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14. ABSTRACT

The hypothesis of this proposal is that Latent TGF-beta Binding Protein 1 (LTBP1) is a pro-metastatic factor in breast cancer. To test this hypothesis we proposed three aims 1) Determine the utility of LTBP1 expression as a biomarker in human breast cancer 2) Determine the consequences of modulating LTBP1 levels on cell motility, invasion and metastasis in breast cancer cell lines 3) Investigate the pathobiological effects of gain and loss of LTBP1 in genetically engineered mouse models. Our most important findings in this cycle are that 1) elevated LTBP1 expression is associated with poor outcome in Basal and HER2 positive ER-negative breast cancers; 2) highly metastatic variants breast cancer cells express elevated LTBP1 expression compared to parental lines; 3) that high LTBP1S expression occurs in mesenchymal-like triple negative breast cancer cell lines; and 4) that lowering expression level within these cells significantly reduces their invasive capability. This was achieved by determining the expression of total LTBP1 as well as LTBP1S and LTBP1L isoforms by qPCR in a large panel of cells and their metastatic variants and by lentiviral knock down.

15. SUBJECT TERMS
Cell Adhesion, Involution, Metastasis, Latent TGFbeta Binding Protein, Pregnancy-associated Breast Cancer

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Table of Contents

Page

1. INTRODUCTION: Our previous results showed that Latent TGF-beta binding protein 1 (LTBP1) encases the permanent breast ductal system positioning latent forms TGF-beta in a poised manner to control breast stem cell activity. We also found LTBP1 elevated during involution, a developmental window associated with increased risk for highly metastatic forms of breast cancer. LTBP is essential for integrin-mediated stretch activation of TGF-beta, which acts as a tumor suppressor early in cancer formation but later on promotes metastasis. The goal of this proposal is to test whether elevated LTBP1 expression induces metastasis. The aims of this project are to examine LTBP1 expression in breast cancer and to test functionally the effect of modulating LTBP1 on invasion and metastasis in breast cancer cells and murine breast cancer models.

2. KEYWORDS: Cell-matrix Adhesion, Involution, Metastasis, Latent TGF-beta Binding Protein, Pregnancy-associated Breast Cancer

3. ACCOMPLISHMENTS:

- **What were the major goals of the project:**

Task 1) Determine the utility of LTBP1 expression as a biomarker in human breast cancer. (50 % complete)
Task 2) Determine the consequences of modulating LTBP1 levels on cell motility, invasion and metastasis in human and murine breast cell lines. (90% complete)
Task 3) Determine the pathological significance of gain and loss of LTBP1 in genetically engineered mouse models. (50% complete)

What was accomplished under these goals:

- This year our major focus has been on carrying out the experiments outlined in Task 2

Task 1. Determine the expression of LTBP1 in human and mouse breast cancers and breast cancer cell lines (50% complete)

Previously, we reported that luminal cells produce LTBP leading us to propose that the cellular seed of breast cancers secrete their own prometastatic soil. We performed preliminary in silico analyses, which showed that high Ltbp1 expression was associated with poor outcome in terms of both recurrence-free and distant metastasis-free survival (Report year 1, Fig. 1). This association was seen in all subtypes but was strongly significant for Basal and HER2 subtypes (report year 1, Fig. 2). We analyzed a small panel of breast cancer cell lines and found higher LTBP1 mRNA expression in Basal B and Luminal HER2-positive ER-negative cell lines compared to other subtypes (Report year 1 Fig 3). Thus LTBP1 expression is associated with cell lines derived from breast cancers with the worst outcome and are prevalent in young women of reproductive age.

This year we extended the in silico analyses by examining larger publicly available datasets of tumors and cell lines. Our results below show that LTBP1 is prometastatic factor correlated with triple negative status, particularly the absence of Estrogen Receptor and confirm that it has prognostic significance for basal and Her2 subtypes. We proceeded to investigate the two isoforms of LTBP1 (short (LTBP1S) and long (LTBP1L) separately. Our results show that they are differentially expressed in human breast cancer cell lines. Of particular interest LTBP1S is specifically expressed in the

mesenchymal subtype of triple negative breast cancer, which is associated with motility and invasive gene signatures.

To examine LTBP1 expression in different breast cancer subtypes we used publicly available gene expression datasets in the Cancer Genomic Browser (https://genome-cancer.ucsc.edu). This contains multiple breast cancer datasets with various associated clinical data. Mostly we used The Cancer Genome Atlas dataset as it has extensive supporting data but, where possible, also mined other datasets to independently confirm the findings. Ltbp1 expression is consistently higher in Basal and Her2 cancers than Luminal A and B (ANOVA $p<0.0001$, Figure 1).

LTBP1 expression is higher in Estrogen Receptor (ER) negative tumors ($p<0.0001$) and this finding is repeated in nine other data sets. LTBP1 is also significantly higher in triple-negative breast cancers (Figure 1). LTBP1 expression did not correlate with Her2 expression, Progesterone Receptor expression or lymph node involvement at diagnosis (data not shown).

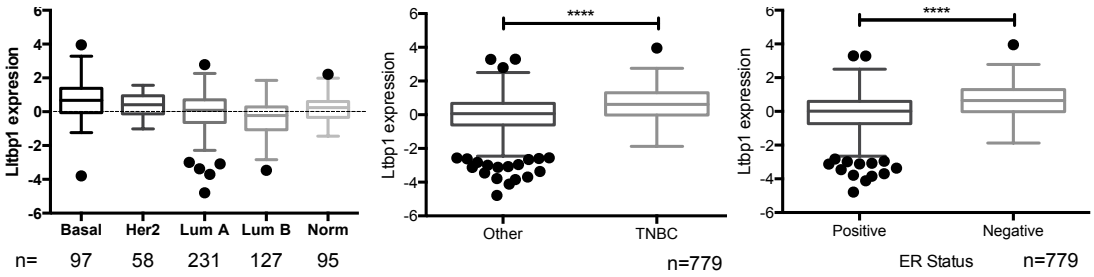


Figure 1: Expression of LTBP1 in microarray data correlated with subtype and hormone receptor status. Left: LTBP1 expression normalized across breast caner subtypes (ANOVA $p<0.0001$). Middle: LTBP1 expression in triple-negative tumors versus all other breast cancers. Right: LTBP1 expression is estrogen receptor positive vs negative tumors. **** = $p<0.0001$

Overall this data reinforces the concept that LTBP1 expression is a biomarker for poor prognosis in breast cancer, particularly Basal and Her2 subtypes.

LTBP1 isoform expression in a panel of human breast cancer cell lines

This year we investigated a larger panel of human breast cancer cells lines that were carefully selected to represent as many subtypes and variations found *in vitro* as possible (see table below). There are only three subtypes found *in vitro* as compared to five *in vivo*. Luminal cell lines generally have an epithelial-like morphology, are non-invasive and express hormone receptors while Basal B cell lines are triple-negative and highly invasive with a more transformed morphology. LTBP1 has two isoforms transcribed from two transcriptional start sites in the same gene a short and a long isoform (LTBP1S and LTBP1L). Importantly the bioinformatics data derived from human tumors presented above cannot distinguish between these isoforms as the probes are located within the shared region of both. In contrast, when analyzing cell lines, we were able to design primers that specifically detect the long or the short isoforms.

Cell Line	Subtype	Hormone Receptors
MCF7	Luminal	ER
T47D	Luminal	ER
ZR751	Luminal	ER
MDA-MB-361	Luminal	ER Her2
ZR7530	Luminal	ER Her2
AU565	Luminal	Her2
HCC1569	Basal A	Her2
HCC1954	Basal A	Her2
MDA-MB-468	Basal A	
BT549	Basal B	
HS578T	Basal B	
MDA-MB-231	Basal B	

qPCR analysis was performed on the panel of breast cancer cell lines tabulated above. All cell lines expressed LTBP1L although an average the Luminal and Basal A subtypes showed higher expression than that of the Basal B subtype. In contrast, LTBP1S was highly expressed in Basal B subtypes with little to no expression detected in the Luminal and Basal A lines (Figure 2).

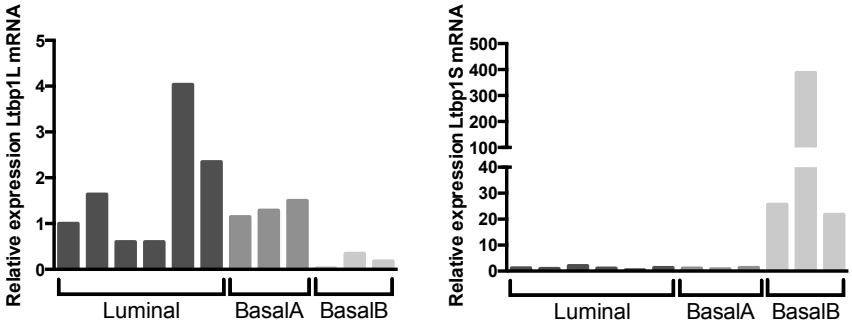


Figure 2: Expression of LTBP1L and LTBP1S in a panel of human breast cancer cell lines. qPCR analysis of LTBP1L and LTBP1S expression in breast cancer cell lines. Expression is relative to first luminal sample on each graph. Data is representative of triplicate experiments.

LTBP1 expression in TNBC subtypes

To further explore whether LTBP1S expression is linked to triple-negative cell lines in general or to the Basal B subtype in particular we investigated a larger panel of triple-negative breast cancer (TNBC) cell lines. Recently, TNBC cell lines have been categorized into three subtypes: Basal-like, Mesenchymal-like and Luminal Androgen Receptor. All the Basal B lines used in our initial panel were categorized as Mesenchymal-like and the one other TNBC cell line included in the panel (MDA-MB-468) is Basal-like. We acquired a larger panel of TNBC cell lines (see table below) and performed qPCR for LTBP1S. 4 out of the 5 Mesenchymal-like lines showed increased

LTBP1S expression. LTBP1S was not seen in any of the others (Figure 3). This suggests that LTBP1S is specifically expressed within the Mesenchymal-like subtype of TNBC.

Cell Line	TNBC subtype	Traditional subtype
HCC1937	Basal-like	Basal A
HCC1143	Basal-like	Basal A
MDA-MB-468	Basal-like	Basal A
HCC38	Basal-like	Basal B
HCC1187	Basal-like	Basal A
BT549	Mesenchymal-like	Basal B
HS578T	Mesenchymal-like	Basal B
SUM159PT	Mesenchymal-like	Basal B
MDA-MB-231	Mesenchymal-like	Basal B
MDA-MB-157	Mesenchymal-like	Basal B
MDA-MB-157	LAR	Luminal

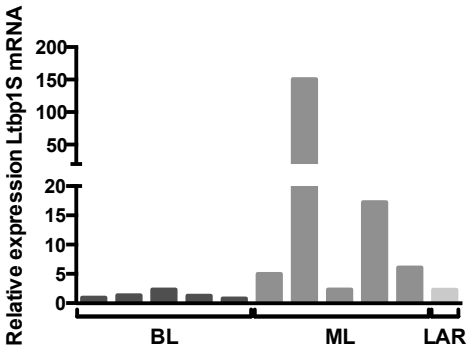


Figure 3: LTBP1S expression in triple-negative breast cancer cell line panel. BL: Basal-like, ML: Mesenchymal-like, LAR: Luminal Androgen Receptor. Data representative of three repeats.

To assess if LTBP1 expression correlates with TNBC subtypes in tumors as well as cell lines we used a recently published data set and online subtyping tool of TNBC (TNBCtype, http://cbc.mc.vanderbilt.edu/tnbc/index.php). After subdividing tumors into the seven TNBC subtypes we looked at total LTBP1 expression. No significant correlation was observed. However, this result is not surprising as this dataset cannot distinguish between LTBP1L and LTBP1S expression and we observed no overall trend for total LTBP1L.

Continued collection of tumors and PABC

Previously we reported the evaluation of conditions for use of LTBP antibodies, as well as antibodies detecting partner proteins elastin and fibrillin, on human tissue (Report year 1 Fig. 4). Immunochemical detection of LTBP in formalin fixed tissue continues to be problematic but works on frozen tissue. Therefore, in light of the in silico findings above, we have focussed the efforts of the NYU biorepository core on the collection of human Basal and Luminal HER2-positive ER-negative breast cancers and will use normal tissue from the Susan G Komen bank as controls.

Conclusions: Taken together our results show that LTBP1L is associated with poor outcome particularly in basal and HER2 positive subtypes, LTBP1L and LTBP1S show

distinct expression patterns in human breast cancer cell lines. LTBP1S is highly expressed within the mesenchymal-like subset of TNBC cell lines. Collectively these results suggest it will be important to know if LTBP1S expression correlated with specific TNBC tumor subtypes *in vivo* and if that in turn relates to survival.

Task 2 Determine the consequences of modulating LTBP1 level in breast cancer cell lines on motility, invasion and metastasis (90% complete).

a) LTBP1 expression in highly metastatic variants of breast cancer cell lines.
To test the hypothesis that LTBP1 is a prometastatic factor we examined LTBP1 expression in two metastatic variants of the MDA-MB-231 cell line. These were selected for increased ability to form lung (231-LM2) and brain (231-AdM) metastasis compared to the parental line. The brain metastatic line was recently re-assigned as an adrenal metastasis line. These lines together with their parental cell line were purchased from Dr. Juan Massague (Memorial Sloan Kettering Cancer Center) and analyzed by qPCR for total LTBP1, LTBP1S and LTBP1L. Both metastatic variants showed increased expression of LTBP1L and LTBP1S was up-regulated within the LM2 line (Figure 4). A qPCR probe that detects both isoforms recapitulates that of LTBP1S suggesting that LTBP1S is the major isoform expressed in MDA-MB-231 cells. This analysis supports our hypothesis by showing a correlation between increased LTBP expression and enhanced metastasis and established that MDA-MB-231 cells and their metastatic variant LM2 provide a good in vitro model in subsequent experiments for testing the effect of downregulating LTBP1 expression on migration and invasion.

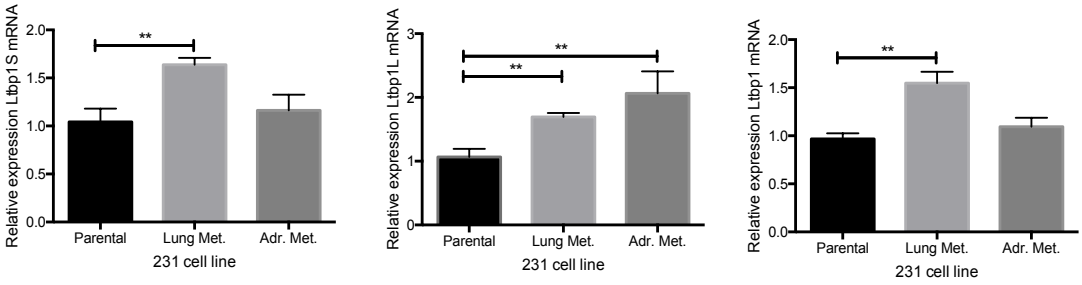


Figure 4: LTBP1 expression in MDA-MB-231 parental line and lung and adrenal metastatic variants. LTBP1S (left), LTBP1L (center) and total LTBP1 (right) mRNA was measured by qPCR. Data is presented relative to parental line. ** t-test $p<0.01$

b) Generation of an inducible lentiviral LTBP1 knock down system.
Previously we reported the cloning of two LTBP1 hairpins (sh1 within the coding region, sh2 in the 3'UTR) and scrambled controls into pLKO-GFP and pLKO-RFP vectors respectively, confirmed their ability to reduce LTBP1 expression and noted extensive cell death of infected MCF10A cells (Report year 1 Fig. 6). To circumvent this issue we recloned the hairpins and Scr control into an inducible system, pLKO Tet-on puro (Addgene 21915), which allows expression of the hairpins to be induced by the

addition of doxycycline to the media. It also confers puromycin resistance to allow selection of a pure population after infection with the vector.

We infected MDA-MB-231-LM2 cells with these inducible vectors, selected with puromycin and induced knockdown by adding doxycycline to the cells every 2 days for 5 days. Knockdown was assessed by PCR.

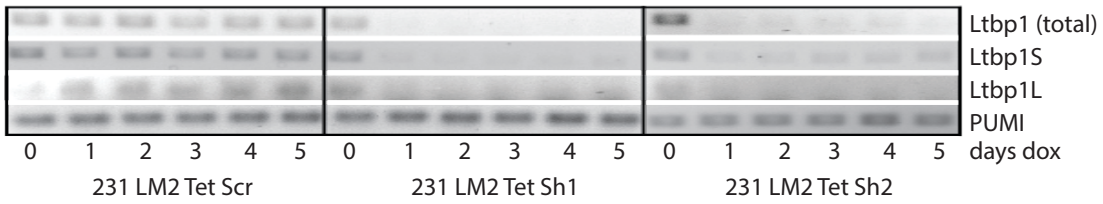
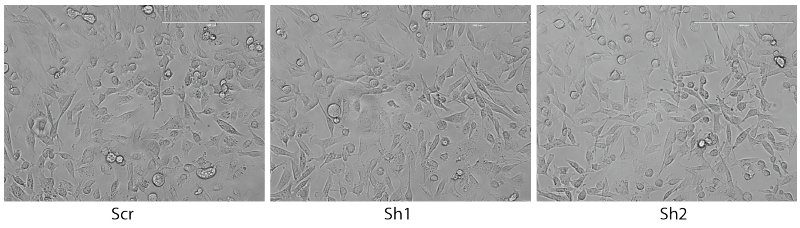


Figure 5: PCR of LTBP1 in a 5 day time course of doxycycline treated MDA-MB-231-LM2 cells containing Scr or LTBP1 knockdown vectors to show efficient knockdown is maintained.

Efficient knockdown of LTBP1 was observed within 24 hours and maintained throughout the time course examined. Knockdown of both the long and short isoforms of LTBP1 was confirmed using isoform specific primers (Figure 5). No morphological changes or cell death was observed (Figure 6).



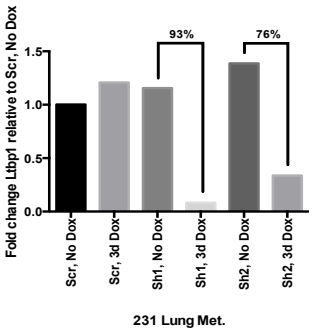


Figure 6: Light microscopy images of doxycycline treated MDA-MB-231-LM2 cells after 5 days shows no morphological changes (left). qPCR of LTBP1 after 3 days of doxycycline treatment to assess knockdown efficiency (right).

c) Effects of LTBP knock down on cell proliferation

Sh1 showed the strongest knockdown though both are over 75% reduced (Figure 6) but all results were confirmed to also be true for Sh2 eliminating concerns of off-target effects. Before using these knockdown cells to assess changes in adhesion and motility it was important to establish if proliferation was changed. Proliferation was assessed over 4 days and no change in cell division was found between knockdown and control cells (Figure 7).

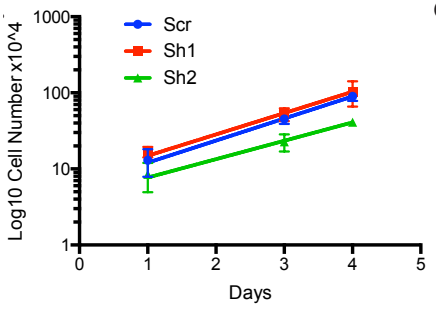


Figure 7: Cell growth of mDA-MB-231-LM2 cells over 4 days after 3 days of doxycycline treatment. Cells were counted using hemocytometer.

d) Effects of LTBP1 knock on cell adhesion

To assess the effects of LTBP1 knockdown on cell adhesion, MDA-MB-231-LM2 cells were treated with doxycycline for 3 days. 50,000 cells were plated per well of a 24 well plate, which was coated with Fibronectin or left either uncoated. After an hour non-adherent cells were removed by washing and attached cells were fixed, stained and counted. No difference in adhesion was observed on uncoated plastic. There is a minimal increase in adhesion onto Fibronectin (Figure 8).

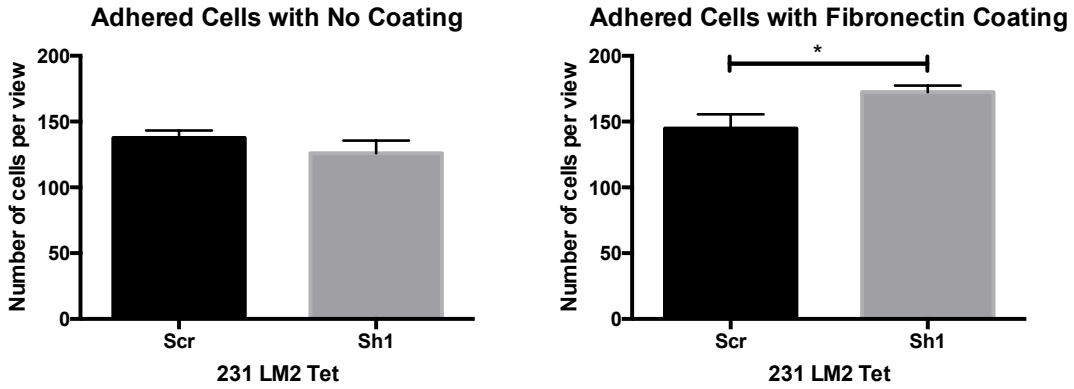


Figure 8: Number of cells adhered to uncoated or Fibronectin coated plastic after 1 hour. Three fields of view were counted per well and each conditioned was performed in triplicate (* $p<0.05$).

d) Effects of LTBP1 knock down on cell motility

To assess changes in migratory and invasive behavior of LTBP1 knockdown in MDA-MB-231-LM2 cells we first performed scratch assays. Cells were grown in the presence of doxycycline for 3 days to confluence. A pipette tip was then used to create a wound through the cells. Cells were imaged every 4 hours to calculate wound closure rate. No difference in the wound closure rate was observed between the cells lines suggesting that knockdown of LTBP1 has no effect on MDA-MB-231-LM2 cell migration *in vitro* (Figure 9).

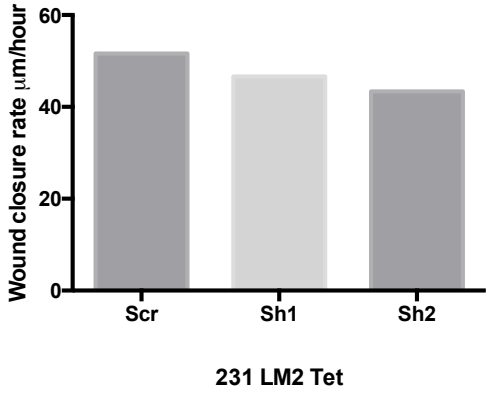


Figure 9: Closure rate of an artificially introduced wound. Size of remaining gap was measured over 8 hours and the closure rate calculated. Results represent three independent experiments. No significant difference.

However, MDA-MB-231-LM2 cells are not epithelial in morphology making scratch assays difficult to evaluate as they do not migrate as a sheet but as single cells, some of which bridge the wound very quickly while others hardly move. Therefore we sought to investigate this further by using a transwell assay. Cells that had been treated with doxycycline for 3 days were plated in low serum conditions above a well with normal serum. The cells were allowed to migrate for 3 hours through the transwell and then collected and stained. Again no difference was observed confirming that knockdown of LTBP1 does not affect migration (Figure 10).

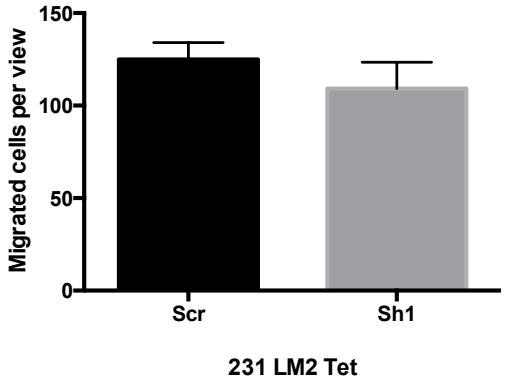


Figure 10: Transwell migration assay. Number of cells passing through a transwell in response to a chemoattractant gradient over 3 hours. Three fields of view were counted per well and each condition was performed in triplicate. Results representative of three independent experiments. No significant difference.

e) Effects on cell invasion

To examine if LTBP1 knockdown affects the invasive behavior of these cells we performed transwell assays where the transwells were coated with matrigel. Cells were plated in low serum above the matrigel and with high serum below. After 18 hours cells that had invaded through the matrigel were fixed, stained and counted.

Knockdown of LTBP1 lowered the invasion efficiency of MDA-MB-231-LM2 cells (Figure 8). This is consistent with the bioinformatics data present in Aim 1 showing LTBP1 is associated with poor prognosis, which in turn is due to invasive cancer.

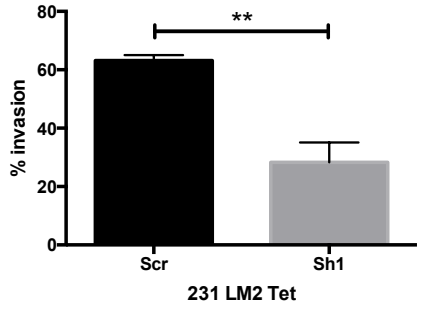


Figure 11: Invasion assay. MDA-MB-231-LM2 cells were plated into transwells coated with matrigel and allowed to invade over 18 hours. Cells were stained and counted. Three fields of view per well were counted and each condition was run in triplicate. Result representative of three independent experiments ($p<0.01$).**

Task 3: Determine the pathological significance of gain and loss of LTBP1 in mice (50% complete).

a) Generate the Ltbp1 transgenic mouse model (months 1-24)

Previously we reported cloning the MMTV-LTR, rabbit beta-globin gene, and polyA sequence into pGEM5z. We have encountered problems in cloning human LTBP1 into pHIV-ZsGreen prior to excision of LTBP-GFP and completion of the transgene. We think that the LTBP1 sequence may be too large for the lentiviral vector and have now obtained a tagged version from a group at McGill and are in the process of completing the transgene construct.

b) Generate lentiviral particles for inducible overexpression and knockdown of Ltbp1 (months 1-24)

Accordingly as Lentiviral overexpression is unfeasible we now plans to achieve overexpression using conventional plasmid vectors. The inducible lentiviral sh1 described above in Task 2b has been designed to work in both murine and human cell lines.

c) Introduce lentiviral constructs into poorly MMTV-Neu/ErbB and highly metastatic MMTV-PyMT MECS and transplant into cleared fatpads.

The PyMT and MMTV-neu mice have begun to develop tumors and we have harvested them and prepared primary tumor mammary epithelial cells and frozen these as a source for infection with the constructs to determine the effects of modulating LTBP1 on tumor progression.

- **What opportunities for training and professional development has the project provided.**

1. Dr. Gann has taken a course in "Scientific Ethics".
2. Dr. Gann has acquired skills in qPCR, and FACS analysis.
3. Drs. Gann and Oliver have acquired skills in assessing Lentiviral knock down.
4. Dr. Oliver obtained a fellowship from The Susan G Komen Foundation

- **How were the results disseminated to communities of interest**

1. Dr. Cowin presented this work to the Developmental Biology Program, Institute of Biotechnology, Helsinki, Finland on Feb 12 2015.
2. Dr. Cowin presented this work to the Skirball Institute Faculty Works in Progress bimonthly meeting.
3. This work has been presented to the NYU Molecular Pathology and Immunology Works in Progress meeting.
4. This work was presented as a poster at the GRC on Cell Contact and Adhesion, Proctor Academy, NH, June 2015
5. This work was presented to our scientist breast cancer survivor advovcates: Dr. Dr. Cara Gottardi, Northwestern University and Dr. Juliet Daniels, McMaster University in June 2015.

- **What do you plan to accomplish during the next reporting period to accomplish the goals and objectives**

1. Stain fresh frozen samples from the biorepository with LTBP1 antibodies.
2. Test human MDA-MB-231-LM2 cell lines lentivirally expressing LTBP1 hairpins for their ability to metastasize in vivo
3. Infect tumorigenic mammary epithelial cells (MECS) from MMTV-PyMT with lentiviral LTBP hairpins and test for their ability to reduce cell invasion and metastasis.

4. IMPACT

- **What was the impact on the development of the principal discipline of the project.**
- Our results show that LTBP1L is associated with poor outcome particularly in basal and HER2 positive subtypes, LTBP1L and LTBP1S show distinct expression patterns in human breast cancer cell lines. LTBP1S is highly expressed within the mesenchymal-like subset of TNBC cell lines. There are no studies on LTBP1, in breast cancer so this has opened up a new field. Our unpublished data reported herein introduce the concept that LTBP1 expression has value as a prognostic indicator of patient outcome in HER2 and Basal type breast cancer. Our in vitro studies support the concept that LTBP aids cell invasion.

- **What was the impact on other disciplines**

Our in vitro studies support the concept that LTBP aids cell invasion this could be relevant in many epithelial tissues and cancers.

- **What was the impact on technology transfer**

Nothing to report

- **What was the impact on society beyond science and technology**

Nothing to report as yet but linking breast development factor to breast cancer risk opens the door to preventative strategies linked to reproductive history.

4. CHANGES/PROBLEMS

- **Delays in approach and reasons for delay**
  We have been delayed in generating the MMTV-LTBP1 transgenic due to problems in cloning human LTBP1 into pHIV-ZsGreen prior to excision of LTBP-GFP. We think that the LTBP1 sequence may be too large for the lentiviral vector and have now obtained a tagged version from a group at McGill and are in the process of completing the transgene construct.

- **Actual anticipated problems or delays and actions or plans to resolve them** – Previously we reported the cloning of two LTBP1 hairpins (sh1 within the coding region, sh2 in the 3'UTR) and scrambled controls into pLKO-GFP and pLKO-RFP vectors respectively, confirmed their ability to reduce LTBP expression and noted extensive cell death of infected MCF10A cells (Report year 1 Fig. 6). To circumvent this issue we recloned the hairpins and Scr control into an inducible system, pLKO Tet-on puro (Addgene 21915), which allows expression of the hairpins to be induced by the addition of doxycycline to the media. It also confers puromycin resistance to allow selection of a pure population after infection with the vector.

- **Changes that had a significant impact on expenditures -** There was a delay in hiring a suitable postdoctoral fellow.

- **Significant changes in use or care of human subjects, animals, biohazards or select agents** - Nothing to report

5. PRODUCTS

- **Publications, conference papers and presentations**

- ***Manuscripts:*** We are currently writing up the in vitro work detailed in Task 2 above and plan to submit to Breast Cancer Research

- ***Books etc:*** Nothing to report

- ***Other publications, conference papers and presentations***

1. Dr. Cowin presented this work to the Developmental Biology Program, Institute of Biotechnology, Helsinki, Finland on Feb 12 2015. ***Acknowledgement of Federal Support:*** YES

2. This work was presented as a poster at the GRC on Cell Contact and Adhesion, Proctor Academy, NH, June 2015. ***Acknowledgement of Federal Support:*** YES

- **Website(s) or other internet site (s):** Nothing to report

- **Technologies or Techniques:** Nothing to report

- **Inventions, patent applications and/or licenses:** Nothing to report

- **Other products:**
  - **Research material:** Generation of inducible lentiviral LTBP1, LTBP1L and LTBP1S hairpin constructs

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS
Individuals working on the project:

Name	Pamela Cowin
Project Role	P.I.
Research Identifier	
Nearest person month worked	12
Contribution to project	Directed research
Funding Support	DOD BC123572 20%

Name	Catina Crismale Gann
Project Role	Postdoctoral fellow
Research Identifier	
Nearest person month worked	3
Contribution to project	Performed work on Aim 1
Funding Support	DOD BC123572 100%

Name	Carrie Oliver
Project Role	Postdoctoral fellow
Research Identifier	
Nearest person month worked	3
Contribution to project	Performed work on Aim 2
Funding Support	Susan G Komen Fellowship 100%

- **Has there been a change in the active support of the PI or senior key personnel since the last reporting period**

P.I. Dr. Pamela Cowin – No change

Postdoctoral Fellow – Dr. Catina Crismale Gann received support from NIH postdoctoral training grant
Postdoctoral Fellow – Dr. Carrie Oliver received a Susan G Komen fellowship and will be replaced on this project by a new hire.

- **What other organizations were involved as partners?**

None

8. SPECIAL REPORTING REQUIREMENTS N/A

9. APPENDICES

None