Award Number: W81XWH-08-1-0345

TITLE: Regulation and Action of SKP2 in Cell and Tumor Models: Mechanisms Underlying Aggressive Growth in Basal-Like Breast Cancer

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REPORT DATE: December 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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**Title:** Regulation and Action of SKP2 in Cell and Tumor Models: Mechanisms Underlying Aggressive Growth in Basal-Like Breast Cancer

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**Sponsoring Agency:**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**Report Date:** December 2011

**Report Type:** Annual Summary

**Dates Covered:** 15 May 2008 – 14 November 2011

**Grant Number:** W81XWH-08-1-0345

**Abstract:**
The objective of this research is to further our understanding of the molecular mechanisms underlying the aggressive growth of estrogen receptor (ER)-negative, basal-like breast tumors. My goal is to determine if SKP2 is a viable new therapeutic target to specifically treat patients who have tumors that are independent of ER signaling. The most significant result was determining that knockdown of SKP2 in TMX2-28 cells shifted the cell cycle resulting in a significant increase in the percentage of cells in the G1/G0 phase, as well as a significant decrease in the percentage of cells in the S-phase of the cell cycle.

**Subject Terms:**
ER-negative, Breast Cancer, SKP2

**Security Classification:**
U

**Distribution/Availability Statement:**
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Introduction:

The objective of this research is to further our understanding of the cellular and molecular mechanisms underlying the aggressive growth of ER-negative, basal-like tumors. The goal is to identify new therapeutic targets to specifically treat patients that have tumors that are independent of ER signaling as these tumors are more often ER-negative. Past work from our lab and others has suggested that S-phase kinase-associated protein 2 (SKP2) plays an important role in breast tumorigenesis and would make a good therapeutic target. By utilizing three models (human tissue, animal models, and tissue culture) in which to characterize the role of SKP2 in breast cancer, we can obtain a better understanding of the molecular mechanisms underlying the aggressive tumor growth of basal-like breast tumors. It is anticipated that results from these studies will show that SKP2 would make a good therapeutic target for the treatment of women with basal-like tumors that are often associated with poor clinical outcome and tend to be ER-negative.

Body:

Task 1: I have received 25 ER-negative FFPE tissue cases, 18 of which are triple negative, from the Cancer Tissue Bank at UMASS Worcester, and will be receiving ~70 additional cases from their archives. These tissues have been stained for SKP2 and its associated proteins and are currently being scored in collaboration with Dr. Otis at Baystate Medical Center.

Task 2: I have successfully created a mixed population, as well as a number of single clone populations of TMX2-28 cells that has been stably transfected with the negative control SKP2-shRNA vector. Additionally, I have successfully created a mass culture population of TMX2-28 cells that have been stably transfected with SKP2-shRNA vector. Single clone populations of the SKP2-shRNA transfected cell line have been established.

Task 3: Alterations in cell cycle have been studied in the single clone population of SKP2 knockdown TMX2-28 cells compared to negative control-shRNA transfected cells. Cell cycle analyses of a mass culture of population of knockdown cells were also studied to confirm knockdown effects.

Task 4: In vivo studies of cell proliferation upon knockdown of SKP2 have begun.

Task 5: Preparations of dissertation and publication manuscripts have begun.

Key Research Accomplishments:

Training Accomplishments:

- Continue collaborations with Dr. Christopher Otis, Director of Surgical Pathology at Baystate Medical Center; Dr. Brian Pentecost, New York Department of Health,; Dr. Sallie Smith-Schneider, Pioneer Valley Life Sciences Institute; and Dr. Douglas Anderton, Associate Dean for Research Affairs, Director of Social and Demographic Research Institute
- Current and active member of AACR, AAAS, and SACNAS
- Continue to talk and meet with my mentor Dr. Kathleen Arcaro on a daily basis
- Attend weekly cancer and chemoprevention journal club, apoptosis journal club, molecular and cellular biology seminar and colloquia, animal biotechnology and biomedical science seminar
- Attended and presented research at a number of cancer research conferences

Research accomplishments:

- Obtained 25 ER-negative FFPE tissue cases, and will obtain ~70 additional cases in order to evaluate SKP2 and its associated protein’s expression.
- Continued pathological studies of SKP2 pathway protein in human breast cancer samples
• Determined alterations in cell cycle resulting from SKP2 knockdown in TMX2-28 cells
• Began in vivo studies on growth and metastasis using TMX2-28 cells as well as SKP2 knockdown TMX2-28 cells

Reportable Outcomes:

To study the role of SKP2 in triple-negative and basal-like breast cancer, the tamoxifen-selected breast cancer cell line, TMX2-28, was used as a model for aggressive growth and invasion. TMX2-28 cells are triple-negative with a basal cytokeratin expression pattern. In contrast to the ER-positive, parent cell line, MCF-7, TMX2-28 cells display aggressive growth and increased invasiveness as evidenced by a reduced doubling time, prolonged S-phase, and invasion through a transwell assay.

We found SKP2 to be overexpressed in 7 out of 30 frozen breast carcinoma samples and to be higher in tumors that were ER-negative and expressed basal cytokeratins 5 and/or 17. Moreover, I found SKP2 to be highly expressed in 46% of ER-negative tumors, 24% of ER-positive tumors, and 18% of reduction mammoplasty tissues. Importantly, SKP2 was highly expressed in 77% of triple negative breast cancers. Currently, I am analyzing additional triple-negative breast cancer tissues in order to further delineate these findings.

I determined that SKP2 mRNA and protein are overexpressed in TMX2-28. Additionally, TMX2-28 cells overexpress a number of cell cycle genes associated with SKP2, including p27, CDK2, and cyclin E. Transient knockdown of SKP2 expression did not significantly alter gene expression of the associated genes.
Knockdown of SKP2 in TMX2-28 cells shifted the cell cycle resulting in a significant increase in the percentage of cells in the G1/G0 phase, as well as a significant decrease in the percentage of cells in the S-phase of the cell cycle. However, there was not a significant difference in the percentage of cells in the G2/M phase of the cell cycle.

**Figure 2:** Gene expression was determined using real time qRT-PCR (One-way ANOVA, p27: p=0.0326; CDK2: p<0.0001; Cyclin E: p<0.0001)

**Figure 3:** TMX2-28 cells were transiently transfected with siRNA targeting SKP2 or a scrambled (SCR) version of the sequence (negative control) using a lipid based transfection agent. Forty-eight hours post transfection RNA was isolated and gene expression was determined using real time qRT-PCR (Unpaired T Test with Welch's correction).
am currently using these stable knockdown cell lines for in vivo studies of tumor growth and metastasis.

Conclusion:

Triple-negative and basal-like breast cancer continues to pose a major challenge to clinicians. Given that triple-negative and basal-like breast cancer patients are without targeted therapies, clinicians are left to rely on non-specific, cytotoxic agents. To develop targeted therapies, the approach must be geared towards the molecular biology of the tumor. Additionally, development of predictive markers can optimize the success of therapeutics. Overexpression of SKP2 can serve as a predictive marker for women at risk for aggressive tumor growth. SKP2 provides a potential target for therapeutics in which triple-negative and basal-like breast cancer patients can benefit.

The final year of this study has led to the continuation of my training through collaborations and interactions with a number of clinicians, pathologists, bench scientists and epidemiologists. Additionally, I have completed cell cycle analysis studies and continued work on immunohistochemical, gene/protein expression cell cycle analysis, and in vivo work. Finally, preparations of dissertation and publication manuscripts have begun.

References: none

Appendices: Curriculum vitae, Era of Hope poster, AACR Advancements in Breast Cancer Research Poster
Bibliography:


Personnel (not salaries) receiving pay from the research effort: None
BUSINESS INFORMATION
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Fax: (413) 545-5731
Email: kfaganso@mc.b.umass.edu

EDUCATION
2005-Present University of Massachusetts Amherst
Doctor of Philosophy, Molecular and Cellular Biology

2000-2004 State University Of New York at Albany
Bachelor of Science, Biological Chemistry and Molecular Biology

EXPERIENCE
2005- Present
Research Assistant in the Molecular and Cellular Biology Program at UMASS Amherst
Research Project: Regulation and action of SKP2 in cell and tumor models: Mechanisms underlying aggressive growth in basal-like breast cancer
Subproject: Characterizing the invasion mechanism utilized by the TMX2-28 breast cancer cell line
Skills Acquired:
- Cell culture techniques
- Fluorescence microscopy
- Nucleotide and protein isolation and quantification
- Mammalian and bacterial cloning
- Cell cycle analysis
- Immunohistochemistry
- RNAi
- PCR (quantitative, real time, reverse transcription)
- Gel electrophoresis
- Zymography
- Tissue microarray construction
- Grant writing

Presented research projects in numerous posters and power point presentations, a number of which were at research conferences.
Summer 2005  
**Summer Program for Undergraduate Research, UMASS Amherst**  
Research Project: Isolation of Adult Mammary Stem Cells from Breast Milk  
Skills Acquired:  
- Cell culture techniques  
- Magnetic cell sorting  
- Fluorescence microscopy  
- RNA isolation  
Presented research project at poster session

Summer 2002  
**Ronald E. McNair Post-Baccalaureate Achievement Program, SUNY Albany**  
Research Project: The Effects of Blocking Intracellular Steroid Receptors in the Hippocampus or Amygdala on Learning and Memory  
Skills Acquired:  
- Animal husbandry  
- Inter- cannulae infusion, hormone inserts and injections  
- Determination of behavioral estrus in rats  
- Inhibitory avoidance testing  
Presented research project at McNair Scholars’ Day

TEACHING ASSISTANTSHIPS:  
- Introductory Biology I Laboratory (BIO 100) Fall ‘06

MENTORSHIP (of incoming graduate students)  
- Northeast Alliance Graduate Mentorship Program Fall ’06, Fall ‘08  
- Molecular and Cellular Biology Graduate Program Fall ’08, Fall ‘11  
- Arcaro Lab Fall ’08-present

AWARDS  
- Carl Storm Underrepresented Minority Fellowship August ‘06  
- SACNAS Travel Scholarship October ‘06  
- AACR Minority Scholar in Cancer Research Award April ’07, October ‘11  
- Northeast Alliance Graduate Fellowship Fall ’06,’07,’11; Spring ’06,’08, ‘12  
- Research Assistantship ‘06-Present  
- Preparing Future Faculty Summer Institute June ‘07

POSTER ABSTRACTS AND ORAL PRESENTATIONS  

Chicanos and Native Americans in Science National Conference, October 26- October 29, 2006. Tampa, FL


POSTER PUBLICATIONS


Katerina D. Fagan-Solis, Christopher M. Otis, Sallie W. Smith-Schneider, Kathleen F. Arcaro. S-Phase Kinase-Associated Protein 2 in Triple-Negative and Basal-Like Breast Cancer [abstract]. In: Proceedings of the Meeting of Advances in Breast Cancer Research: Genetics,

PUBLICATIONS


PROFESSIONAL DEVELOPMENT
Preparing Future Faculty Summer Institute June 2007

PROFESSIONAL SOCIETY MEMBERSHIPS
Society for Advancement of Chicanos and Native Americans in Science 2006-present
American Association for Cancer Research 2006-present
American Association for Cancer Research: Women in Cancer Research 2006-present
American Association for Cancer Research: Minorities in Cancer Research 2006-present
American Association for the Advancement of Science 2008-present

RESEARCH GRANTS
Recent: Department of Defense Predoctoral Traineeship Award ; 2008-2011
  Role: Principal Investigator (100% effort)
Recent: Rays of Hope; Baystate Medical Center ; 2009-2010
  Role: Co-Principal Investigator (100% effort)
Recent: Rays of Hope; Baystate Medical Center ; 2007-2008
  Role: Co-Principal Investigator (100% effort)
Recent: Rays of Hope; Baystate Medical Center ; 2006-2007
  Role: Supported researcher (100% effort)
Recent: Rays of Hope; Baystate Medical Center ; 2005-2006
  Role: Supported researcher (100% effort)
S-Phase Kinase-Associated Protein 2 in Triple-Negative and Basal-Like Breast Cancer

Katerina D. Fagan-Solis1,4, Christopher N. Otis2, Kathleen F. Arcaro3,4
1Molecular and Cellular Biology Program, 2Director of Surgical Pathology, Baystate Medical Center, Springfield, MA, 3Department of Veterinary and Animal Science, 4University of Massachusetts, Amherst

RESULTS

MCF-7

CDK2 mRNA Levels

Cyclin E mRNA Levels

(normalized to HPRT)

0
5
1.0
1.0
1.5

0.0
0.25
0.50
0.75

Frozen Breast Carcinoma Specimens

SKP2 mRNA Levels

(normalized to HPRT)

G0/G1

TMX2-28

Figure 2: Gene expression of SKP2 was determined in 30 frozen breast carcinoma samples using qRT-PCR. Tumors were sorted by SKP2 expression. Tumors with Cki-5 and/or Cki-6 positivity were classified as ER-positive tumors with the number of case 1-8 while ER-negative tumors were assigned 19-36.

Figure 4: mRNA expression of ERα, PR, and HER2 was determined by real time qRT-PCR. Protein expression of ERα, PR, and HER2 was determined by immunohistochemistry.

Table 1: Cki mRNA expression in TMX2-28 Cells. Data are expressed as fold change in TMX2-28 compared to MCF-7 cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>mRNA Expression</th>
<th>Protein Expression</th>
</tr>
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<tbody>
<tr>
<td>SKP2</td>
<td>Decrease (~75%)</td>
<td>Decrease (~75%)</td>
</tr>
<tr>
<td>p27</td>
<td>No significant change</td>
<td>Currently being determined</td>
</tr>
<tr>
<td>CDK2</td>
<td>No significant change</td>
<td>Currently being determined</td>
</tr>
<tr>
<td>CYCLIND1</td>
<td>No significant change</td>
<td>Currently being determined</td>
</tr>
<tr>
<td>CYCLIN E</td>
<td>No significant change</td>
<td>Currently being determined</td>
</tr>
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Figure 6: Gene expression was determined using real-time qRT-PCR.

Table 2: mRNA and protein expression of SKP2 and its associated genes in TMX2-28 cells. 48 hours post transient knockdown of SKP2.

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</tr>
<tr>
<td>CYCLIN E</td>
<td>No significant change</td>
<td>Currently being determined</td>
</tr>
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Figure 8: Schematic representation of ongoing in vivo studies.

CONCLUSIONS

• Numerous researchers have suggested that SKP2 may provide a good target for therapy.
• SKP2 is highly expressed in 46% of ER-negative and 77% of triple-negative and basal-like breast cancers.
• TMX2-28 cells are a Tamoxifen-selected, MCF-7 variant that have a triple-negative and basal-like expression pattern.
• TMX2-28 cells overexpress SKP2 as well as a number of other cell cycle associated genes including p27, CDK2, and cyclin E.
• Knockdown of SKP2 in TMX2-28 cells shifted the cell cycle towards a significant increase in the percentage of cells in the G0/G1 phase, as well as a significant decrease in the percentage of cells in the S phase of the cell cycle.
• Current data suggest that overexpression of SKP2 and the subsequent dysregulation of the cell cycle play a role in the development of the highly proliferative and aggressive nature of triple-negative and basal-like breast cancers.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-08-1-0585 and the Razi Institute organization.

FUNDING
BACKGROUND

• Breast cancer is a heterogeneous disease that varies in its biology and response to therapy.
• Hormonally, receptor-negative (ER) is the most important prognostic factor in breast cancer, dictating a patient’s therapeutic regimen.
• Currently, breast tumors are further classified into subtypes based on their gene expression patterns.
  - Triple-negative tumors are a subtype typically lacking ER, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression.
  - Basal-like tumors are associated with positive basal cytokeratin (CK) 5, 14, and 17 expression patterns.
• Patients with these tumors whose breast tumors are especially prone to late recurrence and/or metastasis to the axillary lymph nodes.

STUDY OF HUMAN TISSUE

• Through study of human tissue, cell culture, and animal models, expression patterns of SKP2 and its associated proteins in breast cancer can be determined.

STUDY OF CELL CULTURE

• TMX2-28 cells are triple-negative and have a mixed basal/luminal cytokeratin RNA expression.

RESULTS

SKP2 mRNA is Overexpressed in Basal-Like Breast Tumors

Figure 2: Gene expression of SKP2 was determined in 38 frozen breast carcinoma samples using real-time qRT-PCR. Tumors were sorted by SKP2 expression. Tumors with CK5 and/or 17 positivity are shaded. ER-positive tumors were assigned the numbers 1-10, while ER-negative tumors were assigned 11-38.

SKP2 Protein is Highly Expressed in 46% of ER-Negative and 77% of Triple-Negative Breast Cancers

Figure 3: SKP2 promotes progression into the S-phase of the cell cycle by regulating p27. (A) In proliferating cells, SKP2 targets p27 for ubiquitination and proteasomal degradation. In targeting p27 for degradation, SKP2 promotes progression into the S-phase of the cell cycle. (B) The absence of SKP2, p27 alleviates the actions of cyclin/CDK complexes thereby permitting the G1/S transition and inhibiting the cell cycle.

TMX2-28 Cells Overexpress a Number of Cell Cycle Genes Associated With SKP2

Table 2: mRNA and protein expression of SKP2 and its associated genes in TMX2-28 cells, 48 hours post transient knockdown of SKP2.

SKP2 Gene Expression

Figure 7: TMX2-28 cells were subjected to transfection with siRNA targeting SKP2 or negative control siRNA. mRNA was isolated from both siRNA TF56 (TMX2-28-S2mc) and a clonally selected TMX2-28 (S2) knockdown population. Gene expression was determined using real-time qRT-PCR. Protein expression was determined by western immunoblot analysis. Cell cycle analysis was determined by flow cytometry (FACS) analysis.

SKP2 PATHWAY

REFERENCES


8. Journal of Clinical Investigation

9. Journal of Clinical Oncology

FUNDING

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0345, the Rays of Hope organization, and the Northeastern Alliance for Graduate Education and the Professorate.