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TITLE: Cotargeting the lncRNA-PIP3 Interaction and AKT/PI3K Signaling Axis: A Novel Paradigm for Treating Triple-Negative Breast Cancer

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14. ABSTRACT
Patients with triple-negative breast cancer (TNBC) have a high incidence of early relapse and metastasis; currently, chemotherapy and targeted therapies are the main treatment modalities for TNBC, but one-third of patients develop recurrence and drug resistance within 3 years of therapy. Recently, we have discovered that LINK-A (Lipid-Interacting Noncoding RNA for Kinase Activation), a breast cancer-upregulated lncRNA, interacts with PtdIns (3,4,5)P3. In vitro and in vivo experiments demonstrated that LINK-A is critical for breast cancer cell invasiveness and metastasis via its functional role in regulating the PI3K-AKT signaling pathway. Importantly, the pan-cancer analysis of LINK-A expression in TCGA reveals strong correlation with TNBC and its potential for metastasis. One important goal of the proposed study would be to establish LINK-A as a novel prognostic biomarker that can reliably stratify patients with TNBC according to clinical outcomes. With the aim to work on ''precision medicine'', we propose to investigate a novel lncRNA-dependent noncanonical PI3K-AKT pathway underlying the metastatic progression of TNBC. Therefore, combinations of PI3K-AKT pathway inhibitors with a LNA-based lncRNA targeting strategy tested in this application may deliver maximum efficacy in treating breast cancer metastasis.

15. SUBJECT TERMS
TNBC, Long non-coding RNA, PIP3, AKT, Phosphorylation, Locked Nucleic Acids, LNA, AKT inhibitor, Tumorigenesis

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INTRODUCTION

TNBC patients have a high incidence of early relapse and metastasis; currently, chemotherapy and targeted therapies (*e.g.* PI3K-AKT inhibition) are the main treatment modalities for TNBC, but one-third of patients develop recurrence and drug resistance within 3 years of therapy. Despite the critical need, the molecular basis for recurrence and drug resistance remains poorly understood. Recently, we have discovered that *LINK-A*, a breast cancer-upregulated lncRNA, interacts with PIP_3. *In vitro* and *in vivo* experiments demonstrated that *LINK-A* is critical for breast cancer cell invasiveness and metastasis via its functional role in regulating the PI3K-AKT signaling pathway. Surprisingly, the pan-cancer analysis of *LINK-A* expression in TCGA reveals strong correlation with TNBC and its potential for metastasis. One important goal of the study is to identify *LINK-A* as a novel prognostic biomarker that can reliably stratify patients with TNBC according to clinical outcomes. With the aim of contributing to "precision medicine", a key priority area highlighted by the Department of Defense, Breast Cancer Research Program overarching challenges, we propose to investigate a novel lncRNA-dependent non-canonical PI3K-AKT pathway underlying the metastatic progression of TNBC by using new technologies including RNAScope®, high-throughput sequencing, *in vivo*-grade locked nucleic acids (LNAs), and orthotopic xenograft models of human breast cancer metastasis. Therefore, combinations of PI3K-AKT pathway inhibitors with an LNA-based lncRNA targeting strategy tested in this application may deliver maximum efficacy in TNBC.

KEY WORDS

TNBC, Long non-coding RNA, PIP3, AKT, Phosphorylation, Locked Nucleic Acids, LNA, AKT inhibitor, Tumorigenesis

ACCOMPLISHMENTS

Major goas of the project – *Our central hypothesis is that LINK-A functions to regulate the WPI3K-AKT signaling pathway via its interaction with PIP_3 in TNBC cells and that this mechanism may impact the efficacy of PI3K-AKT inhibition.* **Due to the extended evaluation and approval period regarding our animal protocol and human subjects, the grant was not set up until September 15, 2017, which is 12 months later than the proposed start date. During this period of time (09/15/2016-9/14/2017), we have established multiple collaborations nation-wide, collected breast cancer tissues, established stable cell lines based on collaboration and generated a transgenic mouse model with support from the PI's start-up funds. The research accomplishments are described under each major task of the proposed research work.**

Major Task 1: Characterization of interaction between PIP_3, *LINK-A* and AKT. We plan to characterize the interaction between PIP_3, *LINK-A*, and AKT in vitro and in vivo. We will also perform functional rescue experiments to demonstrate that LINK-A-PIP3 interaction is required

for AKT activation and downstream cellular activities. To facilitate research investigating the functional role of *LINK-A* in cells, we have genetically edited the *LINK-A* gene into breast cancer cell lines, which will provide a reliable, reproducible, and convenient system. Gene Editing/Cellular Model Core Facility at MD Anderson has generated a genomic deletion of *LINK-A* using CRISPR/Cas9 technology based on collaboration. The single colonies derived from MDA-MB-231 cells have undergone genomic deletions of the 36 nucleotides that are required for PIP_3 binding (referred to as *LINK-A*$^{\Delta PIP3}$) (**Figure 1**).

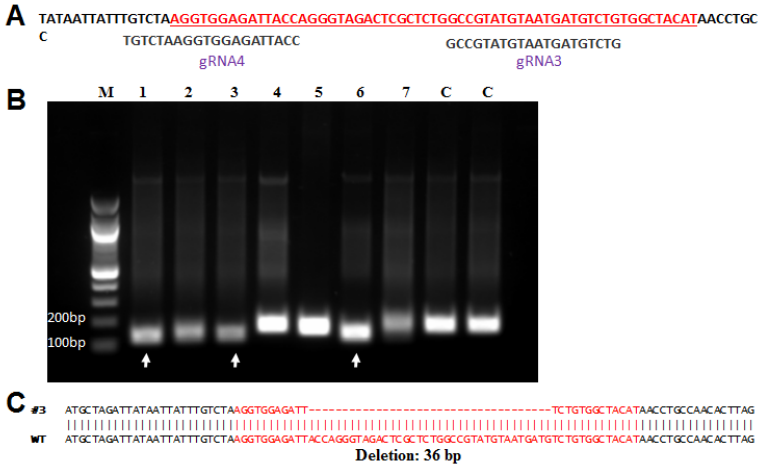

Figure 1. Generation of *LINK-A* deletion mutant using CRISPR/Cas9 technology. A, Illustration of genomic deletion region of *LINK-A* (Chr1: 238644075-238644134) using two sgRNA as shown. **B,** DNA agarose gel detection of deletion of genomic DNA in MDA-MB-231 cells. **C,** Sanger sequencing of clone #3 indicating 36 nucleotides deletion.

To understand how LINK-A associates with AKT and facilitates AKT activation and the association between AKT and PIP_3, we have consulted Lei Zheng, Ph.D., Associate Professor of the University of Texas, School of Medicine, regarding the 3 dimensional structure of the AKT PH domain and computational modeling of the potential *LINK-A*-AKT-PIP_3 interaction. With his insight and advice, we were better able to understand that LINK-A may associate with loop 1 and loop 2 of the AKT PH domain. Crystallographic analysis indicated that the AKT PH domain harbors three variable loops (referred to as L1: aa. 16-21; L2: aa. 40-52; L3: aa. 80-81) (**Figure 2**). The phospho-groups of IP_4 form hydrogen bonds with Lys14 and Arg23, which flank L1. We hypothesize that the formation of the *LINK-A*-PIP_3-AKT complex may cause a conformational change of the AKT PH domain, which can be studied by limited proteolysis (**Figure 2**). This information will faciliate the understanding of the molecular mechansims of LINK-A-dependent AKT activation.

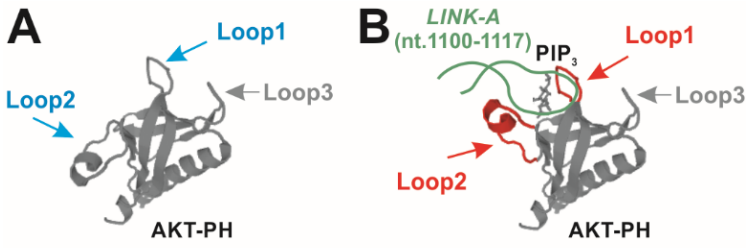

Figure 2. Graphic illustration of *LINK-A*-AKT-PIP_3 interaction. (**A**) In the absence of *LINK-A*, Loop 2 of the AKT-PH domain is free. (**B**) In the presence of *LINK-A*, Loop 2 of the AKT PH domain may be subject to conformational change for enhanced AKT-PIP_3 interaction.

What opportunities for training and professional development has the project provided?
In the process of establishing and arranging all of the necessary collaborative efforts that are essential for the project, the PI is being connected with a diverse group of experts across the nation. Additionally, the acquired tissues broaden the tools available for future research efforts in the PI's laboratory. Although a postdoctoral fellow has yet to be named, there are an abundance of resources that foster professional development throughout the institution of MD Anderson Cancer Center and the Texas Medical Center in Houston. The postdoctoral fellow will be mentored in the research techniques and methods frequently utilized in the lab and encouraged to attend the various seminars and conferences that are held by research powerhouses in the area.

How were the results disseminated to communities of interest?
The PI was awarded the Wilson S. Stone Memorial Award by MD Anderson Cancer Center, celebrating the PI's excellence in conducting research that promotes the biomedical sciences. In accepting this award, the PI presented his research focus on noncoding RNA and cancer to the broader institutional community of students, fellows, nurses, physicians, and scientists. Also, we have been maintaining close communication with our four advocates, Furjen Deng, Susan Rafte, Bree Sandlin, and Anne Meyn, as they relay our work to their respective advocacy groups.

What do you plan to do during the next reporting period to accomplish the goals?
We will strictly adhere to the statement of work to complete all outlined tasks by the three years, although the timeline will be shifted back by one year. Thus, we plan to accomplish all goals that have been proposed for the first year.

IMPACT

What was the impact on the development of the principle disciplines of the project?
In continued efforts to direct all of the collaborative efforts that had been proposed, the PI has been able to grow his network of partner scientists in their respective fields. Additionally, the PI is establishing an impressive array of tissues that can be used as a comprehensive tool in studying various cancer types.
The proposed studies will dissect the underlying mechanisms through which lncRNAs promote tumorigenesis *in vivo*, which is a new direction for the PI. The proposed research work would serve as preliminary data for PI's next grant application.
As supported by the Breakthrough Award, the PI is being shaped into a leader in the fields of lncRNA and breast cancer research, as recognized by the Wilson S. Stone Memorial Award for research in the biomedical sciences. The PI's goal is to continue to serve as a leader within the breast cancer community; build a breast cancer noncoding RNA research/education program for junior investigators; and proactively guide breast cancer science and its dissemination.

What was the impact on other disciplines?
Our research will reveal the fundamental contribution of noncoding RNAs to various disease states, broadening interest in noncoding RNA involvement in various disease processes.

What was the impact on technology transfer?
We will provide the *LINK-A* ΔPIP3 stable cell line and pertinent *LINK-A* transgenic animal models to laboratories that are interested in further investigating this topic, after we conclude our research and publish our findings.

What was the impact on society beyond science and technology?
Therapeutic options for TNBC patients have been limited due to the interwoven signaling pathways that complicate the development of targeted therapies for TNBC. The proposed study will dissect the molecular mechanisms of lncRNA-dependent resistance to AKT inhibitors, which will highlight their clinical potential as diagnostic indicators, stratification markers, and therapeutic targets. Clinically, the lncRNA-directed targeted therapy using LNAs could serve as a promising strategy to improve outcomes for TNBC patients. This proposal will impact the field of breast cancer research by elucidating genetic evidence for the contribution of lncRNAs as oncogenes that promote breast cancer initiation and progression. Successful completion of this research will contribute to pioneering efforts to develop and mature the field of personalized medicine, specifically with regards to LNAs against TNBC relevant lncRNAs.

CHANGES/PROBLEMS:

Actual or anticipated problems or delays and actions or plan to resolve them
We experienced about a year long delay in the start date of this grant proposal in addressing sensitive matters related to the inclusion of human subjects and animal research. Correspondence between a numbers of different parties has delayed the process more than anticipated. All concerns have been addressed and the project will officially begin on September 15, 2017.

PRODUCT

Publications, conference papers, and presentations

Publication: We expect to publish high-impact publications at the end of the grant period.

Invited Presentations:

2016 Wilson S. Stone Award presentation “LncRNA Wire Up Cancer Signaling”, "Cancer Evolution: Mechanisms of Vulnerability and Resistance", MD Anderson, Houston, TX

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Liuqing Yang*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-6518-474X*
Nearest person month worked:	*0*
Contribution to Project:	*Based on collaboration, PI established stable cell line to deplete PIP3 binding motif of LINK-A*
Funding Support:	*Startup funds of PI*

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

No

What other organizations were involved as partners?

Not applicable

SPECIAL REPORTING REQUIREMENTS

Not applicable

APPENDICES

Not applicable