Award Number: W81XWH-15-1-0349

TITLE: Evaluation of Alternative Splicing Regulators As Targets For Selective Therapy of Triple Negative (Basal) Breast Carcinoma

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REPORT DATE: October 2016

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sep 2015 - 29 Sep 2016

4. TITLE AND SUBTITLE

Evaluation of Alternative Splicing Regulators As Targets For Selective Therapy of Triple Negative (Basal) Breast Carcinoma

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0349

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

WEST VIRGINIA UNIVERSITY
RESEARCH CORPORALAN B
MARTIN886 CHESTNUT
RIDGEMORGANTOWN WV 26506

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We established cellular models in MDA-MB-231 and immortalized primary epithelial cells to study the effect of KHDRBS3 and SRSF12 on tumor progression and metastasis (**Task1**). We analyzed the effect of KHDRBS3 depletion on the growth and migration properties of the MDA-BD-231 cells in vitro (**Task 1**) and their ability to form tumors and metastasize in mouse xenograft models (**Task 2**). The results of similar experiments for SRPK1 were recently published and demonstrated a role for SRPK1 in migration and tumor formation. Instead of repeating the published work we expanded **Task 3** to include a KHDRBS3 knockout mouse model in addition to the SRPK1 knockout mouse in the experiments aimed to determine their role for tumor initiation, progression and metastasis in vivo. The mouse strains are at hand and we will start analyzing the effects of the knockouts on tumor growth during the second year of this project. Continued analysis of the splicing factor expression in primary tumor samples further supports strong association of KHDRBS3 with triple negative breast carcinoma (**Task 4**). Finally, RNASeq was used to determine the effect of KHDRBS3 and SRPK1 depletion on the transcriptome of MDA-MB-231 cells (**Task 6**).

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

The goal of this proposal is to evaluate the role of splicing regulators upregulated in triple negative breast cancer in the tumorigenesis process and identify potential therapeutic targets. This will be accomplished through series of experiments in vitro and on animal models of the disease that are designed to determine the effect of splicing factor depletion on tumor initiation, growth and metastasis.

Keywords

Pre-mRNA splicing, breast cancer, KHDRBS3, SRPK1, SRSF12, metastasis

Accomplishments

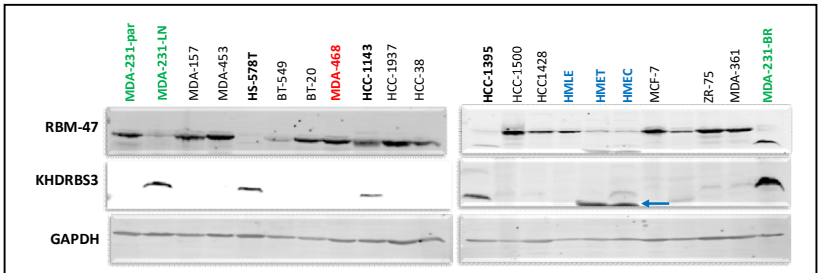

Figure 1. KHDRBS3 expression in cancer cell lines. MDA468 cells (red) express the luminal marker RBM47 but not KHDRBS3. Primary mammary epithelial cells and their derivatives (blue) do not express KHDRBS3. The band detected by the antibody (blue arrow) is not KHDRBS3 and is not sensitive to KHDRBS3 shRNA knockdown. Metastatic derivatives of the MDA-MB-231 line (green), MDA-231LN and MDA-231-BR, express KHDRBS3 unlike the parental line (MDA-231-par).

Major goals

Major Task 1 *Determine the effect of SRPK1, KHDRBS3 and SRSF12 on the malignant properties of the basal type breast cancer cells in vitro.*
Major Task 2 *Determine the effect of SRPK1, KHDRBS3 and SRSF12 on tumor initiation and growth in xenografts.*
Major Task 3 *Demonstrate that reduced SRPK1 and* ***KHDRBS3*** *levels suppress tumor initiation, growth and lung metastasis*
Major Task 4 *Analyze SRPK1, KHDRBS3 and SRSF12 expression in Splicing factor expression in normal and malignant tissue samples*
Major Task 5 *Analysis of the regulation of splicing factor expression by the PI3K pathway and cMyc*
Major Task 6 *Analysis of the regulation of alternative splicing in mammary basal epithelial cells by SRPK1, KHDRBS3 and SRSF12*

Specific objectives and activities

Major Task 1 *Determine the effect of SRPK1, KHDRBS3 and SRSF12 on the malignant properties of the basal type breast cancer cells in vitro.*

We examined the expression of KHDRBS3 in a panel of breast cancer cell lines. As expected we observed expression of the protein in basal type cell lines. We did not detect KHDRBS3 in MDA-MB-468 cells, one of the cell lines we proposed to use in this project, despite their classification as a basal type. Instead MDA-MB-468 cells expressed the luminal RNA binding protein RBM47. We observed correlation of KHDRBS3 expression with the metastatic potential of MDA-MB-231 cells. KHDRBS3 transcript and protein levels were upregulated in MDA-MB-231 cells selected for metastasis to the lymph node (MDA-MB-231-LN), bone marrow (MDA-MB-231-BoM), lung (MDA-MB-231-Lu) and brain (MDA-MB-231-BR) (Figure 2). Analysis of publicly available RNASeq data showed that elevated KHDRBS3 levels are also associated with metastatic properties of at least one more breast cancer cell line, CN34.
After knockdown of KHDRBS3 in MDA-MB-231-LN cells we did not observe significant change in the growth rate (Figure 3A

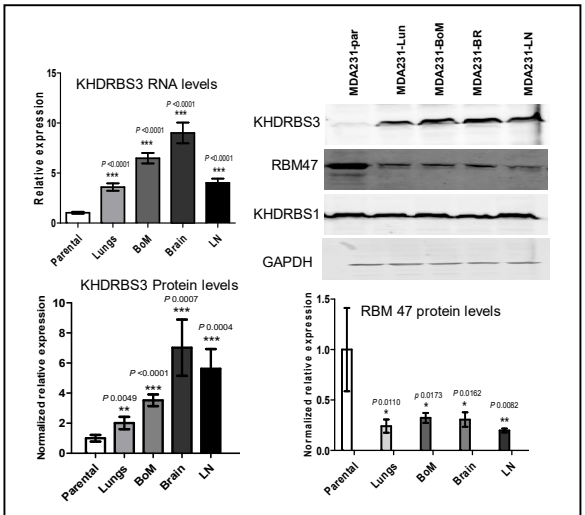

Figure 2. KHDRBS3 and RBM47 levels are reciprocally regulated and KHDRBS3 is upregulated in the metastatic derivatives. Levels of the related KHDRBS1 protein are not affected.

and B). Chemotaxis through collagen gel using FBS as chemoattractant (Figure 3C and D) and migration in Boyden chamber assays (not shown) were also not affected by the knockdown. While we see some changes in cell migration in cells expressing shKHDRBS3#1 we do not consider these to be due to the depletion of KHDRBS3 as shKHDRBS3#4 which is as efficient in depleting the protein has no effect on the migration properties. The effect of shKHDRBS3#1 is likely due to off-target effects, consistent with its significantly larger impact on overall gene expression compared to shKHDRBS3#4.

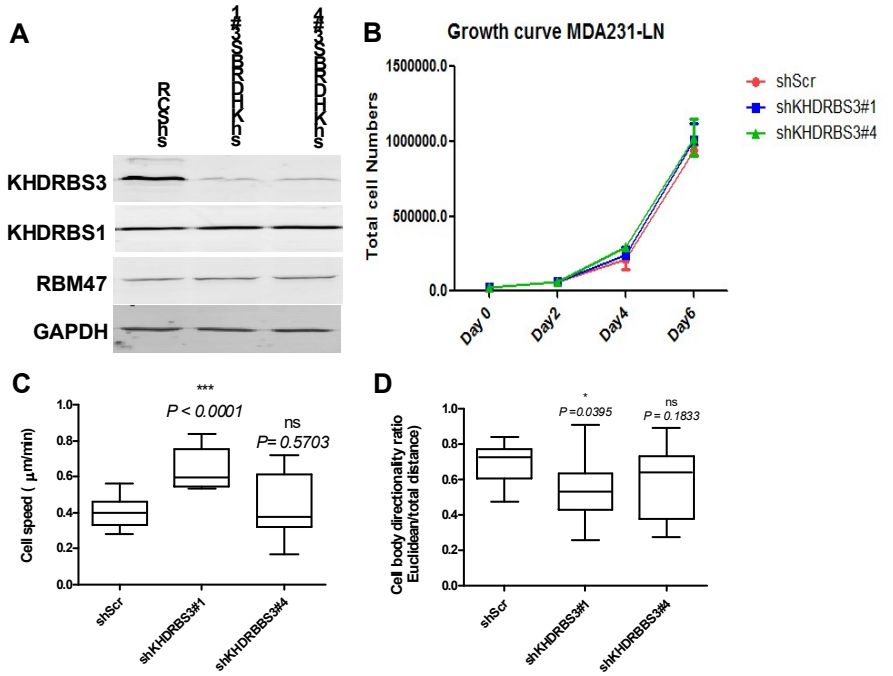

Figure 3. Effect of KHDRBS3 knockdown in MDA-MB-231-LN cells on cell growth and chemotaxis in vitro. (**A**) KHDRBS3 is depleted by shRNA knockdown. The related KHDBRS1 protein and the luminal RBM47 protein are not affected by the KHDRS3 knockdown. (**B**) Depletion of KHDRBS3 does not affect growth in vitro. (**C**) Speed of migration in presence of chemoattractant (fetal bovine serum) is affected by only one of two KHDRBS3 shRNAs. (**D**) Direction of chemotaxis, as measured by the ratio of Euclidian to total distance traveled towards in the chemoattractant, is negatively affected only by one of the shRNAs (shKHDRBS3#1). The impact of shKHDRBS3#1 on migration is likely due to off-target effects of shKHDRBS3#1 as it unlike shKHDRBS3#1 shows broad effect on gene expression.

We decided not to carry out in vitro experiments for SRPK1 as nearly identical work was recently completed by Dr. van de Water's group, convincingly showing that SRPK1 is critical for cell migration in vitro, and tumor growth and metastasis in xenograft models (van Roosmalen et al. 2015). We redirected resources planned for this research towards expanding Major Task 3 by adding a KHDRBS3 knockout model which recently become available (Traunmüller et al. 2016). This would allow us to evaluate the effect of KHDRBS3 on tumor initiation, which is not possible with the xenograft approach. In addition, these experiments will allow us to evaluate the effect of KHDRBS3 in conditions more closely resembling human tumors and rule out potential artifacts due to the nature of the cultured cell line models.

We attempted to raise a peptide antibody against SRSF12. Unfortunately, our antibody did not specifically recognize SRSF12. Commercially available anti-SRSF12 antibodies also failed to detect the protein even when it was overexpressed. The lack of suitable antibody delayed the planned in vitro experiments using SRSF12 knockdown cell lines. As an alternative we established two cell lines (MDA-MB23-LN and immortalized primary breast epithelial cells) that ectopically express SRSF12. As MDA-MB-231 cells do not express endogenous SRSF12, we will use the derivative cell line with ectopic expression of the protein to carry out the planed in vitro experiments in the second year of this project

Major Task 2 *Determine the effect of SRPK1, KHDRBS3 and SRSF12 on tumor initiation and growth in xenografts.*

We used the two KHDRBS3 knockdown lines to evaluate the effect of KHDRBS3 depletion on tumor growth and metastasis in xenograft models. Orthotopic injections of the cell lines in the mammary fat pad of NSG mice were used to evaluate to effect of the knockdown on tumor growth. We monitored the tumor growth by bioluminescence and caliper measurements during a five-week time course and determine the weight at the end of the experiment. We did not observe any effect of the KHDRBS3 knockdown on tumor growth (Figure 4).

To determine the effect on the ability of the knockdown cell lines to colonize distant tissues and metastasize

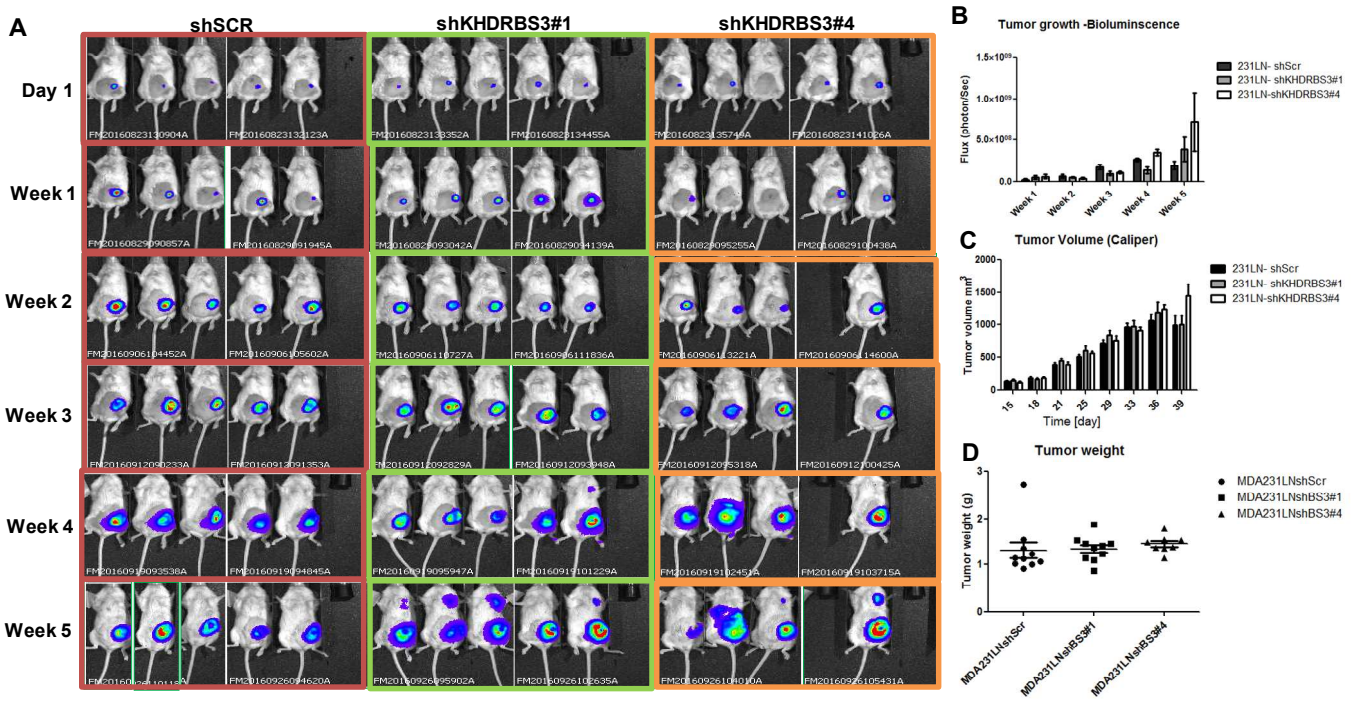

Figure 4. Effect of KHDRBS3 knockdown on the growth of MDA-MB-231-LN orthotopic xenografts in the mammary fat pad. (**A**) The growth of the of the xenografts was monitored by bioluminescence for five weeks (one out of two experiments, each encompassing 5 animals per group is shown). (**B**) Quantification of the bioluminescence measurements (n= 9 to 10). (**C**) Tumor growth was also monitored by caliper measurements of the tumor volume. (**D**) At five weeks the animals were sacrificed, the tumor were dissected and weighted (n= 9 to 10).

from the primary site we analyzed the formation of lung metastasis after tail vein injection or spontaneous migration from the orthotropic injection site (Figure 5). Bioluminescence measurements and histology examination of the lungs failed to detect significant difference between the KHDRBS3 knockdowns and the control (Figure 5).

Major Task 3 *Demonstrate that reduced SRPK1 and **KHDRBS3** levels suppress tumor initiation, growth and lung metastasis*

The plan for this task is to establish SRPK1(+/-) mice, back-cross a C3.1-Tag mouse model of breast cancer and SRPK1 knockout allele to SV129 background and then bring the two alleles together to determine the effect of reduction of SRPK1 protein levels on tumor initiation, growth and metastasis. To reduce the time needed to complete the backcrossing we are using a SNP genotyping service (Dart Mouse) which allows us the select mice with the highest percentage of the desired background for breeding and reduce the number of necessary generations from five to four. We have established the SRPK1 knockout allele and the process of backcrossing it to SV129 background should complete within 3 months (January/February 2017). We experienced some delay due to an error in designing the targeting and genotyping strategies made by the group that created the SRPK1 knockout mice (Wang et al. 2014). We have cleared these issues and confirmed the knockout by sequencing the SRPK1 genomic locus of the mice and developed new genotyping strategy.
We have completed backcrossing the C3.1-Tag allele to SV129 background.
We also obtained a KHDRBS3 knockout mouse which is being backcrossed to SV129 background. The back-crossing process should complete in March/April 2016. KHDRBS3 knockout mice are viable and fertile. Thus, we will be able to evaluate the effect of the complete knockout on tumor initiation, progression and metastasis. We are on track to completing these experiments by the end of year 3 of this proposal.

Major Task 4 *Analyze SRPK1, KHDRBS3 and SRSF12 expression in Splicing factor expression in normal and malignant tissue samples*

We continue to evaluate the KHDRBS3 expression in tumor samples. We stained 15 samples consisting of primary breast cancer samples and patient derived xenografts for KHDRBS3 expression (Figure 6). As suggested by the reviewers we also obtained two tumor tissue arrays (US Biomax) with over 200 breast cancer and normal tissue samples and probed them for KHDRBS3 expression. We observed KHDRBS3 expression consistent with triple negative status. However, the commercial tissue arrays proved to be of low quality and did not yield reproducible results. The arrays were chosen so that some of the samples overlapped, providing a control for the reproducibility of the staining. Four samples that stained positive on one of the arrays (BR2028a) did not produce any signal on the other (BR1921a). In addition, KHDRBS3 stains some cells of hematopoietic origin (lymphocytes/macrophages), which serve as an internal control. In many of the samples we failed to detect signal in these cells, providing further evidence that the antigen may have been lost due to issues with processing and storage by the supplier. We are now testing tissue array samples from a different supplier (Protein biotech), we have identified 7 additional samples from Dr. Pugacheva's (WVU cancer institute) collection of primary tumors and patient derived xenografts, and we are working with the WVU Tissue Bank to identify archive samples from the collection. Both the WVU Tissue Bank and Dr. Pugacheva continue to collect patient tumor samples under stablished protocols and will be sharing those with us as they become available. We are confident that we will have sufficient number of samples to determine the KHDRBS3 expression in patient tumors.

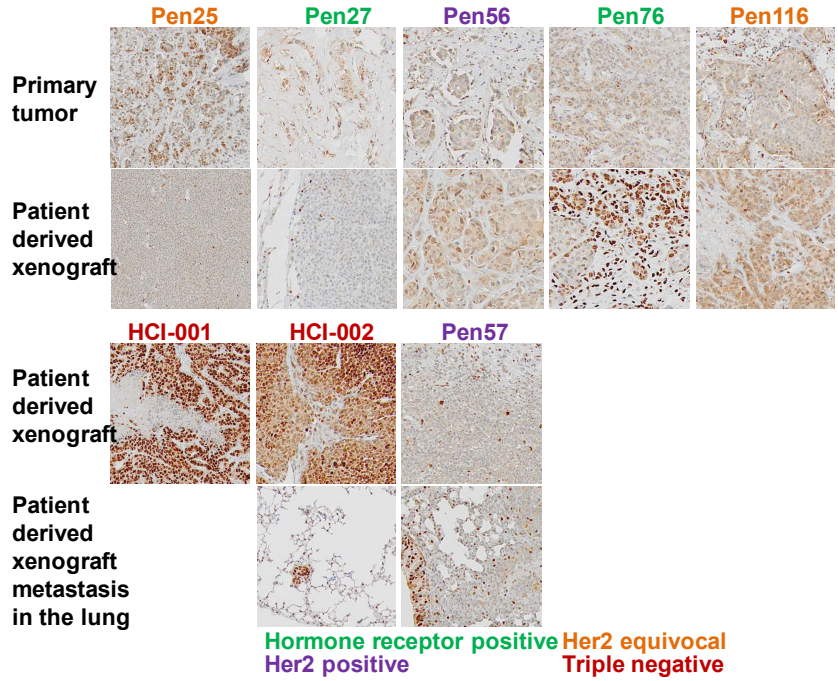

Figure 6. KHDRBS3 expression in patient tumors and patient derived xenografts (PDX)

Evaluation of SRPK1 on over 300 patient tumors has recently shown that SRPK1 levels are elevated and correlates with poor prognosis (van Roosmalen et al. 2015). Consequently, we opt not to duplicate this research. Instead we will redirect the resources towards expanding **Major Task 3** with the addition of KHDRBS3 knockout mouse.

Evaluation of SRSF12 expression has been thwarted due to the lack of suitable antibodies. We consider this to be temporary setback. We will solve the problems by raising new antibodies using different antigens.

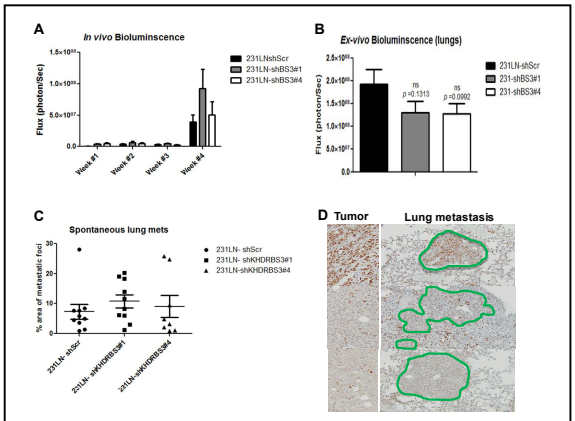

Figure 5. Effect of KHDRBS3 on metastasis of MDA-MB231-LN mouse xenografts. (**A**) Growth of lung metastasis after tail vein injection monitored by bioluminescence. (**B**) Overall metastasis load of lungs 5 weeks post tail vein injection, determined by bioluminescence of dissected lungs. (**C**) Relative area of spontaneous metastasis to the lungs of orthotopic xenografts in the mammary fat pad (5 weeks post injection). The metastasis beds were identified by cell morphology on H&E stained sections and normalized to the total cell area of the lung. (**D**) IHC staining of orthotopic tumors (left) and lung metastasis (right, green outline) by KHDRBS3 antibody, shows stable KHDRBS3 knockdown.

Major Task 5 *Analysis of the regulation of splicing factor expression by the PI3K pathway and cMyc*

We will commence work on this task during the second year of this project.

Major Task 6 *Analysis of the regulation of alternative splicing in mammary basal epithelial cells by SRPK1, KHDRBS3 and SRSF12*

We completed the analysis of the effect of KHDRBS3 and SRPK1 knockdown on splicing in metastatic derivatives of the MDA-MB-231 line by RNA-Seq. We did not see effect of the knockdown on alternative splicing. Gene expression analysis showed dramatic effect of one of the KHDRBS3 shRNAs (shKHDRBS3#1) on gene expression (450 genes showed altered expression levels). We attribute these changes to off-target effects as the second shRNA (shKHDRBS3#4), which is as effective in knocking down the protein affected the expression of only 39 genes. We have prepared RNA samples for RNA-Seq from immortalized breast epithelial cells expressing KHDRBS3 and SRSF12. We expect these samples to be sequenced and analyzed by March 2017.

Major findings

1. KHDRBS3 is expressed in basal type cancers.
2. Elevated KHDRBS3 protein levels correlate with increased metastatic potential in two cell lines models (MDA-MB-231 and CN34).
3. Depletion of KHDRBS3 by shRNA knockdown does not affect the mobility and growth of MDA-MB-231 cells both in vivo and in mouse xenografts.
4. Depletion of SRPK1 and KHDRBS3 in MDA-MB-231 cell line derivatives does not affect global splicing.

Training and professional development opportunities

Fatimah Matalkah (MSc, Research assistant) was trained in the use of xenograft models and in vivo imaging by bioluminescence. These are advanced techniques that require considerable skill and Fatimah has become an expert in performing and troubleshooting them.

Dissemination of results

Nothing to report

Plans for next reporting period

Major task 1. We will raise SRSF12 antibodies, which will enable us to initiate the in vitro experiments planned for this protein in the third quarter of the reporting period and complete them in the third year of the project.

Major task 2. No plans for next reporting period. The work on xenografts using SRSF12 knockout lines will be carried out in the third year of the project.

Major task 3. We will cross our knockout mouse cell lines to the C3.1-Tag transgene to produce KHDRBS3(-/-);C3.1-Tag(+), KHDRBS3(+/+);C3.1-Tag(+), SRPK1(+/-);C3.1-Tag(+) and SRPK1(+/+);C3.1-Tag(+) female mice. The goal is to generate 10 to 20 mice from each genotype. These mice will be monitored over the course of year for tumor development and the metastasis to the lungs will be analyzed after the animals have been sacrificed. The monitoring process is expected to complete by the third quarter of year 3.

Major Task 4. We have ordered from Protein Biotech new tissue arrays that encompass over 200 breast cancer samples. We will analyze the expression of KHDRBS3 in these samples as well as 7 samples from Dr Pugacheva's collection. We are working with the WVU Tissue Core to identify additional breast cancer samples from their collection for IHC staining. We expect that the WVU tissue core will be able to provide 10 to 15 additional samples.

Major task 5. We expect to complete this task in the next reporting period. The work will involve analysis of the role of the PI3K pathway and Myc transcription factor in the regulation of expression of KHDRBS3, SRPK1 and SRSF12. PI3K pathway inhibitors will be used to interfere with the pathway in HME53TR cells,

which are available in the lab. The role of Myc will be assessed after establishing a cell line stably expressing Myc by lentiviral transduction. If our hypothesis that Myc controls the expression of the three splicing factors is proven correct, we will use luciferase reporters and chromatin IP to show direct regulation and identify the control elements.

Major Task 6. We will complete the RNASeq analysis of cell lines expressing ectopic KHDRBS3 and SRSF12 to identify alternative exons regulated by the two splicing factors. Based on the RNASeq results we will generate minigenes. Site directed mutagenesis of the minigenes along with electrophoretic mobility shift assays will be used in year 3 of the project to map the cis-acting elements recognized by the two splicing factors.

Impact

This proposal will impact the development of targeted therapy for triple negative breast cancer by identifying novel therapeutic targets. So far we have shown that one of the proposed targets, KHDRBS3, is strongly associated with metastasis and is expressed in triple negative breast cancers. Regardless of these associations, KHDRBS3 depletion has little effect on the malignant properties of a cancer cell line in vitro and in xenograft models. Another proposed target, SRPK1, was shown by others to be critical for tumor development and metastasis in the same type of cells. To conclusively prove the suitability of these targets for drug development, we still need to evaluate their effect on mouse models of breast cancer, which more closely resemble the human disease than cultured cell lines. The actual impact of the current proposal on breast cancer treatment cannot be determined until these experiments are completed.

Changes/Problems

Changes in approach:

1. We are dropping the proposed in vitro and xenograft experiments for SRPK1 as data from similar experiments was recently published by others.
2. We are introducing KHDRBS3 knockout model to Major Task 3 to evaluate the effect of KHDRBS3 depletion on the tumor initiation, growth and metastasis in a mouse model of triple negative breast cancer. The experiments will follow the design proposed for SRPK1. All necessary changes to the animal protocol have been approved by WVU ACUC. We will not need additional funds as the extra cost will be covered by the resources freed from the in vitro and xenograft experiments proposed for SRPK1.

Encountered problems:

1. We were unable to obtain a working antibody to SRSF12. This is a technical problem which we will solve by raising custom antibodies using multiple peptide antigens.
2. We had issues with low quality tissue arrays from one supplier (US Biomax). We are in the process of evaluating arrays from a different supplier (Protein Biotech). In addition, we continue to collect tumor samples from the WVU Tissue bank and DR. Pugacheva's collection of patient derived xenografts. Again, this is a technical issue that will be resolved.
3. To our surprise we found that depletion of SRPK1 and KHDRBS3 in MDA-MB-231 cell line derivatives did not affect alternative splicing. We are confident that this is not an analysis artifact, as we have checked our software on several data sets, including one from KHDRBS3 knockout mouse, and found them to work as designed. The most likely explanation is that in this particular cell line the loss of SRPK1 and KHDRBS3 is compensated by the closely related SRPK2 and KHDRBS1. To address this issue, we will analyze effect of KHDRBS3 ectopic expression on splicing in immortalized breast epithelial cells.

Products

Nothing to report

Participants & Other Collaborating Organizations

Participants:
Name: Dr. Peter Stoilov
Project Role: Principle Investigator
Researcher Identifier: orcid.org/0000-0003-1108-7271
Nearest person months worked: 3

Name: Fatimah Matalkah
Project Role: Research Assistant
Nearest person months worked: 7

Changes in active other support:

Grant/Role/Amount	Years
NIH/NEI 1R01EY025536-01A1; Ramamurthy and Stoilov MPI; Effort 20%	
Photoreceptor neuron specific alternative splicing of messenger RNA	2016-2020
$1,192,000	

Other Organizations:
Nothing to report

Special reporting requirements

Nothing to report

Appendices

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