TITLE: Define the Twist-ATX-LPAR1 Signaling Axis in Promoting Obesity-Associated Triple Negative Breast Cancer

PRINCIPAL INVESTIGATOR: YIWEI LIN

CONTRACTING ORGANIZATION: UNIVERSITY OF KENTUCKY
Lexington, KY 40526

REPORT DATE: May 2017

TYPE OF REPORT: ANNUAL

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Define the Twist-ATX-LPAR1 Signaling Axis in Promoting Obesity-Associated Triple Negative Breast Cancer

Breast cancer remains the second leading cause of cancer-related death in women worldwide. Triple negative breast cancer (TNBC) carries a poorer prognosis, given its higher genomic instability, tendency toward early metastasis, and lack of effective targeted therapies. Obesity is a risk factor for TNBC so understanding the link between TNBC and obesity is crucial to the development of novel prevention and treatment strategies. TNBC activates the epithelial-mesenchymal transition (EMT) program and a key EMT inducer, the transcription factor Twist is highly expressed in TNBC. Autotaxin (ATX) and LPAR1 were dramatically increased in Twist-overexpressing breast cancer and adipose cells. Encoded by the ENPP2 gene, ATX is a secreted enzyme that produces most of the extracellular lysophosphatidic acid (LPA), which signals through its receptors (LPAR1-6) to mediate a wide range of inflammatory processes including wound healing, fibrosis and metastasis. Adipose is an important source for the synthesis and secretion of ATX, so ATX level/activity are increased during obesity-associated adipose tissue expansion. Accordingly, we propose that Twist activation intensifies the ATX-LPAR1 signaling to promote the development and progression of obesity-associated TNBC. We are testing this hypothesis using genetic and pharmacological approaches in cell and animal models of breast cancer.
## Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>1</td>
</tr>
<tr>
<td>3. Accomplishments</td>
<td>1</td>
</tr>
<tr>
<td>4. Impact</td>
<td>7</td>
</tr>
<tr>
<td>5. Changes/Problems</td>
<td>7</td>
</tr>
<tr>
<td>6. Products</td>
<td>7</td>
</tr>
<tr>
<td>7. Participants &amp; Other Collaborating Organizations</td>
<td>7</td>
</tr>
<tr>
<td>8. Special Reporting Requirements</td>
<td>10</td>
</tr>
<tr>
<td>9. Appendices</td>
<td>10</td>
</tr>
</tbody>
</table>
1. Introduction

Triple-negative breast cancer (TNBC) carries the poorest prognosis among breast cancer subtypes, given its high genomic instability, tendency toward early and recurrent metastases, and lack of effective targeted therapies. Standard surgery with adjuvant chemotherapy and radiotherapy offers limited efficacy once the tumor cells begin to metastasize. Epidemiological evidence strongly indicates the co-morbidities of TNBC and obesity; women with overweight/obesity are at a significantly higher risk of developing TNBC. The obesity rate has been increasing rapidly in the U.S. population over recent decades, posing another daunting threat to TNBC prevention and treatment. This study aims to elucidate the mechanistic linkage between TNBC and obesity for the development of novel targeted therapies. Specifically we found that a transcription factor called TWIST that is increased in both breast tumor cells and in breast adipose tissue increases expression of genes encoding Autotaxin, an enzyme that generates a bioactive lipid called lysophosphatidic acid (LPA) and a particular LPA selective G protein coupled receptor LPAR1. This supports our hypothesis that Twist activation during inflammatory breast cancer development and progression exacerbates development of obesity associated TBBC. We propose studies to test this hypothesis using cell and animal models.

2. Keywords

ATX: Autotaxin
LPA: Lysophosphatidic acid
TNBC: Triple negative breast cancer

3. Accomplishments

3.1. Major goals and accomplishments

During the first year of this project we have focused our efforts on Subtasks 1b, 1c, 2a and 3b as defined in the statement of work. With regards to Subtask 1b which is to determine effects of Twist activation on ATX and LPAR1 expression we have developed assays to measure ATX protein and activity in vitro that can be used to monitor ATX expression and activity in cells and tissues. We have developed recombinant baculovirus vectors for expression of tagged forms of ATX that we can purify for use as controls for these studies. Some of these proteins contain His tags for purification and streptavidin binding peptide tag for detection (Figure 1). In addition, we have applied recently developed CRISPR technology to examine if Twist enhances ATX and LPAR1 expression. Specifically, we performed lentiviral transduction of Twist-targeting gRNA into breast cancer cells MDA-MB-578 and SUM-1315, and selected single cell colony with Twist knockout. We chose CRISPR-gRNA over the shRNA system which was originally proposed, as CRISPR provides higher specificity and fewer off-target effects. To verify knockout of Twist, we first performed mismatch cleavage assay using the KAPA HIFI hot start PCR kit and T7E1 enzyme from NEB. We designed two independent primer sets and performed PCR to detect single base mismatches or small insertions/deletions harbored in the selected single cell colonies. Gel electrophoresis showed cleaved products from Twist knockout cell samples compared to parental control, indicating the presence of...
heteroduplexes that can be recognized by T7E1 (Figure 2). Next, we performed Western blot to examine the expression of Twist as well as Vimentin, which is one of known Twist target genes. As expected, Twist expression was almost completely abolished, and Vimentin expression was significantly decreased in these selected single cell colonies (Figure 3). Furthermore, we performed RT-PCR and confirmed the downregulation of Wnt5a and DDR2, as well as upregulation of CDH1, all of which are already known Twist target genes (Figure 4). Interestingly, knockout of Twist significantly changed the morphology of MDA-MB-578 cells, as they notably lost branching compared to parental control (Figure 5). As knockout of Twist has been confirmed, we continued to examine the expression of ENPP2 and LPAR1 and found both were partially downregulated (Figure 6). We are currently optimizing RT-PCR conditions to verify the downregulation of ENPP2 and LPAR1 upon knockout of Twist. Possibilities cannot be excluded that the transcription of ENPP2 and LPAR1 are co-regulated by other transcription factors and/or epigenetic machineries, which remain to be identified in the coming year. In summary, establishment of Twist knockout single cell colonies would be useful in studying Twist regulation of ENPP2 and LPAR1.

With regards to Subtask 1c which is to determine whether ENPP2 and LPAR1 are direct target genes of Twist, we performed analysis on the promoters of both genes and confirmed the presence of Twist-responsive E-boxes. We also refined the promoter regions to within 2000bp upstream of translation starting sites (Figure 7). We have purchased Bacterial artificial chromosome (BAC) clones encoding the two promoter regions and designed primer sets to generate full length as well as deletions of promoter-luciferase constructs. We are currently performing luciferase reporter assay to examine whether ectopic expression of Twist can enhance
luciferase activity. Once the regions required for Twist regulation are determined, we will generate mutants of E-boxes located within the specific regions and perform luciferase reporter assay to finally identify the critical E-boxes.

With regards to subtask 2a which is to characterize effects of ATX and LPA on TNBC and adipocyte cells we have generated recombinant ATX for use in these studies. This includes a site directed mutant of ATX that lacks LPA generating enzymatic activity for use as a control in these studies. We have developed and refined mass spectrometry based methods to measure LPA and autotaxin substrates. These methods employ UPLC coupled electrospray ionization tandem mass spectrometry using a shimadzu UPLC system, a waters BEH C8 column and an ABSciex 6500 Q-trap mass spectrometer system operated in selected ion monitoring more (Figure 8).

**Figure 5** Morphology of MDA-MB-578 cells with knockout of Twist.

**Figure 6** Western blot examining the expression of LPAR1 and ATX in MDA-MB-578 cells with Twist knockout.

**Figure 7** Graphic illustration of presence of E-box and sequence deletion promoter luciferase constructs.
With regards to Subtask 3b, in addition to the ATX transgenic mice described in the proposal we have also generated mice with adipocyte specific inactivation of the ENPP2 gene encoding ATX by crossing mice expressing a cre recombinase transgene under control of the adiponectin promoter with mice carrying a “floxed” ENPP2 allele. These animals show significant systemic reductions in ATX levels in comparison to controls and these reductions are amplified in models of diet dependent obesity. Figure 9 shows ATX mRNA levels in visceral and subcutaneous adipose tissue from control (fl/fl) mice and the same strain expressing cre recombinase under control of the adiponectin promoter which is active after differentiation of fat cells. Mice harboring the cre deleted allele exhibited significant decreases in ATX (ENPP2) mRNA in subcutaneous and visceral fat depots.

3.2. Opportunities for professional development

A. Grant review/study section service

Dr. Morris served on and/or chaired the following NIH/CSR study sections:

05/23/2016 at NHLBI
Meeting 2016/10 HLBP  SRO: Kristin Goltry

06/08/2016 at NIDDK (Chair)
Meeting 2016/10 ZDK1-GRB-2-O1 SRO: THOMAS TATHAM

06/09/2016 at NIDDK (Chair)
Meeting 2016/10 ZDK1-GRB-2-O2  SRO: THOMAS TATHAM

06/23/2016 at CSR

Meeting 2016/08 ZRG1-BST-U-50  SRO: Kee Pyon

10/18/2016 at NIDDK (Chair)

Meeting 2017/01 ZDK1-GRB-2-J2  SRO: THOMAS TATHAM
Meeting 2017/01 ZDK1-GRB-2-J1  SRO: THOMAS TATHAM

10/20/2016 at NIDDK

Meeting 2017/01 ZDK1-GRB-S-J1  SRO: NAJMA BEGUM

10/25/2016 at CSR (Chair)

Meeting 2017/01 ZRG1-BST-U-50  SRO: Kee Pyon

10/27/2016 at CSR (Chair)

Meeting 2017/01 ZRG1-VH-J-02  SRO: Luis Espinoza

02/16/2017 at NIDDK (Chair)

Meeting 2017/05 ZDK1-GRB-7-M4  SRO: JIAN YANG
Meeting 2017/05 ZDK1-GRB-7-M2  SRO: JIAN YANG

03/22/2017 at NIDDK

Meeting 2017/05 ZDK1-GRB-2-M3  SRO: THOMAS TATHAM

05/24/2017 at NIDDK

Meeting 2017/10 ZDK1-GRB-S-O2  SRO: NAJMA BEGUM

B. Journal reviewing/editorial Board service

Dr. Morris

Associate Editor: Molecular Pharmacology

Dr. Lin

Dr. Lin served as scientific reviewer of multiple journals including Oncogene, Scientific Reports and PLoS ONE.
3.3. Dissemination of research results

A. Presentations

We have been disseminating the results of our study to the research community through invited talks at the universities and scientific conferences in the past 12 months.

Research presentations made by Dr. Morris

Feb 26-March 2 2017 Keystone Conference on Lipidomics and Bioactive Lipids in Metabolism and Disease. Lysophosphatidic acid metabolism and signaling in atherosclerosis. Morris, Smyth

NIEHS FEST, Durham NC December 5-8 Interactions between Diet and Toxicant Exposure Lead to Increased Circulating Levels of the Cardiometabolic Disease Biomarker TMAO. Petreillo, Hennig, Morris

PCB Workshop Kobe Japan Oct 7-13 Interactions between Diet and Toxicant Exposure Lead to Increased Circulating Levels of the Cardiometabolic Disease Biomarker TMAO. Petreillo, Hennig, Morris

Dioxin 2016 Florence Italy July 28-Sept 1 Interactions between Diet and Toxicant Exposure Lead to Increased Circulating Levels of the Cardiometabolic Disease Biomarker TMAO. Petreillo, Hennig, Morris


As a junior faculty, Dr. Lin has attended multiple conferences and workshops including AACR annual meeting, University of Kentucky Markey Cancer Research Meeting and Annual Breast Cancer Symposium, which provided great opportunities for scientific communications and collaborations.

B. Publications of relevance to the project

One paper reports studies of a new class of hybrid ATX inhibitor that take advantage of our finding that bile acids bind to and modulate ATX activity. These new inhibitors may have improved bioavailability. The second paper is a commentary about the role of circulating lipoproteins in LPA metabolism and signaling. The third paper reports a deubiquitinase that targets EMT transcription factor Snail. The discovery gives indications that as another important EMT transcription factor, TWIST may be the target of deubiquitinases and becomes stabilized during breast cancer metastasis.


3.4. Future plans.

We will continue to address the goals of the funded proposal. We will continue to develop new ATX inhibitors, analytical methods and animal models to provide orthogonal experimental approaches to those described in the proposal.
4. Impact.  
TNBC is the most aggressive breast cancer subtype. TNBC is aggressive metastatic and more likely to recur than other breast cancer subtypes. TMBC does not respond to receptor targeted therapies. Currently TNBC is treated by surgery with adjuvant chemo or radiation therapy. Obesity is a risk factor for TNBC and a poor prognostic factor. The major impact of the research we are conducting is that it will identify a mechanism linking obesity to the development and progression of TNBC. The pathways we propose to study may then be targets for pharmacological intervention in TNBC.

5. Changes/ Problems  
We have not encountered any problems with the research as presented in the original proposal and as defined in the statement of work.

6. Products  
We have generated mice with tissue specific inactivation of the ENPP2 gene in adipose tissue. We were also involved in the design and characterization of a new class of "hybrid" small molecule autotaxin inhibitors.

7. Participants and collaborating organizations  

What individuals have worked on the project?

*Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name: Andrew J Morris  
Project role: PI  
Researcher identifier: PI at UK  
Nearest person months worked: 1  
Contribution to project: Directing the project

Name: Suchismita Halder  
Project role: Biochemist  
Researcher identifier: researchers at UK  
Nearest person months worked: 6  
Contribution to project: Generation and analysis of mouse strains, measurements of proteins and lipids

Name: Yiwei Lin  
Project role: PI  
Research identifier: PI at UK  
Nearest person months worked: 12  
Contribution to project: Mechanistic characterization of the Twist-ATX-LPAR1 signaling axis.

Has there been a change in the active other support of the PD/PI(s) or senior key personnel during the last reporting period?

Dr. Morris has received some new research funding while other sources of funding have expired or been renewed. None of this new support overlaps with this DOD grant. Current other support for Dr. Morris is listed below.
Lipid phosphate phosphatase 3 as a novel atherosclerosis suppressor
The goal of this project is determine how allelic variation of intronic enhancer sequences expression of the PPAP2B gene encoding lipid phosphate phosphatase 3 to determine cardiovascular disease risk using functional genomics gene knockout and gene editing approaches in cells and mouse models. Role: PI (MPI)

Twist-ATX-LPAR1 signaling axis in promoting obesity-associated triple negative breast cancer
The goal of this project is to test the hypothesis that transcriptional regulation of autotaxin and lysophosphatidic acid receptors by twist is important for obesity associated risk and progression of triple negative breast cancer. Role: PI

Adipose autotaxin: a novel link between obesity and cardiovascular disease.
The goal of this project is to test the hypothesis that adipocytes are a source of the enzyme autotaxin which promotes cardiovascular disease in obese mouse models. Role Co-Investigator

Lysophosphatidic acid and cardiovascular disease risk
The goal of this project is to test the hypothesis that association of the bioactive lipid lysophosphatidic acid with atherogenic lipoproteins is a determinant of cardiovascular disease risk. This is a renewal of BX001984 that has been approved for funding with a projected start date of 01/01/2017 Role: PI

Triple quadrupole mass spectrometer system
This award funds acquisition of an ABSciex triple quadrupole HPLC coupled mass spectrometer system to replace a ~9 year old workhorse instrument in the mass spectrometry facility core at the University of Kentucky. Role: PI

"University of Kentucky Center for Cancer and Metabolism"
Goals: To strengthen UK’s cancer research enterprise by providing a thematically focused multidisciplinary infrastructure dedicated to defining the contribution of metabolism in the development and treatment of cancer and to use this novel multidisciplinary platform to develop promising early-stage
investigators with enhanced skills in an exciting new area of cancer research. This is the project under consideration for funding.

Role: Mentor

P20 GM103527 (Cassis, L) 09/08/08-07/31/18 0.60 cal months NIH/NIGMS

Center of Research in Obesity and Cardiovascular Disease: Administrative & Analytical Core
I direct an analytical core of this center grant and serve as a mentor to junior faculty investigators supported by this award. This award provides $75,000 annual direct costs to the core and no support for my personal research program.

Role: Core director, mentor.

P42 ES007380 (Hennig, B) 04/07/97-03/31/19 0.70 cal months NIH/NIEHS

Superfund Basic Research Program: Research Support Core
I direct this core which provides Bioanalytical and Bioinformatics support to investigators of the University of Kentucky Superfund basic research program. This award provides $223,000 in direct costs to the core for the current budget period.

Role: Core director

P42 ES007380 (Hennig, B) 04/07/97-03/31/19 1.00 cal months NIH/NIEHS

Superfund Chemicals, Nutrition, and Endothelial Cell Dysfunction
The goal of this study is to identify mechanisms by which environmental pollutants impair vascular endothelial cell function to promote cardiovascular disease. This sub-project received $225,000 in direct costs for the current budget period.

Role: Co-Investigator.

R01 ES023470 (Zhou, C) 09/26/13-06/30/18 0.48 cal months NIH/NIEHS

Endocrine disruptor mediated activation of PXR causes dyslipidemia
The goal of this study is to define the role of environmental toxins that serve as ligands for PXR as regulators of pathological hyperlipidemia and cardiovascular disease. I will make mass spectrometry based measurements of lipids and environmental toxins. This award provides no support for my personal research program.

Role: Co-Investigator.

R01 DK107646 (Kern, P) 09/21/15-07/31/18 0.0 cal months NIH/NIDDK

Cold Induced Changes in Human Subcutaneous White Adipose
The goal of this study is to define the mechanisms and physiological consequences of cold-induced “browning” of white adipose tissue. I will make measurements of lipids and related metabolites for this project. I will make measurements using stable isotope tracers to monitor glucose and fatty acid metabolism for this project. This award provides no support for my personal research program.

Role: Other Significant Contributor

R01 HL123358 (Zhou, C) 08/01/15 – 05/31/19 0.35 cal months NIH/NHLBI

A novel mechanism for ART-associated dyslipidemia and atherosclerosis
The goal of this project is to test the hypothesis that certain anti-viral drugs increase heart disease risk but altering the clearance of low density lipoproteins from the circulation. I will make measurement of these drugs and certain lipids for this project.

Role: Co-Investigator

R21 ES022745-02 (Zhou, C) 04/01/14-03/31/17 0.0 cal months
Mechanisms of atherogenic effects of bisphenol A
The goal of this project is to determine how the environmental pollutant bisphenol A promotes cardiovascular disease. I am making measurements of bisphenol A and its metabolites using mass spectrometry based methods. This award provides no support for my personal research program.
Role: Other Significant Contributor

OVERLAP

None

Dr. Lin does not have any change in the active other support to report.

W81XWH-16-1-0066 (Lin, Y) 04/15/16-04/14/19 12.0 cal months DOD/USAMRAA
Twist-ATX-LPAR1 signaling axis in promoting obesity-associated triple negative breast cancer
The goal of this project is to test the hypothesis that transcriptional regulation of autotaxin and lysophosphatidic acid receptors by twist is important for obesity associated risk and progression of triple negative breast cancer.
Role: PI

8. Special reporting requirements
N/A

9. Appendices
N/A