogene homologue; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GLI, Gli-Kruppel family member; IGF, insulin-like growth factor; IGFR, insulin-like growth factor receptor; LEF, lymphoid enhancer-binding protein; MSX, muscle segmentation homeobox; NRG, neuregulin; PTHLH, parathyroid hormone-like hormone; PTHR1, parathyroid hormone receptor; TBX, T-box; WNT, wingless-related MMTV integration site²⁸.
Figure 3. Epithelial-mesenchymal transition (EMT). An EMT involves a functional transition of polarized epithelial cells into mobile and ECM component-secreting mesenchymal cells. The epithelial and mesenchymal cell markers commonly used by EMT researchers are listed. Colocalization of these two sets of distinct markers defines an intermediated phenotype of EMT, indicating cells that have passed only partly through an EMT. Detection of cells expressing both sets of markers makes it impossible to identify all mesenchymal cells that originate from the epithelia via EMT, as many mesenchymal cells likely shed all epithelial markers once transition is completed. For this reason, most studies in mice use irreversible epithelial cell-lineage tagging to address the full range of EMT-induced changes. ZO-1, zona occludens 1; MUC1, mucin1, cell surface associated; miR200, microRNA200; SIP1, survival of motor neuron protein interacting protein 1; FOXC2, forkhead box C2.41.
Figure 4. Snail genes occupy a central position in triggering EMT in physiological and pathological situations. Different signalling molecules have been implicated in the activation of Snail genes in several processes that subsequently lead to the conversion of epithelial cells into mesenchymal cells. Although the action of Snail in the epithelial–mesenchymal transition (EMT) as a direct transcriptional regulator (repressor) has been shown only for E-cadherin, different in vitro and in vivo approaches point to a series of target genes that are directly or indirectly regulated by these transcription factors. BMP, bone morphogenetic protein; FGF, fibroblast growth factor; ILK, integrin-linked kinase; PTH(rP)R, parathyroid-hormone-related peptide receptor; TGF-β, transforming growth factor-β.
Snail and active nuclear ERK1/2 study

Figure 5.

<table>
<thead>
<tr>
<th>A.</th>
<th>B.</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47-D Ctrl</td>
<td>T47-D Snail siRNA</td>
</tr>
<tr>
<td>Ctrl</td>
<td>Snail siRNA</td>
</tr>
<tr>
<td>Snail</td>
<td>Snail</td>
</tr>
<tr>
<td>p-ERK</td>
<td>p-ERK</td>
</tr>
<tr>
<td>ERK</td>
<td>ERK</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Vimentin</td>
</tr>
<tr>
<td>β-actin</td>
<td>β-actin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47-D Ctrl</td>
</tr>
<tr>
<td>Ctrl</td>
</tr>
<tr>
<td>Snail</td>
</tr>
<tr>
<td>p-ERK</td>
</tr>
<tr>
<td>ERK</td>
</tr>
</tbody>
</table>

Figure 5. Snail regulates ERK activity in breast cancer cells.

Normal immortalized epithelial cells HMEPiC, the adenocarcinoma cell lines MCF-7 and T47-D, and triple negative cell line MDA-MB-231 were utilized to analyze Snail levels by Western blot analysis (A). T47-D and MDA-MB-231 cells were transfected...
transfected with control siRNA or Snail siRNA and expression levels of Snail, phospho-
ERK1/2 (p-ERK), and total ERK1/2 were determined with Western blot (B) and
immunocytochemistry/immunofluorescence (C). Actin was utilized as a loading control
for Western blot analysis; DAPI was used to identify the nuclei in immunofluorescent
analyses. Magnification X20; insert X40. Results are representative of triplicate
experiments performed independently.
Figure 6.

A. MCF-7 Neo vs. MCF-7 Snail

B. Western blot analysis
- Snail
- E-cadherin
- Vimentin
- β-actin

C. Adhesion

D. Migration

E. Tumor Volume
Figure 6. Snail increases EMT and tumorigenicity.

MCF-7 Neo and MCF-7 Snail were grown to 60-70% confluence and images were captured with AxioZeiss microscopy at 63X magnification (A). MCF-7 Neo and MCF-7 Snail were used to analyze expression of Snail, E-cadherin, phospho-ERK1/2 (p-ERK) and total ERK1/2 by Western blot analysis (B). 3x10^4 MCF-7 Neo and MCF-7 Snail were plated in 96 well-plates coated with collagen or fibronectin. MCF-7 Neo displayed higher adhesion on rat tail collagen and human fibronectin (C). 5x10^4 MCF-7 Neo and MCF-7 Snail cells were plated on the top chamber of inserts coated on the outside with either rat tail collagen or human fibronectin in 24 well-plates. Cells that had migrated to the lower chamber after 5 h were fixed and stained with crystal violet. Images of migration were captured with AxioZeiss microscopy at 10X magnification and subsequently stained cells were counted and graphically represented (D). MCF-7 Snail exhibited increased migration on collagen and fibronectin. MCF-7 Neo and MCF-7 Snail cells were subcutaneously implanted into nude female mice (n=6 per group). After two weeks, mice were sacrificed and tumor volumes determined and graphically represented (E). Results are representative of triplicate experiments performed independently. Statistical significance was assessed using GraphPad Prism software by paired Student’s t-test (**p<0.01, ***p<0.001).
Snail were stained with hematoxylin/eosin (H&E) to examine histology of the tissues. Mouse xenografts were stained with Snail primary antibody (A). MCF-7 Neo and MCF-7 Snail nude mice were compared to MCF-7 Neo and MCF-7 Snail nude mice

Figure 7. Snail increases tumorigenicity in vivo. MCF-7 Neo and MCF-7 Snail nude
Figure 8.

A. MCF-7 Neo  MCF-7 Snail

B. MCF-7 Neo  MCF-7 Snail

C. MCF-7 Neo  MCF-7 Snail

p-ERK

DAPI

p-ERK

/p-DAPI

p-ERK

p-EDc-1

p-P90RSK

β-actin

Topol

GAPDH

Snail

p-ERK

ERK

p-Elk-1

Elk-1

p-p90RSK

p90RSK
Figure 8. Snail promotes nuclear translocation of phospho-ERK.

Immunofluorescent analysis (A) and Western blot analysis (B) was performed on MCF-7 Neo and MCF-7 Snail samples utilizing antibodies to Snail, phospho-ERK1/2 (p-ERK) or total ERK1/2. Western blot analysis was performed on nuclear and cytoplasmic fractions prepared from MCF-7 Neo and MCF-7 Snail cells. The blots were probed with Snail, phospho-ERK1/2 (p-ERK), total ERK1/2, phospho-Elk-1(p-Elk-1), total Elk-1, phospho-p90RSK (p-p90RSK), and total p90RSK primary antibodies. Actin was used as the loading control for Western blot analysis and DAPI was used to identify nuclei for immunofluorescence studies. Immunofluorescence images were visualized with AxioZeiss fluorescent microscopy. Images were acquired at 20X magnification (inserts 63X). Results are representative of triplicate experiments performed independently.
Figure 9. Phospho-ERK co-localizes with NUP98 nuclear membrane marker in MCF-7 parental cells.

ICC was performed on parental MCF-7 cells to examine where nuclear ERK activity was localized. Co-staining with DAPI nuclear marker and NUP98 nuclear membrane marker suggested that phospho-ERK activity was predominantly found at the nuclear membrane since there was strong co-localization (white color) with NUP98.
Figure 10. Snail knockdown correlates with nucleo-cytoplasmic translocalization of phospho-Elk-1

MDA-MB-231 breast cancer cells were transfected with either control siRNA or Snail siRNA. The knockdown resulted in translocation of phospho-Elk-1 (A) from the nucleus to the cytoplasm. Total Elk-1 was used to normalize phospho-Elk-1 during ICC fluorescence staining (B).
Figure 11. Inhibition of ERK activity decreases Snail and partially reverts EMT independent of proteasomal degradation. MCF-7 Neo and MCF-7 Snail untreated or treated with UO126 (20 μM) for 30 min, 2 h, 6 h, and 24 h, then analyzed by Western blot for expression of phospho-ERK (p-ERK), total ERK and Snail (A). The morphology of MCF-7 Neo and MCF-7 Snail cells untreated or treated with 20 μM UO126 for 24 h was visualized by light microscopy (B). DAPI staining was performed on MCF-7 Neo
and MCF-7 Snail cells treated with 20 μM UO126 for 24 h to show that cells were still alive and the nucleus was intact after treatment. Cells were visualized with AxioZeiss fluorescent microscopy (C). Western blot analysis was performed on MCF-7, MCF-7 Neo and MCF-7 Snail cells untreated or treated with 20 μM UO126 for 24 h to analyze the expression of E-cadherin and vimentin EMT markers (D). MCF-7 Neo and MCF-7 Snail cells were treated with UO126, MG132, or UO126 plus MG132. Expression of p-ERK, ERK and Snail was determined by Western blot analysis (E). Actin was used as the loading control and DMSO vehicle was used for control samples during UO126 treatments. Results are representative of triplicate experiments performed independently. Magnification X40.
Figure 12. Differential role of ERK1 and ERK2 isoforms. MCF-7 Neo and MCF-7 Snail were transiently transfected with control siRNA, ERK1 siRNA or ERK2 siRNA and expression levels of phospho-ERK (p-ERK), ERK, Snail and E-cadherin were determined by Western blot analysis (A). Result of the Western blot was quantified utilizing ImageJ software from NIH (B). Actin was utilized as the loading control. Results are representative of triplicate experiments performed independently.
Figure 13. Differential functional roles of ERK activity in MCF-7 Neo vs MCF-7 Snail. MCF-7 Neo and MCF-7 Snail were left untreated or treated with UO126 for 24 h. Subsequently, 5x10^4 cells were plated into 8μm pore inserts coated with rat tail collagen I or human fibronectin (FN) in a 24 well plate and cells allowed to migrate for 5 h. Cells that had migrated were fixed and stained with crystal violet and cells counted to obtain relative cell migration (A). MCF-7 Neo and MCF-7 Snail were left untreated or treated with UO126 for 24 h. 3x10^4 cells were plated into 96 well plates coated with rat tail collagen I or human fibronectin, and allowed to adhere for 20 min. Cells that adhered were stained with crystal violet, solubilized with Sorenson solution, and OD measured at 590 nm to obtain relative cell adhesion (B). Results are representative of triplicate
experiments performed independently. Statistical significance was assessed using
GraphPad Prism software by paired Student’s t-test (*p<0.05, **p<0.01, ***p<0.001).
Snail and hydroxytamoxifen resistance study

Figure 14.

A.

<table>
<thead>
<tr>
<th></th>
<th>MCF-7 Neo</th>
<th>MCF-7 Snail</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

MCF-7 Neo  MCF-7 Snail

20X 40X

Figure 14. Snail decreases estrogen receptor-α expression *in vitro* and *in vivo*

Expression of ER-α was determined by Western blot in MCF-7 Neo and MCF-7 Snail lysates (A). MCF-7 Neo and MCF-7 Snail nude mouse xenografts were stained with ER-α primary antibody (B). MCF-7 Neo and MCF-7 Snail were stained with hematoxylin/eosin (H&E) to examine histology of the tissues. β-actin was used as the loading control.
Figure 15.

A. MCF-7 Neo MCF-7 Snail

- Complete RPMI
- 17-β-estradiol
- 4-OHT
- 17-β-estradiol and 4-OHT
Figure 15. Hydroxytamoxifen therapy does not affect MCF-7 Snail morphology or Snail expression.

7.5X10^4 cells were treated for 72 hours with 5% DCC, 17-β-estradiol, 4-OHT, and 17-β-estradiol plus 4-OHT (A). Morphology was visualized at 10X magnification. MCF-7 Neo and MCF-7 Snail lysates were immunobotted and probed for Snail (B) and ER-α (C). β-actin was used as the loading control for Western blot analysis.
Figure 16. Sustained UO126 and 4-OHT combination treatments effectively reduce MCF-7 Snail viability.

MCF-7 Neo and MCF-7 Snail were treated with UO126 and/or 4-OHT at various concentrations (1μM, 10μM, or 20μM) for 48 hours, then viability was quantitated by measuring accumulation of resazurin dye (A). Cytotoxicity was assessed after treatments
by quantitating the dead/live cell signal (B). Values at p<0.05 were considered statistically significant.
Figure 17.

A.

Staining with TMRM mitochondrial dye – MCF-7-Neo (48 hr)

- Untreated
- UO126 – 20 μM
- UO126 – 10 μM / TAM 1 μM
- Hydroxytamoxifen (TAM) 1 μM
- Hydroxytamoxifen (TAM) 10 μM
- CCCP (positive control for apoptosis)

B.

Staining with TMRM mitochondrial dye – MCF-7-Snail (48 hr)

- Untreated
- UO126 – 10 μM
- UO126 – 10 μM / TAM 1 μM
- Hydroxytamoxifen (TAM) 1 μM
- Hydroxytamoxifen (TAM) 10 μM
- UO126 – 20 μM
- UO126 – 10 μM / TAM 10 μM
- Dim
- CCCP (positive control for apoptosis)
Figure 17. Mitochondrial permeability is not responsive to UO126 and/or 4-OHT treatments. MCF-7 Neo and MCF-7 Snail were treated with various concentrations of UO126 and/or 4-OHT for 48 hours, then mitochondrial permeability was analyzed after TMRM assay was performed (A,B) Immunofluorescence microscopy was used to visualize cells after treatment. Flow cytometry analysis was used to quantitate TMRM results (C). Lines indicated represent different treatments and concentrations: UO126 (20μM), UO126 (10μM) plus 4-OHT (10μM), 4-OHT (10μM), no treatment, no significance.
Figure 18. Cell apoptosis induced by UO126 or UO126/4-OHT is caspase-independent. MCF-7 Neo and MCF-7 Snail treated with UO126 and/or 4-OHT at various concentrations for 48 hours, were also stained with Caspase-Glo® 3/7 reagent and activity was quantitated using a plate reader.
References


42. Giordano, S., Update on locally advanced breast cancer Oncologist 2003, 8, 521-530.


64. (a) Paget, S., The distribution of cancer metastasis: the 'seed and soil' hypothesis revisited.


85. (a) W. A. Paznekas, e. a., Genomic organization, expression, and chromosome localization of the human Snail gene (Snai1) and a related processed pseudogene (Snai1P). *Genomics* 1999, 62 (1), 42-49; (b) A. V. Pinho, I. R., F.X. Real. p53-dependent regulation of growth, epithelial-mesenchymal transition and sternness in normal pancreatic epithelial cells. *Cell Cycle* 2011, 10 (8), 1312-1321.

87. AR Ramjaun, e. a., Upregulation of two BH3-only proteins, Bmf and Bim, during TGF beta-induced apoptosis. *Oncogene* 2007, 26 (7), 970-981.


