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14. ABSTRACT

Triple negative breast cancer (TNBC) represents 20-25% of sporadic breast cancers, lacks ER, PR, and overexpressed Her2 – and thus has no targeted treatment options. TNBC is the most clinically challenging subtype with exceptionally poor prognosis, high recurrence and metastases and currently represents an unmet medical need. While classified pathologically by negative criteria, TNBC is a heterogeneous group of breast cancers in need of better molecular classification. Numerous studies have linked EGFR signaling to TNBC, but paradoxically, TNBC is refractory to the well-used EGFR inhibitors that have been efficacious in other cancers. In addition, Wnt signaling has been linked to TNBC, but its relationship to EGFR signaling and inhibitor resistance has not been explored. In the current work, the HBP1 transcriptional repressor may be a new and unappreciated bridge to both TNBC pathways. Previously, we have shown that decreases in HBP1: 1) trigger an increase in both Wnt and EGFR signaling to regulate proliferation and senescence; and 2) were coincident with exceptional increased tumor growth and invasiveness in preclinical models of breast cancer. Our new data show that HBP1 levels are significantly reduced in TNBC relative to other breast cancer subtypes in clinical specimens. Strikingly, a new combined EGFR and HBP1 gene signature predicted 90+% of TNBC patients in 4 large patient databases and predicted a poor patient prognosis in non-TNBC patients. To understand how HBP1 decreases trigger a poor prognosis, a whole genome analysis revealed a surprising change in several hundred genes to enact a Warburg-like metabolic reprogramming. The Warburg effect is characterized by increased glycolytic flux with increased biosynthesis of amino acids, lipids, and nucleic acids—all to provide for the increased growth and proliferation demands of a tumor cell. This grant seeks to understand how HBP1, EGFR and Wnt signaling trigger metabolic reprogramming in the context of TNBC.

15. SUBJECT TERMS- : none listed

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The text of the report must include all sections addressed in the table of contents to include:

1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research. Triple negative breast cancer (TNBC) represents 20-25% of sporadic breast cancers and is the most clinically challenging breast cancer subtype. TNBC has an exceptionally poor prognosis with high recurrence and metastases. TNBC lacks expression of the three key molecular parameters of breast cancer – ER, PR, and overexpressed Her2 – and thus have no targeted treatment options. While classified pathologically by negative criteria, TNBC is also heterogeneous group of breast cancers, currently sub-classified by anecdotal clinical experience. TNBC has significant mortality and currently represents an unmet medical need. We hypothesize that the HBP1 transcription factor regulates a novel metabolic re-programming through the Wnt and EGFR pathways, both of which contribute to TNBC. In this grant, we will apply a dual gene expression and metabolic approach to delineate new insights into TNBC. We aim to dissect the molecular framework in the context of TNBC properties that contribute to the exceptionally poor prognosis of this disease and translate the findings into a new gene-and metabolite-based platform to expedite the discovery of new therapeutic and diagnostic strategies. The objective of this grant is to understand the mechanism by which HBP1, Wnt and EGFR signaling may regulate a critical metabolic reprogramming in breast cancer. Given the lack of integrated gene and NMR-based metabolic data in specific disease parameters, delineating gene-based and metabolic alterations in should open a door into new insights for TNBC classification and treatment

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words). Breast Cancer, Warburg metabolism, Wnt signaling

3. OVERALL PROJECT SUMMARY (adapted from submitted SOW)

Goals: We postulate that decreases in HBP1 associated with TNBC result in elevated Wnt and EGFR signaling, which enact a metabolic re-programming akin to the Warburg effect, and contribute to the aggressive properties of TNBC. The Wnt signaling pathway has not been previously associated with a Warburg effect and HBP1 may also be an integral factor in EGFR inhibitor resistance. Using pre-clinical models of TNBC that recapitulate human disease, we will investigate how HBP1, Wnt and EGFR signaling may regulate a critical metabolic reprogramming in breast cancer, using both gene-based signaling and NMR-based approaches. Given the lack of integrated gene and NMR-based metabolic data in specific disease parameters, delineating gene-based and metabolic alterations in the associated networks should give new insights into therapeutic and diagnostic strategies for TNBC.

Specific Aims. SA1. HBP1 and Metabolic Signaling Mechanisms in TNBC. A surprising preliminary result shows that several hundred genes (involved in glycolysis, TCA cycle, oxidative phosphorylation and intermediary metabolism) are altered upon HBP1 decreases. Based on the striking metabolic pattern, we hypothesize that the decreases in HBP1 trigger a Warburg-like metabolic re-programming that is orchestrated by the induction of Wnt and EGFR signaling, both of which have been previously associated with TNBC. These mechanisms will be investigated in pre-clinical models that stably re-capitulate the pathology of the original human TNBC tumors with resulting metastases. Some pre-clinical therapeutic combinations aimed at Wnt and EGFR signaling will be investigated for new effects in restoring a normal metabolism.

SA2. NMR-based Investigations of Metabolic Reprogramming in TNBC. The objective is to use NMR-based metabolomic analysis in TNBC models to complement the gene-based analysis. We will elaborate clinically relevant metabolite ratios in the context of informative models of TNBC and build a metabolic signaling framework for TNBC. This metabolic signaling framework provides a new venue for evaluating new therapeutic paradigms and characterizing TNBC properties.

Progress to date.

Specific Aim 1. HBP1 and Metabolic Signaling Mechanisms in TNBC (months 1-36). We have previously shown that HBP1 decreases are associated with a poor prognosis and the acquisition of invasiveness and preliminary finding show that decreases in HBP1 is associated with TNBC. Surprising preliminary results show that several hundred genes involved in glycolysis, TCA cycle, oxidative phosphorylation and intermediary metabolism are altered upon HBP1 decreases in the HMEC tert. We hypothesize that the decreases in HBP1 lead to a Warburg-like metabolic re-programming that is orchestrated by the induction of Wnt and EGFR signaling, both of which have been previously associated with TNBC. The objective of this aim is to elaborate the molecular basis with respect to metabolic reprogramming and key signaling pathways in TNBC. These mechanisms will be investigated in pre-clinical models that that stably re-capitulate the pathology of the original human TNBC tumors with resulting metastases

Tasks 1A. (months 1-6)

- Implement all TNBC xenograft models (Months 1-6).
- Optimize, if possible, MDA-MB-231 (LM1) with surgical resection for detection of metastases. Currently, there are lung metastases in two of six mice after surgical resection. Cells are tagged with luciferase and will be visualized by imaging after luciferin IP injection (months 1-3).
- Implement Huntsman Cancer Institute/Univ. Utah human xenograft models HCI-002, 009-010 (months 1-6)

Optimize conditions for tumor detection and metastases by immunostaining and immunoblot. Confirm the pathology. (months 1-6).
Stably passage human xenografts over repeated implantation, dissection, re-implantation in NOD-SCID mice (months 1-24)

Year 1 Progress. The first two subtasks are completed. We have optimized an MDA-MB-231 using another variant obtained from Prof. Gail Sonenshein. The cells are GFP and luciferase tagged. Briefly, cells are implanted in the mammary gland as specified in the grant. When palpable, we perform a surgical resection and wait 6-8 weeks. We have observed that the mice have metastases in the lung and in the brain. One underwent a seizure. In the process of optimizing the xenograft model, we now have a new model for brain metastases from the primary site and for tumor-induced seizures. We are currently optimizing detection of the primary tumor and metastases, since the luciferase imaging is ineffective for detected metastases. The Tufts imaging facility has both CT and MRI that may be effective for detection of the brain and lung metastases. We are currently optimizing detection Wnt signaling parameters in the tissues. For the final subtask, we have now obtained an MTA agreement between Tufts and Huntsman. The PDX tumors fragments for implantation have arrived. A core facility will be deriving the PDX models and tumors and providing the tumors to us.

Tasks 1B. (months 1-18).

- Conduct signaling and gene analysis outlined in Aim1 and Figure 1. All reagents and procedures are in place, except for the tissue chromatin IP assay.
- Establish a good baseline for effects in cells, tumors and in metastases. Use HMEC-tert cells with HBP1 KD; in MDA-MB231 TNBC cells and xenografts with HBP1KD and in human TNBC xenografts.
- Optimize published chromatin IP assays for TNBC xenograft tumor tissues.

Methods to be used. mRNA expression. All primers for qPCR have been validated with conditions with SYBR green for linear and quantitative detection of all genes in Figure 1.
Protein expression. Antibodies for HBP1, EGFR, AKT, β-catenin, GSK3β, MYC, HIF1α, HK2, PKM2, LDH1, PFKFB3 from Cell Signaling Technologies (CST) (with exception of HBP1 (active motif)) have been validated for immunoblot and immunofluorescence.
Signaling activity. Antibodies for phosphorylated GSK3β(Ser9), AKT(S473), S6K(T389), EGFR(Tyr1173) from CST will be used to detect activity of the indicated signaling molecules.
MYC and HIF1α activity. Chromatin IP assays on the HK2 and LDHA promoters[2,11,28] will be used.

- Test the impact of high signaling by EGFR, Wnt, or both.
  - HBP1 KD. Use HMEC-tert and MDA-MB231 cells in the presence and absence of HBP1 KD (by lentiviral infection with two different shRNA (denoted 76 and 74). The impact on Wnt signaling and EGFR signaling has been previously characterized in our lab.
  - HBP1 KD on metastases in MDA-MB231 after surgical resection. Determine if the metastases also have high Wnt and EGFR signaling by the criteria in Task 1A.
- Assess all signaling and gene expression parameters in the Human TNBC xenograft models by immunoblot and tissue immune staining. Determine relative levels of Wnt and EGFR signaling by examining the specific activity of the indicated kinases and gene levels.

Year 1 Progress. The assays for detection are established. We have created the HBP1 KD cells and have assessed the impact on primary tumorigenesis, but have not yet begun the analysis for metastases. We must first optimize the tissue-base staining with the above reagents. We do see changes in Wnt signaling. For this period, we have focused on the characterization of the metabolic aspects of the diagram in Aim 1.

Task 1C (months 18-36)

- Investigate drug combinations with focus on blocking Wnt signaling and EGFR signaling. Emphasize erlotinib and EGCG.
  - MDA-MB231. Empirical maximum dose for erlotinib in NOD scid-mice is 0.5 mg/kg. Empirical maximum for EGCG (16.5 mg/kg; human equivalent of 800 mg/day, an attainable level).
  - Assess effects by the above assays in both the primary tumor and metastases.
  - Human TNBC xenograft models. Same analysis.
- Alternative. Test. Tankyrase inhibitors (AV839 and endo IWR1, Tocris/R&D Biosystems) for inhibition of Wnt signaling and collaboration with erlotinib in HMEC-tert and MDA-MB231 TNBC cells. Examine gene and metabolic criteria.
  - Caveat: inhibitors only used in cell culture.
  - Alternative—test by use of Tankyrase KD in MDA-MB231 tumors (approach used in non small cell lung cancer cell xenografts-Casas-Selves, M. et al (2012) Cancer Research, epub 2012/06/29.)

Year 1 Progress. Task 1C was scheduled for Years 2 and 3, but we have already made progress. A combination of EGCG and Decitabine reduces both primary tumors and importantly diminishes brain metastases in the new pre-clinical model. This combination also blocks Wnt signaling (by β-catenin levels) and proliferation (by ki67 staining) in treated primary tumors. We also see some reversal of the Warburg metabolic effects—to be explored further. A notable aspect of the experiment

Using a previous TNBC model (before we optimized for metastases), one experiment with erlotinib was attempted and there was no effect alone (as is the case in human breast cancer patients. However, a combination of EGCG/DAC and erlotinib lead to tumor reduction, but the mice were only borderline healthy by IACUC animal criteria. We are contemplating dropping the erlotinib experiments, given the above success with EGCG.

Specific Aim 2. NMR-based Investigations of Metabolic Reprogramming in TNBC (months 1-36). The objective is to use NMR-based metabolite analysis in TNBC models to complement the gene-based analyze. These new TNBC models provide an excellent opportunity to metabolic profiling to complement the metabolic-and gene-based framework of Aim 1 to determine the impact on primary TNBC tumors and resulting metastases. Together, the applications of NMR for metabolite analysis with the molecular studies on metabolic signaling from Aim 1 create a opportunity to classify TNBC and with future applications to MRI/MRS imaging. Our NMR-based metabolic analysis provides an excellent entry for improving the information that can be gained by MRI/MRS imaging. Together, the integrated gene-based and NMR-based metabolic studies in this grant should provide new insights for future applications of NMR-based imaging and diagnostic strategies for TNBC.

Tasks 2A: months 1-18

- Fully implement J-resolved spectra and its analysis using Chenomx software suite.
- Finish quantifying metabolites from the 1H-NMR analyses of HMEC-tert cell lines, and MDA-MB-231 cell lines and tumor xenografts with appropriate statistical tests (Student T-test, Metaboanalyst) to ascertain significant changes in individual compound concentrations and ratios of concentrations
- Full implementation of pattern and pathway recognition using IPA software both for metabolites alone and in combination with gene array analyses.
  - Begin 13C flux analysis in cell line models, optimizing data collection time points for optimal time course analysis
  - Begin 13C NMR flux analyses in xenograft tumors of human TNBC and metastases, if apparent.
  - Optimize extract isolation from metastases for NMR analyses (simultaneous with optimization of extract for gene-based analyses in Aim 1)

Year 1 Progress. We have made good progress on the NMR analysis. All conditions for tumor analysis have been optimized, including the extraction procedures. We have implemented the Chenomx analysis, Initially, we performed cell line analysis, but are now only focusing on xenograft tumors. We now routinely pulse with 13C-glucose prior to isolating the tumors, enabling us to derive both steady state and metabolic flux data. We are analyzing a set of 5-6 tumor analyses with 1H NMR and 13C NMR. The results indicate that lactate, glutathione and lipids are all elevated in the TNBC tumors. Under conditions of EGCG/DAC treatment, all these characteristic Warburg metabolic processes are decreased. We are refining the analyses with statistical consultations and with Metaboanalyst.

Tasks 2B. (months 12-24).

- Analyze xenograft tumors in the presence and absence of HBP1 KD for metabolic consequences.
- Generate and test any new hypotheses based on the gene and NMR analyses. For example, significant changes in TCA cycle compounds may initiate targeted gene expression experiments such as fumarate hydratase; alternatively, pattern of changes identified in expression analysis could initiate specific NMR experiments, such as flux analysis using fumarate.

Year 1 Progress. This task is slated for year 2.

Tasks 2C. (months 18-36).

- Use NMR on extracts to assess the impact of treatments defined in Aim 1 on the baseline NMR spectra for both 1H and 13C analysis.
- Investigate access to local in vivo animal MRI and MRS facilities (Children's, Beth Israel, and Massachusetts General Hospital, Brigham & Women's Hospital).
- Pilot experiment with in vivo MRS on tumor xenografts derived from MDA-MB231 and human TNBC xenografts (months 30-36).

Year 1 Progress. This task is slated for years 2 and 3. We have only investigated access to in vivo animal MRI facilities. WE are in discussions with both Boston University and with MGH for access to their facilities, once we identify critical NMR peaks in Task2A.

Manuscript on initial gene and NMR characterization of cells and MDA-MB231 xenografts (months12-18).
Manuscript on initial gene and NMR characterization in the metastases of the MDA-MB231 xenografts after surgical resection (months 12-24).
Manuscript on gene and NMR-based analysis the human TNBC xenografts and metastases (months 18-36).

Year 1 Progress. These tasks are slated for years 2 and 3.

Communication Plan. As described above, the research plan has inputs from gene-expression/signaling, NMR, and breast cancer clinical perspectives—united together in a research program. The PI and team are all located on the Tufts Medical School Boston campus. All are part of the monthly Cancer Center Breast Cancer working group that brings together clinicians and basic research scientists. Dr. Erban brings a wealth of clinical expertise and is one of the most desired breast oncologists in the area—and he appreciates the science behind the findings. Thus, he is an expert consultant in configuring our pre-clinical project. As an example of our past and ongoing work together, Drs. Erban, Paulson and Yee are working towards bringing a green tea/EGCG based drug combination into Phase 1 clinical trials for treating TNBC and other aggressive breast cancers. Drs. Yee, Paulson, and Baleja, whose laboratories are within 75 feet of each other on the same floor, will continue to have team meetings every two weeks to discuss progress and directions. We also have a shared DropBox account for sharing primary research data. The high level of personal trust, flexibility, and excellent communication has allowed us to bring our respective expertises to the common goal of improving TNBC patient outcomes.

Year 1 Progress. Drs. Baleja, Paulson and Yee meet weekly to discuss the results of this project and the progress of joint students on the project. We consult Dr. Erban as needed. Dr. Erban connected us with a breast radiologist, Dr. Shital Makim, who has been helpful in advising on general breast MRI issues for primary and metastatic tumor detection and the key considerations for an in vivo MRI/MRS for animals.

4. KEY RESEARCH ACCOMPLISHMENTS.

- Developed a feasible pre-clinical model for TNBC and metastatic progression.
- Using a signaling and metabolic framework to develop hypotheses for investigation

- Optimized comprehensive NMR metabolomic analysis for breast tumors to identify potential MRS markers.

5. CONCLUSION: In year 1, we have optimized the TNBC pre-clinical model to better reflect the human course of TNBC. We include a surgical resection to extend the tumor progression time course and to maximize the appearance of distant metastases to lung and brain. To further mimic the course of human disease, the brain tumor bearing mice exhibit seizures. For the human metastatic breast cancer patients, there has been a rising prevalence in fatal brain metastases. These patients often have seizures that are often refractory to standard anti-seizure drugs. Thus, in the course of the proposed experiments, a value-added bonus of the field is the optimization for a pre-clinical model that reflects human breast cancer and which will be useful in future studies to treat fatal brain metastases and associated seizures that interfere with the quality of limited life. We are making progress towards identifying key metabolites and NMR spectral regions for future testing of MRI/MRS markers. Such delineation requires the biochemistry of signaling and basic metabolism. We are currently exploring venues for pre-clinical MRI/MRS testing in preparation for translating the finding of our laboratory studies. The plans for year 2 will be 1) to examine both primary tumors and brain metastases; 2) continue to delineate potential therapeutic vulnerabilities through detailed signaling investigations; 3) continue to identify key NMR peaks and develop potential MRS biomarkers.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.
    (1) Lay Press: none
    (2) Peer-Reviewed Scientific Journals:none
    (3) Invited Articles: none
    (4) Abstracts: AACR Breast Cancer Meeting, San Diego, CA October 2013.

7. **INVENTIONS, PATENTS AND LICENSES:** none.

8. **REPORTABLE OUTCOMES:** Optimized a preclinical model for TNBC to include brain metastases, which is a significant problem for patients.

9. **OTHER ACHIEVEMENTS:** The studies of this grant were the topics of masters' research and undergraduate research theses. Brian Pedro graduates with highest research honors as a result of the work for this grant.

10. **REFERENCES:** None

11. **APPENDICES:** none