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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.					
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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 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.

Year 2 Progress. The Animal Imaging core facility has taken over the derivation of the PDX tumors from the Huntsman Cancer Institute MTA. There have been some difficulties in their Nod-Scid gamma mouse colonies. We are monitoring the progress and will step in with assistance, as needed. The major and new goal would be testing the efficacy of EGCG/DAC (see Task 1c) on PDX tumors. None of the PDX tumors metastasize to the brain, although some spread to lung and liver. If PDX tumors could be established (as published), then assessing efficacy of EGCG/DAC should be feasible, since doses have already been established.

We are continuing to optimize detection and production of metastases. We have improved the surgical resection methods to improve survival of the tumor-bearing animals. Typically, we had been performing resection when tumors reached palpable size or about 1 mm. Currently, we have had recurrences in the lymph nodes that have forced us to sacrifice the animals before any metastases could be assessed. We are contemplating the performance of resection at even smaller tumor sizes.

We have sought imaging methods to detect metastases in vivo without dissecting the tumors and sacrificing the mice. The Animal Imaging facility now has implemented a more sensitive IVIS imaging machine for scanning luciferase-tagged tumors. We are currently testing the machine of better detection of metastases to the lung and to the liver. The brain metastases have been difficult to detect, except after dissection of the brain. For the detection of brain metastases, we have attempted to use MRI to detect the brain tumors in a live animal but have not been successful. We guess that the brain metastases are small and below the detection limit. Upon installation of the new IVIS imager for luciferase-tagged tumors, the Perkin Elmer technician told us that they routinely image brain tumors with about 100 cells in mice whose head hair was removed by Nair or equivalent chemical products. We are in the process of performing the imaging with tumor-bearing mice, whose head hair has been shaved. We also learned that repeated Nair use is detrimental to the mice and are using a small shaver to gently remove the hair.

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, GSK3 β , MYC, HIF1 α , 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 GSK3 β (Ser9), AKT(S473), S6K(T389), EGFR(Tyr1173) from CST will be used to detect activity of the indicated signaling molecules.

MYC and HIF1 α activity. Chromatin IP assays on the HK2 and LDHA promoters^{2,8,9} 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.

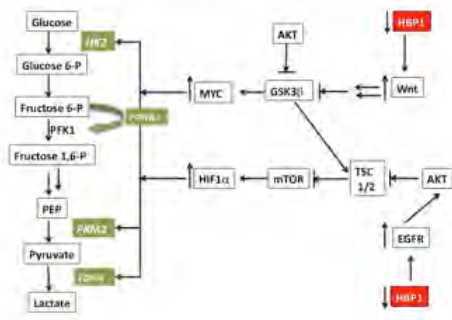


Figure 1: A Hypothesis for Metabolic Signaling. We postulate that the decreases in HBP1 activate Wnt and EGFR/mTOR signaling to increase the MYC and HIF1 α transcription factors and to increase metabolic re-programming through some of the following genes:

HK2. Hexokinases catalyze the conversion of glucose to glucose 6-P and is the first committed step in glycolysis. HK2 is increased in several cancer cells and in other tissues exhibiting a Warburg effect. GSK3 β inhibition in Wnt signaling is associated with HK2¹, which is regulated by MYC and HIF1 α ^{2,3}. **PFKFB3.** PFKFB3 is a proliferation-associated isoform of PFK 2^{4,5}, which catalyzes the synthesis of fructose 2,6 bisphosphate (F2, 6P), an allosteric activator of PFK1, the rate-limiting step of glycolysis. Thus, elevated PFKFB3 activity should increase PFK1 activity and overall glycolysis. **PKM2.** Pyruvate Kinase M2 is a proliferation-associated isoform of pyruvate kinase that catalyzes the conversion of phosphoenolpyruvate to pyruvate in glycolysis. PKM2 was the first glycolytic enzyme to be associated with the Warburg effect in cancer cells⁶. PKM2 also promotes the interaction of β -catenin with TCF4, an essential step of Wnt signaling⁷. **LDHA.** Lactate Dehydrogenase A is an isoform of lactate dehydrogenase, which catalyzes the conversion of pyruvate to lactate. LDHA is expressed in cancer cells and is directly regulated by MYC and HIF1 α ⁸. B. HK2 mRNA and protein expression in response to HBP1 KD

Year 2 Progress. We have focused on the metabolic consequences of the HBP1 KD tumors and have used the gene expression and NMR analysis to discover an alteration in lipid metabolism. The results are summarized in Figure 1. In addition, Wnt signaling was reported to be a major contributor to a Warburg metabolic re-arrangement in 2014^{10,11}. This work nicely frames our studies with HBP1 and the altered metabolic re-arrangements. Figure 1 summarizes the progress to date for Task 1B, 2A and 2B with respect to the HBP1 KD tumors. The metabolomics are a compilation of the ¹H (steady state) and ¹³C-glucose (pulse) NMR studies and of the protein and gene expression of the indicated metabolic enzymes. Drs. Eric Paulson and Jim Baleja, both supported by the grant, led the analyses.

Increased Lipid Synthesis in HBP1 KD TNBC Tumors

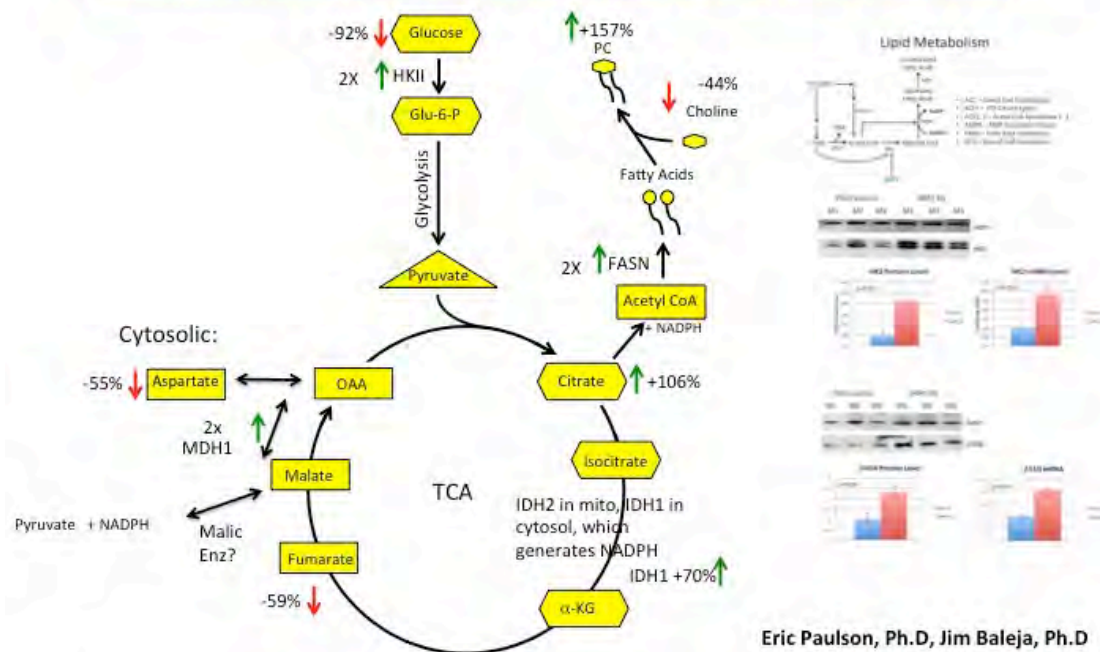


Figure 2. Extending the Warburg effect: HBP1 Elevates HK2, FASN, and Lipid metabolism? A. Glycolytic and Lipid Biosynthetic Pathways Altered by HBP1 KD. MDA-MB-231 TNBC cells with a stable HBP1 KD, or with a control virus, were grown as tumors in NOD/SCID mice. Tumors were excised on ice and snap-frozen in liquid nitrogen. Polar and nonpolar metabolites were extracted from tumor tissue (~0.2g) with H₂O:MeOH:CHCl₃ (1:3:3) and quantified using NMR analysis. Metabolic enzymes were measured by western blot and qRT-PCR. Increased glycolysis is suggested by increased glucose utilization (decreased glucose, -92%, p=0.05; increased HKII enzyme, +410%, p=0.001). Increased lipid biosynthesis is suggested by increased citrate (+106%, p=0.016), the substrate for citrate lyase, and increased biosynthetic enzymes (e.g. FASN, +125%, p=0.016). Measurable lipid products include an increase in phosphatidylcholine (+157%, p=0.043), which correlates with a decrease in head-group choline (sn-glycero-3-phosphocholine, -41%, p=0.12). B. Glycolysis Altered by HBP1 KD. Protein from control or HBP1 KD MDA-MB-231 TNBC tumors were analyzed by western blot for HK2 enzyme and Actin as a protein control. Three representative control and 3 HBP1 KD tumors were analyzed by immunoblot and quantified using ImageJ. RNA was also extracted and analyzed by qRT-PCR using validated primers for HK2 and 18S as a control. C. Lipid Biosynthesis Altered by HBP1 KD. Extracts from control or HBP1 KD MDA-MB-231 TNBC tumors (Fig. 1A) were analyzed by western blot for α -FASN with Actin as a loading control. 3 representative control and 3 HBP1 KD tumors were analyzed by immunoblot and quantified with ImageJ. RNA was extracted and analyzed by qRT-PCR using validated primers for FASN and 18S as a control.

In the course of this project, we have shown our data to Dr. Craig Thompson and Dr. Lew Cantley, who are leaders in the cancer metabolism field. Both expressed that the data was very strong, but raised questions on the fate of OAA in driving the lipid biosynthetic changes via the TCA cycle. We are currently pursuing re-analysis of the NMR data to address this question, coincidentally raised by these two eminent experts. However, this summary illustrates that we can successfully execute a multi-disciplinary analysis and discover new aspects to metabolic regulation by HBP1 and by Wnt signaling. Some recent studies have highlighted altered lipid metabolism as a consequence of the enhanced glucose flux in a Warburg-like metabolism.

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. 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.

Year 2 Progress. We decided to refocus the EGCG/DAC treatment of the TNBC tumors to address possible metabolic changes and to obtain a comprehensive view of EGCG/DAC treatment. Please note that both doses are at or below the doses that are in clinical trial use or are FDA approved. Two important conclusion arose from this re-direction. Our previous studies had shown that EGCG/DAC treatment in the animal model decreased the primary TNBC tumor and also resulting brain metastases (see Figure 2). The recent work showed that 1) the combination of EGCG/DAC treatment appears to solidly inhibit Wnt signaling with a compilation of biochemical and gene expression evidence. 2) treatment of the primary tumors with EGCG/DAC appeared to reverse a Warburg like metabolism. The NMR studies will be summarized in Aim 2, task 2B. These two complementary discoveries underscore the role of Wnt signaling in coordination of a Warburg-like metabolism.

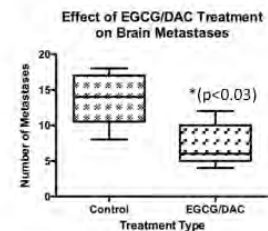
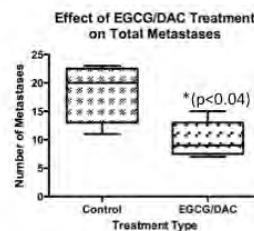
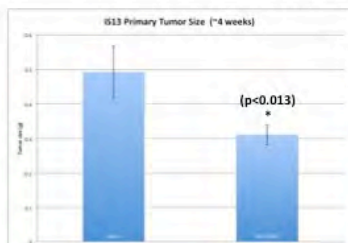
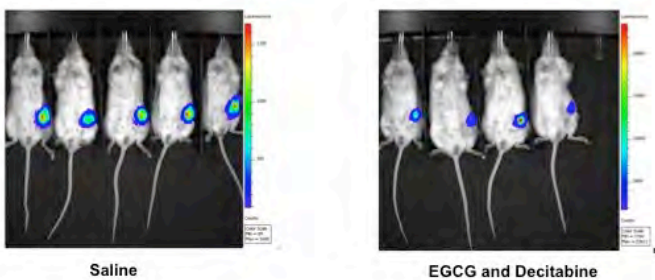


Figure 3. EGCG/DAC suppresses TNBC Tumors and Brain Metastases. A. The IS13 subclones of MDA-MB231 cells tagged with luciferase were implanted orthotopically into NOD-SCID mice. At least 5 mice per group were treated with either vehicle or with EGCG (16.5 mg/kg) and DAC (0.5 mg/kg) for 4 weeks. Tumors were measured with calipers. B. Control and treated mice were imaged after injection with luciferin. C and D. Orthotopic implants of the IS13 brain-metastasizing variant of MDA-MB231 cells, tagged with GFP, were treated with EGCG/DAC or saline for 6 weeks. GFP-labeled metastatic foci were counted on dissected brain, lung, and liver. The data shown as a box plot were analyzed with an unpaired two-tail t-test in Prism (graphpad).

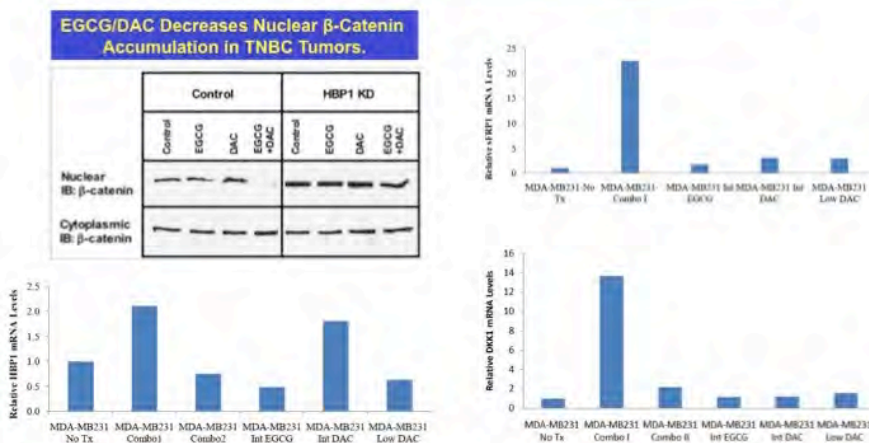


Figure 4. EGCG/DAC treatment leads to induction of Wnt pathway inhibitors and an overall blockade of Wnt Signaling. The LM1 subclone of MDA-MB231 cells tagged with luciferase were implanted orthotopically into NOD-SCID mice. At least 5 mice per group were treated with either saline or with the indicated dosages for 4 weeks. Combo 1 is EGCG (16.5 mg/kg) and DAC (0.5 mg/kg) for 4 weeks on alternating days. Combo 2 is EGCG (16.5 mg/kg) and DAC (0.25 mg/kg) for 4 weeks on alternating days. Int. EGCG is EGCG (16.5 mg/kg). Int. DAC is DAC (0.5 mg/kg). Lo DAC is (0.25 mg/kg). The tumors were harvested and extracts prepared as directed by Pierce. The nuclear β -catenin levels were determined by immunoblot from nuclear extract with antisera from Millipore. The mRNA levels were determined by qRT-PCR with primers optimized for detecting the mouse HBP1, SFRP1, and DKK genes.

In presenting the work, the most frequent questions queried the spectrum of biochemical consequences with EGCG/DAC treatment. Thus, we conducted an RNA-Seq analysis comparing control and EGCG/DAC treated TNBC xenograft tumors. While our biochemical analysis showed that Wnt signaling was blocked with treatment, we thought that an unbiased comprehensive snapshot would be useful to complete the work for publication. While we are still analyzing the data using various bioinformatics tools, the Wnt signaling pathway has already emerged as a top candidate, consistent with our biochemical analysis. We are currently analyzing the data to test at least 1-2 other candidate pathways or processes. This will be a final figure in a manuscript delineating the effects of EGCG/DAC on TNBC.

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.

Year 2 Progress. We have made substantial progress in implementing NMR analysis for tumors and integrating the data with the ongoing gene and protein analysis—all to assemble a comprehensive biochemical picture of the gene expression and metabolic alterations with treatment. For the analysis of the HBP1 KD TNBC tumors, see Figure 1 above. Our previous data (submitted with the application)

showed that HBP1 knockdown led to a metabolic rearrangement of over 300 genes. The recent progress indicates a specific and unexpected alteration in lipid metabolism.

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).

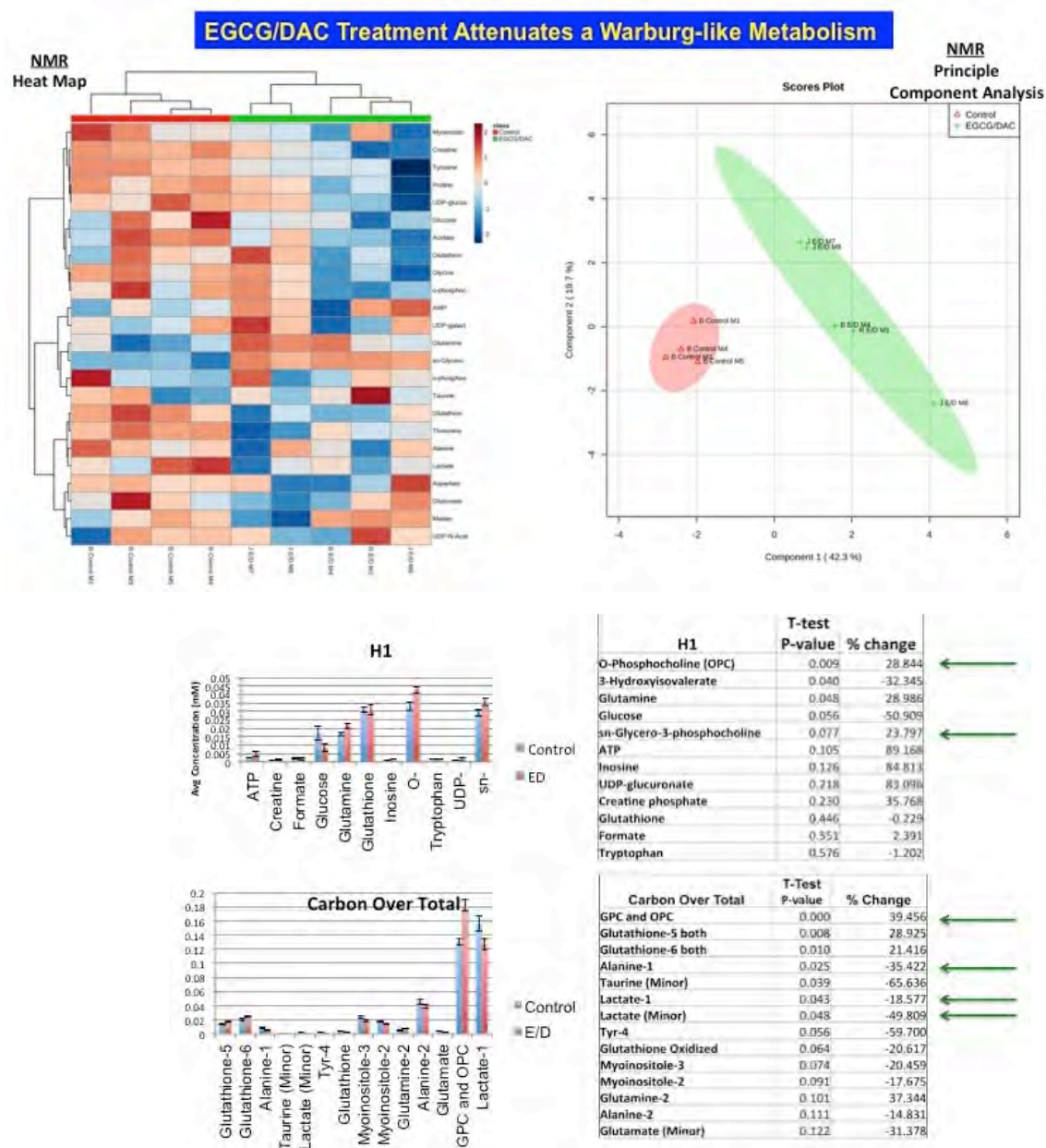


Figure 4. Metabolomic Analysis of EGCG/DAC Treated Tumors. The IS13 subclone of MDA-MB231 cells tagged with luciferase were implanted orthotopically into NOD-SCID mice. At least 5 mice per group were treated with either vehicle or with EGCG (16.5 mg/kg) and DAC (0.5 mg/kg) for 4 weeks. The tumors were dissected and immediately snap frozen in nitrogen. For the ¹³C-glucose labeling, the mice were injected with uniformly labeled ¹³C glucose (135 mg/kg) for 30 minutes before sacrifice. The tumors were dissected and immediately snap frozen in nitrogen. Extracts for NMR were prepared and then subjected to NMR. The chemical peaks were identified using Chenomx. The output was analysed by Metaboanalyst with selection for heat maps and principle component analysis. The relative magnitudes were compared using Excel and Graphpad software for statistical significance. At least 3 samples were analysed for control and for treated groups.

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.

Year 2 Progress. Based on Figure 1, phosphocholine and citrate may be a two candidate markers for further MRS development. We are now consulting with Dr. Shital Makim, our expert breast radiologist on the feasibility of the detected NMR spectra for MRS marker development. We are also exploring MRI facilities at MGH for testing MRS detection in tumors from live animals.

We used NMR analysis to discern the impact of EGCG on the Warburg-like metabolism upon EGCG/DAC treatment. Using principle component analysis and non-hierarchical clustering, we found that the treated tumors were definitively different than the control tumors in the detected metabolite profiles, suggesting that treatment had a dramatic consequence not only is reducing tumor size, but in the biochemical properties of tumor metabolism. We are still analyzing the differences, but there is a wide re-arrangement in amino acid, glucose and other metabolic pathways with EGCG/DAC treatment. An initial conclusion is that there is diminution of the Warburg effect with a large decrease in lactate production and glucose utilization as two consequences of EGCG/DAC treatment.

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.

Year 2 Progress. We have been meeting weekly as a research group to discuss results and refine experiments. The aligned efforts have allowed the development of feasible functional and metabolic frameworks. We are also meeting regularly to configure the manuscripts.

Manuscript on initial gene and NMR characterization of cells and MDA-MB231 xenografts (months 12-18).

4. KEY RESEARCH ACCOMPLISHMENTS.

Year 1

- 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.

Year 2

- Refining a pre-clinical model for TNBC and metastatic progression.
- Implemented a signaling and metabolic framework to develop hypotheses for investigating metabolic vulnerabilities.
- Implemented comprehensive NMR metabolomic analysis for breast tumors. Initial identification of potential metabolites for development as MRS markers

- Discovery of a drug combination that appears to alter a Warburg metabolism, diminish Wnt signaling, and diminish brain metastases.

5. CONCLUSION:

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.

Year 2. We have made progress towards developing a metabolic picture of TNBC primary tumors in the xenograft models. Because of a change in direction to de-emphasize erlotinib and EGFR signaling, this created a better focus on the already-efficacious EGCG/DAC regimen for primary tumors and metastases. We discovered that EGCG/DAC is a very useful tool to decrease Wnt signaling. Together with newly published findings relating Wnt signaling to metabolic re-programming, we investigated and found that EGCG/DAC may reverse aspects of a Warburg metabolism. Such new insights were possible with the refinement of the integrated NMR and gene expression approaches that we used to develop the profile of tumors with diminished HBP1 expression, as modeled by a xenograft tumors of the HBP1KD TNBC xenograft.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

- 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.
 - Lay Press: none
 - Peer-Reviewed Scientific Journals:none
 - Invited Articles: HBP1 Review for Gene.
 - Abstracts: AACR Breast Cancer Meeting, San Diego, CA October 2013.
 - Speaker at Northeast Breast Cancer Symposia, Dartmouth Medical Scholl. May 2015.

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:**

Year 1. The studies of this grant were the topics of masters' research and undergraduate research theses. Brian Pedro graduated with highest research honors as a result of the work for this grant.

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