

AWARD NUMBER: **W81XWH-16-1-0067**

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

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14. ABSTRACT 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 <i>ENPP2</i> 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.					
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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 the 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 TNBC. We propose studies to test this hypothesis using cell and animal models.

2. Keywords

ATX: Autotaxin

LPA: Lysophosphatidic acid

LPAR1: Lysophosphatidic acid receptor 1

TNBC: Triple negative breast cancer

3. Accomplishments

3.1. Major goals and accomplishments

We hypothesize that Twist activation intensifies the inflammatory ATX-LPAR1 signaling to promote the development and progression of obesity-associated triple negative breast cancer (TNBC). The overall objective of this proposal is to delineate the function and regulation of Twist, and to explore the therapeutic potential of targeting Twist-ATX-LPAR1 axis in TNBC and obesity. Accordingly, we have defined three **Major Tasks: 1 Characterize the function of Twist in regulating ATX and LPAR1 expression; 2 Delineate the role of Twist-ATX-LPAR1 axis during TNBC cell-adipocyte crosstalk; 3 Define the Twist-ATX-LPAR1 signaling axis in promoting obesity-associated TNBC *in vivo*.** During the second year of this project we focused on Major Tasks 1 and 2 as defined, and the data generated are summarized below.

Progress related to Major Task 1, Subtask 1a: Determine whether Twist acetylation correlates with adipogenic differentiation

We have successfully used an *in vitro* adipogenesis model as proposed. Figure 1 shows the changes in adipocyte morphology before and after induction of differentiation. Quantification of triglyceride (TG) content is shown in Figure 2. Expression of adipogenesis markers Pref-1 (pre-adipocyte exclusive) and Fabp4 (surface marker of mature adipocyte) was also quantified by RT-PCR (Figure 3).

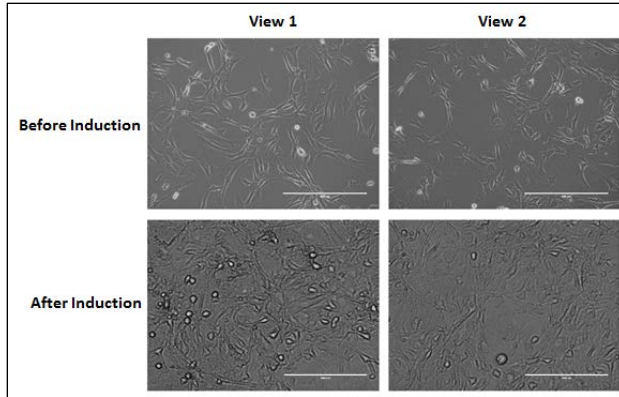


Figure 1 *In vitro* adipogenesis. Pre-adipocytes were induced according to adipogenesis protocol and images were taken under microscope.

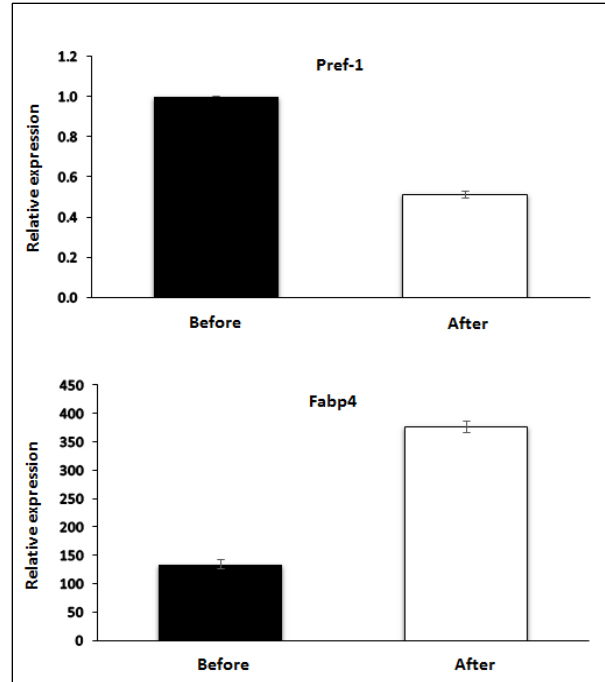
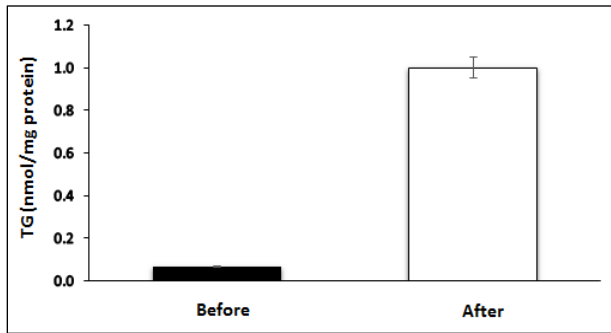


Figure 3 RT-PCR results indicate the relative mRNA levels of Pref-1 and Fabp4 before and after adipocyte differentiation. Gapdh was used as internal control.

Figure 2 Quantification of TG content before and after induction of adipocyte differentiation.

After induction of adipogenic differentiation, samples were collected at different time points and Twist protein was enriched through immunoprecipitation. We found that Twist expression was dynamically regulated during adipogenesis, with its level reaching a peak at 48 hours post induction. In addition, Twist was significantly upregulated when cells were treated with interferons (Figure 4). At 48 hours after induction, Twist was immunoprecipitated and the level of di-acetylation was determined by Western blotting using a specific antibody developed by us (Figure 5). We found that diacetyl-twist was not detected in undifferentiated cells but the level of diacetyl-twist was increased in differentiated adipocytes, which is consistent with our hypothesis.

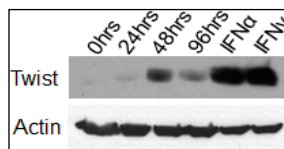


Figure 4 Pre-adipocytes were induced for differentiation and treated with interferons. Twist expression was examined at different time points.

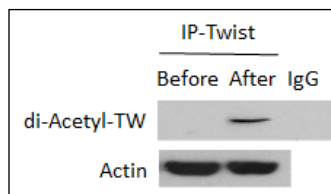


Figure 5 Before and after adipocyte differentiation, Twist was immunoprecipitated and the level of di-acetylation was detected.

Progress related to Major Task 1, Subtask 1b: Determine whether Twist activation regulates ATX synthesis and secretion by adipocytes/TNBC cells

In the previous annual report, we have successfully established Twist knockout breast cancer cell lines 1315-Twist-KO and 578-Twist-KO using CRISPR technology, and found the expression of LPAR1 and ATX was decreased in Twist knockout cells. To further examine whether LPAR1 and ATX expression is regulated by Twist di-acetylation and its interaction with BRD4, we treated cells with TNF-alpha (to increase Twist di-acetylation) or the BET inhibitor JQ1 (to disrupt Twist-BRD4 interaction). We failed to detect significant change of expression of LPAR1 and ATX by Western blotting, due to limited sensitivity of commercial available antibodies. However, RT-PCR showed that TNF-alpha increased, while JQ1 decreased the expression of LPAR1 and ATX in adipocytes as well as breast cancer cells SUM1315 and Hs578T (Figure 6), which was consistent with our hypothesis. To determine the level of ATX more quantitatively, we plan to measure ATX activity by LC-MS as proposed. We are optimizing the treatment conditions of JQ1 and TNF-alpha (drug concentration and duration of treatment) on adipocytes and various breast cancer cells (MDA-MB-231, SUM1315, BT549 and MDA-MB-157) for LC-MS analysis.

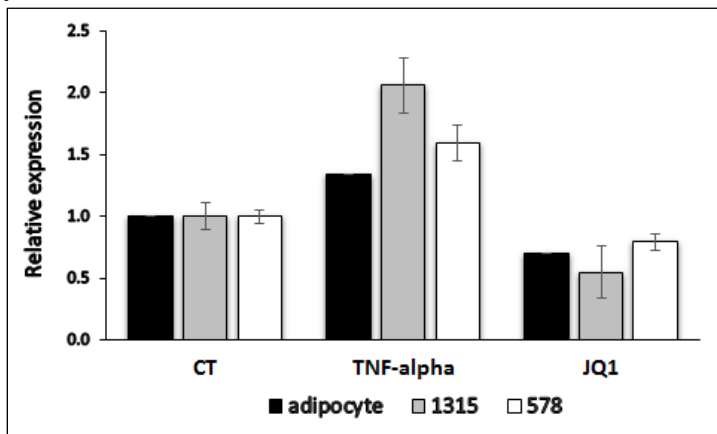


Figure 6 Human breast cancer cells and adipocytes were treated with TNF-alpha and JQ1, and the mRNA level of ENPP2 was examined by RT-PCR. GAPDH was used as internal control.

Progress related to Major Task 1, Subtask 1c: Determine whether ENPP2 and LPAR1 are direct target genes of the Twist-BRD4 complex

Originally we planned to perform luciferase assay as well as ChIP assay to determine whether Twist directly regulates ENPP2 and LPAR1 expression. An alternative approach, as mentioned in the proposal, would be to perform human gene 2.0 ST array analyses in combination with deep sequencing (ChIP-Seq). This approach can determine direct targeting of ENPP2 and LPAR1 by Twist as well. In addition, we hypothesize that Twist can regulate the expression of other genes critical in the development of obesity-associated TNBC. The array-ChIP-Seq combination approach can provide comprehensive and unbiased data and better address this hypothesis. Furthermore, through gene set enrichment analysis (GSEA) of these data, we will be able to identify potential signaling pathways correlated to the Twist-ATX-LPAR1 axis. Therefore, application of the array-ChIP-Seq analysis is highly relevant to this project and advantageous compared to luciferase and ChIP assays. We have already completed the human gene 2.0 ST array analyses. We have also evaluated several commercial available Twist antibodies for ChIP-Seq and optimized experimental conditions, and will submit the samples for analysis in the coming months.

Progress related to Major Task 2, Subtask 2a: Determine the role of Twist-ATX-LPAR1 axis in modulating the properties of TNBC cells and adipocytes

To determine whether the crosstalk between adipocytes and breast cancer cells is dependent on the Twist-ATX-LPAR1 axis, we performed adipocyte-breast cancer cell co-culture. In brief we cultured breast cancer cells in the upper chamber and mouse adipocyte 3T3-L1 in the bottom chamber. JQ1, TNF-alpha or control solvent (DMSO) was added to the culture medium to regulate the expression of ATX and LPAR1.

After co-culture, invasion assay was performed, as breast cancer cells passing through basement membrane layer and clinging to the bottom of the insert membrane were stained with crystal violet and quantified. All experiments were performed in triplicate. SUM1315 and Hs578T cells with Twist knockout were used as control. Since expression of ATX and LPAR1 is regulated by Twist, the Twist-ATX-LPAR1 axis is disrupted in these Twist knockout cells. As expected, TNF-alpha increased and JQ1 decreased the invasion of breast cancer cells, and knockout of Twist compromised the effect of TNF-alpha (Figure 7). We are also planning to use the co-culture system to test the effect of a highly specific ATX inhibitor PF8380 as well as LPAR1 antagonists on breast cancer cell invasion. In brief, experimental groups will be set up as (1) culture medium with LPA as positive control; (2) culture medium with ATX and its substrate (in order to generate LPA) as another positive control; (3) culture medium with only ATX substrates; and (4) culture medium with ATX substrates and ATX inhibitor/LPAR1 antagonist. We expect to find that addition of ATX inhibitor or LPAR1 antagonist will inhibit breast cancer cell invasion (experimental group (3) vs group (4)). Furthermore, we performed mammosphere formation assay using SUM1315 and Hs578T cells with or without Twist knockout. During culture, cells were treated with JQ1. Consistent with our hypothesis, JQ1

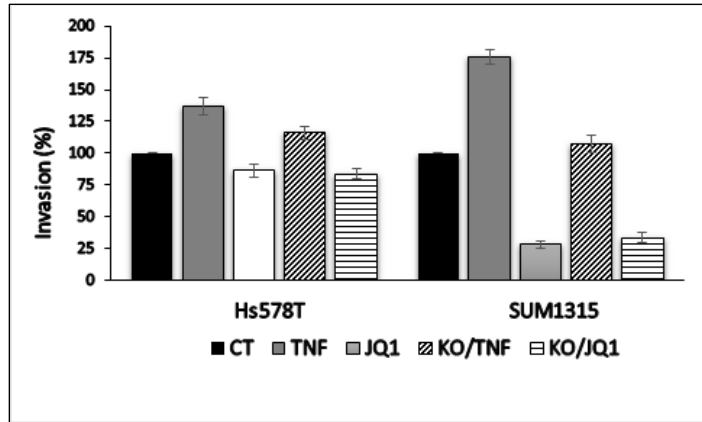


Figure 7 Hs578T and SUM1315 cells were treated with TNF-alpha and JQ1 and cell invasion assay was performed.

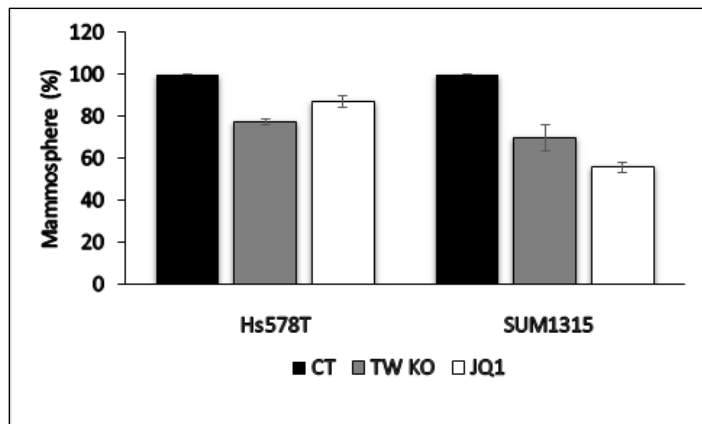


Figure 8 Hs578T and SUM1315 cells with or without Twist knockout were treated with JQ1 and mammosphere formation was examined.

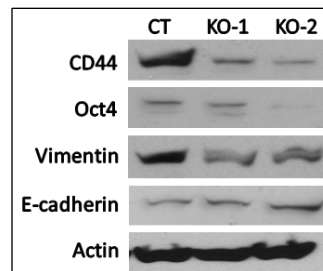


Figure 9 Western blot examining expression of EMT and CSC markers in SUM1315 cells with or without knockout of Twist.

treatment or Twist knockout could inhibit mammosphere formation (Figure 8). Furthermore, we performed Western blotting and RT-PCR to examine the expression of EMT markers and cancer stem cell (CSC) markers in the abovementioned cells. As expected, we found that Twist knockout reduced the expression of CD44, Oct4 and Vimentin, and increased the expression of E-cadherin (Figure 9).

It was found that breast cancer cells could induce delipidation (decrease of cellular TG content) of co-cultivated adipocytes. Adipocyte delipidation may happen to the tumor's benefit since it provides extra source of energy to fuel tumor growth and metastasis. To examine whether TNBC cell-conditioned media can cause delipidation of adipocytes, we performed TG quantification of 3T3-L1 adipocytes. Unexpectedly we only found modest changes of TG content in adipocytes cultured in conditioned media from TNBC cells with Twist knockout or JQ1 treatment compared to control (Figure 10). We will continue to optimize experimental conditions to confirm our hypothesis. Lastly, we are now collecting samples of SUM1315 cells cultured in (1) regular medium; (2) regular medium with JQ1; (3) adipocyte-conditioned medium and (4) adipocyte-conditioned medium with JQ1 for human inflammatory cytokines & receptors PCR array analysis. Taken together, we found that the Twist-ATX-LPAR1 axis is critical in mediating the crosstalk and properties of TNBC cells and adipocytes.

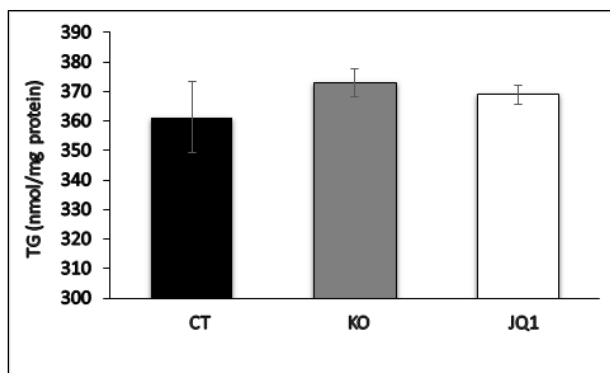


Figure 10 TG content was quantified in adipocytes cultured in TNBC cell-conditioned media.

Progress related to Major Task 3, Subtask 3b: Define the role of Twist-ATX-LPAR1 axis in tumorigenesis and metastasis of TNC cells *in vivo*

The broad goal of this task is to generate mice with adipocyte specific deficiency of ATX. These mice will then be used to determine the role of adipose derived ATX in breast cancer tumorigenesis and metastasis. Because we intend to explore the effect of diet induced obesity on breast cancer growth in these models we also examined the effect of feeding these mice an obesity promoting high fat diet.

We have established two animal models with reduced ATX levels. Deletion of the *Enpp2* gene, encoding ATX, is embryonically lethal, therefore systemic ATX reduction was achieved postnatally by breeding mice with exons 3 and 4 of *Enpp2* flanked by lox-P sites (fl/fl) to mice carrying the Cre recombinase under the control of the MX-1 promoter and activating the promoter in neonatal pups with synthetic double-stranded RNA (MX1- Δ mice). Adipocyte-specific loss of ATX was achieved with Cre recombinase under the control of the adiponectin promoter (Adipoq- Δ mice). To elicit diet-induced obesity, littermate mice with or without Cre recombinase were placed on HFD for 20 weeks.

Global reduction in *Enpp2* expression was observed in MX1- Δ mice (open bars, Figure 11A), with significantly lower levels of gene expression in kidney and spleen, and to a lesser extent in heart and lung as compared to identically treated fl/fl littermate controls (dark bars; Figure 1A). ATX protein in subcutaneous and visceral adipose tissue was also lower in MX1- Δ as compared to fl/fl controls (Figure 11B). ATX activity was reduced by approximately 50% in plasma from

MX1-Δ mice (Figure 11C). Together, these results confirm the effectiveness of the post-natal strategy to reduce *Enpp2* expression and ATX levels. No significant difference was observed between the MX1-Δ and fl/fl mice in weight gain on HFD (Figure 1D) or fat and lean mass after 20 weeks of diet (Figure 11E). No difference in heart, liver, lung or spleen weight was observed

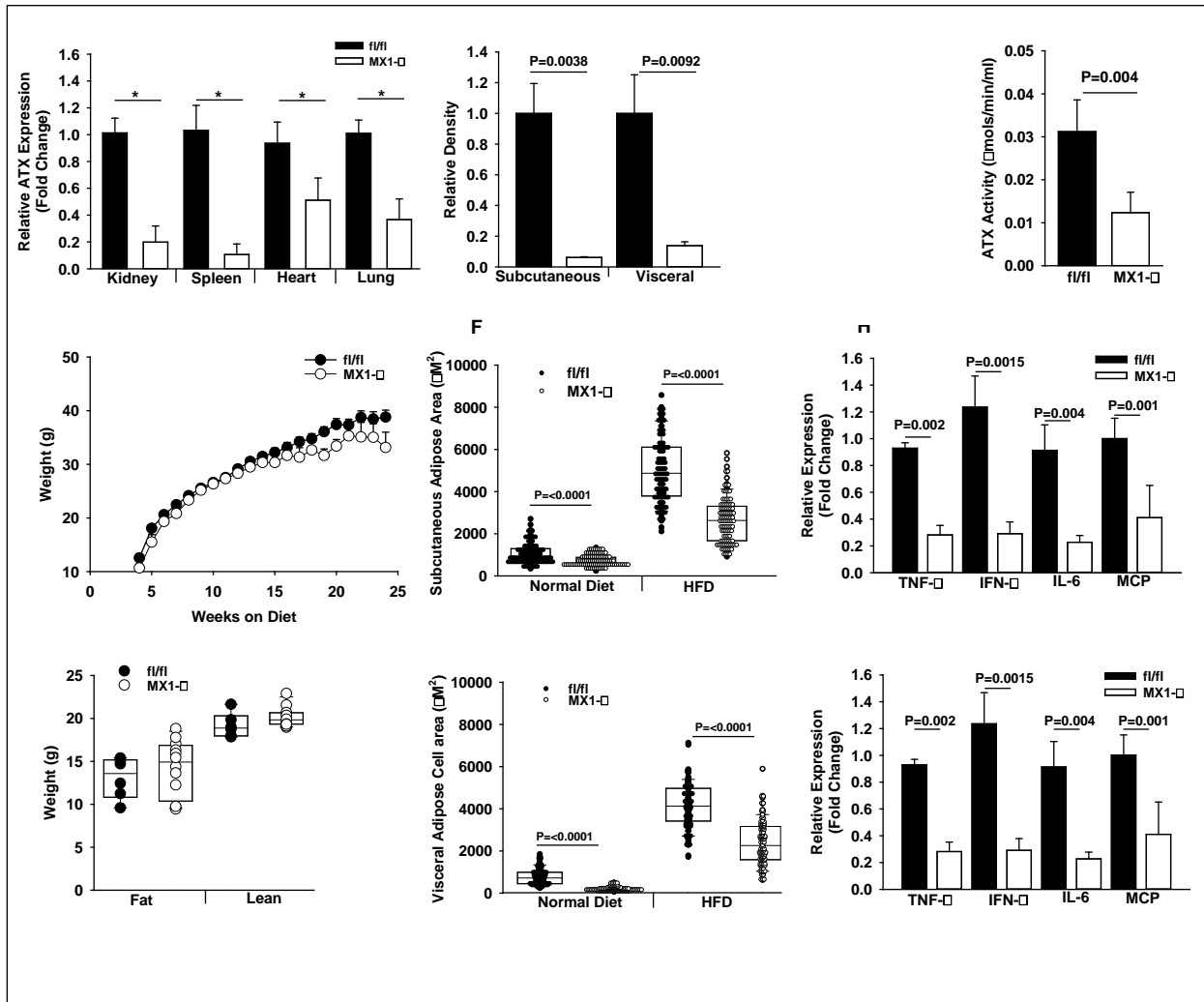


Figure 11 Characterization of mice with global post-natal reductions in ATX expression: Reduction in adipocyte size and adipose tissue inflammation without an effect on body weight after HFD. **A.)** Relative gene expression in different tissues in fl/fl (dark bars) and MX1-Δ (open bars) male mice (n=7). **B.)** ATX protein expression in subcutaneous and visceral fat. **C.)** Plasma ATX activity (μmols/min/ml) in fl/fl (dark bars) and MX1-Δ (open bars) male mice (n=7). **D.)** Average body weight in fl/fl (dark circles) and MX1-Δ (open bars) male mice (n=7) at the indicated times. Mice were placed on HFD at 4 weeks of age. **E.)** Body composition by fat and lean weight in fl/fl (dark circles) and MX1-Δ (open bars) male mice (n=5) after 20 weeks on HFD. **F.)** Cell area in subcutaneous adipose tissue in fl/fl (dark circles) and MX1-Δ (open bars) male mice (n=5) on normal chow or after 20 weeks on HFD. **G.)** Cell area in visceral adipose tissue in fl/fl (dark circles) and MX1-Δ (open bars) male mice (n=5) on normal chow or after 20 weeks on HFD. **H.)** Gene expression in subcutaneous adipose tissue from fl/fl (dark circles) and MX1-Δ (open bars) male mice after 20 weeks on HFD. **I.)** Inflammatory gene expression in visceral adipose tissue from fl/fl (dark circles) and MX1-Δ (open bars) male mice after 20 weeks on HFD. Comparison between genotypes was performed by t-test. * = P<0.05.

(data not shown). Interestingly, adipocyte cell size in both subcutaneous (Figure 11F) and visceral (Figure 11G) fat was significantly smaller in the MX1-Δ mice. In parallel with the changes in cell size, inflammatory marker expression in subcutaneous (Figure 11H) and visceral (Figure 11I)

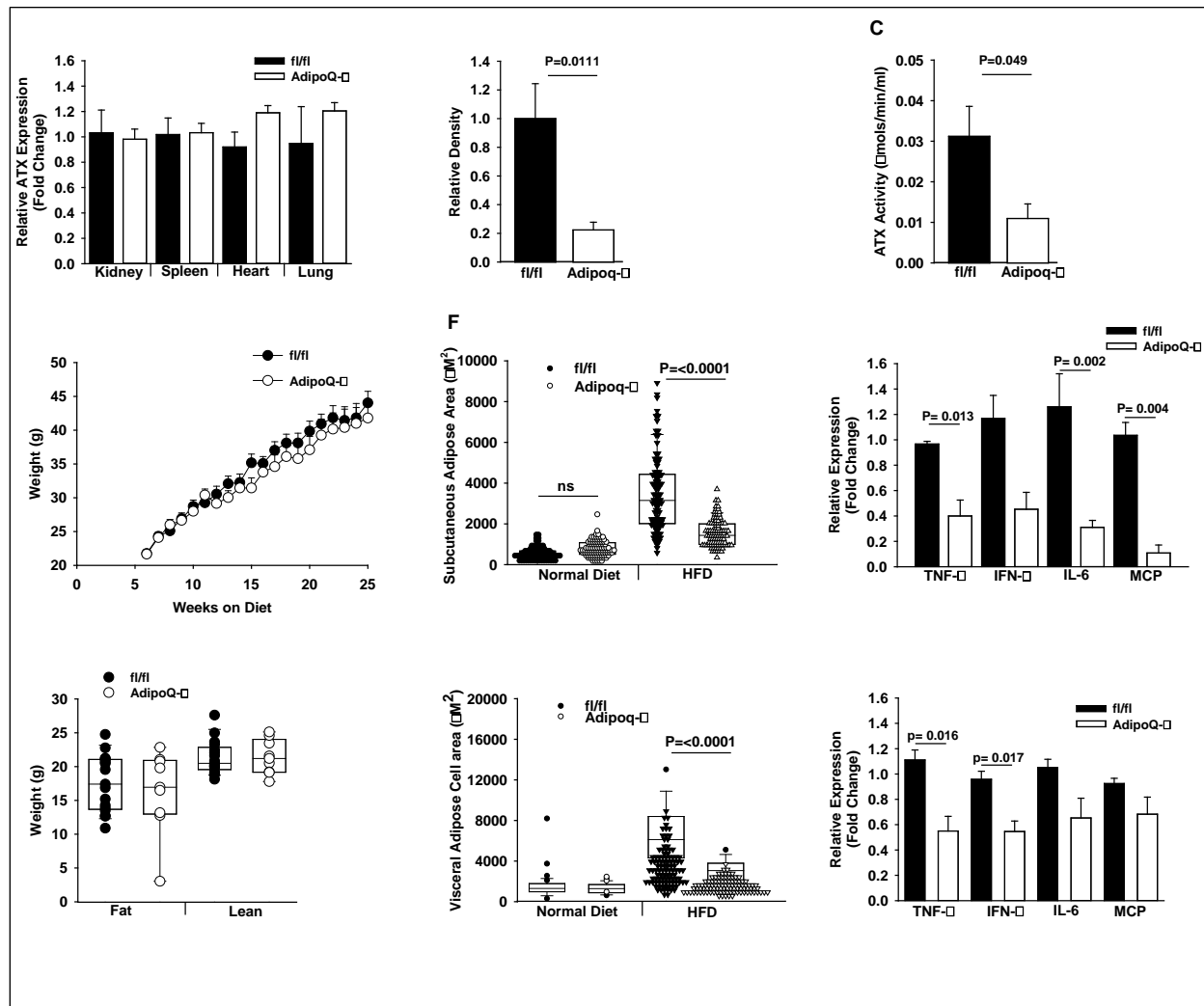


Figure 12 Characterization of mice with adipose specific reductions in ATX expression: Reduction in adipocyte size and adipose tissue inflammation without an effect on body weight after HFD. **A.)** Relative gene expression in different tissues in fl/fl (dark bars) and Adipoq-Δ (open bars) male mice (n=5). **B.)** ATX protein expression in subcutaneous and visceral fat. **C.)** Plasma ATX activity (μmols/min/ml) in fl/fl (dark bars) and Adipoq-Δ (open bars) male mice (n=5). **D.)** Average body weight in fl/fl (dark circles) and Adipoq-Δ (open bars) male mice (n=5) at the indicated times. Mice were placed on HFD at 4 weeks of age. **E.)** Body composition by fat and lean weight in fl/fl (dark circles) and Adipoq-Δ (open bars) male mice (n=5) after 20 weeks on HFD. **F.)** Cell area in subcutaneous adipose tissue in fl/fl (dark circles) and Adipoq-Δ (open bars) male mice (n=5) on normal chow or after 20 weeks on HFD. **G.)** Cell area in visceral adipose tissue in fl/fl (dark circles) and Adipoq-Δ (open bars) male mice (n=5) on normal chow or after 20 weeks on HFD. **H.)** Gene expression in subcutaneous adipose tissue from fl/fl (dark circles) and Adipoq-Δ (open bars) male mice after 20 weeks on HFD. **I.)** Inflammatory gene expression in visceral adipose tissue from fl/fl (dark circles) and Adipoq-Δ (open bars) male mice after 20 weeks on HFD. Comparison between genotypes was performed by t-test. * = P<0.05.

adipose tissue was also lower in MX1- Δ animals. In particular, gene expression for tumor necrosis factor alpha (*Tnfa*), interferon gamma (*Ifny*), interleukin-6 (*IL-6*) and chemokine CC motif ligand 2 (*Mcp1*) were all reduced in the MX1- Δ mice. These findings suggest a change in adipose response to diet-induced obesity in mice with reduced ATX levels.

We also examined the phenotype of Adipoq- Δ mice fed HFD for 20 weeks. No difference in kidney, spleen, lung, or heart *Enpp2* expression was observed between Adipoq- Δ mice and their fl/fl littermate controls (Figure 12A). ATX levels in subcutaneous fat were significantly reduced in Adipoq- Δ mice (Figure 12B), as was plasma ATX activity (Figure 12C). No difference in weight gain over 20 weeks on HFD diet was noted between the two groups (Figure 12D), and lean and fat body masses were similar (Figure 12E). Organs weights were also not different. As was observed with systemic ATX reduction, adipocyte-specific targeting of ATX resulted in smaller adipocyte size in mice fed HFD (Figure 12F and 12G), although not in mice on normal chow. Subcutaneous adipose (Figure 12H) and visceral adipose (Figure 12I) from Adipoq- Δ mice also displayed lower levels of some but not all inflammatory cytokine gene expression. In particular, gene expression for *Tnfa*, *IL-6*, and *Mcp-1* were lower in Adipoq- Δ subcutaneous fat (Figure 4H) and for *Tnfa* and *Ifny* in Adipoq- Δ visceral fat (Figure 12I).

3.2. Opportunities for professional development

A. Grant review/study section service

NIH peer review (Dr. Morris)

10/20/2016 at NIDDK Meeting 2017/01 ZDK1-GRB-S-J1 SRO: NAJMA BEGUM

10/25/2016 at CSR Meeting 2017/01 ZRG1-BST-U-50 SRO: Kee Pyon

10/27/2016 at CSR Meeting 2017/01 ZRG1-VH-J-02 SRO: Luis Espinoza

02/16/2017 at NIDDK 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

Editorial Board: Journal of Lipid Research

Ad hoc peer review for several commercial and societal journals.

Dr. Lin

Scientific reviewer of multiple journals including Oncogene, Scientific Reports and PLoS ONE.

3.3. Dissemination of research results

A. Presentations (Dr. Morris)

Feb 26-March 2 2017 Keystone Conference Lipidomics and Bioactive Lipids in Metabolism and Disease, Lake Tahoe, CA “Functional validation of PPAP2B gene variants as determinants of coronary artery disease risk.”

August 18-20, Dioxin 2017 Vancouver Canada “Relationship between serum Trimethylamine N-oxide and exposure to dioxin-like pollutants”

August 20-25, 2017 FASEB Conference Lysophospholipid and Related Mediators - From Bench to Clinic, New Orleans LA. Promoter enhancer interactions regulating PLPP3 gene expression.

December 6-8 2017, NIEHS Superfund Annual Meeting, Philadelphia PA. “Lipidomic analysis of PCB induced hepatic steatosis”

B. Publications of relevance to the project

Kraemer MP, Halder S, Smyth SS, Morris AJ. Measurement of Lysophosphatidic Acid and Sphingosine-1-Phosphate by Liquid Chromatography-Coupled Electrospray Ionization Tandem Mass Spectrometry. *Methods Mol Biol.* 2018; 1697:31-42. doi: 10.1007/7651_2017_55. PubMed PMID: 28770493.

Yiwei Lin, Yu Wang, Qing Shi, Qian Yu, Cuicui Liu, Jing Fang, Jiong Deng, B.Mark Evers, Binhua P. Zhou, Yadi Wu. Stabilization of the transcription factors slug and twist by the deubiquitinase dub3 is a key requirement for tumor metastasis. *Oncotarget* (2017) 8(43), 75127-75140.

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

Mice with inducible post-natal and adipose specific inactivation of the ENPP2 gene.

7. Participants and collaborating organizations

What individuals have worked on the project?

Andrew J Morris (no change)

Suchismita Halder (no change)

Yiwei Lin (no change)

Has there been a change in the active other support during the last reporting period?

2I01CX001550 (Dr. Morris) 01/01/17-12/31/21 3.00 cal months
VA CSR&D Merit Review \$150,000 +5/8 GS15 salary/benefits
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.
Role: PI

1S10OD021753-01A1 (Dr. Morris, A.) 3/15/17-3/14/18 0 cal months
NIH/NCATS Shared instrumentation grant program \$352,381
Triple quadrupole mass spectrometer system
This award funds acquisition of a Thermo Q-Exactive hybrid quadrupole orbitrap mass spectrometer system to replace a ~9 year old workhorse instrument in the mass spectrometry facility core at the University of Kentucky.
Role: PI

P30 ES026529 (Shi, X) 04/01/17-03/31/22 1.20 cal months
NIH/NIEHS \$1,000,000
Center for Appalachian Research in Environmental Sciences
The overall goal of this application is to support an integrated core center to increase the efficiency and impact of environmental disease research at the University of Kentucky. Dr. Morris's role is to direct an Analytical Core that provides bioanalytical and computational services to center-affiliated investigators which has a budget of \$150,000/year.
Role: Core Lead (Analytical Core)

P20 GM121327 (MPI: St. Clair, D; Zhou, B.P.) 12/01/16-11/30/21 0.60 cal months
NIH/NIGMS \$1,500,000
"University of Kentucky Center for Cancer and Metabolism"
The goal is 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. Dr. Morris is a mentor for one of the junior investigators supported by this award.
Role: Mentor

8. Special reporting requirements

N/A

9. Appendices

N/A