Regulation and Action of Skp2 and Rhoa in Cell and Tumor Models: Investigation into the Molecular Mechanisms Responsible for the Aggressive Phenotype of Triplenegative Breast Cancer

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REGULATION AND ACTION OF SKP2 AND RHOA IN CELL AND TUMOR MODELS: INVESTIGATION INTO THE MOLECULAR MECHANISMS RESPONSIBLE FOR THE AGGRESSIVE PHENOTYPE OF TRIPLE-NEGATIVE BREAST CANCER

A Dissertation Presented

by

KATERINA D. FAGAN-SOLIS

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Molecular and Cellular Biology Graduate Program
Regulation and Action of SKP2 and RhoA in Cell and Tumor Models: Investigation into the Molecular Mechanisms Responsible for the Aggressive Phenotype of Triple-Negative Breast Cancer

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DEDICATION

To my Mama, Vivian Solis-Fagan and my best friend and sister Karlie Vavrinek. Without the two of you I would not have started or finished this journey. Here’s to boats, retirement, and a family compound!
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My sincere thanks go first to my advisor, Dr. Kathleen Arcaro. Kathleen took me on in her lab after a twenty minute “interview” that was a surprise to both of us, and all we talked about was that I was from Albany, NY and did my undergraduate studies at SUNY Albany and she did her post-graduate work at SUNY Albany. Absolutely no science talk occurred, however when asked to take me on for the summer as part of a research program she did not hesitate. Kathleen has been a great mentor and a model of the type of scientist that I wish to be. She has been supportive, understating, attentive, and instrumental in my graduate training.

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everything. She was always there with a kind word or a motivating comment when I
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Last but not least I’d like to thank God for without my faith I would not be the
person I am today. Glory be to God in the highest.
ABSTRACT

REGULATION AND ACTION OF SKP2 AND RHOA IN CELL AND TUMOR MODELS: INVESTIGATION INTO THE MOLECULAR MECHANISMS RESPONSIBLE FOR THE AGGRESSIVE PHENOTYPE OF TRIPLE-NEGATIVE BREAST CANCER

FEBRUARY 2013

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Directed by: Professor Kathleen F. Arcaro

Breast cancer tops the list of new cancer cases and is predicted to be the second leading cause of cancer deaths in women in 2012. The primary objective of the present study was to provide insights into the molecular mechanisms underlying the aggressive growth and metastasis of triple-negative and basal-like breast cancers. To study increased growth and invasive behavior in triple-negative and basal-like breast cancers we utilize both an interesting and relevant cell culture model and examination of human tissue.

In this study, we use the Tamoxifen-selected, MCF-7 derivative, TMX2-28 breast cancer cell line. TMX2-28 cells are triple-negative in that they lack expression of the estrogen receptor alpha (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). They also have acquired a mixed basal/luminal cytokeratin profile, suggestive of a more basal-like phenotype. TMX2-28 cells are highly proliferative and invasive.
In addition to our cell culture model, we also examine human tissue. Thirty frozen breast carcinoma samples were evaluated for mRNA expression. Additionally, I analyzed protein expression, using immunohistochemistry (IHC), of 50 benign reduction mammoplasty and 188 breast tumors (formalin-fixed paraffin embedded). Of the 188 breast tumors, 93 were ERα-positive and 95 were ERα-negative. Of the 95 ERα-negative samples, 24 were further classified as non-triple negative (either PR or HER2 positive), 49 were classified as triple-negative, and 22 were not further classified due to unavailability of HER2 status and were used only in analyses of ERα-negative tumors. Thirty-seven of the 188 tumor samples were ductal carcinoma in situ, 138 were invasive ductal carcinomas, and 13 were classified as other. Lastly, 23 of the 188 tumors were grade 1, 48 were grade 2, 105 were grade 3, and 12 did not have grade data available.

S-phase kinase-associated protein 2 (SKP2) plays an important role in cell cycle regulation by targeting p27 for degradation. The cyclin-dependent kinase (CDK) inhibitor p27 regulates G1/S transition by binding cyclin/CDK complexes and abrogating its activity. By targeting p27 for degradation, SKP2 frees the complexes needed to progress into the S phase of the cell cycle. Evaluation of SKP2 expression in TMX2-28 revealed significantly higher levels than in other breast cancer cell lines. Despite the high levels of SKP2 expression, p27 protein was not reduced. However, levels of the Serine 10 phosphorylated form of p27 (pSer10p27), which has been associated with increased proliferation rates, was found to be increased. Furthermore, suppression of SKP2 completely eliminated the pSer10p27 and slowed cycle progression confirming the role of SKP2 in the aggressive growth of TMX2-28 cells.
Assessment of mRNA from 30 frozen human breast cancers demonstrated that SKP2 is more highly expressed in ERα-negative and basal-like breast cancers. Immunohistochemical analysis of 188 breast cancers and 50 benign reduction mammoplasty tissues confirmed that SKP2 is more highly expressed in ERα-negative breast cancers and for the first time demonstrated that triple-negative breast cancers are more likely to overexpress SKP2 than are non-triple-negative, but still ERα-negative, tumors. In contrast to some previous reports, we did not observe an inverse relationship between SKP2 and p27 expression. Only 11% of tumors expressed high SKP2 and low p27, while 32% of tumors had high SKP2 and high p27. Although no significant relationship between SKP2 and p27 expression was observed in human breast cancers, a significant positive relationship was discovered between SKP2 and pSer10p27. Furthermore, high levels of SKP2 and pSer10p27 were observed significantly more often in ERα-negative and triple negative breast tumors than in ERα-positive breast cancers. Based on these results and those of the cell culture experiments showing complete elimination of pSer10p27 after suppression of SKP2 it appears that levels of pSer10p27 may be a better indicator of SKP2-dependent p27 degradation than are levels of p27. Therefore, that inhibiting SKP2 in triple-negative breast cancers expressing high levels of both SKP2 and pSer10p27 regardless of p27 levels may be a valid therapeutic approach.

A foremost threat to patients is tumor invasion and metastasis, with the greatest risk to patients diagnosed with triple-negative and basal-like breast cancers. Two distinct morphological/functional mechanisms are known for single cell migration in tissues: mesenchymal and amoeboid invasion. Mesenchymal movement involves the use of proteases that cause cellular lysis in tissues, thereby creating a path through which cells
can invade. Amoeboid movement is protease-independent; cells find paths through the ECM by pushing and squeezing through regions of adequate size. Despite their invasive phenotype, TMX2-28 retains morphology similar to non-aggressive MCF-7 cells, suggesting that their invasion may be proteolytic-independent.

We determined that TMX2-28 lack MMP-1 mRNA, and MMP-2/MMP-9 protein expression; each of which is important in protease-dependent invasion. Furthermore, TMX2-28 cells have low expression of other genes key to protease-dependent invasion including Slug, Zeb 1, Zeb 2, Vimentin, Fibronectin and N-cadherin. RhoA is a member of the Rho superfamily of GTPases that acts as a molecular switch to control signal transduction and is critical to the amoeboid invasion mechanism. TMX2-28 cells have high expression of protease-independent invasion genes such as RhoA, ROCK 1, ROCK 2, and E-cadherin. Finally, treating TMX2-28 cells with a RhoA pathway inhibitor or an shRNA targeting RhoA significantly reduces their invasiveness. These data suggest that TMX2-28 cells use a RhoA-dependent, proteolytic-independent invasion mechanism. Collectively, the data presented here demonstrate the roles of SKP2 and RhoA in triple-negative and basal-like breast cancers, making both genes, as well as their pathways, desirable therapeutic targets.
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CHAPTER 1

INTRODUCTION

Breast Biology and Breast Cancer Statistics

The breast is composed of glands, named lobules, which are responsible for milk production, and ducts that connect the lobules to the nipple (Figure 1.1). The remainder of the breast is made up of fatty, connective, and lymphatic tissue (1). Cancer is a disease characterized by uncontrolled growth and spreading of abnormal cells. Breast cancer originates in the breast in either lobular or ductal cells (2, 3). Two types of breast cancer exist; *in situ* cancers are confined within ducts or lobules while invasive or infiltrating breast cancers began in lobules or ducts but has succeeded in migrating beyond the site of origin (2, 3).

Breast cancer is estimated to top the list of new cancer cases and to be the second leading cause of cancer deaths in women in 2012. An estimated 227,000 women will be diagnosed with breast cancer this year and approximately 40,000 will die as a result of their breast cancer (Figure 1.2) (4). In recent years, incidence rates of breast cancer have been steadily increasing among all races (Figure 1.3 Top). Incidence rates are higher in non-Hispanic white women compared to African Americans in most age groups. However African American women have higher incidence rates before the age of forty and are more likely to die of breast cancer at every age. (Figure 1.3 Bottom) (5).

The Discovery of Breast Cancer Subtypes
The development of gene expression profiling in 2000 using cDNA microarray technology has led to the identification of five breast tumor subtypes with distinct molecular signatures and each correlated with different clinical outcomes (Figure 1.4) (6-10). Luminal A and luminal B subtypes are defined by expression of estrogen receptor α (ERα) and luminal cell cytokeratins (CK) 8 and 18. The luminal A subtype typically has greater ERα expression and has a favorable outcome while the luminal B subtype expresses less ERα and has intermediate outcome. The ERBB2+ subtype is characterized by over expression of human epidermal growth factor receptor 2 (HER2) and has poor overall outcome. The normal-like tumor subtype is ERα-negative and has high expression of some basal epithelium markers including CK 5 and 17. Lastly, the basal or basal-like tumors are ERα negative and have high expression of basal cell markers including CK 5 and 17. Basal-like tumors are highly aggressive and generally have poor patient outcome (7, 11).

A new subtype of breast cancer, termed the triple-negative subtype, was brought to the forefront of research in 2005. Triple-negative breast cancers were defined as tumors that lack expression of ERα, Progesterone Receptor (PR), and HER2 (12). While the majority of triple-negative tumors also express basal cell markers, and the majority of basal-like cancers are also triple-negative (13-15), it is noteworthy to mention that clinical, microarray, and immunohistochemical data show that these tumor types are not entirely the same and thus cannot be lumped together into one subtype (16). Patients with triple-negative breast cancer face poor prognosis, as their tumors will not respond to anti-estrogen nor anti-HER2 therapies (17, 18). Rather than conventional cytotoxic therapies, designing individualized treatment for specific subgroups of disease is
necessary and requires targeting genes or pathways actively engaged in the 
pathophysiology of the breast cancer subtype.

The Hallmarks of Cancer

In 2000, Douglas Hanahan and Robert Weinberg proposed that six biological 
capabilities are acquired during the development of human cancer (Figure 1.5). These 
hallmarks of cancer are distinctive and complimentary and enable cancer cells to grow 
and metastasize (19). Importantly, they provide cancer researchers with a foundation in 
which to understand the diverse biology of cancer. The six hallmarks consist of: 1. 
Sustaining proliferative signaling. 2. Evading growth suppressors. 3. Activating 
invasion and metastasis. 4. Enabling replicative immortality. 5. Inducing angiogenesis. 
6. Resisting cell death. Since this this landmark publication, a number of other cell 
processes have emerged as potential hallmarks including reprogramming of energy 
metabolism, and evading immune destruction (20). The research described in this 
dissertation involves sustaining proliferation signaling through dysregulation of the cell 
cycle and activating invasion and metastasis.

The Cell Cycle and SKP2

The cell cycle comprises a series of tightly controlled events that drive the 
replication of DNA and cell division. Cyclins, cyclin dependent kinases (CDK) and 
CDK inhibitors regulate cell cycle progression by controlling the transitions between cell 
cycle phases. Formation of specific cyclin/CDK complexes is required for phase 
transitioning as these complexes target substrates involved in the transition process (21- 
23). CDK inhibitors are negative regulators of the cell cycle. They act through binding
to CDKs and blocking the formation of cyclin/CDK complexes, thus preventing the progression of the cell cycle. Dysregulation of the cell cycle results in uncontrolled cell proliferation in particular the G\textsubscript{1}/S transition. Abnormal regulation of the proteins involved in these processes is more than likely to be pivotally involved in cancer development and progression (19, 24).

S-phase kinase-associated protein 2 (SKP2) is an F-box protein and is component of the SKP1-Cullin1-F-box (SCF)/E3-ligase complex (25). The F-box proteins of SCF complexes are variable and function as receptors for specific target proteins and promote their degradation (26). SKP2 is the specific factor of the SCF\textsuperscript{SKP2}/E3 ligase (Figure 1.6 A) and is involved in cell cycle progression through degradation of p27 (25, 27). In normal cells, SKP2 targets a number of proteins including the negative cell cycle regulator p27 for ubiquitin-mediated degradation (28). During the transition from G\textsubscript{1} to S phase, p27 undergoes phosphorylation modifications at three major sites, Serine 10 (Ser10), Threonine 187 (Thr187) and Threonine 198 (Thr198) (29). Phosphorylation at Ser10, catalyzed by KIS and MIRK kinases, stabilizes p27 in quiescent cells; however, this phosphorylation event has been shown to promote cell proliferation (29-32). Thr198 phosphorylation is catalyzed by ROCK 1 kinase, only when Ser10 is already phosphorylated, and results in the inactivation of both free and CDK bound p27 (33, 34). Lastly, phosphorylation of Thr187 is catalyzed by SKP2 and promotes the ubiquitin ligase-mediated degradation of p27 (Figure 1.7) (21, 28, 35-39). p27 is a cyclin dependent kinase (CDK) inhibitor that regulates the G\textsubscript{1}/S transition by binding cyclin/CDK complexes, specifically Cyclin E/CDK2 and Cyclin A/CDK2 complexes, and abrogating their actions, thus promoting progression into the S phase of the cell cycle.
Because SKP2 is responsible for the degradation of p27, SKP2 is thought to function as an oncoprotein and high protein levels have been associated with triple-negative and basal-like tumors (16, 40).

**Invasion, Metastasis and RhoA**

Metastasis is dependent upon cancer cells being able to invade surrounding extracellular matrix (ECM) and adapt to different microenvironments within the primary tumor, the ECM, blood and/or lymphatic systems, and finally, to establish a new niche in a distant tissue (41-43). The proteins that control cell-cell and ECM interactions are thought to play a role in tumor cell invasion and metastasis by controlling cell morphology, motility, and interactions with the tumor microenvironment (41, 44). Thus, such proteins provide attractive therapeutic targets.

Two distinct morphological/functional mechanisms are known for single cell migration in tissues: fibroblast-type mesenchymal invasion, and leucocyte-type amoeboid invasion. Mesenchymal movement is characterized by an elongated cellular shape, and involves the use of proteases that cause cellular lysis in tissues, thereby creating a path through which cells can invade (44-48). In this kind of motility, cell speed is relatively slow (0.1-1 µm/min) and is dependent on actin polarization and lamellipodium formation for directionality of cell movement (49, 50). Amoeboid migrating cells have a rounded morphology and their movement is protease–independent (Figure 1.8). Instead, cells find paths through the ECM by pushing and squeezing through regions of adequate size (43, 44, 47, 48, 51, 52). In this kind of motility, cells exploit the propulsory forces resulting from acto-myosin cytoskeleton contractility, which results in very high migratory speeds (up to 4 µm/min). Amoeboid movement occurs independently of cell polarization but
requires RhoA-ROCK signaling to promote the rapid remodeling of the cell cortex (45, 51, 53, 54).

Migration of cancer cells is dependent on Ras Homolog Gene Family, Member A (RhoA) signaling (43, 44, 47, 51, 52). RhoA is a member of the Rho superfamily of GTPases that acts as a molecular switch to control signal transduction (Figure 1.9 A) (55). It carries out its actions through activation of its two targets: Rho-associated, coiled-coil containing protein kinase (ROCK) 1 and ROCK 2, which in turn regulate different facets of actin dynamics including stress fiber assembly, cell contraction, actin-filament stabilization, and focal adhesion organization (Figure 1.9 B) (56-59).

The role of RhoA and its effector ROCK in mesenchymal motility is complex; their activity needs to be reduced to extend protrusion at the front of the cell but they promote the retraction of the lagging end (50, 60). However, the overall effect of inhibiting RhoA/ROCK in mesenchymal cells is often minimal (51). Conversely, RhoA activity is critical to the amoeboid invasion mechanism (53, 61-63). Up-regulation of RhoA mRNA and protein is well documented for a variety of human cancers and has been positively correlated with cancer metastasis (64-66).

**A Triple-Negative Breast Cancer Cell Line Model**

In this study, we use the Tamoxifen-selected, MCF-7 derivative, TMX2-28 breast cancer cell line. TMX2-28 cells have lost expression of the estrogen receptor (ERα) and have acquired a mixed basal/luminal cytokeratin profile, suggestive of a more basal-like phenotype (67-69). Morphologically, TMX2-28 cells retained an epithelial cell shape similar to MCF-7. Behaviorally, TMX2-28 cells are highly proliferative (Figure 1.10 A) and invasive (Figure 1.10 B) (67, 68). It is these characteristics that make TMX2-28 cells
an interesting and relevant model for studying increased growth and invasive behavior in triple-negative and basal-like breast cancers. Studies of TMX2-28 cell cultures have guided our investigation of proteins expressed in human breast cancer.
Figure 1.1 Anatomical structure of the human breast (1).
Figure 1.2 Leading new cancer cases and deaths; 2012 estimates. Breast cancer is estimated to top the list of leading new cancer cases and to be the second leading cause of cancer deaths in women in 2012. An estimated 227,000 women will be diagnosed with breast cancer this year and approximately 40,000 will die as a result of their breast cancer (4, 5).
Figure 1.3 Breast cancer incidence and mortality rates. (Top) Age-specific female breast cancer incidence (2004 – 2008) and mortality (2003 – 2007) rates. Incidence rates of breast cancer have been steadily increasing among all races. (Bottom) Trends in female breast cancer incidence by race and ethnicity, US, 1975 – 2008. Rates are higher in non-Hispanic white women compared to African Americans in most age groups. African American women have higher incidence rates before the age of forty and higher death rates at all ages (5).
Figure 1.4 Identification of five breast tumor subtypes with distinct molecular signatures.

Expression of ERα and luminal cell CKs 8 and 18 define luminal A and luminal B subtypes. The luminal A subtype typically has greater ERα expression and has a favorable outcome while the luminal B subtype expresses less ERα expression and has intermediate outcome. The ERBB2+ subtype is characterized by over expression of HER2 and has poor overall outcome. The normal-like tumor subtype is ERα-negative and has high expression of some basal epithelium markers including CK 5 and 17. Lastly, the basal or basal-like tumors are ER negative status and have high expression of basal cell markers including CK 5 and 17. Basal-like tumors are highly aggressive and generally have poor patient outcome (9).
Figure 1.5 The Hallmarks of Cancer (19).
Figure 1.6 SKP2 protein structure and mechanism. (A) SKP2 is a component of the SCF$^{SKP2}$ complex and promotes progression into the S-phase of the cell cycle by regulating p27. (B) In proliferating cells, SKP2 targets p27 for ubiquitin-mediated degradation. By targeting p27 for degradation, SKP2 promotes progression into the S-phase of the cell cycle. p27 abrogates the actions of cyclin/CDK complexes thereby preventing the G$_1$-S transition and inhibiting the cell cycle (70, 71).
Figure 1.7 Model of p27 phosphorylation. During the transition from G\textsubscript{1} to S phase, p27 undergoes phosphorylation modifications at three major sites, Serine 10 (Ser10), Threonine 187 (Thr187) and Threonine 198 (Thr198) (29). Phosphorylation at Ser10, catalyzed by KIS and MIRK kinases, stabilizes p27 in quiescent cells; however, this phosphorylation event has been shown to promote cell proliferation (29-32). Thr198 phosphorylation is catalyzed by ROCK 1 kinase, only when Ser10 is already phosphorylated, and results in the inactivation of both free and CDK bound p27 (33, 34). Lastly, phosphorylation of Thr187 is catalyzed by SKP2 and promotes the ubiquitin ligase-mediated degradation of p27 (21, 28, 35-39).
Figure 1.8 Morphological/functional mechanisms for single cell migration in tissues, fibroblast-type mesenchymal invasion and leucocyte-type amoeboid invasion (72).
Figure 1.9 RhoA protein structure and mechanism. (A) Protein structure of RhoA kinase. (B) Rho acts as a molecular switch to control signal transduction in cells. Its main targets are the Rocks (1 & 2), which regulate difference facets of actin dynamics (73, 74).
Figure 1.10 TMX2-28 cells are (A) highly proliferative and (B) invasive (67, 68).

(Gozgit et. al. 2006; 2007)
CHAPTER 2

S-PHASE KINASE-ASSOCIATED PROTEIN 2 (SKP2) OVEREXPRESSION IS ASSOCIATED WITH INCREASED SERINE 10-PHosphorylated p27 (pSer10p27) IN TRIPLE-NEGATIVE BREAST CANCERS

Introduction

Breast cancer is the most common female malignancy, and the second leading cause of cancer related death in the U.S. (75). Breast cancer patients with disease of similar stage and grade often respond differently to therapy resulting in disparate clinical outcomes. The development of gene expression profiling, using cDNA microarray technology, has led to the identification of several subgroups of breast cancers with distinct molecular signatures (7, 11). Historically, only estrogen receptor α (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) have been widely accepted as predictive factors and are used to dictate a patient’s therapeutic regime (76).

Approximately 70% of breast cancers express ERα and are termed ERα-positive (77). For these patients, targeted endocrine therapy with anti-estrogens is available and their tumors are often responsive. The remaining 30% of breast cancers do not express ERα and are classified as ERα-negative (77). ERα-negative breast cancers are more aggressive, and have been further classified by molecular profiling into two biologically different subtypes, triple-negative and basal-like subtypes (7, 11). Triple-negative tumors lack ERα, PR, and HER2 expression while basal-like tumors are typically triple-negative but are also associated with positive basal cytokeratin (CK) 5, 14, and/or 17 expression
patterns (7, 16, 78-80). Basal or basal-like tumors are also ERα-negative but have high expression of basal cell markers including CK 5 and 17. Triple-negative and basal-like tumors are highly aggressive and generally have poor patient outcome as patients with these subtypes have tumors that will not be responsive to anti-estrogen or anti-HER2 therapies (7, 11, 17, 18). Rather than treating patients with conventional cytotoxic therapies, designing individualized treatment for specific subgroups of disease is necessary and requires targeting genes or pathways actively engaged in the pathophysiology of breast cancer.

Dysregulation of the cell cycle results in uncontrolled cell proliferation. In particular, the G\textsubscript{1}/S transition, and aberrant regulation of the proteins involved in this process are pivotally involved in cancer development and progression (19, 24). S-phase kinase-associated protein 2 (SKP2) plays an important role in cell cycle regulation, and was originally identified as part of a SCF ubiquitin-protein ligase complex though its interaction with Cyclin A during S phase of the cell cycle (35). SCF complexes have three core components, SKP1, Cullin/cdc53, Rbx1/Roc, and an F-box containing protein that confers its name (Figure 1.6). SKP2 serves as the F-box component of the SCF\textsubscript{SKP2} complex and governs its specificity (81). p27 is a cyclin dependent kinase (CDK) inhibitor that regulates the G\textsubscript{1}/S transition by binding cyclin/CDK complexes, specifically Cyclin E/CDK2 and Cyclin A/CDK2 complexes, and abrogating their actions. In normal cells, SKP2 targets a number of proteins including p27 for ubiquitin-mediated degradation, thus promoting progression into the S phase of the cell cycle (21, 28, 35, 36). During the transition from G\textsubscript{1} to S phase, p27 undergoes phosphorylation modifications at three major sites, Serine 10 (Ser10), Threonine 187 (Thr187) and
Threonine 198 (Thr198) (29). Phosphorylation at Ser10 (pSer10p27) stabilizes p27 in quiescent cells, however pSer10p27 has been shown to promote cell proliferation (29-32). Thr198 phosphorylation (pThr198p27) is catalyzed by ROCK 1 kinase, only when Ser10 is already phosphorylated, and results in the inactivation of both free and CDK bound p27 (33, 34). Lastly, phosphorylation of Thr187 (pThr187p27) is catalyzed by SKP2 and promotes the ubiquitin ligase-mediated degradation of p27 (21, 28, 35-39).

SKP2 has oncogenic potential and its overexpression has been found in several cancers, including thyroid, oral epithelial, colorectal, lymphomas, non-small cell lung carcinoma, melanoma, and Kaposi’s sarcomas (40, 82-89). Emerging evidence has demonstrated that SKP2 overexpression significantly promotes the transition from G1 to S phase of the cell cycle, resulting in accelerated proliferation, making this process critically involved in the pathogenesis of breast cancer (89-93). Overexpression of SKP2 has been associated with poor prognosis on its own (94) and in combination with high Cyclin E expression (92) as well as other unfavorable prognostic factors including increased tumor grade, lack of expression of ERα and PR, and HER2 overexpression (95, 96). High SKP2 expression has been shown to be a hallmark of triple-negative, basal-like tumors (16). Additionally, SKP2 expression was found to have an inverse relationship to p27 expression levels (94, 95, 97-99). However, to date eight published studies have examined the relation between SKP2 and p27 expression in breast cancer and reported mixed results. Of the two largest studies, one did not observe an inverse relationship between SKP2 and p27 (92, 100), while other found the relationship to have little prognostic value (96).
Given the inconsistency in the literature regarding the relationship between SKP2, p27 and prognosis, the extent to which overexpression of SKP2 results in increased phosphorylation of p27 and cell proliferation in breast cancer is uncertain. Therefore, we directly examined SKP2, p27, and Ser10-phosphorylated p27 (pSer10p27) in human breast cancers. We also examined the expression of the related pathway cell cycle genes, CDK2 and Cyclin E, as well as Cyclin D1. Additionally, we examined SKP2 and related proteins in MCF-7-derived TMX2-28 cells that are triple-negative and have a mixed basal/luminal CK profile (68, 69). Suppression of SKP2 in these cells provided an opportunity to examine the function of SKP2 in the development of triple-negative, basal-like breast cancers.

**Materials and Methods**

**Cell Culture:** TMX2-28 and MCF-7 cells were maintained in T-75 culture flasks in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with cosmic calf serum (5%), insulin (10 µg/ml), non-essential amino acids (100X), penicillin-streptomycin (10,000 µg/ml), and L-glutamine (200 mM) at 37°C and 5% CO₂. TMX2-28-NC, TMX2-28-S2, and TMX2-28-MC cells were maintained as above with the addition of 2.5 µg/ml Puromycin to the culture medium. MDA-MB-231 cells were maintained in L-15 medium supplemented with 10% FBS at 37°C and 0% CO₂. SKBR-3 cells were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. All cells were passaged when near 80% confluence.

**RNA and Protein Isolation:** Total RNA (n=3 biological samples) was isolated with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to
manufacturer’s suggestions and protocols as previously described (67, 68, 101). RNA was treated with Turbo DNA-Free (Ambion, Austin, TX) to remove any DNA contamination; RNA quality was assessed by 260/280 nm spectrophotometer readings (Nanodrop 8000; Thermo Scientific, Wilmington, DE). Protein- containing cell lysates were isolated from cell cultures (n=3 biological samples) with pre-chilled SDS lysis buffer (1% SDS, 0.06 M Tris-HCL, and 10% glycerol) according to our standard laboratory protocols (67, 68, 101). Extracts were used for Western immunoblotting.

**Western Immunoblotting:** Protein lysates were mixed with NuPage sample buffer and reducing agent (Invitrogen, Carlsbad, CA), heated at 70°C for 10 min, separated on Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA) and then transferred to an Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) according to manufacturer’s protocols. Membranes were incubated in blocking buffer (5% nonfat dry milk/Tris buffered saline and 0.1% Tween 20) for 1 hour at room temperature with gentle shaking. Membranes were then incubated with primary antibodies, [SKP2 (1:200; sc-7164), Cyclin D1 (1:200; sc-753), Cyclin E (1:200; sc-198), p27 (1:200; sc-528) (polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA); CDK2 (1:500; ab6538; polyclonal) and pSer10p27 (1:10,000; ab62364; monoclonal) (Abcam, Cambridge, MA)], overnight at 4°C, followed by incubation with the secondary antibody, anti-rabbit IgG linked to horseradish peroxidase (1:1000; 7074S; Cell Signaling Technology, Danvers, MA), for 1 hour at room temperature. Chemiluminescent signals were detected with SuperSignal West Pico Kit (Peirce, Rockford, IL), and imaged using the G.BOX Chemi HR-16 (Syngene, Fredrick, MD). Membranes were stripped using Restore stripping
buffer (Thermo Scientific, Rockford, IL) and reprobed for glyceraldehyde-3-phosphate (GAPDH; 1:10,000; 5174; Cell Signaling Technology, Danvers, MA).

**Quantitative Real-Time Reverse Transcriptase PCR (qRT-PCR):** RNA samples (n=3 biological samples) were reverse transcribed and amplified using the One Step RT-PCR kit (Qiagen, Valencia, CA) in the Roche LightCycler (Roche, Indianapolis, IN). Total RNA (75 ng) was incubated with Qiagen RT-PCR master mix including primers (25µM each) and SYBR Green I nucleic acid stain (2X; Invitrogen, Carlsbad, CA, product number S7563) in pre-cooled capillaries (Roche, Indianapolis, IN) and was reversed transcribed (50°C for 30 min). Following reverse transcription, samples were heated to 95°C for 15 min to activate the HotStar Taq DNA polymerase and to simultaneously inactivate the reverse transcriptase. The generation of amplified products was monitored over 45 PCR cycles by fluorescence of intercalating SYBR Green. Each cycle consisted of the following steps: 1. Denaturation at 95°C for 15 sec. 2. Annealing at 60°C for 15 sec. 3. Extension at 72°C for 30 sec. Relative mRNA levels were normalized to hypoxanthine ribosyltransferase (HPRT) levels to control for RNA quality and concentration. Gene specific primers that span an exon-exon junction, designed using Primer3 software (http://frodo.wi.mit.edu/), and purchased from Integrated DNA Technologies, Inc. (Coralville, IA), were used for ERα, PR, HER2, SKP2, p27, CDK2, Cyclin E, Cyclin D1, and HPRT (Table 2.1).

**RNA Interference:** pGIPz lentiviral shRNAmir (Open Biosystems, Lafayette, CO) targeted against SKP2 (Source ID: V2LHS_199552) or a negative control (random
sequence with no significant homology to any endogenous human, mouse or rat gene) pGIPz lentiviral shRNAmir were transfected into TMX2-28 cells using Superfect Transfection Reagent (Qiagen, Valencia, CA) per manufacturer’s instructions. Forty-eight hours post transfection; cells were treated with selection medium (DMEM supplemented with 2.5 µg/ml Puromycin). Five days following initial selection, cells transfected with the negative control shRNAmir were seeded at low density into 100 mm culture dishes and cultured until small colonies formed (<50 cells). Cloning cylinders (BellCo Glass, Inc. Vineland, NJ) were used to isolate colonies resulting from a single clone to create the TMX2-28-NC (Negative Control) cell line. Cells transfected with the shRNAmir targeting SKP2 were split into two populations. One population of cells was kept and termed TMX2-28-MC (Mass Culture), and the other population was seeded at low density into 100 mm culture dishes and cultured until small colonies formed (<50 cells). Cloning cylinders (BellCo Glass, Inc. Vineland, NJ) were used to isolate colonies resulting from a single clone to create the TMX2-28-S2 (SKP2 knockdown) cell line.

**Cell Cycle Analysis:** Adherent cells cultures were harvested by trypsinization, resuspended in phosphate buffered saline (PBS), and fixed with 95% ethanol (ETOH) at 4°C overnight. Fixed cells were resuspended in staining solution (PBS, 2 mM MgCl₂, 10 µg/ml RNase A, 100 µg/ml propidium iodide) and incubated at 37°C for 20 minutes. Single cell populations (1x10⁶ cells/ml) were analyzed using FACSDiva 4.1 and cell cycle analysis was performed using BD FACSDiva software (Becton-Dickinson Bioscience, Franklin Lakes, NJ).
**Human Tissue:** Institutional Review Board approval was obtained from Baystate Medical Center and the University of Massachusetts at Amherst. Samples were identified numerically to maintain patient anonymity. For the mRNA analysis of SKP2 and cytokeratins, 30 frozen breast tumor samples were retrieved from Baystate Medical Center, Department of Surgical Pathology as previously described (67, 68, 101). For the immunohistochemistry of SKP2 and related proteins, 159 tumor and 40 reduction mammoplasty (RM) tissues were stained. These tissues were obtained as follows: six sections were cut from each of five tissue microarrays (TMAs) containing a total of 101 tumor and 40 RM cases represented in triplicate as previously described (101), and six slides from each of 58 formalin-fixed paraffin-embedded (FFPE) breast tumors were purchased from University of Massachusetts Medical School Cancer Tissue Bank, Worcester, MA. An additional 29 tumor cases and 10 RM blocks were stained and scored for SKP2 only as previously described (102). The combined tumor cases = 188 and RM = 50. Clinical pathology reports accompanied all cases providing data on age, tissue type, histological grade, and ERα, PR, and HER2 status when available. Table 2.2 provides an overview of the clinical and pathological characteristics of the reduction mammoplasty and breast carcinoma patients.

**Immunohistochemistry (IHC):** Immunohistochemical study was performed using the Dakocytomation LSAB2 System-HRP kit (Dako, Carpinteria, CA) as previously described (101). Primary antibodies for the IHC were the same as those used for the Western immunoblotting, with IHC-specific dilutions as follows: SKP2 (1:25), Cyclin D1 (1:200), Cyclin E (1:200), p27 (1:200) (polyclonal; Santa Cruz Biotechnology,
Santa Cruz, CA); CDK2 (1:1000; polyclonal) and p27 (phosphor S10; 1:50; monoclonal; Abcam, Cambridge, MA), with secondary antibody anti-rabbit from Dako (Dako polymer K4003; Dako, Carpinteria, CA). Slides were stained with diaminobenzidine (DAB) chromogen for 10 minutes. The slides were then counterstained for 15 seconds in Mayer’s hematoxylin, transferred to glacial acetic acid water for 15 seconds, and then to ammonia water to blue. Finally, slides were dehydrated in ETOH and xylene before manual coverslipping.

The performance of each antibody was optimized through the use of negative (no primary antibody) and positive (tonsil, skeletal muscle, or breast cancer tissues with known protein positivity) controls and by testing a series of dilutions bracketing the manufacturer’s suggested dilutions. Antigen retrieval methods (Sodium citrate, pH, EDTA) were also optimized. Western immunoblotting was performed to confirm protein specificity based on protein size.

**Review of IHC Sample Staining and Pathology:** Scoring was conducted by one anatomic pathologist (CNO) without knowledge of the hormone receptor status of samples. TMAs and tissue slides were scored for immunoreactivity of all six proteins, assigning values of negative, weak, moderate, or strong for the intensity of staining. Tissues in which immunoreactivity was scored as weak, moderate or strong were further evaluated for the distribution of the staining and received descriptors of focal or diffuse for high and low percentage of cells stained respectively. While the number of cells showing immunoreactivity was not counted, tissue in which it appeared that greater than 50% of the cells were stained was scored as diffuse. Tissue in which it appeared that less
than 50% of the cells were stained was scored as focal, and in general these tissues had less than 10% of the cells stained. Tissues scored as negative were not evaluated for focal versus diffuse distribution. Scoring occurred over twelve sessions during which a single observer (KDF-S) recorded all evaluations. Periodically, previously evaluated slides were included in the blinded scoring session resulting in a total of ten different tumors being independently scored three times (three tumors for SKP2, two for p27, two for pSer10p27 and one each for Cyclin E, CDK2 and Cyclin D1). Of these thirty repeat scores, there was only one discrepancy: a tumor was scored once as moderate/diffuse and twice as strong/diffuse for p27.

**Statistical Analyses:** Cell cycle and mRNA expression data were analyzed and graphed with GraphPad Prism Version 3.02 (GraphPad Software, Inc., San Diego, CA). For ANOVA and post hoc two-tailed comparisons, significance was set at p<0.05. A Bonferonni correction was used to adjust the p-value whenever multiple t-tests were conducted.

For analysis of the immunohistochemistry results the intensity scores of negative, weak, moderate and strong were converted to two metrics: positive (including weak, moderate and strong) versus negative (including negative only) and high (including moderate and strong) versus low (including negative and weak). For each case in the TMAs, the highest score given to any of the three punches was used for analysis. For the slides, scores were based on evaluation of all tumor or RM tissue. Analysis also was conducted on the descriptors of diffuse and focal, as well as the original scores of negative, weak, moderate and strong, providing a total of four metrics that were analyzed.
Relationships between protein levels and hormone status, tumor grade, and tumor type were assessed with two-tailed Fisher Exact tests and p-values are presented. For tumor type and grade the original evaluations of negative, weak, moderate and strong were used in the analysis. Relationships between protein levels and age were assessed with regression analysis on the original evaluations of negative, weak, moderate and strong. STATA 11.2 (Statacorp LP, College Station, TX) was used for analysis of IHC data.

Results

Characterization of TMX2-28 receptor status

ER\(\alpha\), PR, and HER2 expression are the most important prognostic factors for breast cancer, dictating a patient’s therapeutic regime. Prior studies have shown that TMX2-28 cells lack expression of ER\(\alpha\) and have many characteristics of basal-like breast tumors (Chapter 1) however the status of PR and HER2 expression was not examined (67, 69). To determine the triple-negative receptor status of TMX2-28 cells, ER\(\alpha\), PR, and HER2 expression was analyzed by qRT-PCR and IHC. When compared to its ER\(\alpha\)/PR positive parent cell line, MCF-7, triple-negative cell line MDA-MB-231, and the ER\(\alpha\)/PR negative, HER2 overexpressing cell line SKBR-3, it is clear that TMX2-28 cells not only lack expression of ER\(\alpha\), but also lack expression of PR and HER2 (Figure 2.1), making them a triple-negative breast cancer cell line.

SKP2 is overexpressed in the triple-negative breast cancer cell line, TMX2-28

High levels of SKP2 protein in breast cancers have been associated with poor prognosis, ER\(\alpha\)- and HER2-negative status, and basal cell markers (92, 94, 99). To determine whether the triple-negative, Tamoxifen-selected TMX2-28 cells have
increased levels of SKP2 we compared mRNA and protein levels among three breast cancer cell lines, TMX2-28, MCF-7, and MDA-MB-231. The mRNA and Western immunoblot analyses demonstrated that SKP2 expression was significantly higher in TMX2-28 cells (Figure 2.2 A). SKP2 mRNA levels in TMX2-28 cells were roughly six times greater than in the parent cell line MCF-7 and the aggressive MDA-MB-231 cell line. These data suggest TMX2-28 may be a good model for studying the aggressive nature of a subset of triple-negative breast cancers that have high SKP2 and basal-like phenotypes.

**Overexpression of SKP2 regulates expression of its pathway genes in TMX2-28**

SKP2 controls cell cycle progression through regulation of p27. SKP2 targets p27 for degradation, which allows the formation of CDK2/Cyclin E complexes and entrance of the cell into the S phase of the cell cycle (16-18). We have shown that TMX2-28 cells overexpress SKP2, next we sought to determine the expression of other genes involved in this pathway. The mRNA and protein expression of the SKP2 pathway genes, p27, pSer10p27 (protein only), CDK2, and Cyclin E, as well as the expression of Cyclin D1, a cell cycle gene not involved in the SKP2 pathway, was determined using qRT-PCR and Western immunoblotting. Since TMX2-28 cells have higher expression of SKP2 than do MCF-7, we expected TMX2-28 to have lower levels of p27 protein, due to increased phosphorylation. Contrary to our prediction, TMX2-28 cells had significantly higher p27 mRNA and protein levels than did either MCF-7 or MDA-MB-231 (p<0.05; Figure 2.3 A). As expected, however, protein levels of pSer10p27, the form of p27 tagged for degradation, was present at substantially higher levels in TMX2-28 than in other cell lines (Figure 2.3 A). The targets of p27 inhibition, Cyclin E and CDK2 were
also overexpressed in TMX2-28 as compared to MCF-7 cells (p<0.05 & p<0.01 respectively; Figure 2.3 B & C), while the levels of the non-SKP2 pathway gene, Cyclin D1, were similar among all cell lines (p>0.05; Figure 2.3 D).

To determine whether the overexpression of SKP2 alters the expression of genes in the p27 S phase transition, we assessed the effects of suppressing SKP2 expression in TMX2-28 cells. For this study, TMX2-28 cells were stably transfected with either a negative control shRNA or an shRNA targeted against SKP2. The resulting lines were termed TMX2-28-NC (negative control), TMX2-28-S2 (single clone SKP2 knockdown), and TMX2-28-MC (mass culture SKP2 knockdown). As shown in Figure 2.2 B, we were able to reduce SKP2 gene expression by approximately 83% in both knockdown lines; suppression was confirmed by Western immunoblotting. Expression of p27, pSer10p27, CDK2, Cyclin E, and Cyclin D1 was determined in the three cell lines. Knockdown of SKP2 resulted in a significant (p<0.001) increase of p27 gene expression that was reflected in an increase in protein expression (Figure 2.3 A). In agreement with this finding, pSer10p27 protein levels decreased in TMX2-28-S2 cells compared to the negative control. Although not significant, there was a slight increase in Cyclin E gene expression and protein levels confirmed this (Figure 2.3 B). SKP2 knockdown in TMX2-28 cells also resulted in a significant increase in CDK2 gene and protein expression and as expected, gene and protein expression of Cyclin D1 remained consistent (p>0.05; Figure 2.3). TMX2-28-MC cells mirrored TMX2-28-S2 cells in their expression profiles.
Knockdown of SKP2 alters cell cycle in TMX2-28

To further investigate the role played by SKP2 overexpression in the control of cell cycle in breast cancer, TMX2-28-NC, TMX2-28-S2, and TMX2-28-MC cells were subjected to fixation, staining with propidium iodide, and subsequent cell cycle analysis. Suppression of SKP2 in both the clonally selected and mass culture cell lines resulted in a noticeable shift of the cell cycle towards G\(_0\)/G\(_1\) (Figure 2.4). We found a significant increase in the percentage of cells in the G\(_0\)/G\(_1\) phase and a significant decrease in the percentage of cells in the S phase of the cell cycle. We did not, however, see a significant change in the percentage of cells in the G\(_2\)/M phase of the cell cycle. The results from this experiment point to a prominent role of SKP2 in the proliferative potential of TMX2-28 cells.

SKP2 gene expression is higher in basal-like human breast cancers

We examined SKP2 mRNA expression in 30 frozen breast carcinoma samples by qRT-PCR to determine the extent to which expression patterns detected in the triple-negative, basal-like TMX2-28 cells reflect those of breast cancer. Eighteen ER\(\alpha\)-positive and twelve ER\(\alpha\)-negative breast cancers were assessed for mRNA expression of SKP2 and basal cytokeratins 5 and 17 (Figure 2.5). Tumors expressing CK5 and/or 17 were classified as basal cytokeratin expressing. ER\(\alpha\)-negative tumors that also expressed CK 5 and/or 17 were classified as basal-like tumors. Data revealed that there was a marginally significant trend towards higher SKP2 expression in ER\(\alpha\)-negative tumors (n=12) over ER\(\alpha\)-positive (n=18; \(p=0.067\)). When tumors were classified by expression of basal cytokeratins, tumors expressing CK 5 and/or 17 (n=11) also expressed significantly higher SKP2 mRNA levels than tumors that did not express basal cytokeratins (n=19;
Additionally, significantly higher SKP2 expression was found in tumors classified as basal-like (ERα-negative and expressing basal cytokeratins) (n=6) than non-basal-like (n=24; p=0.043). These results correspond well with the SKP2 overexpression in our TMX2-28 cell model.

**SKP2 and phosphorylated p27 are overexpressed in triple-negative breast cancers**

SKP2, p27, pSer10p27, CDK2, Cyclin E, and Cyclin D1 expression was examined immunohistochemically using tissue microarrays and tissue slides. We evaluated protein nuclear staining characteristics of benign RM epithelial cells and of tumor epithelial cells. Figure 2.6 provides examples of breast cancer cases that received scores of negative, weak, moderate, or strong based on staining intensity.

Table 2.3 provides a summary of the protein expression in human breast tissue. Scores of negative, weak, moderate, and strong were converted to two metrics: positive versus negative and high versus low (described in detail in the methods section). Tissues with immunoreactivity were further evaluated for distribution of staining and received scores of diffuse or focal, representing high and low percentage of cells stained respectively. Immunohistochemical analysis of 238 breast tissue specimens revealed that SKP2 protein is predominantly expressed in breast cancer (59%; 111 out of 188) as compared to RM tissue (8%; 4 out of 50; p<0.001). Of the tumors evaluated for distribution of SKP2 immunoreactivity, 21% (21 out of 98) were scored as diffuse.

Breast cancer cases were classified by ERα status and ERα-negative tumors were further stratified by triple-negative status. Note that for 22 ERα-negative tumors, HER2 status was unavailable and therefore these cases were excluded from triple-negative analyses. SKP2 was expressed significantly more often in ERα-negative tumors than in
ERα-positive tumors: 84% of ERα-negative tumors were positive for SKP2 while only 33% of ERα-positive tumors were positive for SKP2 (p<0.001). Similar results were obtained when immunoreactivity was analyzed by high/low: 63% of ERα-negative tumors had high SKP2 expression while only 16% of ERα-positive tumors had high SKP2 (p< 0.001). Additionally, of the tumors scored for staining distribution, 28% of ERα-negative tumors had diffuse staining whereas only 6% of ERα-positive tumors were scored diffuse (Table 2.3).

Among ERα-negative tumors, triple-negative tumors were more likely to be positive for SKP2 than were non-triple-negative tumors: 98% versus 75%, respectively (p=0.002), and the staining intensity was more frequently scored as high in triple-negative tumors than it was in non-triple negative tumors (80% versus 58%, respectively; p=0.056). Furthermore, triple-negative tumors were significantly more likely to be scored as having a high percent of cells with immunoreactivity for SKP2: 40% of triple-negative tumors were scored as having diffuse SKP2 expression, while 0% of non-triple negative were scored as having diffuse SKP2 expression (p=0.003).

SKP2 expression has been inversely correlated with p27 levels in subsets of breast cancer with poor prognosis (94, 95, 97-99). Therefore, since we observed higher levels of SKP2 in ERα-negative and triple-negative tumors, we expected these groups of tumors to have low p27 immunoreactivity. In contrast to our expectation, p27 expression was not lower in ERα-negative or triple-negative tumors; indeed there was no significant relationship between any of the p27 scoring metrics and hormone receptor status. The lack of relationship between p27 and hormone receptor status was not due to low p27 expression, as tumors were scored positive (92%), high (69%) and diffuse (61%) for p27.
immunoreactivity. In contrast to SKP2, p27 expression was common in RM tissue but still at significantly lower levels than in cancer when analyzed as both positive/negative and high/low (p=0.040 and p<0.001, respectively; Table 2.3).

SKP2 promotes the ubiquitin-mediated proteolysis of p27 through phosphorylation, which targets it for degradation via the proteasome pathway (28), yet no study has examined levels of phosphorylated p27 in breast cancer. Analysis in 192 breast tissue specimens showed that pSer10p27 protein is more frequently expressed in breast cancer (50%) as opposed to RM tissue (24%; p= 0.006). Of the 80 tumors scored positive for pSer10p27, 34% had diffuse staining.

Given the high levels of SKP2 in ERα-negative and triple-negative tumors, we predicted that pSer10p27 would also be high in these tumors. As predicted, pSer10p27 expression was significantly greater in ERα-negative than in ERα-positive tumors when assessed by either positive/negative or high/low immunoreactivity (Table 2.3: p<0.001 for both comparisons). However, staining distribution (diffuse versus focal) did not differ with ERα status. When categorized by triple-negative status, there were no significant differences in pSer10p27 expression or staining distribution.

All three of the remaining cell cycle genes, Cyclin E, CDK2 and Cyclin D1, were expressed primarily in cancer as opposed to RM tissue (Table 2.3). Despite the higher levels in cancer, when evaluated as high/low, most tumors, regardless of hormone status, were scored as low for Cyclin E. Likewise the majority of tumors were scored low for CDK2. In contrast, Cyclin D1 was scored as positive and high more frequently in ERα-positive cancers, and was more likely to be scored as low in triple-negative cancers.
Expression of SKP2 and phosphorylated p27 are highly correlated

Relationships between SKP2 and other proteins are presented in Table 2.4 in which the total number of tumors staining positive or negative, and high or low for SKP2 are presented, along with the percentage and number of those tumors that were scored positive (left side of table) or high (right side of the table) for each of the cell cycle genes. Analysis of 159 tumors in which data were available for both SKP2 and p27 revealed no significant relationship between the two proteins, either when the analysis was conducted on positive/negative or high/low scores. In contrast, phosphorylated p27 levels were positively associated with SKP2 expression. Comparing the relationship between SKP2 and pSer10p27 for high/low staining shows that of the 98 SKP2-positive tumors, 66 (67%) were also positive for pSer10p27. In contrast, of the 61 SKP2-negative tumors, only 14 (23%) were positive for pSer10p27. Another way of expressing the relationship between SKP2 and pSer10p27 is to consider that of the 80 tumors positive for pSer10p27, 66 or 83% were positive for SKP2. The positive relationship between SKP2 and pSer10p27 was highly significant (p<0.001; n=159) as was the relationship when data were analyzed using high/low scores (p< 0.001; n=159). Furthermore, ERα-negative and triple negative tumors were more likely to be scored high for both SKP2 and pSer10p27 than were ER-positive cancers (data not shown; Fisher’s Exact < 0.001 for both comparisons).

Higher levels of SKP2 were associated with increased Cyclin E expression, for both metrics analyzed (p=0.035 and p=0.04). CDK2 on the other hand, was more likely to be scored high in tumors that were scored low for SKP2 (p=0.031). There was no relationship between SKP2 and Cyclin D1 expression. Examples of the
immunoreactivity observed for SKP2 and related proteins are shown in Figures 2.7 through 2.10; cases are provided of both when SKP2 expression was high and low.

Previous research suggests that high levels of SKP2 and Cyclin E together with low levels of p27 may occur more frequently in triple negative tumors and may be a biomarker for poor prognosis (92). While we did not find a relationship between p27 and SKP2, it remains feasible that the combined expression pattern could be an indicator of prognosis. Despite small sample sizes, we found that the pattern of high SKP2 and Cyclin E with low p27 was significantly more common in triple negative tumors than in ERα-negative tumors that were not triple negative (data not shown; Fisher’s Exact = 0.008).

**SKP2 is more highly expressed in ERα-negative and triple-negative tumors from younger women while pSer10p27, Cyclin D1, p27, and CDK2 are more highly expressed in tumors from older women**

The relationship between strong protein expression and age are presented in Table 2.5. SKP2 expression in tumors was inversely correlated with age (p=0.005; n=159). When tumors were categorized by ERα status, SKP2 expression was inversely correlated with age in ERα-negative tumors only (p= 0.014; n=75). Further stratification of ERα-negative tumors revealed that SKP2 immunoreactivity was more frequently scored as strong in younger women with triple-negative tumors (p=0.029; n=49), but not in younger women with non-triple negative tumors (p=0.84; n=18). While expression of SKP2 was generally stronger in younger women, age explained little of the variability in
SKP2 expression, either when the analysis was conducted on all tumors ($R^2=0.05$), ER$\alpha$-negative tumors ($R^2=0.08$), or triple-negative tumors ($R^2=0.1$).

In contrast to SKP2, immunoreactivity of p27, pSer10p27, CDK2 and Cyclin D1 was positively correlated with age. Analyses on the total tumor set revealed a trend for stronger staining in older women for pSer10p27 ($p=0.003$; $n=159$) and Cyclin D1 ($p=0.012$; $n=158$), this trend was also significant in ER$\alpha$-negative tumors (pSer10p27: $p=0.004$; $n=75$ and Cyclin D1: $p=0.006$; $n=84$), and for Cyclin D1 in triple-negative ($p=0.017$; $n=49$). Strong immunoreactivity of p27 was positively correlated with age among non-triple negative tumors only ($p=0.018$; $n=18$), while the association between strong CDK2 immunoreactivity and age was significant among ER$\alpha$-negative tumors only ($p=0.032$; $n=71$). There was no significant relationship between Cyclin E expression and age.

**SKP2 is more highly expressed in invasive and high grade tumors**

The relationship between tumor type or grade and protein immunoreactivity is presented in Table 2.6 and Table 2.7 respectively. Invasive ductal carcinomas (IDC) were more likely to have strong SKP2 staining then were ductal carcinomas in situ (DCIS; $p=0.018$; $n=188$). Furthermore, strong SKP2 expression was more frequently associated with grade three tumors than with lower grade tumors ($p=0.010$; $n=188$). The only other association between cell cycle protein expression and tumor type or grade was for CDK2, which was more likely to be strongly expressed in DCIS than in IDC tumors ($p<0.001$; $n=159$).
Discussion

Patients with triple-negative and basal-like tumors face poor prognosis, as their tumors are not responsive to anti-estrogen or anti-HER2 therapies (17, 18). The progression of these hormone receptor negative breast cancers may be significantly impacted by dysregulation of cell cycle regulatory proteins. SKP2 is a component of the E3 ligase SCF$^{SKP2}$ complex, and functions as a receptor to specific target proteins initiating their degradation and promoting cell cycle progression (25, 27, 103). High expression of SKP2 has been associated with aggressive cancers including the basal-like breast cancer subtype (16, 94-96). While several researchers have suggested that SKP2 is a suitable therapeutic target for breast cancer, the subset of breast cancers that will respond to treatment with inhibitors of SKP2 has not been clearly identified.

The primary objective of the present study was to provide insight into the oncogenic role of SKP2 in triple-negative and basal-like breast cancers. To accomplish this, we first validated the triple-negative status of the ER$\alpha$-negative, basal-like breast cancer cell line, TMX2-28. Next we learned that, unlike the parent MCF-7 cell line, or the triple-negative, aggressive MDA-MB-231 cell line, TMX2-28 express high levels of SKP2, befitting of a triple-negative and basal-like breast tumor model. Based on the well-known role of SKP2 in p27 degradation we expected TMX2-28 cells to have low levels of p27 protein. However, both mRNA and protein levels of p27 were significantly higher in TMX2-28 cells than in either of the cell lines with low SKP2 expression. This finding led us to ask whether SKP2 was targeting p27 for degradation in TMX2-28 cell cultures. We found significantly higher levels of pSer10p27 in TMX2-28 as compared to other cell lines. Furthermore, suppression of SKP2 in TMX2-28 cells using RNAi
techniques resulted in significantly decreased levels of pSer10p27, increased levels of p27, and importantly, a slowing of cell cycle progression as evidenced by an increase in the percentage of cells in the G1/G0 and a decrease in the percentage of cells in S phase of the cell cycle.

To our knowledge, TMX2-28 is the only breast cancer cell line that is triple-negative and naturally expresses high levels of SKP2, a hallmark of basal-like phenotype (16). In a study of four breast cancer cell lines transfected with SKP2, Sonoda and colleagues (99) reported a ‘marked’ decrease in p27 protein. However, from the Western blots shown in the publication, the decrease in p27 protein appears to be quite modest. The continued, albeit lower, expression of p27 protein in the presence of high SKP2 expression is in agreement with our findings from TMX2-28 cells indicating that levels of p27 protein may not be the best biomarker of SKP2-dependent degradation of p27. On the other hand, the results may simply highlight the need for more accurate quantification of p27 protein levels; a problem we will encounter again with the IHC data. However, as we showed in Figure 2.2A, while suppression of SKP2 results in a detectable increase in p27 protein, it also results in the complete elimination of pSer10p27, clearly demonstrating the value of assessing pSer10p27 levels.

TMX2-28 cells were derived from MCF-7 cells treated with Tamoxifen. To some extent, this selection process mimicked the progression that occurs in many women, for whom after prolonged treatment with Tamoxifen their tumors become resistant to the drug and they develop ERα-negative breast cancer. Signoretti and colleagues (94) reported that MCF-7 cells arrested in G1 by combined Tamoxifen treatment and estrogen deprivation showed increased expression of SKP2, and suggested that deregulated SKP2
may play a role in the development of resistance to anti-estrogens. The extent to which overexpression of SKP2 in TMX2-28 cells was critical to the development of Tamoxifen resistance or is a consequence of loss of ERα is unknown. However, in human tissue SKP2 overexpression has been associated, although not consistently, with ERα-negative breast cancers. In the present study, using real time qRT-PCR and tissue from 30 frozen breast cancers, we found SKP2 mRNA more frequently overexpressed in ERα-negative, basal-cytokeratin-expressing, and basal-like breast cancers. Since SKP2’s role in regulating p27 expression occurs post translation, we next examined protein expression in formalin-fixed breast tissue.

Based on the published literature, the relationship between SKP2 and p27 protein expression in breast cancer subtypes and its relevance to prognosis is unclear. In 2002 Signoretti and colleagues (94) examined cDNA microarrays of 89 breast cancers and discovered that high levels of SKP2 were more frequent in ER-negative (11 of 18) as compared to ER-positive tumors (16 of 71), and that a small subset of cancers (five) with the highest levels of SKP2 were ER-, PR- and HER2-negative. They then used IHC to examine a new set of 84 breast cancers for expression of SKP2, p27, Ki67, and ER proteins (but not HER2). Again, higher levels of SKP2 were observed in ER-negative tumors (12 of 18) as compared to ER-positive tumors (7 of 47). Among the 19 tumors that had high levels of SKP2 expression, p27 was significantly more likely to be low (18 of 19). Both high SKP2 and low p27 expression were associated with poor survival, but a univariate analysis including expression of both proteins found no independent prognostic value of SKP2 expression, and no improved prognostic ability of the combination. Still, the authors concluded that SKP2 has oncogenic potential that it is
overexpressed in a subset of ER- and HER2-negative breast tumors, that inhibition of SKP2 is a valid therapeutic goal, and that more studies are needed.

Since the publication by Signoretti et. al., seven additional studies examining the expression of SKP2 and p27 in breast cancer have been published. In 2005, two studies of 82 (97) and 50 (98) breast cancers found expression of SKP2 to be significantly higher in ER-negative tumors, and to be inversely correlated with p27 expression. A year later Traub and colleagues (100) reported that 34% of the 338 breast cancers they examined had high SKP2 and low p27 and this combined ‘risk factor’ was significantly associated with a poorer outcome as compared to tumors with all other combinations of SKP2 and p27 expression patterns. However, contrary to the first three studies, they found no association between SKP2 expression and ER status. Furthermore, they found SKP2 levels to be higher in HER2-positive breast cancers. In another study published the same year, Sonoda and coworkers (99) examined SKP2 mRNA levels in 167 breast cancers, of which they selected 137 for IHC analysis of both SKP2 and p27. Based on the IHC analysis they reported SKP2 expression to be inversely correlated with p27, and based on the mRNA analysis they reported SKP2 to be associated with poorer survival and to be an independent indicator of prognosis. They also were the first to report higher SKP2 expression in tumors of younger women. In the three studies published in 2008, one study reported no relationship between SKP2 and p27 expression among 438 breast cancers (92), another study reported an inverse relationship between SKP2 and p27 among 40 breast cancers (95), while the third and largest study of 1598 breast cancers reported a significant inverse relationship: among the 313 breast cancers positive for SKP2 a greater percentage (66 %) had low levels of p27 (96). Interestingly, the authors
of the largest study found no predictive or prognostic value of the inverse relationship and did not recommend routine assessment of SKP2 and p27 for node-negative early breast cancer.

In our analysis of 159 tumors for which we had IHC results for both SKP2 and p27, we found no relationship between the expression levels of SKP2 and p27. Eleven percent of tumors expressed high SKP2 and low p27, while 32% of tumors had high SKP2 and high p27. Furthermore, there was no relationship between p27 and ERα or triple-negative status, even though our sample included 95 ERα-negative and 49 triple-negative breast cancers (the largest sample of triple-negative and third largest sample of ERα-negative breast cancers among published results). Given the range of previous results discussed above, our findings are in agreement with some aspects of some previous reports, but not others. For example, we are the first to confirm the findings of Sonoda and colleagues showing that younger women tend to have higher expression of SKP2 (99).

Our most important finding, however, was not about the relationship between SKP2 and p27 expression. In contrast to our findings on p27, we found expression of SKP2 to be significantly positively correlated with pSer10p27 in human breast cancers. Furthermore, the combination of high levels of SKP2 and high pSer10p27 were observed significantly more often in ERα-negative and triple negative breast tumors than in ERα-positive breast cancers. We learned from the cell culture data that suppressing SKP2 in our triple-negative breast cancer model TMX2-28 reduced the phosphorylation of p27 at Ser10 and slowed the cell cycle, while the level of p27 was high both before and after SKP2 knockdown. Therefore, inhibiting SKP2 in triple-negative and basal-like breast
cancers that express high levels of both SKP2 and pSer10p27, regardless of p27 levels, may be an appropriate therapeutic approach.

It appears from the cell culture and IHC studies that levels pSer10p27 may be a better indicator of SKP2-dependent p27 degradation than are levels of p27. But why are pSer10p27 levels more closely associated with SKP2 expression than are p27 levels? We considered whether inconsistencies in relationship between SKP2 and p27 in the published literature could be due to differences and difficulties in scoring immunoreactivity (See Appendix A). In all of the eight studies that examined p27 and SKP2 expression in breast cancer, “low p27” was defined as less than 50% of the cells showing anti-p27-immunoreactivity (92, 94-100). However, the cutoffs used to define SKP2 expression varied among these same eight studies. Depending upon the study, SKP2 expression was considered “high” or “positive” if greater than 5% (100), 10% (92, 94-96, 98) or 50% (97, 99) of cells showed immunoreactivity to the SKP2 antibody. Considerable variability was observed in number of tumors expressing SKP2 and p27. The percentage of tumors that were scored low for p27 ranged from 20 (95) to 64 (100), while the percentage of tumors scored high for SKP2 ranged from 20 (96) to 65 (97). Surprisingly, one of the papers with the highest cut-off for defining SKP2 immunoreactive cells (>50%) reported the greatest percentage of tumors with high SKP2 (97). In none of these studies was intensity of the immunoreactivity considered even though intensity of staining is part of the validated “Allred score” for evaluating hormone receptor status in breast cancer (104) and intensity is routinely used in analysis of protein expression in pathological tissue (67, 68, 101, 105).
In the present study, immunoreactivity of all six proteins was scored for both intensity (negative, weak, moderate, strong) and distribution (diffuse and focal), providing a total of four metrics for analysis (described in detail in the methods). Our metric of diffuse and focal corresponds roughly with greater and less than 50% of cells showing immunoreactivity; therefore it useful to compare results from this metric with published reports. Limiting our results to diffuse and focal we still find no significant relationship between SKP2 and p27 and a highly positive relationship between SKP2 and the pSer10p27. It is unlikely that inconsistencies in scoring alone account for the closer association between SKP2 and pSer10p27 or the differences in results among the published studies.

Other factors, such as low levels but hyperactive SKP2, have been proposed to explain the inconsistent relationship between SKP2 and p27 and prognosis. Additionally p27 inactivation could occur through cytoplasmic sequestration, which is known to occur in other tumors (106-108) and which would result in low nuclear p27 staining that was independent of SKP2 expression. Alternatively p27 expression in breast cancer may be partially regulated by currently unknown mechanisms. Decreased expression of p27 is associated with poor clinical outcome in breast cancer (109-111). Decreased expression of p27 could be due to increased SKP2 and in those cases targeting SKP2 would be appropriate. However, decreased levels of p27 in tumor tissue may be unrelated to SKP2 expression, in which case inhibitors of SKP2 would likely have little effect on tumor proliferation. Results from the TMX2-28 cell model demonstrate that SKP2-mediated cell cycle entry is occurring in the presence of continued high levels of p27 and that inhibition of SKP2 eliminates pSer10p27 and slows cell cycle while only modestly
reducing p27. These results suggest that the combined high levels of SKP2 and pSer10p27 may be a good indicator of whether inhibiting SKP2 will result in slowing cell cycle and proliferation.

The relationship between SKP2 and pSer10p27 is a strong, but not perfect correlation. SKP2 does not directly affect the phosphorylation of p27 at Ser10, thus, as with the relationship between SKP2 and p27 the limited correlation could be due to other factors that affect Ser10 phosphorylation, including but not limited to expression of KIS/MIRK. However the strong relationship between SKP2 and pSer10p27 does suggest that SKP2 is indirectly regulating this phosphorylation event.

The results from the present IHC analysis identified a subset of tumors with poor prognosis and no available therapy, triple-negative tumors, which could respond to inhibitors of SKP2. Agents that can specifically decrease SKP2 expression or activity will provide a significant therapeutic impact and new, targeted therapeutic options for patients who otherwise solely depend on the radiation and chemotherapy available to them. Two studies have shown that the mTOR inhibitor rapamycin can also abrogate SKP2’s actions by increasing degradation of SKP2 protein and interfering with its transcription (112, 113). Additionally, one small molecule, Compound A, has been shown to overcome chemotherapeutic resistance, specifically to dexamethasone, doxorubicin and melphalan (114, 115). Compound A also inhibits cell growth via cell cycle arrest in multiple myeloma cells by blocking recruitment of SKP2 to SCF complexes (114). Another small molecule, SMIP0004 has been shown to decrease SKP2 expression in prostate cancer cells (115). Recently, a number of natural inhibitors of SKP2 expression have been studied in breast cancer cell cultures. These compounds
include curcumin, lycopene, pentagalloylglucose, quercetin, epigallocatechin-3-gallate (EGCG), and gallic acid (116-118). However, further preclinical and clinical studies are needed to verify the efficacy of these compounds.

The results of the present study provide additional and important information about patients with triple-negative and basal-like breast cancers, and provide insight into the importance of alterations in the cell cycle regulatory gene SKP2 on tumor progression. Our findings suggest that SKP2 overexpression could modulate the malignant phenotype of triple-negative and basal-like breast cancers, through regulation of p27 phosphorylation. The precise molecular characterization of p27, direct and indirect phosphorylation by SKP2 will be relevant to delineate the properties of these breast cancer subtypes and identify patients with triple-negative and/or basal-like tumors driven by SKP2 pathway dysregulation. These patients could possibly improve their prognostic outcomes if treated with a targeted molecular therapy aiming to reduce increased SKP2 levels by either small molecules and/or natural inhibitors.
<table>
<thead>
<tr>
<th>Gene</th>
<th>RefSeq Number</th>
<th>Forward Sequence (5'-3')</th>
<th>Reverse Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKP2</td>
<td>NM_005983</td>
<td>CATTTCAGCCCT TTTGGTG</td>
<td>GGGGAAATTCA GAGAATCCA</td>
</tr>
<tr>
<td>p27</td>
<td>NM_004064</td>
<td>TGCAACCGACGA TTCTTCTA</td>
<td>TTCCATGAAGT CAGCGATATG</td>
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<td>CDK2</td>
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<td>TTGTCAAGCTGC TGGATGTC</td>
<td>TGATGAGGGGA AGAGGAATG</td>
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<td>Cyclin E</td>
<td>NM_001238</td>
<td>CCGTACTGAGC TGGGCAA</td>
<td>CAAGCTCACCT CCATTAACCA</td>
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<td>Cyclin D1</td>
<td>NM_053056</td>
<td>TGGCCACAGAT GTGAAGTT</td>
<td>CGGGTCACACT TGATCACTC</td>
</tr>
<tr>
<td>HPRT</td>
<td>NM_000194</td>
<td>ACCCCACGAAGT GTTGGATA</td>
<td>AAGCAGATGGA CACAGAACT</td>
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</table>
### Table 2.2
Clinical and pathological characteristics of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (Range)</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (in years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction Mammoplasty</td>
<td>25.5 (15-55)</td>
<td>40 (80)*</td>
</tr>
<tr>
<td>All Tumors</td>
<td>55 (25-89)</td>
<td>188 (100)</td>
</tr>
<tr>
<td>ERα+</td>
<td>55 (25-89)</td>
<td>93 (49)</td>
</tr>
<tr>
<td>ERα-</td>
<td>54 (29-87)</td>
<td>95 (51)</td>
</tr>
<tr>
<td>NTN†</td>
<td>58 (32-85)</td>
<td>24 (25)</td>
</tr>
<tr>
<td>TN</td>
<td>53 (29-87)</td>
<td>49 (52)</td>
</tr>
<tr>
<td>N/A‡</td>
<td>59.5 (38-86)</td>
<td>22 (23)</td>
</tr>
</tbody>
</table>

**Histology**
- DCIS: 37 (20)%
- IDC: 138 (73)%
- Other: 13 (7)%

**Tumor Grade**
- G1: 23 (12)%
- G2: 48 (26)%
- G3: 105 (56)%
- N/A: 12 (6)%

*Age was not available for 10 reduction mammoplasty tissues; †ERα-negative but either PR or HER2 positive; ‡ERα-negative tumors that did not have HER2 status available
Table 2.3
Expression of SKP2, p27, pSer10p27, Cyclin E, CDK2 and Cyclin D1 in breast tumors and reduction mammoplasty (RM)

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Positive % (N)</th>
<th>P-value</th>
<th>High % (N)</th>
<th>P-value</th>
<th>Diffusea % (N)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SKP2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM</td>
<td>8 (4 of 50)</td>
<td>&lt;0.001</td>
<td>0 (0 of 46)c</td>
<td>&lt;0.001</td>
<td>b</td>
<td>-</td>
</tr>
<tr>
<td>Cancer</td>
<td>59 (111 of 188)</td>
<td></td>
<td>40 (75 of 188)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERα+</td>
<td>33 (31 of 93)</td>
<td>&lt;0.001</td>
<td>16 (15 of 93)</td>
<td>&lt;0.001</td>
<td>6 (2 of 31)</td>
<td>0.016</td>
</tr>
<tr>
<td>ERα-</td>
<td>84 (80 of 95)</td>
<td></td>
<td>63 (60 of 95)</td>
<td></td>
<td>28 (19 of 67)b</td>
<td></td>
</tr>
<tr>
<td>NTNd</td>
<td>75 (18 of 24)</td>
<td>0.002</td>
<td>58 (14 of 24)</td>
<td>0.056</td>
<td>0 (0 of 14)b</td>
<td>0.003</td>
</tr>
<tr>
<td>TNc</td>
<td>98 (48 of 49)</td>
<td></td>
<td>80 (39 of 49)</td>
<td></td>
<td>40 (19 of 48)</td>
<td></td>
</tr>
<tr>
<td><strong>p27</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM</td>
<td>79 (23 of 29)</td>
<td>0.040</td>
<td>69 (109 of 159)</td>
<td>&lt;0.001</td>
<td>61 (95 of 146)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cancer</td>
<td>92 (146 of 159)</td>
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<td></td>
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</tr>
<tr>
<td>ERα+</td>
<td>93 (78 of 84)</td>
<td>0.615</td>
<td>69 (58 of 84)</td>
<td>0.887</td>
<td>71 (55 of 78)</td>
<td>0.165</td>
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<tr>
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<td>68 (51 of 75)</td>
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<td>59 (40 of 68)</td>
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<tr>
<td>NTNd</td>
<td>100 (18 of 18)</td>
<td>0.120</td>
<td>67 (12 of 18)</td>
<td>0.917</td>
<td>56 (10 of 18)</td>
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</tr>
<tr>
<td>TNc</td>
<td>88 (43 of 49)</td>
<td></td>
<td>65 (32 of 49)</td>
<td></td>
<td>58 (25 of 43)</td>
<td></td>
</tr>
<tr>
<td><strong>pSer10p27</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM</td>
<td>24 (8 of 33)</td>
<td>0.006</td>
<td>12 (4 of 33)</td>
<td>0.015</td>
<td>12 (1 of 8)</td>
<td>0.427</td>
</tr>
<tr>
<td>Cancer</td>
<td>50 (80 of 159)</td>
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<td>33 (53 of 159)</td>
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<td>34 (27 of 80)</td>
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<tr>
<td>ERα+</td>
<td>36 (30 of 84)</td>
<td>&lt;0.001</td>
<td>15 (13 of 84)</td>
<td>&lt;0.001</td>
<td>33 (10 of 30)</td>
<td>1.0</td>
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<tr>
<td>ERα-</td>
<td>67 (50 of 75)</td>
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<tr>
<td>NTNd</td>
<td>56 (10 of 18)</td>
<td>0.372</td>
<td>56 (10 of 18)</td>
<td>0.856</td>
<td>30 (3 of 10)</td>
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<td>Cyclin E</td>
<td>Cyclin D1</td>
<td></td>
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<tr>
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<td>70 (59 of 84)</td>
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<tr>
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<td>61 (97 of 158)</td>
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<tr>
<td>ERα⁺</td>
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<td>12 (10 of 84)</td>
<td>70 (59 of 84)</td>
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<td></td>
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<tr>
<td>ERα⁻</td>
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<td>51 (38 of 74)</td>
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<tr>
<td>NTN⁺d</td>
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</tr>
<tr>
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<td>39 (19 of 49)</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cancer</strong></td>
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<td>19 (30 of 157)</td>
<td>61 (97 of 158)</td>
<td></td>
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<tr>
<td>ERα⁺</td>
<td>22 (18 of 82)</td>
<td>12 (10 of 84)</td>
<td>70 (59 of 84)</td>
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</tr>
<tr>
<td>ERα⁻</td>
<td>6 (4 of 71)</td>
<td>27 (20 of 73)</td>
<td>51 (38 of 74)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTN⁺d</td>
<td>0 (0 of 17)</td>
<td>24 (4 of 17)</td>
<td>71 (12 of 17)</td>
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</tr>
<tr>
<td>TN⁺c</td>
<td>6 (3 of 48)</td>
<td>29 (14 of 49)</td>
<td>39 (19 of 49)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Diffuse and Focal staining was evaluated only for tissues that scored positive

*Thirteen tumors and 4 RM tissues with SKP2 reactivity were not evaluated for distribution of staining, therefore the numbers for diffuse and focal are reduced

*Four RM tissues were scored as positive/negative only, therefore the numbers for high/low are reduced

*NTN: Non-triple-negative, tumors that were negative for ERα, but positive for PR and/or HER2 receptors

*TN: Triple-negative, tumors that were negative for ERα, PR and HER2 receptors
Table 2.4  
Relationship between SKP2 expression in breast cancer and cell cycle genes

<table>
<thead>
<tr>
<th>SKP2 Expression % (N)</th>
<th>SKP2 Expression % (N)</th>
<th>P-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive</strong></td>
<td><strong>Negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27 Positive</td>
<td>94 (92 of 98)</td>
<td>89</td>
<td>0.231</td>
</tr>
<tr>
<td>pSer10p27 Positive</td>
<td>67 (66 of 98)</td>
<td>23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CDK2 Positive</td>
<td>35 (33 of 94)</td>
<td>42</td>
<td>0.367</td>
</tr>
<tr>
<td>Cyclin E Positive</td>
<td>56 (54 of 97)</td>
<td>40</td>
<td>0.035</td>
</tr>
<tr>
<td>Cyclin D1 Positive</td>
<td>78 (76 of 97)</td>
<td>84</td>
<td>0.418</td>
</tr>
<tr>
<td><strong>Positive</strong></td>
<td><strong>Negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27 Positive</td>
<td>74 (51 of 69)</td>
<td>64</td>
<td>0.202</td>
</tr>
<tr>
<td>pSer10p27 Positive</td>
<td>55 (38 of 69)</td>
<td>17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CDK2 Positive</td>
<td>7 (5 of 67)</td>
<td>20</td>
<td>0.031</td>
</tr>
<tr>
<td>Cyclin E Positive</td>
<td>26 (18 of 68)</td>
<td>13</td>
<td>0.040</td>
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<td>Cyclin D1 Positive</td>
<td>65 (44 of 68)</td>
<td>59</td>
<td>0.457</td>
</tr>
</tbody>
</table>

p-values reflect results of $\chi^2$ testing the relationship between expression of SKP2 and one other cell cycle gene.

Note: The percentages (and numbers) provided are based on the positive and high expression of specific cell cycle genes in tumors when SKP2 was evaluated as positive/negative and as high/low.
### Table 2.5

**Relationship between strong protein expression and age**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Relationship</th>
<th>N (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SKP2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total tumors</td>
<td>Inverse</td>
<td>159 (0.005)</td>
</tr>
<tr>
<td>ERα+</td>
<td>84 (0.261)</td>
<td></td>
</tr>
<tr>
<td>ERα-</td>
<td>75 (0.014)</td>
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</tr>
<tr>
<td>NTN</td>
<td>18 (0.835)</td>
<td></td>
</tr>
<tr>
<td>TN</td>
<td>49 (0.029)</td>
<td></td>
</tr>
<tr>
<td><strong>p27</strong></td>
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<td></td>
</tr>
<tr>
<td>Total tumors</td>
<td>Inverse</td>
<td>159 (0.530)</td>
</tr>
<tr>
<td>ERα+</td>
<td>84 (0.929)</td>
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</tr>
<tr>
<td>ERα-</td>
<td>75 (0.440)</td>
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</tr>
<tr>
<td>NTN</td>
<td>Positive</td>
<td>18 (0.018)</td>
</tr>
<tr>
<td>TN</td>
<td>49 (0.219)</td>
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</tr>
<tr>
<td><strong>pSer10p27</strong></td>
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<td></td>
</tr>
<tr>
<td>Total tumors</td>
<td>Positive</td>
<td>159 (0.003)</td>
</tr>
<tr>
<td>ERα+</td>
<td>84 (0.237)</td>
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</tr>
<tr>
<td>ERα-</td>
<td>75 (0.004)</td>
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</tr>
<tr>
<td>NTN</td>
<td>Positive</td>
<td>18 (0.021)</td>
</tr>
<tr>
<td>TN</td>
<td>Positive</td>
<td>49 (0.020)</td>
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<tr>
<td><strong>CDK2</strong></td>
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<td></td>
</tr>
<tr>
<td>Total tumors</td>
<td>Positive</td>
<td>153 (0.212)</td>
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<tr>
<td>ERα+</td>
<td>82 (0.759)</td>
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<tr>
<td>ERα-</td>
<td>Positive</td>
<td>71 (0.032)</td>
</tr>
<tr>
<td>NTN</td>
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</tr>
<tr>
<td>TN</td>
<td>48 (0.103)</td>
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</tr>
<tr>
<td><strong>Cyclin E</strong></td>
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</tr>
<tr>
<td>Total tumors</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>ERα+</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>ERα-</td>
<td>73 (0.612)</td>
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<tr>
<td>NTN</td>
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<tr>
<td>TN</td>
<td>49 (0.471)</td>
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<tr>
<td><strong>Cyclin D1</strong></td>
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<td>Total tumors</td>
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<td>ERα+</td>
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<tr>
<td>NTN</td>
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<td>TN</td>
<td>Positive</td>
<td>49 (0.017)</td>
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* Relationship could not be established due to zero tumors with strong positive staining.
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<tr>
<th>Cell Cycle Gene</th>
<th>DCIS % (N)</th>
<th>IDC % (N)</th>
<th>P-value</th>
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<tr>
<td>SKP2</td>
<td>3 (1 of 37)</td>
<td>19 (26 of 138)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>p27</td>
<td>38 (14 of 37)</td>
<td>33 (36 of 109)</td>
<td>0.955</td>
</tr>
<tr>
<td>pSer10p27</td>
<td>11 (4 of 37)</td>
<td>17 (19 of 109)</td>
<td>0.467</td>
</tr>
<tr>
<td>CDK2</td>
<td>27 (9 of 33)</td>
<td>1 (1 of 107)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>0 (0 of 36)</td>
<td>1 (1 of 108)</td>
<td>0.106</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>27 (10 of 37)</td>
<td>30 (32 of 108)</td>
<td>0.009</td>
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Table 2.7

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<th>Grade 2 % (N)</th>
<th>Grade 3 % (N)</th>
<th>P-value</th>
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<td>26 (27 of 105)</td>
<td>0.004</td>
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<td>p27</td>
<td>3 (6 of 20)</td>
<td>28 (11 of 40)</td>
<td>37 (32 of 87)</td>
<td>0.690</td>
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<tr>
<td>pSer10p27</td>
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<td>8 (3 of 40)</td>
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<tr>
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<td>5 (4 of 85)</td>
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<td>1 (1 of 86)</td>
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<td>Cyclin D1</td>
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<td>38 (15 of 40)</td>
<td>26 (23 of 87)</td>
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</table>
Figure 2.1

ERα mRNA Levels (Normalized to HPRT)

- TMX2-28
- MCF-7
- MDA-MB-231
- SKBR-3

(Figure 2.1)
B

PR mRNA Levels (Normalized to HPRT)

TMX2-28  MCF-7  MDA-MB-231  SKBR-3

(Figure 2.1)
(Figure 2.1)

C

HER2 mRNA Levels (Normalized to HPRT)

**HER2**

***

- TMX-2-28
- MCF-7
- MDA-MB-231
- SKBR-3
**Figure 2.1** TMX2-28 is a triple-negative derivative of the MCF-7 breast cancer cell line. Upper panel: Relative mRNA expression of (A) ERα, (B) PR, and (C) HER2 were determined by real time qRT-PCR and normalized to HPRT. Differences among cell lines were analyzed with one-way ANOVA and post hoc t-tests with Bonferroni correction; *p<0.05, ***p<0.001; Panel A & B: MCF-7 differed significantly from all other cell lines Panel C: SKBR-3 differed significantly from all other cell lines. Lower panel: ERα, PR, and HER2 protein expression was determined by immunohistochemistry.
(Figure 2.2)
Figure 2.2 SKP2 is overexpressed in TMX2-28 cells. (A) Relative SKP2 mRNA expression in TMX2-28, MCF-7, and MDA-MB-231 cells was determined by real time qRT-PCR. SKP2 protein expression was determined by Western immunoblotting (GAPDH as loading control). (B) TMX2-28 cells were stably transfected with either a negative control pGIPZ shRNAmir (TMX2-28-NC), or a pGIPZ shRNAmir targeted against SKP2 (TMX2-28-S2, TMX2-28-MC). Relative SKP2 mRNA and protein expression was determined as in panel A. Differences in mRNA levels among cell lines were analyzed using one-way ANOVA and post hoc t-tests with a Bonferroni correction (*p<0.05; **p<0.01); Panel A: TMX2-28 differed significantly from other cell lines; Panel B: TMX2-28 and TMX2-28-NC differed significantly from other groups but not from each other.
Figure 2.3

(A) Relative p27 mRNA Expression

(B) Cyclin E mRNA Levels (Normalized to HPRT)

(C) Relative CDK2 mRNA Expression

(Figure 2.3)
Figure 2.3 Expression of SKP2 pathway genes in TMX2-28 cells. Relative mRNA expression of (A) p27, (B) Cyclin E, (C) CDK2, and (D) Cyclin D1 were determined by real time qRT-PCR and normalized to HPRT. Protein expression of (A) p27, (B) Cyclin E, (C) CDK2, and (D) Cyclin D1 were determined by Western immunoblotting (GAPDH as loading control). Differences in mRNA levels among cell lines were analyzed using one-way ANOVA and post hoc t-tests with a Bonferroni correction for comparisons between TMX2-28 cells and MCF-7 cells †p<0.005; for comparisons between TMX2-28 cells and MDA-MB-231 cells ‡p<0.05; for comparisons between TMX2-28-NC and TMX2-28-S2 or TMX2-28-MC cells **p<0.01, ***p<0.001.
Figure 2.4 SKP2 knockdown decreased the percentage of cells in the S-phase of the cell cycle. TMX2-28-NC, TMX2-28-S2, and TMX2-28-MC cells were fixed and stained with propidium iodide. Cell cycle analysis was assessed by flow cytometry (*p<0.05; **p<0.01).
Figure 2.5 SKP2 is overexpressed in ERα-negative, basal-like breast tumors. Relative SKP2 mRNA expression was determined in 30 frozen breast carcinoma samples using real time qRT-PCR normalized to HPRT. Numbers 1-18 indicates ERα-positive tumors, while ERα-negative tumors were assigned numbers 19-30. ERα-positive tumors that had CK 5 and/or CK17 positivity were classified as basal-cytokeratin-expressing tumors and are represented by striped bars. ERα-negative tumors that also had expression of CK 5 and/or 17 were termed basal-like and are represented by black bars.
Figure 2.6 Scoring system used to evaluate cell cycle proteins in human tissue. Intensity of immunoreactivity of epithelial cells was used to assign scores of negative, weak, moderate, and strong staining. Images are from tumor samples with insets show increasing intensity of protein staining. Scale bar = 100 μm.
Figure 2.7
Figure 2.7 Expression of SKP2 in breast cancer tumors is highly correlated with pSer10p27 (Case 1). Breast tumors TMAs and tissue slides were prepared, stained, and scored as described in the Methods. Representative images of tumor tissue show the relationships between the expression of SKP2, p27, pSer10p27, CDK2, Cyclin E, and Cyclin D1 when SKP2 expression is high. Scale bar =100 μm.
(Figure 2.8)
**Figure 2.8** Expression of SKP2 in breast cancer tumors is highly correlated with pSer10p27 (*Case 2*). Breast tumors TMAs and tissue slides were prepared, stained, and scored as described in the Methods. Representative images of tumor tissue show the relationships between the expression of SKP2, p27, pSer10p27, CDK2, Cyclin E, and Cyclin D1 when SKP2 expression is high. Scale bar =100 µm.
(Figure 2.9)
Figure 2.9 Expression of SKP2 in breast cancer tumors is highly correlated with pSer10p27 (Case 3). Breast tumors TMAs and tissue slides were prepared, stained, and scored as described in the Methods. Representative images of tumor tissue show the relationships between the expression of SKP2, p27, pSer10p27, CDK2, Cyclin E, and Cyclin D1 when SKP2 expression low. Scale bar =100 μm.
(Figure 2.10)
Figure 2.10 Expression of SKP2 in breast cancer tumors is highly correlated with pSer10p27 (Case 4). Breast tumors and RM TMAs and tissue slides were prepared, stained, and scored as described in the Methods. Representative images of tumor tissue show the relationships between the expression of SKP2, p27, pSer10p27, CDK2, Cyclin E, and Cyclin D1 when SKP2 expression low. Scale bar =100 µm.
CHAPTER 3

THE RAS HOMOLOG GENE FAMILY, MEMBER A (RHOA) PATHWAY MEDIATES PROTEASE-INDEPENDENT INVASIVE BEHAVIOR IN A TRIPLE-NEGATIVE BREAST CANCER CELL LINE

Introduction

Metastasis is dependent upon cancer cells being able to invade surrounding extracellular matrix (ECM) and adapt to different microenvironments within the primary tumor, the ECM, blood and/or lymphatic systems, and finally, to establish a new niche in a distant tissue (41-43). The proteins that control cell-cell and ECM interactions are thought to play a role in tumor cell invasion and metastasis by controlling cell morphology, motility, and interactions with the tumor microenvironment (41, 44). Thus, such proteins provide attractive therapeutic targets.

Two distinct morphological/functional mechanisms are known for single cell migration in tissues: fibroblast-type mesenchymal invasion, and leucocyte-type amoeboid invasion. Mesenchymal movement is characterized by an elongated cellular shape, and involves the use of proteases that cause cellular lysis in tissues, thereby creating a path through which cells can invade (44-47). Amoeboid migrating cells have a rounded morphology and their movement is protease–independent. Instead, cells find paths through the ECM by pushing and squeezing through regions of adequate size (43, 44, 47, 51, 52).

Matrix Metalloproteinases (MMPs) are a family of endopeptidases capable of degrading ECM components. MMPs are synthesized and secreted as zinc dependent
proenzymes that require activation prior to becoming proteolytically active (119). It is known that MMP-1, MMP-2, and MMP-9 are responsible for degrading fibrillar collagen as well as collagen type IV, the primary components of the connective tissue matrix and basement membranes respectively (120-122). A number of studies have shown that there is an association between high MMP-2 and MMP-9 expression and the invasiveness of tumors when compared to normal breast epithelium. Cells undergoing epithelial to mesenchymal transition (EMT) in particular have high expression of MMP-2 and MMP-9 (123-128).

Migration of cancer cells is dependent on Ras Homolog Gene Family, Member A (RhoA) signaling (43, 44, 47, 51, 52). RhoA is a member of the Rho superfamily of GTPases that acts as a molecular switch to control signal transduction (55). It carries out its actions through activation of its two targets: Rho-associated, coiled-coil containing protein kinase (ROCK) 1 and ROCK 2, which in turn regulate different facets of actin dynamics including stress fiber assembly, cell contraction, actin-filament stabilization, and focal adhesion organization (56-59). Up-regulation of RhoA mRNA and protein is well documented for a variety of human cancers and has been positively correlated with cancer metastasis (64-66).

In this study, we use the Tamoxifen-selected, MCF-7 derivative, TMX2-28 breast cancer cell line. TMX2-28 cells are triple-negative (Figure 2.1) and have acquired a mixed basal/luminal cytokeratin profile, suggestive of a more basal-like phenotype (67-69). Morphologically, TMX2-28 cells retained an epithelial cell shape similar to MCF-7. Behaviorally, TMX2-28 cells are highly invasive, as assessed through the ability to invade though Matrigel, when compared to MCF-7 and MDA-MB-231 breast cancer
cells (67). These phenotypic characteristics suggest that these cells may exhibit an amoeboid mode of invasion. However, the importance of matrix metalloproteinases (MMPs) in invasion was not experimentally excluded nor was evidence of amoeboid invasion by these cells demonstrated.

A cDNA microarray comparing gene expression of TMX2-28 cells to their parent cell line, MCF-7, indicated that the Rho family member, RhoE is downregulated in TMX2-28 (Table 3.1). RhoE was the first member of the Rho kinase family to be identified (129). Unlike the other family members, RhoE does not act as a classic GTPase switch, as it does not hydrolyze GTP (129-131). RhoE functions by binding to and inhibiting the RhoA effector ROCK 1 (but not ROCK 2). This interaction also results in the phosphorylation of RhoE by ROCK 1, which increases RhoE’s stability and activity (73, 132). In addition, RhoE binds to p190RhoGAP and increases its activity toward RhoA, thus promoting the formation of inactive GDP-bound RhoA (133). This data led to the question, since TMX2-28 cells downregulate the expression of RhoA inhibitor RhoE, do TMX2-28 upregulated RhoA, and if so, is the upregulation of RhoA resulting in the increased invasiveness of these cells? In the present study we seek to further define the molecular aspects underlying their invasive mechanism.

**Materials and Methods**

**Cell Culture:** TMX2-28, MCF-7, and HeLa cells were maintained in T-75 culture flasks at 37°C and 5% CO₂, in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with calf serum (5%), insulin (10 µg/ml), non-essential amino acids (100X), penicillin-streptomycin (10,000 µg/ml), and L-glutamine (200 mM). MDA-MB-
231 cells were maintained in Leibovitz-15 (L-15) medium supplemented with 10% FBS at 37°C and 0% CO₂. All cells were passaged when near 80% confluence.

**RNA and Protein Isolation:** Total RNA (n=3 biological samples) was isolated with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer’s suggestions and protocols as previously described (67, 68, 101). RNA was then treated with Turbo DNA-Free (Ambion, Austin, TX) to remove any DNA contamination. The quality of the RNA was assessed by 260/280 nm spectrophotometer readings (Nanodrop 8000; Thermo Scientific, Wilmington, DE). Protein-containing cell lysates were isolated from cell cultures (n=3 biological samples) with pre-chilled SDS lysis buffer (1% SDS, 0.06 M Tris-HCL, and 10% glycerol) according to our standard laboratory protocols (67, 68, 101). Extracts were used for Western immunoblotting.

**Western Immunoblotting:** Protein lysates were mixed with NuPage sample buffer and reducing agent (Invitrogen, Carlsbad, CA), heated at 70°C for 10 min, separated on a Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA) and then transferred to an Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) according to manufacturer’s protocols. Membranes were incubated in blocking buffer (5% nonfat dry milk/Tris buffered saline and 0.1% Tween 20) for 1 hour at room temperature with gentle shaking. Membranes were then incubated with anti-RhoA rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) diluted 1:1000 overnight at 4°C, followed by incubation with the secondary antibody, anti-rabbit IgG linked to horseradish peroxidase; diluted 1:1000 (Cell Signaling Technology, Danvers,
MA), for 1 hour at room temperature. Chemiluminescent signals were detected with SuperSignal West Pico Kit and protocol (Peirce, Rockford, IL), and imaged using the G.BOX Chemi HR-16 (Syngene, Fredrick, MD). Membranes were stripped using Restore stripping buffer (Thermo Scientific, Rockford, IL) and reprobed for glyceraldehyde-3-phosphate (Cell Signaling Technology, Danvers, MA; 1:10,000).

**Quantitative Real Time Reverse Transcriptase PCR (qRT-PCR):** RNA samples (n=3 biological samples) were reverse transcribed and amplified using the One Step RT-PCR kit (Qiagen, Valencia, CA) in the Roche LightCycler (Roche, Indianapolis, IN). Total RNA (75 ng) was incubated with Qiagen RT-PCR master mix including primers (25µM each) and SYBR Green I nucleic acid stain (2X; Invitrogen, Carlsbad, CA, product number S7563) in pre-cooled capillaries (Roche, Indianapolis, IN) and was reversed transcribed (50°C for 30 min). Following reverse transcription, samples were heated to 95°C for 15 min to activate the HotStar Taq DNA polymerase and to simultaneously inactivate the reverse transcriptase. The generation of amplified products was monitored over 45 PCR cycles by fluorescence of intercalating SYBR Green. Each cycle consisted of the following steps: 1. Denaturation at 95°C for 15 sec. 2. Annealing at 60°C for 15 sec. 3. Extension at 72°C for 30 sec. Relative mRNA levels were normalized to hypoxanthine ribosyltransferase (HPRT) levels to control for RNA quality and concentration. Gene specific primers, designed using Primer3 software (http://frodo.wi.mit.edu/), and purchased from Integrated DNA Technologies, Inc. (Coralville, IA), were used for MMP-1, MMP-2, MMP-9, SLUG, ZEB 1, ZEB 2, Vimentin, Fibronectin, N-Cadherin, RhoA, ROCK 1, ROCK 2, and HPRT (Table 3.2).
**Matrigel Invasion Assay:** BD BioCoat Matrigel Invasion Chambers and 8.0 μm pore size PET track-etched membranes (Becton Dickinson, Franklin Lakes, NJ) were used according to the manufacturer’s protocol. Cells (5 x 10^4 cells total; n=3 biological samples) were plated in the top chamber containing a basal medium (DMEM). A medium rich in nutrients (DMEM supplemented with 10% FBS) was used in the bottom chamber as a chemoattractant. Twenty-two to twenty-four hours later, cells were fixed and stained using 10% formalin and crystal violet respectively. Cell numbers were determined from microphotographs taken over four (non-overlapping) areas of the membrane.

**Zymography:** Gelatin zymography was performed using precast polyacrylamide gels containing 10% gelatin (Bio-Rad, Hercules, CA). Collected serum free, conditioned medium (n=3 biological samples) was mixed in equal volumes with sample buffer (0.5 M Tris-HCL, glycerol, 10% SDS, 0.1% Bromophenol blue). To prepare serum free conditioned medium, cells were seeded into 24-well tissue culture plates at a concentration of 1x10^5 cells/well in normal culture medium. Twenty-four hours post seeding, cells were washed three times with phosphate-buffered saline followed by incubation in serum free medium. Serum free conditioned medium was collected 48 hours later and stored at -20°C until use. Twenty to fifty microliters of mixed sample was loaded into the gel and electrophoresed at 100 V for 90 min. Following electrophoresis, gels were rinsed with 1X renaturing buffer (2.5% Triton X-100) for 30 min with gentle agitation. Gels were then transferred to 1X developing buffer (50 mM
Tris base, 200 mM NaCl, 5 mM CaCl₂, 0.02% Brij 35) for 30 min at room temperature with gentle agitation to equilibrate the gel. Gels were then incubated overnight at 37°C with gentle agitation in fresh 1X developing buffer. Gels were then stained for 1 hour with 0.5% Coomassie Blue, de-stained twice for 30 min each with de-staining solution [Methanol: Acetic Acid: Water (50:10:40)], and rehydrated in H₂O for 10 min.

**Inhibitor and siRNA Treatments:** For inhibitor assays, cells (n=3 biological samples) were treated with the Rho Kinase pathway inhibitor H-1152 (Calbiochem, Billerica, MA) at 0, 20, and 100 µM concentrations for 24 hours (134, 135). Concentrations were determined by dose response testing (See Appendix B). Following treatment, either RNA was isolated as mentioned above, or cells were seeded into invasion chambers in the respective concentrations of inhibitor.

For siRNA assays, RhoA specific and negative control (random sequence without homology to any human gene) siRNAs (RhoA-siRNA and NC-siRNA respectively) were designed using Ambion’s (Austin, TX) siRNA Template Design Tool (RhoA sense: CCUUAUAGGUACUGUGUAATT; antisense: UUACACAGUAACUAUAAAGGTA; negative control sense: UUAUCGGCAAAUUCUUAUCGGACAGAG; antisense: UUGAUAAAGGAAUUGGCGAUGGACAGAG). DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and siRNA generated using Silencer® siRNA Construction Kit (Ambion, Austin, TX) per manufactures’ protocols. TMX2-28, MCF-7 (non-invasive control) and HeLa (RhoA dependent invasion control) cells were seeded into 100 mm dishes at a concentration of 10⁶ cells/dish and allowed to attach overnight (n=3 biological samples). On the day of
transfection, HiPerFect transfection agent (Qiagen, Valencia, CA) and 25 mg/well of a
RhoA specific or a negative control siRNA was diluted into Opti-MEM (Invitorgen,
Carlsbad, CA) and added to cells. Following incubation under normal growth conditions
for 48 hours, cells were harvested for both RNA and Protein isolation or invasion assays.

Statistical Analyses: Data were analyzed and graphed with GraphPad Prism
Version 3.02 (GraphPad Software, Inc., San Diego, CA). For ANOVA and post hoc
comparisons, significance was set at p<0.05.

Results

TMX2-28 cells do not express MMP-1 mRNA or active MMP-2 or MMP-9 protein

In breast cancer, MMPs have been shown to be important players in mesenchymal
invasion, specifically MMP-1, -2, and -9 (120-122, 136). To determine the possible
involvement of MMPs in the invasion mechanism of TMX2-28 cells, MMP-1, -2, and -9
mRNA expression was analyzed by qRT-PCR. As shown in Figure 3.1 A-C, TMX2-28
cells have significantly lower MMP-1 mRNA expression (no detectable level) when
compared to the mesenchymal MDA-MB-231 cells. However, there was no significant
difference in mRNA expression of MMP-2 or MMP-9 among the cell lines. MMPs are
synthesized and secreted as zinc dependent pro-enzymes that require activation (119). To
assess activity of MMP-2 and -9, conditioned media from all cell lines were run on a
zymography gel. It was determined that although TMX2-28 cells have mRNA
expression, they do not express active MMP-2 or MMP-9 enzymes (Figure 3.1 D). These
results indicate that TMX2-28 cells do not rely on MMP activity to degrade the ECM.
TMX2-28 cells have little to no expression of six common EMT genes

Epithelial-mesenchymal transition (EMT) is a well-recognized process facilitating migration and invasion of cancer cells during metastasis (137). An increase in expression of a number of genes including SLUG, ZEB1, ZEB2, Fibronectin, Vimentin, and N-Cadherin is characteristic of this process (138-143). The mRNA levels of these genes were assessed by qRT-PCR to determine whether TMX2-28 cells have increased expression. Gene expression of SLUG, ZEB1, ZEB2, Fibronectin and Vimentin, was found to be low in TMX2-28 cell cultures (Table 3.3), similar to that in the non-invasive parent cell line, MCF-7. The expression of all six genes was significantly higher in the invasive, mesenchymal-like MDA-MB-231 cell line than in either the non-invasive MCF-7 or the invasive TMX2-28 cells. While expression of N-Cadherin in TMX2-28 was slightly above that in MCF-7 cells, it was still significantly less than in MDA-MB-231 cells. Together with results on MMP expression, these data suggest that TMX2-28 cells do not utilize the protease-dependent, mesenchymal invasion mechanism.

RhoA pathway genes are overexpressed in TMX2-28 cells

Next, we questioned whether TMX2-28 cells exploit the Rho kinase pathway, specifically RhoA pathway signaling. RhoA mRNA was overexpressed in TMX2-28 cells at both the mRNA and protein levels (Figure 3.2 A) when compared to levels in MCF-7 and MDA-MB-231 cells. Additionally, the major downstream targets of RhoA, ROCK 1 and ROCK 2, were also found to be overexpressed in these cells (Figure 3.2 B and C). These data suggest that TMX2-28 cells may take advantage of RhoA dependent, protease-independent invasion process.
Blocking RhoA pathway activity decreased migratory and invasive behaviors

We next designed experiments to determine whether blocking the activity of RhoA resulted in impairment of migratory and invasive behavior. For these studies, TMX2-28 cells were treated with the ROCK inhibitor H-1152 to block RhoA pathway signaling. HeLa (RhoA dependent invasion control), and MCF-7 (non-invasive control) cells were also used as positive and negative controls respectively. As expected, blocking RhoA activity in the RhoA dependent HeLa cells resulted in significant abrogation (~66%) of invasive behavior. When RhoA activity was blocked in TMX2-28 cells, there was significant inhibition of migration and invasion (Figure 3.3). Migration was reduced by ~90% and ~94% when cells were treated with 20 and 100 µM of the inhibitor respectively. In contrast, the reduction of invasion was dose-dependent, ~62% inhibition after treatment with 20 µM and ~92% inhibition after treatment with 100 µM.

To further investigate the effect of blocking RhoA signaling on migration and invasion, siRNA specifically targeting RhoA was used to knockdown expression in TMX2-28, MCF-7 and HeLa cells (Figure 3.4 A). Knockdown of RhoA in these cells resulted in a significant decrease in both migratory (50%) and invasive (52%) behavior (Figure 3.4 B), confirming the phenotypic changes observed when RhoA pathway signaling was blocked using H-1152 (Figure 3.3). This effect was specific and was not seen when cells were transfected with the negative control siRNA. Together these data show that RhoA plays an important role in the ability of TMX2-28 cells to be highly invasive and migratory while maintaining an epithelial morphology.
Discussion

Cancer cells can use alternative mechanisms of motility for invasion (45, 47). Two distinct mechanisms have been identified, the more widely utilized, protease-dependent, mesenchymal, and the less utilized, protease-independent, amoeboid movements (44-47). In breast cancer, MMPs have been tied to mesenchymal cell movement (128), however blocking MMP activity was unsuccessful in preventing growth and metastasis of late stage cancers in clinical trials (46, 144). Additionally, a study by Wolf and colleagues showed that when triple-negative, mesenchymal, MDA-MB-231 breast cancer cells were challenged with MMP inhibitors, these cells adapted a round, epithelial-like morphology and continued to be invasive (47). These studies suggest that breast cancer cells have the ability to utilize an amoeboid invasion mechanism. Thus, determining the mechanisms behind this mode of invasion is critical for the development of therapies to block the metastatic process.

The primary objective of the present study was to better define the molecular aspects underlying invasion, in TMX2-28 cells as a model of triple-negative, basal-like breast cancer. We previously reported that the Tamoxifen-selected derivative of MCF-7 cells, TMX2-28, are ERα negative and highly invasive (67-69); comparable to MDA-MB-231 cells, an established breast cancer cell line known for being highly invasive and exploiting the proteolytic-dependent, mesenchymal mode of invasion. The rounded, epithelial-like cell morphology of TMX2-28 cells suggests an amoeboid mode of cellular invasion, however, this was not investigated.

The Tamoxifen-selected, MCF-7 derived TMX2-28 cells were used as a model to study migration and invasion in triple-negative, basal-like breast cancer. Previous studies
of gene expression and IHC identified TMX2-28 cells as triple-negative (Chapter 2) and having a mixed basal/luminal CK expression profile (Chapter 4) (68). In the present paper, our initial inquiry focused on experimentally excluding proteolytic pathways in TMX2-28 invasion. We determined that TMX2-28 cells do not express MMP-1 mRNA or active MMP-2 or -9 protein, nor do they have expression of genes known to increase in expression during EMT. Interestingly, a cDNA microarray comparing gene expression of TMX2-28 cells to their parent cell line, MCF-7, indicated that a number of proteases, MMPs, and adhesion molecules are downregulated in TMX2-28 (Table 3.1) (67, 68). These data together with the lack of MMP-1, -2, or -9 or EMT gene expression indicate that proteases are not critical to TMX2-28’s invasion mechanism and thus they do not utilize a mesenchymal migration mechanism.

The next logical research direction we took was to determine which pathway TMX2-28 cells use to migrate. cDNA microarray analysis indicated the TMX2-28 cells downregulate the RhoE, which acts as an inhibitor of RhoA (133). It is known that RhoA signaling is important for many types of migration, and up-regulation of RhoA in cancer has been associated with cancer metastasis (45, 46, 64-66). It was found that TMX2-28 cells overexpress RhoA mRNA and protein. Additionally, they overexpress mRNA from RhoA’s two main targets ROCK 1 and 2. This provides three potential targets for therapy development against metastasis. Furthermore, inhibition of RhoA pathway activity through the use of either the ROCK inhibitor H-1152, or a RhoA specific siRNA resulted in decreased migratory and invasive behavior.

Based on these observations, we can conclude that TMX2-28 cells do not use protease dependent mesenchymal movements as their primary invasion mechanism. Their
rounded morphology, use of RhoA, and lack of mesenchymal phenotype, supports our hypothesis that TMX2-28 use amoeboid movement. Furthermore, the cDNA microarray mentioned previously, also revealed that TMX2-28 cells expressed mRNA for a number of genes known to regulate cell membrane morphology and control cytoskeletal organization (Table 3.1). These included mitogen inducible gene 2 (MIG2). MIG2 is a component of cell-ECM adhesion sites that control cell shape and spreading. MIG2 promotes cellular dynamics though the recruitment of Migfilin and its interactions with Filamin and actin (67, 145, 146). A study by Gozgit et. al. showed that MIG2 is overexpressed in TMX2-28 cells as well as in breast cancer tissues (67). Knockdown of MIG2 also resulted in reduced invasive behavior. RhoA signaling is known to be involved in focal adhesion formation along with MIG2 (145, 147, 148). From this knowledge one could suggest that RhoA and MIG2 may work collectively to facilitate amoeboid invasion. Further investigation into how these two proteins may interact is necessary for complete understanding of this mechanism.

An understanding of the mechanisms behind cell migration and tumor metastasis is critical given that metastasis accounts for the majority of breast cancer deaths. Collectively, the results of our study suggest that TMX2-28 cells exploit a RhoA dependent, proteolytic-independent invasion mechanism. Targeting the RhoA pathway in triple-negative, basal-like breast cancer cells that have a proteolytic-independent invasion mechanism may provide therapeutic strategies for the treatment of cancer patients with increased risk of metastasis.
Table 3.1
Genes associated with cytoskeleton organization, protease secretion, adhesion, and membrane morphology differentially regulated in TMX2-28 as compared with MCF-7

<table>
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<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Accession Number</th>
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<tbody>
<tr>
<td>ADAM metallopeptidase with thrombospondin type 1 motif</td>
<td>ADAMTS19</td>
<td>NM_133638.1</td>
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<tr>
<td>Matrix metallopeptidase-like 1 (Matrix metallopeptidase 25)</td>
<td>MMPL1</td>
<td>NM_004142.1</td>
</tr>
<tr>
<td></td>
<td>(MMP25)</td>
<td></td>
</tr>
<tr>
<td>Protease, Serine, 23</td>
<td>SPUVE</td>
<td>NM_007173.1</td>
</tr>
<tr>
<td></td>
<td>(PRSS23)</td>
<td></td>
</tr>
<tr>
<td>TIMP metallopeptidase inhibitor 1</td>
<td>TIMP1</td>
<td>NM_003254.1</td>
</tr>
<tr>
<td>TIMP metallopeptidase inhibitor 2</td>
<td>TIMP2</td>
<td>NM_003255.2</td>
</tr>
<tr>
<td>Neuronal cell adhesion molecule</td>
<td>NRCAM</td>
<td>NM_005010.1</td>
</tr>
<tr>
<td>Fibronectin leucine rich transmembrane protein 3</td>
<td>FLRT3</td>
<td>NM_013281.1</td>
</tr>
<tr>
<td>Fibroblast growth factor 13</td>
<td>FGF13</td>
<td>NM_004111.1</td>
</tr>
<tr>
<td>Selectin L</td>
<td>SELL</td>
<td>NM_000655.2</td>
</tr>
<tr>
<td>Ras homolog gene family, member E</td>
<td>RHOE</td>
<td>NM_001254738.1</td>
</tr>
<tr>
<td>EGF-containing fibulin-like extracellular matrix protein 1</td>
<td>EFEMP1</td>
<td>NM_004105.2</td>
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<tr>
<td>Carcinoembryonic antigen-related cell adhesion molecule 5</td>
<td>CEACAM5</td>
<td>NM_004363.1</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>CTSD</td>
<td>NM_001909.4</td>
</tr>
<tr>
<td>Cathepsin F</td>
<td>CTSF</td>
<td>NM_003793.3</td>
</tr>
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<td>CTSS</td>
<td>NM_001199739.1</td>
</tr>
<tr>
<td>Integrin β8</td>
<td>ITGB8</td>
<td>NM_002214.2</td>
</tr>
<tr>
<td>Integrin α6</td>
<td>ITGA6</td>
<td>NM_000210.1</td>
</tr>
<tr>
<td>Laminin, gamma 1</td>
<td>LAMC1</td>
<td>NM_002293.3</td>
</tr>
<tr>
<td>Laminin, beta 1</td>
<td>LAMB1</td>
<td>NM_002291.1</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>VTN</td>
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<tr>
<td>Serpin peptidase inhibitor, clade E member 1</td>
<td>SERPINE1</td>
<td>NM_000602.3</td>
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<tr>
<td>Mitogen inducible gene 2</td>
<td>MIG2</td>
<td>NM_006832</td>
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http://www.nature.com/bjc/journal/v97/n6/suppinfo/6603926s1.html?url=/bjc/journal/v97/n6/full/6603926a.html; (68)
Table 3.2
Primers used to detect relative mRNA expression of genes

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<tr>
<th>Gene</th>
<th>RefSeq Number</th>
<th>Forward Sequence (5'-3')</th>
<th>Reverse Sequence (5'-3')</th>
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<tbody>
<tr>
<td>ERα</td>
<td>NM_000125</td>
<td>ATGATCAACTGGG CGAAGAG</td>
<td>GATCTCCACCATTG CCCTCTA</td>
</tr>
<tr>
<td>PR</td>
<td>NM_000926.4</td>
<td>GGAAGGGCTACGA AGTCAA</td>
<td>TAACTTGCATGAT CTTGTCAAACCA</td>
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<tr>
<td>HER2</td>
<td>M11730</td>
<td>CCCAGGCCTGAAT ATGTTGA</td>
<td>CTCTGGGTTCTCTG CCGTAG</td>
</tr>
<tr>
<td>MMP-1</td>
<td>NM_002421</td>
<td>CGACTCTAGAAAC ACAAGAGCAAGA</td>
<td>AAGGGTAGCTTAC TGCACACGCTT</td>
</tr>
<tr>
<td>MMP-2</td>
<td>NM_004530</td>
<td>GTGCTGAAGGACA CACTAAGAAAGA</td>
<td>TTGCCATCCTTCTC AAAGTTGTAAGG</td>
</tr>
<tr>
<td>MMP-9</td>
<td>NM_004994</td>
<td>CACTGTCCACCCCC TCAGAGC</td>
<td>GCCACTTGTCCGCG GATAAGG</td>
</tr>
<tr>
<td>SLUG</td>
<td>NM_003068.4</td>
<td>CATGCTGTTCATA CCACAAC</td>
<td>GGTGTCAGATGGA GGAAGG</td>
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<tr>
<td>ZEB 1</td>
<td>NM_001128128.2</td>
<td>GGGAGGAGCAGTG AAAGAGA</td>
<td>TTCTTTGCCCTTCC TTCTTG</td>
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<tr>
<td>ZEB 2</td>
<td>NM_014795.3</td>
<td>AAGCCAGGGACAG ATCAGC</td>
<td>CCACACTCTGTGC ATTTTGAACT</td>
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<tr>
<td>Vimentin</td>
<td>NM_003380.3</td>
<td>AAAGTGTGGCTGC CAAGAAC</td>
<td>AGCTCAGAGAGG TCAGCAA</td>
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<tr>
<td>Fibronectin</td>
<td>NM_212482.1</td>
<td>GAACATATGATGCC GAAGAGA</td>
<td>GTTGTGACAGATT TCCTCGT</td>
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<tr>
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<td>NM_001792.3</td>
<td>CACTGCTCAGGAC CCAGAT</td>
<td>TAGCCCGAAGAGATGG TCC</td>
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<tr>
<td>RhoA</td>
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<td>GATGGAAAGCAGG TAGAGTTGG</td>
<td>TCAGTATAACATC GGTATCTGGGTA</td>
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<td>ROCK 1</td>
<td>NM_005406</td>
<td>AATGCTTTGATGCTG GATACACCTTTT</td>
<td>CTGTCAGTAAGGA AGGCACAAA</td>
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<td>ROCK 2</td>
<td>NM_004850</td>
<td>GAAGTGCAAGTGG TTGTCA</td>
<td>GCTATTGGCAAGG GCCATAA</td>
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<tr>
<td>HPRT</td>
<td>NM_000194</td>
<td>ACCCCACGAAAGTG TTGGATA</td>
<td>AAGCAGATGGCCA CAGAACT</td>
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Table 3.3

<table>
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<th>TMX2-28</th>
<th>MCF-7</th>
<th>MDA-MB-231</th>
</tr>
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<tbody>
<tr>
<td>SLUG</td>
<td>0.0039 ± 8.8 \times 10^{-4} ***</td>
<td>0.0060 ± 0.002</td>
<td>1.8748 ± 0.464</td>
</tr>
<tr>
<td>ZEB1</td>
<td>0.0336 ± 0.008 **</td>
<td>0.0098 ± 0.003</td>
<td>0.5865 ± 0.158</td>
</tr>
<tr>
<td>ZEB2</td>
<td>0.0009 ± 3.7 \times 10^{-4} ****</td>
<td>0.0013 ± 6.1 \times 10^{-4}</td>
<td>0.2202 ± 0.041</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0.0090 ± 0.0045^$</td>
<td>1.1013 ± 0.364</td>
<td>0.7001 ± 0.042</td>
</tr>
<tr>
<td>Vimentin</td>
<td>0.0005 ± 3.7 \times 10^{-5} ***</td>
<td>0.0005 ± 1.2 \times 10^{-4}</td>
<td>0.5461 ± 0.139</td>
</tr>
<tr>
<td>N-Cadherin</td>
<td>0.6086 ± 0.282 **</td>
<td>0.1414 ± 0.066</td>
<td>1.4790 ± 0.172</td>
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</tbody>
</table>

Data were analyzed using one-way ANOVAs and t-tests with Bonferroni corrections; for comparisons between TMX2-28 cells and MCF-7 cells \$p<0.005; for comparisons between TMX2-28 cells and MDA-MB-231 cells \*p<0.05, **p<0.01, ***p<0.001
Figure 3.1 TMX2-28 cells do not secrete active MMP-1, -2, and -9. Total RNA was isolated from TMX2-28, MCF-7, and MDA-MB-231 cells at 80% confluence. Relative mRNA expression of MMP-1 (A), MMP-2 (B), and MMP-9 (C) were determined by qRT-PCR and normalized to the housekeeping gene HPRT. Differences among cell lines were analyzed with one-way ANOVA and post hoc t-tests with a Bonferroni correction; *p<0.05. (D) Conditioned media was collected from confluent cell cultures grown in serum-free medium for 48 hours and used for MMP-2 and -9 activity assessment.
**Figure 3.2** Expression of RhoA, ROCK 1, and ROCK 2 are upregulated in TMX2-28 cells. Relative mRNA expression of (A) RhoA, (B) ROCK 1, and (C) ROCK 2 were determined by qRT-PCR and normalized to the housekeeping gene HPRT. Differences among cell lines were analyzed with one-way ANOVA and post hoc t-tests with a Bonferroni correction; **p<0.01. Protein lysates (~20 μg) were probed for RhoA expression by Western immunoblotting (GAPDH used as loading control).
Figure 3.3 Blocking RhoA kinase pathway activity with the ROCK inhibitor H-1152 reduces migratory and invasive behavior. TMX2-28, HeLa (positive control) and MCF-7 (negative control) were treated with H-1152 at 0, 20, and 100 µM concentrations for 24 hours. Cells were then seeded into invasion chambers with basal media containing the respective concentration of inhibitor and incubated for 22-24 hours. Cells were then fixed, stained and counted. Differences among cell lines were analyzed with one-way ANOVA and post hoc t-tests with a Bonferroni correction; *p<0.05, **p<0.01, ***p<0.001.
Figure 3.4 RhoA knockdown in TMX2-28 cells results in inhibition of migration and invasion. TMX2-28 and HeLa cells were transiently transfected with either a negative control siRNA (NC-siRNA) or a RhoA specific siRNA (RhoA-siRNA; MCF-7 cells were not transfected). Forty-eight hours post transfection cells were collected for either RNA/protein isolation or invasion assay. (A) Relative mRNA expression of RhoA was determined by qRT-PCR and normalized to HPRT. Protein lysates (~20 μg) were probed for RhoA expression by Western blotting (GAPDH as loading control). (B) Cells were seeded into invasion chambers, incubated for 22-24 hours, fixed, stained and counted. Differences among cell lines were analyzed with one-way ANOVA and post hoc t-tests with a Bonferroni correction; *p<0.05, **p<0.01, ***p<0.001.
CHAPTER 4

FURTHER CHARACTERIZATION OF THE TMX2-28 BREAST CANCER CELL LINE

Introduction

Human breast tumor cell lines are needed for multidisciplinary research in breast cancer. Cell lines that have been thoroughly characterized are the most useful for research. The TMX2-28 cell line has been used as a model for breast cancer in a number of studies including the research in this dissertation. As mentioned in prior chapters, Fasco and colleagues first characterized these cells following selection of 28 clones resulting from prolonged exposure of MCF-7 cells to the anti-estrogen Tamoxifen (69). While the majority of the clones expressed varying ratios of wildtype ERα and its splice variant ERΔ3, one clone, later termed TMX2-28, had completely lost expression of ERα mRNA and protein. It was noted that these cells grew faster than MCF-7 under standard culture conditions, had the ability to form foci independently of estradiol or Tamoxifen, and thus must rely on ER-independent genes and signaling pathways for cell growth (69).

Studies in the Arcaro laboratory continued characterizing these cells by studying differentially expressed genes resulting from a cDNA microarray comparing TMX2-28 with their parent cell line MCF-7 (68). One thousand four hundred and two transcripts were found to be differentially expressed greater than two fold in TMX2-28 cells (200 upregulated, 1202 downregulated). Results from this array not only confirmed ERα down regulation, but also determined that TMX2-28 cells retained expression of luminal
cytokeratins 8, 18, 19, and 20, and gained expression of basal cytokeratins 5, 14, and 17 (68), giving them a mixed basal/luminal cytokeratin profile.

Studies for this dissertation research further revealed that TMX2-28 cells fall into the triple-negative designation (Chapter 2) in that they not only lack ERα expression, but also lack expression of PR and HER2. Continued investigation revealed that TMX2-28 cells display an altered cell cycle with nearly twice the percentage of cells in the S and G2/M phases of the cell cycle than the G0/G1 phase, as compared to MCF-7 (68). Additionally, TMX2-28 cells were found to be highly invasive, as compared to MCF-7, demonstrated by their ability to invade through Matrigel similar to the highly invasive MDA-MB-231(67).

An in depth study of specific proteins upregulated in TMX2-28 cells have identified a number of potential biomarkers (PLD1, PALM) and therapeutic targets (MIG2, SKP2, RhoA) for ERα-negative and triple-negative breast cancers (Chapter 2 & 3; Ref (67, 68, 101). Alongside the main research described in this dissertation, a number of genes involved in cell cycle, cell proliferation, anti-hormone resistance, cell migration and invasion, or are tumor subtype markers were also assessed in TMX2-28 cells.

Expression of cytokeratins (CK) reflects the epithelial cell type, state of tissue growth, differentiation, and functional status of tissue (149). In normal breast epithelium, the luminal and myoepithelial (basal) cells express different cytokeratin profiles. Luminal cells express CKs 7, 8, 18, and 19, while basal cells express CKs 5, 14, and 17 (150-152). Thus CK 5, 14, and 17 are used to define breast cancer with basal cell origins and tumors with acquired basal cell characteristics.
Epidermal growth factor receptor (EGFR) is a member of the epidermal growth factor receptor superfamily, which promotes tumor cell proliferation and survival in a variety of cancer types including lung, prostate, head and neck, stomach, kidney, brain, pancreatic, and breast (153-160). Recent studies have brought EGFR into the forefront of cancer research due to its high association with poor patient prognosis and its overexpression in triple-negative, basal-like tumors, making it a standard marker for basal cell origin (161-166).

C-myc is a proto-oncogene with the ability to transform cells (167). C-myc encodes a transcription factor that regulates cell proliferation through targeting cyclin/cyclin dependent kinase (CDK) complexes (168-171) and can drive cells through G1/S transition in the absence of growth factors (172). Studies have also shown that C-myc activation can induce apoptosis (167, 173).

The insulin-like growth factor 1 receptor (IGF1R) is a member of the tyrosine kinase receptor superfamily (174). IGF1R is known to control cell proliferation, ERα expression, and it is suggested to be involved with cell motility/adhesion and anti-hormone resistance in breast cancer (175-180). Interference with activation, expression, or signaling of IGF1R results in inhibition of growth and induction of apoptosis in breast cancer cells (181).

The cell cycle is based on two key families of proteins, CDKs and cyclins. CDKs activate downstream signaling through phosphorylation of their target genes; cyclins bind to CDKs and control their ability to phosphorylate target proteins (182-185). CDK4 is expressed and has actions in the G1 of the cell cycle. Progression though G1 depends upon the ability of CDK4 to bind and be activated by Cyclin D (186).
FoxM1 is a member of the forkhead box family of transcription factors and promotes cell cycle progression into the M phase of the cell cycle (187, 188). FoxM1 controls transcription of a number of cell cycle, mitosis and genes responsible for chromosome segregation including Cyclin B1, Cyclin D1, NEK2, CDC25B, Aurora B Kinase, and Surviven (187-190). In breast cancer, FoxM1 is known to be elevated and also to both regulate ERα expression, and to be regulated by ERα (191, 192).

Rac1 and CDC42 are members of the Rho GTPase family. Rac1 induces actin rich surface protrusions (lamellipodia) and cell ruffling, while CDC42 promotes the formation of actin-rich membrane extensions (filopodia) (193-196). CDC42 also controls cell directionality during mesenchymal cell movement through polarizing microtubules (49). p21 activated kinase (PAK) is a downstream target of both Rac1 and CDC42 (197-199). PAK then targets substrates involved in a number of cellular processes including cell proliferation, survival, motility, angiogenesis, EMT, anchorage independent growth, and metabolism (199-205).

E-cadherin is a calcium regulated cell-cell adhesion molecule expressed primarily in epithelial cells (206, 207). Studies have shown that expression of E-cadherin in breast cancer rescues progression and invasiveness of tumors, and formation of metastases (208). Inactivation of E-cadherin is characteristic of invasive breast cancer and has been suggested as a useful prognostic factor for classifying subgroups of triple-negative and basal-like breast cancers (209-214).

The importance of estrogen signaling in breast cancer progression is well described (215-218). Estrogens are steroid hormones that regulate growth, differentiation and function in various tissues throughout the body. Until the discovery of ERβ in 1996,
it was thought that all biological effects of estrogen were mediated through one receptor, ERα (215, 216, 219). Human mammary tissue expresses both ER subtypes (219-221). ERβ is highly homologus to ERα, having 96% amino acid homology at the N-terminus, and 58% amino acid homology at the C-terminus (219). ERβ can bind estradiol with an affinity similar to that of ERα and can stimulate transcriptional activation through estrogen response elements (222). A number of studies have found that ERα-negative breast cancers with ERβ immunoreactivity respond favorably to adjuvant Tamoxifen treatment (223-227). Conversely, a number of studies have shown that ERβ can mediate opposite effects than those of ERα and suppress proliferation (228-230).

The forkhead transcription factor, FoxP3 regulates the development and function of T regulatory cells (231, 232). Until recently, FoxP3 expression was thought to be confined to the T cell lineage (231, 233), however studies have since shown that FoxP3 is expressed in cancer, including breast cancer, melanoma, and cell lines derived from solid tumors (234-237). Studies from Zuo et al. have demonstrated that FoxP3 may also act as a master regulator controlling the expression of the breast cancer oncogenes, SKP2 and HER2 through transcriptional repression (237, 238). Zuo also noted that FoxP3 heterozygous mice show an increase rate of spontaneous mammary cancer (238). Lastly, expression of FoxP3 in breast cancer has also been associated with poor patient survival (239).

Materials and Methods

Cell Culture: TMX2-28 and MCF-7 cells were maintained in T-75 culture flasks at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with calf serum (5%), insulin (10 µg/ml), non-essential amino acids (100X), penicillin-
streptomycin (10,000 µg/ml), and L-glutamine (200 mM). MDA-MB-231 cells were maintained in Leibovitz-15 (L-15) medium supplemented with 10% FBS at 37°C and 0% CO₂. Jurkat cells were maintained in RPMI medium supplemented with 10% FBS at 37°C and 5% CO₂. All cells were passaged when near 80% confluence.

**RNA Isolation:** Total RNA (n=3 biological samples) was isolated with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer’s suggestions and protocols as previously described (67, 68, 101). RNA was then treated with Turbo DNA-Free (Ambion, Austin, TX) to remove any DNA contamination. The quality of the RNA was assessed by 260/280 nm spectrophotometer readings (Nanodrop 8000; Thermo Scientific, Wilmington, DE).

**Quantitative Real Time Reverse Transcriptase PCR (qRT-PCR):** RNA samples (n=3 biological samples) were reverse transcribed and amplified using the One Step RT-PCR kit (Qiagen, Valencia, CA) in the Roche LightCycler (Roche, Indianapolis, IN) as previously described. Gene specific primers, designed using Primer3 software (http://frodo.wi.mit.edu/), and purchased from Integrated DNA Technologies, Inc. (Coralville, IA), were used for CK 5, CK 14, CK17, EGFR, C-myc, IGF1R, CDK4, FoxM1, CDC42, Rac1, PAK, E-Cadherin, ERβ; primers for FoxP3 were purchased from SABiosciences (Valencia, CA; Table 4.1).
Results

Gene expression of markers of basal cells, proliferation, migration/invasion and hormone signaling in TMX2-28

Using qRT-PCR to determine the gene expression of basal markers, it was revealed that TMX2-28 cells have significantly higher relative expression of CKs 5, 14, and 17 than luminal MCF-7 cells and similar levels as basal MDA-MB-231 cells (Table 4.2). Contrary to our prediction, TMX2-28 cells had significantly lower expression of EGFR than MDA-MB-231 cells. The proliferation markers C-myc, IGF1R, CDK4, and FoxM1 were also examined for mRNA expression in TMX2-28 cells. No significant difference in expression of C-myc or FoxM1 was found, however there was a significant decrease in IGF1R expression compared to MCF-7 and a significant increase in expression of CDK4 compared to MDA-MB-231 cells (Table 4.2).

I also examined the expression of four genes involved in cell migration, adhesion and invasion (Rac1, CDC42, PAK, and E-Cadherin). It was determined that TMX2-28 cells express significantly higher levels of Rac1 mRNA than do MCF-7, and similar levels as those of MDA-MB-231 cells. TMX2-28 also had significantly less PAK expression than MCF-7 cells, and although not significant, they have higher expression of PAK than MDA-MB-231 cells. These cells were also found to have significantly less expression of CDC42 than MDA-MB-231 cells (Table 4.2). Lastly, TMX2-28 cells were found to express significantly less E-cadherin mRNA than MCF-7 cells, not significantly different from MDA-MB-231 (Table 4.2; Figure 4.1).

Further gene expression studies revealed that TMX2-28 cells have little to no expression of ERβ, similar to MCF-7, and significantly less than MDA-MB-231 cells,
which are considered ERβ-positive (Table 4.2). Figure 4.2 shows that compared to expression of ERβ in human tissues (prostate and testis), TMX2-28 cells lack ERβ expression. Lastly, relative FoxP3 expression was also determined. FoxP3 was found to be significantly less expressed in TMX2-28 cells when compared to MCF-7 and Jurkat T lymphocyte cells, both of which are known to be FoxP3 expressing cells (Table 4.2; Figure 4.3).

Discussion

A great advantage to cancer research has been the development of the ability to culture tumor cell lines in an in vitro setting. With this technology, researchers have been able to examine the effects of manipulating the levels of oncogenes, proto-oncogenes, and suppressor genes on behavioral properties. They can then compare in vitro properties with those in human cancer tissue, and investigate the mechanisms that differ between them to determine which cell line best mimics the cancer of interest. In this chapter, gene expression of basal cell markers, genes associated with cell proliferation, hormone signaling, migration, adhesion, and invasion were examined in TMX2-28 cells in order to further characterize this cell line as a model for triple-negative and basal-like breast cancers.

It was discovered that TMX2-28 cells express a number of basal cell markers, CK 5, 14, and 17, and the proliferation marker CDK4. Expression of one gene associated with invasion, CDC42, was found to have low expression in TMX2-8 cells. This was not surprising as CDC42 is known to play an important role in protease-dependent, mesenchymal invasion and as I have shown in Chapter 3, TMX2-28 utilizes a protease-independent, amoeboid invasion mechanism. The mRNA expression of the adhesion
protein E-Cadherin was also determined to be at low expression levels in TMX2-28 cells. Gene expression assays also revealed that TMX2-28 cells do not express ERβ, suggesting that they truly rely on ER independent growth mechanisms. Lastly, TMX2-28 cells do not express the transcription factor FoxP3, suggesting that FoxP3 regulates neither SKP2 nor HER2 expression in TMX2-28 cells. The gene expression profiling in this chapter has revealed a number of unique expression patterns in the TMX2-28 breast cancer cells line. These patterns help explain the hormone-independent and Tamoxifen insensitive, uncontrolled aggressive growth phenotype associated with ERα-negative, triple-negative, and basal-like breast cancers.
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<th>Reverse Sequence (5’-3’)</th>
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<td>(Start position lies in exon 12)</td>
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<td>MCF-7</td>
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<td>CK 5</td>
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<td>1.259 ± 0.438†</td>
<td>0.002 ± 0.001</td>
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<td>0.009 ± 0.006†††, ¶</td>
<td>0.604 ± 0.030</td>
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</tbody>
</table>

Data were analyzed using one-way ANOVAs and t-tests with Bonferroni corrections; for comparisons between TMX2-28 cells and MDA-MB-231 cells *p<0.05, **p<0.01, ***p<0.001; for comparisons between TMX2-28 and MCF-7 cells †p<0.05, ††p<0.01, †††p<0.001; for comparisons between TMX2-28 and Jurkat cells ¶p<0.001; §only one biological sample was available for assaying; a Significant ANOVA (p<0.05) but not significant with post hoc t-tests.
Figure 4.1 E-Cadherin is downregulated in TMX2-28 cells. Relative mRNA expression of E-Cadherin was determined by qRT-PCR and normalized to the housekeeping gene HPRT. Differences among cell lines were analyzed with one-way ANOVA and post hoc t-tests with a Bonferroni correction; **p<0.01.
Figure 4.2 ERβ is downregulated in TMX2-28 cells. Relative mRNA expression of ERβ was determined by qRT-PCR and normalized to the housekeeping gene HPRT. Differences among cell lines were analyzed with one-way ANOVA and post hoc t-tests with a Bonferroni correction; NS= not significant. ***p<0.001.
**Figure 4.3** FoxP3 is downregulated in TMX2-28 cells. Relative mRNA expression of FoxP3 was determined by qRT-PCR and normalized to the housekeeping gene HPRT. Differences among cell lines were analyzed with one-way ANOVA and post hoc t-tests with a Bonferroni correction; ***p<0.001.
CHAPTER 5

TUMORIGENICITY AND METASTATIC POTENTIAL OF TMX2-28 CELLS

Introduction

Previous studies have validated that MCF-7 cells respond to estrogen (17β estradiol; E2) in vivo in xenograft model systems in mice by inducing tumor growth (240, 241). Thus, I established a collaboration with the laboratory of Dr. Sallie Smith-Schneider to test TMX2-28 and SKP2 knockdown TMX2-28 cells for their in vivo tumorigenicity and metastasis potential in mouse xenograft models. Based on the highly proliferative and invasive nature of TMX2-28 cells, and the data we have showing that suppressing SKP2 alters their cell cycle and thus decreases proliferative potential, it is predicted that TMX2-28 and TMX2-28-NC (negative control) cells will establish tumors of larger size and volume than MCF-7 and TMX2-28-S2 (SKP2 knockout cells).

Materials and Methods

Xenograft assays for tumorigenicity: For the first set of xenograft studies, two groups of 10, 5-week-old) athymic nude mice (Jackson Laboratories, Bar Harbor, ME) received subcutaneous inoculations of cells without E2 supplementation. For group 1, inoculations consisted of 2 x 10^6 TMX2-28 or MCF-7 cells in 100 μL of Hank’s balanced salt solution, injected into the right and left flank, respectively. For group 2, each inoculation consisted of 2 x 10^6 TMX2-28-NC or TMX2-28-S2 cells in 100 μL of Hank’s balanced salt solution, injected into the right and left flank, respectively. During this
twenty-four day assay, mice were manually palpated beginning at day 3 post injection and every 3-4 days after.

For the second set of xenograft studies, a group of 10, 6-week-old non-obese diabetic background severe combined immune deficient (NOD-SCID) mice (Jackson Laboratories, Bar Harbor, ME) received subcutaneous inoculations of cells without estrogen supplementation. Each inoculation consisted of $2 \times 10^6$ TMX2-28-NC or TMX2-28-S2 cells in 100 µL of Hank’s balanced salt solution, injected into the right and left flank, respectively. During this sixty-two day assay, mice were manually palpated beginning at day 3 post injection and every 3-4 days after. IACUC approval was obtained for all work with animals.

**Results**

**TMX2-28 cells were unable to sustain palpable tumors in xenograft models**

Xenograft studies for tumorigenicity revealed that TMX2-28 cells, regardless of SKP2 expression levels, were unable to sustain appreciable tumor growth. The first set of xenograft studies involved evaluation of tumorigenesis potential of TMX2-28 versus MCF-7 cells (Group 1) and the potential of TMX2-28-NC versus TMX2-28-S2 cells (Group 2) in the absence of $E_2$ in athymic nude mice. Following the twenty-four day study, growth size at the sight of injection steadily decreased from all 20 mice with possible tumors 4 days post injection to 10 (day 12), 5 (day 15), 1 (day 18), and finally zero by day 21. Although none of the mice produced sustainable palpable tumors, upon surgical evaluation, seven mice had small growths at the site of injection, four of which were injected with TMX2-28 cells, and the remaining three were injected with TMX2-
28-S2 cells. These seven growths were collected, fixed and embedded in paraffin along with the lungs and livers of all twenty mice, and are available for further study.

The second set of xenograft studies only involved evaluation of tumorigenesis potential of TMX2-28-NC versus TMX2-28-S2 cells in the absence of E2 in NOD-SCID mice. During this 62-day study, “possible” tumors were manually detected in 8 of the 10 mice by 6 days post injection. This number increased to 9 mice by day 9, however steadily it decreased to 5 (day 13), 3 (day 16), 1 (day 20), and finally 0 mice by day 24. The majority of mice began to develop tumors even though they were not sustainable. Upon surgical evaluation, three mice had small growths at the site of injection, two injected with TMX2-28-S2 and one injected with TMX2-28-NC cells. One of the mice with the TMX2-28-S2 injection site growth also had an abdominal growth present. For this study a number of organs that are frequent sites of breast cancer metastasis (brain, liver, and lungs) were collected along with the four growths; the tissue at the injection site, and the surrounding mammary glands (lower glands). These tissues have been fixed, embedded in paraffin and are available for further study.

Discussion

Xenograft studies for tumorigenicity revealed that TMX2-28 cells, regardless of SKP2 expression levels, were unable to sustain appreciable tumor growth following subcutaneous flank injections into xenograft mouse models. During both studies, possible tumors were manually detected in mice by 6 days post injection, but ultimately all mice lost them by day 24.
Knowing that TMX2-28 cells are triple-negative, have a mixed basal/luminal CK expression profile, and are highly proliferative and invasive when cultured *in vitro*, we would expect this cell line to establish sustainable tumors in a xenograft mouse model. Surprisingly, they did not. One other study has also evaluated the *in vivo* tumorigenicity of TMX2-28 cells. Spink and colleagues inoculated the mammary glands of 6- and 8-week-old SCID mice, with and without E$_2$ supplementation. They too observed that TMX2-28 cells grew poorly as mammary gland xenografts regardless of E$_2$ supplementation (242).

Given that TMX2-28 cells are highly proliferative and invasive *in vitro*, that the majority of mice began to develop tumors even though they were not sustainable, and that both studies had a few mice with small growths upon surgical evaluation, it is reasonable to suggest that these cells may not have formed substantial tumors at the injection site for two reasons: 1. The cells had already left the “primary” tumor site to establish metastatic niches throughout the body or 2. The cells lack expression of genes that are critical to the process of growth in the *in vivo* setting.

To address the first hypothesis, a number of tissues were collected after both xenograft assays and were fixed in formalin and embedded in paraffin. With these tissues we can exploit the fact that the shRNA construct used to stably transfect TMX2-28 cells also contains a GFP gene, which results in GFP expression in the control and SKP2-silenced cell lines (TMX2-28-NC and TMX2-28-S2). Anti-GFP antibodies could be used to evaluate the presence or absence of TMX2-28 cells in the various organs collected. Observations that these cells were able to establish metastatic growths would fit with the
aggressive and highly metastatic nature of triple-negative and basal-like tumors seen in breast cancer patients.

It is equally possible that TMX2-28 cells grew poorly in xenograft models simply because they were unable to successfully establish in an in vivo setting. If this were the case then deciphering why they are unable to grow would be instrumental in designing targetable therapy to combat tumor growth and metastasis. It is known that MCF-7 cells (TMX2-28’s parent cell line) are typically non-invasive but will form tumors in vivo in the presence of E₂ (242-244), so what is lacking in TMX2-28 cells that made them loose this ability? To address this hypothesis, further studies comparing MCF-7 and TMX2-28 cells are needed. Our lab has both cDNA and methylation array data that compare the parent and derivative cell lines. Looking for genes that are related to growth and metastasis and that are downregulated and/or promoter-methylated in TMX2-28 as opposed to MCF-7 would be the first place to start.

Additionally, it may be advantageous to compare these cells with another triple-negative cell line that is known to form xenograft (i.e. MDA-MB-231). Another direction would be to study other Tamoxifen-selected lines such as the TMX2-11 and TMX2-4 cell lines, which were created alongside TMX2-28, or to look at cells during the process of Tamoxifen selection. These cells could shed light on the pathway genes that were altered and are preventing TMX2-28 cells from growing in vivo. If we can determine why these cells do not form primary tumors in vivo, we will have a greater understanding of how to target aggressive types of cancers in patients.
CHAPTER 6

DISCUSSION AND FUTURE DIRECTIONS

Introduction

Breast cancer is a heterogeneous disease that varies in its biology and response to therapy. Homeostasis of non-cancerous cells requires a delicate balance between proliferation, cell cycle arrest and apoptosis; this equilibrium is disrupted during cancer progression. Historically, estrogen receptor (ER\textsubscript{α}), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) are the most important prognostic factors in breast cancer, dictating a patient’s therapeutic options. Currently, breast tumors are further classified into subtypes based on their gene expression patterns (7, 11). One tumor subtype lacks expression of ER\textsubscript{α}, PR, and HER2 and is referred to as triple-negative. The basal-like tumor subtype is associated with positive basal cytokeratin (CK) expression and is often triple-negative (7, 16, 78-80). Patients with these subtypes face poor prognosis, as these cancers are typically aggressive in their growth, highly metastatic and often unresponsive to anti-estrogen and anti-HER2 therapies (17, 18). The data presented here was designed to answer questions specifically related to growth and invasion in triple-negative and basal-like breast cancers, with the objective of furthering our understanding of the molecular mechanisms underlying these processes.

In this study, we use the Tamoxifen-selected, MCF-7 derivative, TMX2-28 breast cancer cell line. TMX2-28 cells have lost expression of ER\textsubscript{α} and have acquired a mixed basal/luminal cytokeratin profile, suggestive of a more basal-like phenotype (32-34). Morphologically, TMX2-28 cells retained an epithelial cell shape similar to MCF-7.
Behaviorally, TMX2-28 cells are highly proliferative and invasive (32, 33). It is these characteristics that make TMX2-28 cells an interesting and relevant model for studying increased growth and invasive behavior in triple-negative and basal-like breast cancers.

It was also discovered that TMX2-28 cells express a number of basal cell markers, CK 5, 14, and 17, and the proliferation marker CDK4. Additionally, low expression (mRNA) of one gene associated with invasion, CDC42, and the adhesion protein E-Cadherin also was found in TMX2-28 cells. Gene expression assays also revealed that TMX2-28 cells do not express ERβ, suggesting that they truly rely on ER-independent growth mechanisms. Lastly, TMX2-28 cells do not express the transcription factor FoxP3, suggesting that SKP2 and HER2 expression is not regulated by FoxP3 in TMX2-28 cells.

**SKP2 Mediates Proliferation In Vitro and is Associated With Aggressive Breast Cancer Subtypes**

S-phase kinase-associated protein 2 (SKP2) plays an important role in cell cycle regulation by targeting p27 for degradation (21, 28, 35, 36). During the transition from G1 to S phase, p27 undergoes phosphorylation modifications at three major sites, Serine 10 (Ser10), Threonine 187 (Thr187) and Threonine 198 (Thr198) (29). Phosphorylation at Ser10 stabilized p27 in quiescent cells, however, this phosphorylation event has been shown to promote cell proliferation (29-32). Thr198 phosphorylation is catalyzed by ROCK 1 kinase, only when Ser10 is already phosphorylated, and results in the inactivation of both free and CDK bound p27 (33, 34). Lastly, phosphorylation of Thr187 is catalyzed by SKP2 and promotes the ubiquitin ligase-mediated degradation of
p27 (21, 28, 35-39). The cyclin-dependent kinase (CDK) inhibitor p27 regulates G1/S transition by binding cyclin/CDK complexes and abrogating its activity (21, 35, 36). Overexpression of SKP2 has been associated with poor prognosis on its own (94) and in combination with high Cyclin E expression (92) as well as other unfavorable prognostic factors including increased tumor grade, lack of expression of ERα and PR, and HER2 overexpression (95, 96).

The primary objective of the present study was to provide insight into the oncogenic role of SKP2 in triple-negative and basal-like breast cancers. To accomplish this, we first evaluated SKP2 expression in a triple-negative, basal-like breast cancer cell line TMX2-28 (Chapter 2). We found significantly higher SKP2 levels in TMX2-28 cells, and that these cells overexpress a number of SKP2 pathway proteins including p27, CDK2, and Cyclin E, but did not overexpress an alternative cell cycle pathway protein Cyclin D1. We also discovered that partially blocking SKP2 expression in TMX2-28 cells using RNAi results in altering their cell cycle pattern by decreasing the percentage of cells in the S phase and increasing the percentage of cells in the G1/G0 phase of the cell cycle.

Another way we could establish that SKP2 overexpression is resulting in the increase of G1/S transition in TMX2-28 cells is to examine the activity of CDK2. CDK2, in complex with Cyclin E is responsible for G1/S transition through the phosphorylation of a number of proteins including the retinoblastoma (Rb) (245). In order for CDK2/Cyclin E to have its kinase actions on Rb, CDK2 must first be activated through phosphorylation of Thr160. Cyclin E binding to CDK2 results in conformational changes that reduced steric hindrance around Thr160 allowing the residue to be available for
phosphorylation by Cyclin Activating Kinase (246-250) Thus to show activation of CDK2, protein expressions of activated CDK2 (Thr160 phosphorylated CDK2) and phosphorylated Rb could be examined.

Additionally, we assessed mRNA and protein expression of SKP2, and its associated proteins, in human breast and breast cancer tissues. SKP2 mRNA was found to be more frequently expressed in ERα-negative, basal cytokeratin expressing, and basal-like breast cancers. Using IHC and TMA methods, we also evaluated the relationships between SKP2 and its pathway genes as well as the relationship between SKP2 and several clinicopathological features. High levels of SKP2 did not always result in low levels of p27. In our study 11% of tumors expressed high SKP2/low p27, however, 32% of tumors with high SKP2 also had high p27. Furthermore, low levels of SKP2 were associated with low p27 in 20% of the tumors. While we found no significant relationship (neither positive nor inverse) between SKP2 and p27 expression, a significant positive relationship was discovered between SKP2 and the phosphorylated form of p27 (pSer10p27), a form of p27 associated with increased proliferation rates (30, 38).

Taken together with the work from other groups, these findings strongly suggest that SKP2 could play a vital role in the progression of triple-negative and basal-like breast cancers. The next step in this study would be the evaluation of p27 phosphorylation in TMX2-28 cells and human breast tissues. The relationships between SKP2 overexpression and pThr187p27, pSer10p27/pThr187p27 double phosphorylation, and pSer10p27/pThr198p27 double phosphorylation needs to be determined.
In vitro studies would involve immunoprecipitation of pSer10p27 using the same anti-pSer10p27 monoclonal antibody used for IHC studies in Chapter 2 (ab62364; Abcam, Cambridge, MA). The resulting protein can then be run out on a Western immunoblotting gel and probed for either pThr187p27 or pThr198p27. For this assay I would suggest using Abcam’s anti-pThr187p27 (ab118644) and anti-pThr198p27 antibodies (ab64949; Abcam, Cambridge, MA). Although Abcam’s antibody is not backed by literature yet, I was unable to optimize the antibody for pThr187p27 that is seen in literature (sc-16324, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for either Western blotting or IHC (251-253).

It would also be advantageous to evaluate the expression of these pThr187p27 and pThr198p27 in breast and breast cancer tissues (as performed in Chapter 2) and see if its expression correlates with age, tumor type, tumor grade, ERα and triple-negative status, and SKP2 expression using the same antibodies as above and consecutive slices of tissue samples. Alternatively, sequential antibody staining of tissue could be performed in order to visualize multiple proteins (Ser10, Thr187, Thr198, and SKP2) within the same tissue slide. This could be done using secondary antibodies with different fluorophores attached to them. Potential problems with this approach include cross contamination, and limitations on the antibodies that are available for the phosphorylated forms of p27.

SKP2 overexpression could modulate the malignant phenotype of triple-negative and basal-like breast cancers through direct and indirect regulation of p27 phosphorylation. SKP2 is known to directly regulated p27 through SCF/E3 ligase activity (resulting in pThr187p27), however SKP2 may be indirectly regulating the p27 inactivation thorough its ability to stimulate RhoA transcription. Increased RhoA leads
to increased activation of its downstream effectors ROCK 1/2 and mDia, which in turn, not only activates SKP2 transcription but ROCK 1 also catalyzes the phosphorylation of pSer10p27 at Thr198 resulting in the inactivation of p27.

The results from these additional studies would help elucidate the extent to which combined high levels of SKP2 and pSer10p27 alone, in combination with pThr187p27 and/or pThr198p27 may be a good indicator of whether inhibiting SKP2 will result in slowing cell cycle and proliferation. Knowledge of these relationships may further delineate SKP2 as a suitable and favorable target for the development of specific therapeutic intervention; providing a molecular signature that would predict which patients will benefit from SKP2-targeted therapy.

**RhoA is Vital to the Invasion Mechanism of TMX2-28 Cells**

A foremost threat to patients is tumor invasion and metastasis, with the greatest risk to patients diagnosed with triple-negative and basal-like breast cancers. To manifest their malignant potential, tumor cells must have the ability to invade the extracellular matrix (ECM), enter the blood or lymphatic system, travel to and invade distant tissues, and establish new tumor sites (41-43). Two distinct morphological/functional mechanisms are known for single cell migration in tissues: fibroblast-type mesenchymal invasion, and leucocyte-type amoeboid invasion. Mesenchymal movement is characterized by an elongated cellular shape, and involves the use of proteases that cause cellular lysis in tissues, thereby creating a path through which cells can invade (44-47). Amoeboid migrating cells have a rounded morphology and their movement is protease–independent; instead, cells find paths through the ECM by pushing and squeezing
through regions of adequate size (43, 44, 47, 51, 52). Proteins that control cell-cell and ECM interactions also are thought to play a role in invasion and metastasis in a proteolytic-independent way, by controlling cell morphology, motility, and interactions with the tumor microenvironment (41, 44).

The primary objective of the present study was to better define the molecular aspects underlying invasion, in TMX2-28 cells as a model of triple-negative, basal-like breast cancer. Despite their aggressive phenotype, TMX2-28 retains morphology similar to non-aggressive MCF-7 cells, suggesting that their invasion may be proteolytic-independent. We determined that TMX2-28 lack MMP-1 mRNA, and MMP-2/MMP-9 protein expression; each of which are important in protease-dependent invasion. It is important to note, however, that the activity of other MMPs (i.e. collagenases, stromelysins, membrane type, and others), as well as other proteases (i.e. serine/threonine proteases), have not yet been examined in TMX2-28.

TMX2-28 cells have low expression of other genes key to protease-dependent invasion such as Slug, Zeb 1, Zeb 2, Vimentin, Fibronectin and N-cadherin. Conversely, these cells have high expression of protease-independent invasion genes such as Rho, ROCK 1, ROCK 2, and E-cadherin. Finally, treating TMX2-28 cells with a RhoA pathway inhibitor or shRNA targeting RhoA, significantly reduces their invasiveness.

These data suggest that TMX2-28 cells use a RhoA dependent, proteolytic-independent invasion mechanism. However, additional studies are still needed to definitively state that TMX2-28 cells move in an amoeboid fashion. One such study would involve live cell microscopy. Live cell microscopy would allow us to observe TMX2-28 cell movement in a 3D matrix and thus determine if movement is characteristic
of amoeboid or mesenchymal invasion mechanisms. For these studies, collaborations were established first with Dr. Dominique Alfandari. However, we were unable to complete these studies because we were unable to replicate optimal culture conditions while cells were at the microscope thus we were unable to keep the cells alive long enough to observe movement.

A new collaboration was established with Dr. Sallie Smith Schneider at the Pioneer Valley Life Sciences Institute to use their microscope that included a heated microscope stage and the ability to regulate CO$_2$ levels. Working with Nicholas Panzarino, we have begun the process of optimizing the conditions for live microscopy (Matrigel thickness, concentration of cells, length of recording time, etc.).

Following microscopy studies, there are a number of directions in which this research can go. If microscopy does not confirm amoeboid movement, then the next step would be to determine whether these cells undergo epithelial-to-mesenchymal transition (EMT) when in a 3D environment. This could be done by examining MMP and protease expression and activation, as well as expression of genes known to change during EMT in TMX2-28 cells cultured in a 3D matrix. However, this would still leave us with the initial problem that targeting mesenchymal movement is insufficient because cells can switch to amoeboid movement when challenged with protease inhibitors (47).

If microscopy confirms amoeboid movement then we would need to further define the possible interacting proteins involved in this invasion mechanism in order to design effective targets. For example, RhoC is another member of the Rho superfamily that has 95% homology to RhoA and a higher affinity and specificity to RhoA’s main target genes, ROCK 1 and ROCK 2 (254-256). The activation of the ROCKs by RhoC
has been shown to lead to the disruption of adherens junctions and increased cell motility and enhanced F-actin assembly (255). Overexpression of RhoC has been observed in bladder, ovarian, head and neck, pancreatic, hepatocellular, esophageal, and breast cancer (257-262). Given that RhoC plays a role in cell motility, at least partially through the same downstream effectors (ROCK 1 & 2) as RhoA, it is possible that RhoC may also be involved in the amoeboid invasion mechanism.

Another example is RhoE. RhoE was the first member of the Rho kinase family to be identified (129). Unlike the other family members, RhoE does not act as a classic GTPase switch, as it does not hydrolyze GTP (129-131). RhoE functions by binding to and inhibiting the RhoA effector ROCK 1 (but not ROCK 2). This interaction also results in the phosphorylation of RhoE by ROCK 1, which increases RhoE’s stability and activity (73, 132). In addition, RhoE binds to p190RhoGAP and increases its activity toward RhoA, thus promoting the formation of inactive GDP-bound RhoA (133).

RhoC appears to work in concert with RhoA to promote cell motility, while RhoE seems to be a negative regulator of motility via inhibition of RhoA. With this knowledge, we can design inhibiting molecules that mimic the actions of RhoE and target RhoA/C resulting in down regulation and abrogation of invasive behaviors. Then we could engineer breast cells that have the ability to grow in an in vivo setting, to express varying levels of the target gene and evaluate the efficacy of the designed inhibitors on these cells. Targeting the Rho pathway may provide therapeutic strategies that could be used individually or in combination with protease inhibitors in patients with increased risk of metastasis.
SKP2 and RhoA Work Jointly to Produce the Aggressive Phenotype of Triple-Negative and Basal-Like Breast Cancers

The research outlined in this dissertation suggests individual and critical roles of SKP2 and RhoA on proliferation and invasion (respectively) of triple-negative and basal-like breast cancers. One point of study that must also be considered and evaluated is that SKP2 and RhoA have been shown to regulate the transcription of each other (263, 264). A number of studies between 1993 and 2001 observed that Rho family proteins were vital for progression through the G\textsubscript{1} phase of the cell cycle. RhoA was reported to down-regulate both p21 and p27 and stimulate Cyclin D1 accumulation (265-269). The downstream effectors causing these affects were unknown until studies by Mammato and colleagues deciphered that RhoA, through its target genes ROCK 1 & 2 and mDia, were stimulating the transcription of SKP2 in late G\textsubscript{1} phase and thus mediating cell growth (263). Another study by Chan et. al. in 2010 demonstrated that SKP2 cooperated with C-myc to induce transcription of RhoA independently of the SCF\textsuperscript{SKP2}/E3 ligase activity (264). SKP2 and C-myc were found to induce transcription of RhoA through recruitment of the transcriptional co-regulators, Miz1 and p300 to the RhoA promoter (264). Data from Mammoto and Chan suggest that SKP2 and RhoA work in concert to regulate cell growth and invasive potential.

In our triple-negative, basal-like breast cancer cell model TMX2-28 both SKP2 and RhoA are overexpressed. Given that SKP2 can induce the transcription of RhoA and vice versa, it is not unreasonable to suggest that there is a positive feedback loop occurring that is driving the overexpression of both genes in this cell line (Figure 6.1). Two questions that need to be evaluated are, when SKP2 is suppressed in TMX2-28 cells,
what happens to the expression of RhoA, and what is the effect on invasion potential (using TMX2-28-S2 and TMX2-28-MC cells)? If this positive feedback loop were in play, than we would expect that knocking down SKP2 would also have resulted in decreased expression of RhoA and inhibition of invasion. Evaluation of the interactions of SKP2 with C-myc, p300, Miz1, and/or the RhoA promoter could be performed in the SKP2 knockdown cells by immunoprecipitation and CHIP analysis. Our studies also indicate that SKP2 overexpression is correlated with triple-negative breast cancers, thus it would be advantageous to see if the relationship between SKP2 and RhoA translates to human samples.

Recently Yamamura et al. discovered that microRNA-34a could suppress the assembly and function of SKP2/C-myc/Miz1 complex (270, 271). Likewise, microRNA-31 has been shown to suppress breast cancer metastasis partially through down regulation of RhoA (272). Studies of the expression and activity of these microRNAs would add to the knowledge of how growth and metastasis is regulated in triple-negative and basal-like breast cancers.

Lastly, findings by Mohanty and colleagues indicated that the potency at which p27 inactivates CDK2 is controlled through C-terminal phosphorylation (Thr197/8) by ROCK but only when p27 was phosphorylated at Ser10 in advance. When p27 was doubly phosphorylated (Ser10 and Thr197/8) it could no longer inactivate CDK2 and thus promoted S phase transition of the cell cycle (253). Our studies elucidated that TMX2-28 cells overexpress ROCK 1 & 2, that suppression of SKP2 in these cells completely abolished pSer10p27 and that there was a positive correlation between SKP2 and pSer10p27 in human triple-negative tumors. Our data, together with Mohanty’s
evokes the question of the role of ROCK phosphorylation of p27 in these breast cancer subsets. ROCK’s involvement in the process of p27 degradation would add to our finding that regulation of p27 is vital to the proliferative nature of triple-negative and basal-like breast cancers.

The results of these studies could provide additional important information about patients with aggressive breast cancer subsets. Collectively, the data presented here demonstrate the roles of SKP2 and RhoA in triple-negative and basal-like breast cancers and that both genes make desirable therapeutic targets. However, additional studies are needed to provide a full understanding of the mechanisms behind cell growth and metastases, and how the proteins involved in these processes interact and regulate each other.
Figure 6.1 Model of how SKP2 and RhoA work in concert to regulate cell growth and invasive potential. SKP2, independent of its SCF/E3 ligase activity, can complex with C-myc and Miz1 to activate the transcription of RhoA. RhoA’s downstream effectors, Rock1/2 and mDia can stimulate the transcription of SKP2. This positive feedback loop could drive the overexpression of both SKP2 and RhoA and subsequent dysregulation of proliferation and invasion.
## APPENDIX A

### DIFFERENCES IN SCORING IMMUNOREACTIVITY OF SKP2 AND p27 IN BREAST CANCER

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Scoring for SKP2 (Intensity; Distribution)</th>
<th>Scoring for p27 (Intensity; Distribution)</th>
<th>N</th>
<th>SKP2 &amp; p27 Relationship</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signoretti</td>
<td>2002</td>
<td>Hi/Lo; &gt;=&lt;10%</td>
<td>Hi/Lo; &gt;=&lt;50%</td>
<td>83</td>
<td>1(4%) 21(25%) 30(36%) 32(35%)</td>
<td>Inverse</td>
</tr>
<tr>
<td>Zheng</td>
<td>2005</td>
<td>Hi/Lo; &lt;=50%</td>
<td>Hi/Lo; &lt;=50%</td>
<td>82</td>
<td>8(10%) 45(55%) 23(28%) 6(7%)</td>
<td>Inverse</td>
</tr>
<tr>
<td>Slotky</td>
<td>2005</td>
<td>Hi/Lo; &lt;=10%</td>
<td>Hi/Lo; &lt;=50%</td>
<td>43</td>
<td>3(7%) 15(35%) 22(51%) 3(7%)</td>
<td>Inverse</td>
</tr>
<tr>
<td>Traub</td>
<td>2006</td>
<td>Pos/Neg; &lt;=5%</td>
<td>Pos/Neg; &lt;=50%</td>
<td>338</td>
<td>63(19%) 116(34%) 45(55%) 114(34%)</td>
<td>None</td>
</tr>
<tr>
<td>Sonoda</td>
<td>2006</td>
<td>Hi/Lo</td>
<td>N/A</td>
<td>137</td>
<td>14(10%) 52(38%) 38(28%) 33(24%)</td>
<td>No stats</td>
</tr>
<tr>
<td>Voduc</td>
<td>2008</td>
<td>Hi/Lo</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A 52(38%) 38(28%) 33(24%)</td>
<td>None</td>
</tr>
<tr>
<td>Davidovich</td>
<td>2008</td>
<td>Hi/Lo</td>
<td>N/A</td>
<td>40</td>
<td>10(25%) 7(17.5%) 22(55%) 12.5%</td>
<td>Inverse</td>
</tr>
<tr>
<td>Ravaoli</td>
<td>2008</td>
<td>Hi/normal/low</td>
<td>Normal/low</td>
<td>159</td>
<td>N/A 208(13%) N/A N/A</td>
<td>Inverse</td>
</tr>
<tr>
<td>ERα-negative</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>208</td>
<td>N/A 119(80%) N/A N/A</td>
<td>None</td>
</tr>
<tr>
<td>ERα-Positive</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>53</td>
<td>N/A 8(17.5%) N/A N/A</td>
<td>None</td>
</tr>
<tr>
<td>Fagan-Solis</td>
<td>2012</td>
<td>Hi/Lo; &gt;=&lt;50%</td>
<td>Hi/Lo; &gt;=&lt;50%</td>
<td>159</td>
<td>51(32%) 18(11%) 58(36%) 32(20%)</td>
<td>None</td>
</tr>
<tr>
<td>2012</td>
<td>Hi/Lo; &gt;=&lt;50%</td>
<td>Hi/Lo; &gt;=&lt;50%</td>
<td>159</td>
<td>92(58%) 6(4%) 54(34%) 7(4%)</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX B

H-1152 INHIBITOR DOSE RESPONSE MIGRATION ASSAYS
Notes: HeLa and TMX2-28 cells were treated with H-1152 at noted concentrations for 48 hours without media change.
APPENDIX C

ZYMOGRAPHY PROTOCOL

Precast gelatin gels-BioRad (Cat# 161-1167)

Buffers:

1. Running Buffer (10x Stock)

Tris Base 15g
Glycine 72g
SDS 5g
Ultra Pure H$_2$O ~500ml

(pH should be around 8.3)

2. Sample Buffer- From BioRad (Cat# 161-0764)
3. Renaturing Buffer- From BioRad (Cat# 161-0765)
4. Developing Buffer- From BioRad (Cat# 161-0766)
6. Staining Buffer- 40% Methanol, 10% Acetic Acid, 0.5% Coomassie Blue, 49.5% Ultra Pure H$_2$O.
7. Destaining Buffer- 40% methanol, 10% Acetic Acid, 50% Ultra Pure H$_2$O

Protocol:

1. Dilute 1 part sample with 1 part sample buffer.
2. Incubate at room temperature for 10 min.
3. Load samples into gel and run at 100V for 90 min (or until dye front reaches bottom of the gel.
4. Place gels in renaturing buffer for 30 min at room temp.
5. Transfer gel to developing buffer and incubate for 30 min at room temp.
6. Transfer gel to fresh developing buffer and incubate at 37°C with gentle shaking overnight.
7. Stain gel with staining buffer for 1 hour at room temp.
8. Destain until clear bands appear against blue background (30-60 min.).
    a. Gel may shrink following detaining. To rehydrate gel prior to photographing, incubate in Ultra Pure H$_2$O until gel regains original size (~10min).


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