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TITLE: A Novel Therapeutic Strategy Targeting BACH1 for Triple-Negative Breast Cancer

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14. ABSTRACT

Currently women have a 12% chance of developing breast cancer during their lifetime. The most aggressive and lethal subtype of breast cancer is triple negative breast cancer (TNBC), yet no approved targeted therapy for this group of patients. My research proposal addresses overarching challenges: (1) to develop therapeutic intervention with the approved drugs that are less toxic. (2) to maximize the efficacy of the approved drug with a combinational treatment that targets a metabolic regulator. With these questions, I will characterize breast tumor metabolism, identify molecular mechanisms beyond, and establish their roles for a novel therapeutic intervention. The goal of this research is to provide a novel therapeutic regimen to give benefits to the patients with breast cancer. My research accomplishment during the first year (2017-2018) identified BACH1 regulates mitochondrial genes at the transcriptional levels by direct binding on the promoter regions in breast cancer cells. These regulations altered metabolic phenotypes such as oxygen consumption rates (OCR) and extracellular acidification rates (ECAR), as well as increasing NAD+/NADH ratio, ATP levels, and mitochondrial ROS levels when BACH1 is depleted. Taken together, my research during the **Year 1** from DOD clearly elucidated metabolic pathway regulation by BACH1 as a transcriptional regulator in TNBC cells.

15. SUBJECT TERMS

BACH1 inhibits mitochondrial genes expression through direct recruitment on promoter regions to suppress mitochondrial oxidative respiration in breast cancer cells.

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INTRODUCTION

The research proposal is to test the hypotheses that (1) depletion of BACH1, either genetically or pharmaceutically, drives metabolic pathways toward mitochondrial oxidative phosphorylation that can be targeted by metformin, a respiratory inhibitor, and (2) the combination of 2 FDA-approved drugs, metformin and panhematin, will be an effective combination for treating high-risk triple-negative breast cancer (TNBC) patients that express high BACH1 levels. The project's specific aims are (1) characterize metabolic pathways regulated by BACH1 in TNBC cells; (2) define the molecular mechanisms by which BACH1 regulates mitochondrial genes; (3) analyze the efficacy of combination treatment targeting BACH1 and mitochondrial exudative phosphorylation (oxphos) for breast cancer.

KEYWORDS
Triple-negative breast cancer, BACH1, hemin, metformin, cancer metabolism, novel combination therapy

ACCOMPLISHMENTS

What were the major goals of the projects?

Major goal of the project is to identify whether BACH1 regulates metabolic pathways in breast cancer. Therefore, depletion of BACH1 either genetically or pharmaceutically drives metabolic pathways to the mitochondrial oxidative phosphorylation that can be further a target by metformin, respiratory inhibitor. Using the approved metabolic drug, metformin, and hemin (active ingredient of panhematin), the combinational treatment of metformin and hemin will be tested in mouse model harboring TNBC tumors. To obtain these goals, I propose specific aims as following;

Aim 1. Define metabolic pathways regulated by BACH1 in TNBC cells (Year 1).

Aim 2. Define the molecular mechanisms by which BACH1 regulates mitochondrial genes (Year 2).

Aim 3. Analyze the efficacy of treating TNBC by targeting both BACH1 and mitochondrial oxidative phosphorylation (Oxphos) (Year 3).

What was accomplished under these goals?

Accomplishment: I accomplished most of the proposed experiments accordingly to the time lines.

Specific Aim 1.

Subtask1-Completed in month **3.**

All the cell lines that were proposed were successfully tested for validation of mitochondrial gene expressions using qRT-PCR. Total RNA from TNBC cells was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Two µg of RNA was adapted for reverse transcriptase PCR (Applied Biosystem) to generate cDNA. Real-time PCR was carried out using LightCycler 96 (Roche) and a Fast Start Essential DNA master mix (2X) reagent. Cq values normalized relative to the expression of endogenous control genes using $2^{(-\Delta\Delta Cq)}$ were plotted. Quantitative RT-PCR validated shBACH1 induction of inner mitochondrial membrane genes (8 out of 10 genes) largely involved in the electron transport (ETC) by quantitative RT-PCR using two human TNBC cell lines that express BACH1, BM1 and MDA-MB-436. COX15, COX18, SLC25A15, SLC25A22, ATP5D, ATP5G3, NDUFA9, and UQCRC1 were significantly increased around 1.5-3 folds upon BACH1 depletion compared to control cells, while PBH and ATP5J mRNA are not changed by BACH1 depletion in BM1 cells (Figure 1A). qRT-PCR experiments were repeated at least three times for statistical analyses using two-tailed t-test with a GraphPad Prism 7.0a software. In addition, protein levels of ETC genes were validated using western blot assays (Figure 1C).

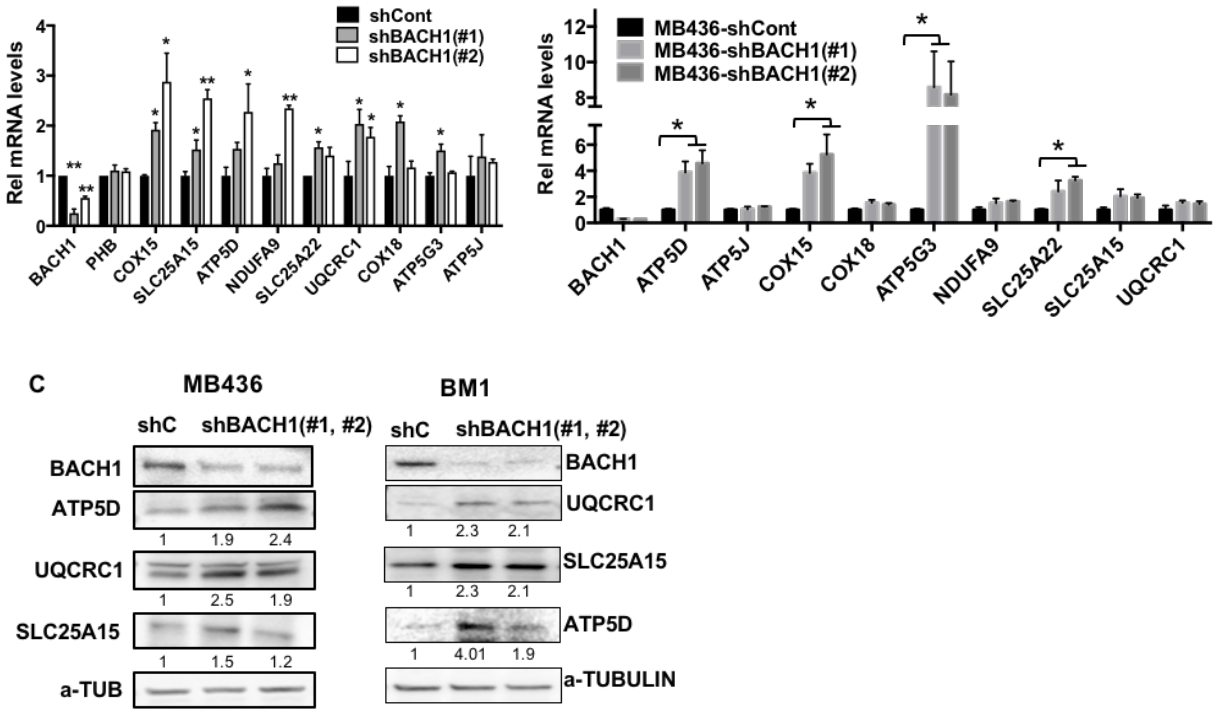

Figure 1. Relative mRNA levels of mitochondrial inner membrane genes in BM1 (left) and MB436-shBACH1 cells (right) with two distinct shBACH1 vectors (#1, #2) compared to the wild type control (shCont) by qRT-PCR. Mean ± s.e.m. of three independent assays with p-values (*p<0.05) determined by two tailed Student's t-test. Representative images of western blots of mitochondrial genes using MB436-shBACH1 and BM1-shBACH1 cell lysates. Samples were probed with antibodies to proteins as indicated. Band density generated using a Licor Odyssey Fc is shown below the blots.

Subtask2- Completed in Month **6**.

I determined whether the changes in ETC gene expression affect metabolic phenotypes in breast cancer cells by measuring both oxygen consumption rates (OCR), an indication of aerobic respiration, and extracellular acidification rates (ECAR), a readout of lactic acid produced from increased glycolysis. ECAR and OCR were monitored by a Seahorse Bioscience Analyzer (Biophysics Core Facility, University of Chicago). Cells were seeded in 24 well plates at a density of 5×10^4 and 96 well plates at a density of 5×10^3 - 8×10^3 cells per well with growth media for at least 18 hours. The following day, media was changed to base media (DMEM, 143 mM NaCl, phenol red, pH 7.35). For ECAR analysis, cells were added with media (2 mM Glutamine, pH 7.35) and monitored every 3 minutes following successive administration of 10 mM of glucose, and inhibitors such as 1 μM of oligomycin and 50 mM of 2-DG. For OCR analysis, cells were added with mito stress test base media (10 mM Glucose, 2 mM Glutamine, 1 mM Pyruvate, pH 7.4) and monitored every 3 minutes following successive administration of inhibitors such as 2 μM of oligomycin, 2 μM of FCCP, and 0.5 μM of Rotenone/AntimycinA. BCA protein assays were used to normalize metabolic rates to cell number. At least six biological replicates of shBACH1 or hemin-treated cells were analyzed to compare to shRNA control cells. P-values were determined by paired two-tailed t-test over time.

TNBC cells depleted of BACH1 displayed increased basal OCR as well as maximum OCR but decreased ECAR relative to the control. These results suggest that BACH1 loss promotes oxidative phosphorylation (Figure 2).

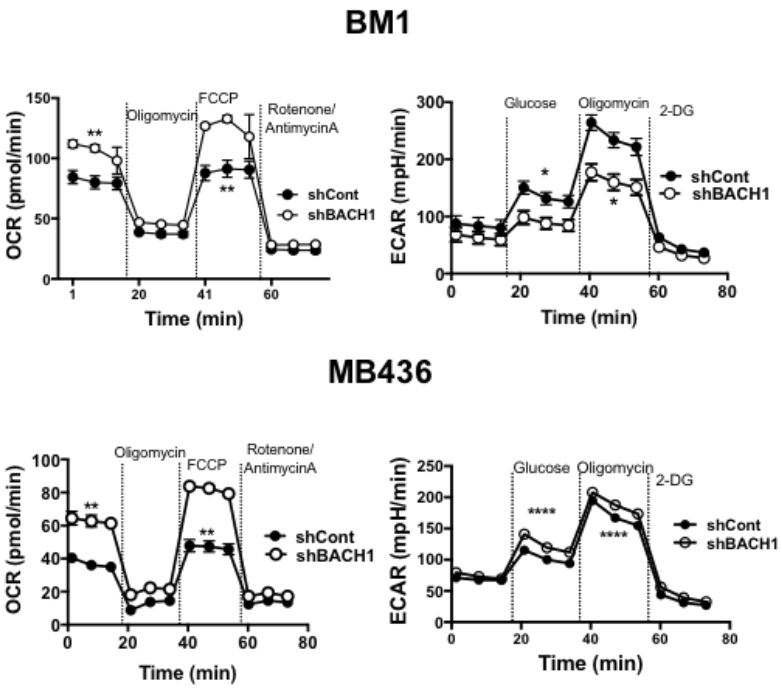

Figure 2. Measurement of OCR and ECAR of BM1 (top) and MB436 cells (bottom) expressing control or shBACH1. Data are mean ± s.e.m. (n=6) with p-value (*p<0.05, **p<0.01) determined by two-tailed student's t-test.

Subtask 3- Completed in Month **10**.

Also energy phenotypes were tested using MDA-MB-436 and BM cells depleted with BACH1 using either shRNA or hemin treatment compared to control cells (subtask 2). Cellular metabolites such as NAD+/NADH ratio were measured using TNBC cell lines using the NAD+/NADH-Glo Assay kit (Promega, G9071) in accordance with the manufacturer's protocol. NAD/NADH ratio was significantly increased in BACH1-depleted cells compared to control cells, indicating increased oxidative phosphorylation in shBACH1 cells.

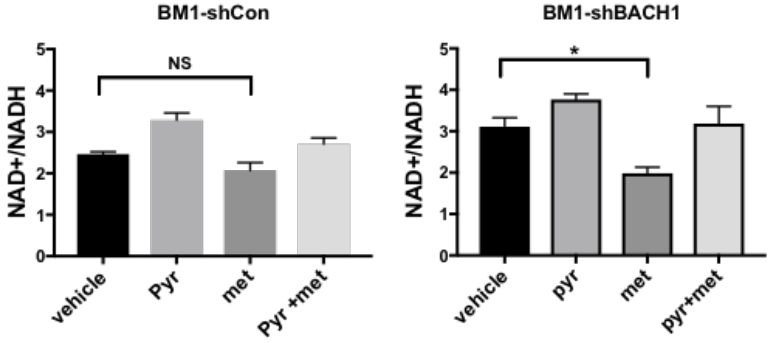

Figure 3. Relative NAD^+/NADH ratios in BACH1-depleted BM1 and its control cells treated with pyruvate (2.5 mM) and/or metformin (5 mM) for 24 hrs. Data are mean ± s.e.m. (n=3) with p-values (*p<0.05) by two-tailed student's t-test.

Subtask 1- Completed in Month **11.**

To determine whether mitochondrial genes are direct BACH1 targets, I analyzed potential BACH1 recruitment sites (MAF recognition elements, TGCTGAC(G)TCAGC) within the promoter regions of these genes (Figure 4). Having identified potential BACH1 binding sites for six mitochondrial genes, *ATP5C, COX15*, *UQCRC1, ATPJ, SLC25A22,* a*nd TIMM8B,* I performed ChIP assays with BACH1 antibody using BM1 and MDA-MB-436 cells as well as MDA-MB-468 which does not express BACH1 as a negative control cells. BACH1-depleted cells (BM1-shBACH1 and MDA-MB-436-shBACH1) were used as negative control for BACH1 recruitment along with IgG. In addition, Heme oxygenase 1 (HMOX1), which is transcriptionally repressed by BACH1, served as positive controls for BACH1 binding specificity. Simply, two million cells were plated on 10 cm plates overnight prior to crosslinking with 10% of formaldehyde for 10 min followed by quenching with 157 mM of glycine for 3 min. After washing cells with cold PBS, total cell lysates in ice were sonicated at 80 % output for 10 seconds with a 10 second pause for 4 cycles and pre-cleared with anti-goat-IgG for 1 hour at 4 °C. Supernatants were precipitated with antibodies against BACH1 (AF5776, R&D System), RNA Pol II (Cell Signaling), H3K273Me (Abcam) antibody or IgG (normal Goat, Santa Cruz) overnight and followed by washing for qPCR.

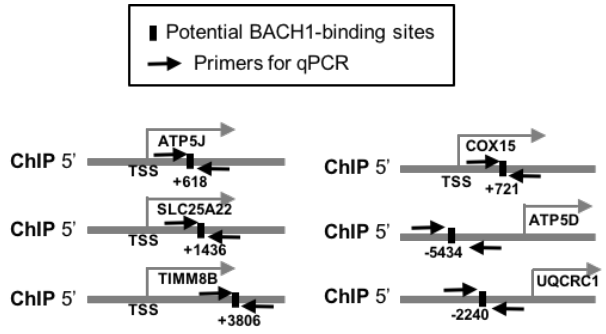

Figure 4. Schematic diagram showing proximal BACH1 binding on the promoter regions of mitochondrial membrane genes. TSS: Transcription Start Site. Arrows: primers used for ChIP-qPCR.

ChIP assays showed a striking enrichment of BACH1 bound to the promoter regions of ETC genes as well as binding of the repressive histone marker H3K27Me3 (Figure 5). These data suggest that BACH1 is a direct suppressor of mitochondrial ETC gene transcription in breast cancer cells. ChIP assays at least three times were analyzed using two-tailed t-test.

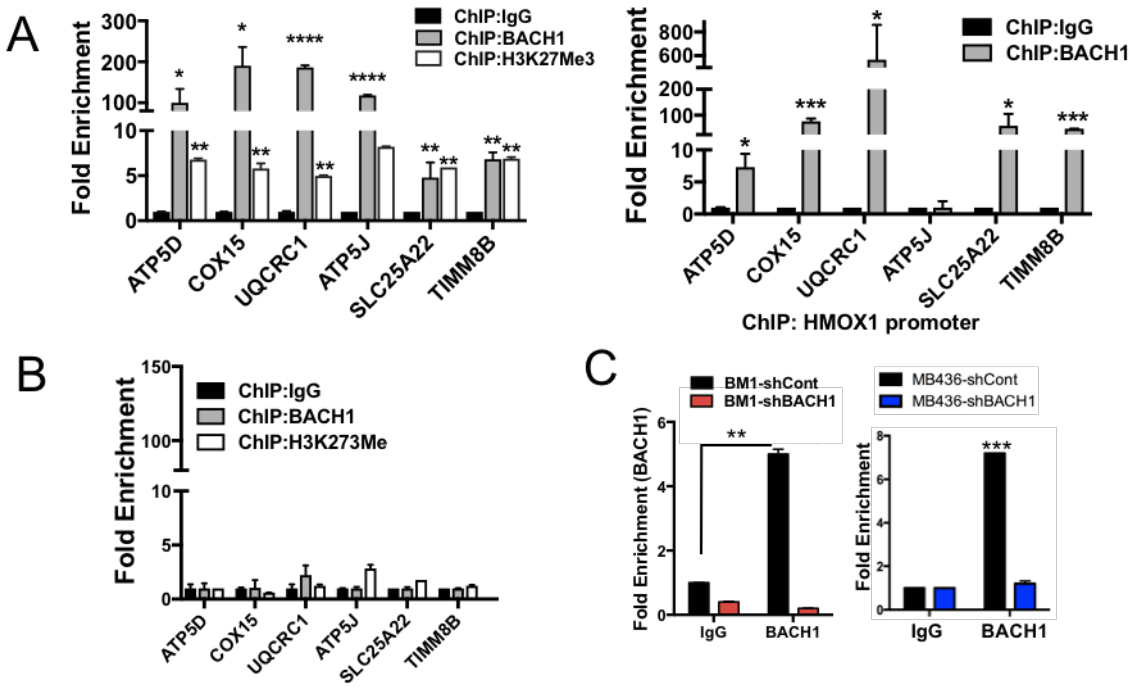

Figure 5. ChIP analyses showing relative fold enrichment of BACH1 recruitment to the mitochondrial gene promoters using BACH1-depleted BM1 (A, left) and MB436(A, right) or MB468 (B) or HMOX1 promoter (C) using BACH1-depleted BM1 or MB436 cells. Data are mean ± s.e.m. (n=3) with p-value (*p<0.05, **p<0.01, ***p<0.001) determined by two-tailed student's t-test.

Specific Aim 3.

Accomplishment: To investigate whether shBACH1 tumors are sensitive to metformin in mouse models, I used C3TAg mice model that generates M6C cells.

Subtask 1-Completed in Month **12.**

Mouse mammary cancer cells such as M6C were treated with metformin to monitor whether these cells are sensitive to metformin independent of BACH1 expression levels. As shown in Figure 6 (left), growth of M6C cells were inhibited with dose dependent manner of metformin (1, 5, and 10mM). Cell viability was measured using Incucyte system to show cellular growth as measured as confluency (%) covered by cells, which is equivalent measurement as CaAM assays. Interestingly, heme treatment with combination with metformin did not change cellular growth indicating that M6C cells are highly sensitive to metformin, and thus not affect by heme (hemin) treatment. Since mouse TNBC cell line, M6C, was sensitive to the treatment of metformin alone, mouse TNBC cell lines will be excluded from the future experiments as well as animal models. Instead, I will use human TNBC xenograft mouse model using two cell lines; MB436 and BM1.

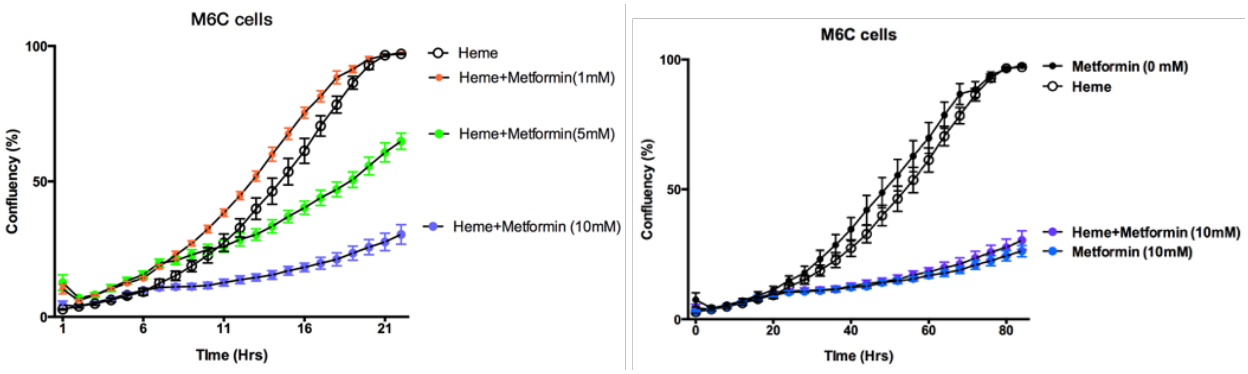

Figure 6. Cellular growth as measured as confluence area (%) covered by cells of BACH1 depleted cells (BM1-shBACH1) and control cells treated with metformin (left) or combination of hemin and metformin (right) using IncuCyte system. Values are mean ± s.e.m. of 6 biological replicates.

Subtask 3-Completed in Month **12.**

I established a xenograft mouse TNBC model using MDA-MB-436 cells. All animal protocols related to mouse experiments were approved by the University of Chicago Institutional Animal Care and Use Committee and by the US Army Medical research and Material Command (USAMRMC), Office of Research Protections (ORP), Animal Care and Use Review Office (ACURO) for review and approval prior to initiation. Two million human breast cancer cells (MDA-MB-436) in 100 µl PBS were injected into the 4th mammary fat pads of 5 to 6 weeks old athymic nude female mice (Charles River Laboratories). When tumors reach about 20-30 mm^3 in volume, mice were randomized into treatment groups (n=8-9/group) for treatment of hemin (50 mg/kg/day) or vehicle (20 mM NaOH in phosphate buffered saline) by intraperitoneal injection daily for 10 days prior to metformin treatment for 8 weeks. Mice that do not develop tumors were excluded from the analysis. Metformin (200 mg/kg/day) was provided in drinking water *ad libitum*. Tumor growth was monitored weekly by caliper measurement weekly in two dimensions to generate ellipsoid volumes using an equation of vol. = 0.4 x (LxW^2). Tumor volume was shown as mean ± s.e.m with significance (p-value) by paired t-test.

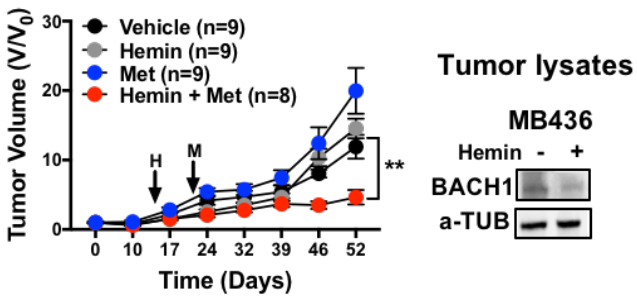

Figure 7. Tumor volume and weight measured from mice xenografted with MB436 cells (2x10^6 cells/mouse) and treated with metformin (200 mg/kg) in drinking water. Representative blot images of Bach1 using tumor lysates are shown (right). Values are shown as mean ± s.e.m. with p-value (**p<0.01) by two-tailed paired student's t-test.

Animal experiments using combination therapy of hemin and metformin indicate that neither hemin nor metformin alone altered tumor size compared to control tumors. However, metformin rapidly suppressed growth of tumors that were treated with hemin combined (Figure 7). BACH1 suppression by hemin treatment using tumor lysates were shown in western blots (Figure 7, right).

In addition, Tumor weight measured at the end of experiment supports that combination treatment using hemin and metformin clearly and significantly reduced tumor weights compared to control or single treatment of hemin or metformin (Figure 8). This was not due to overall toxicity, since all mice in this treatment groups showed no changes in body weight or behavior changes (Figure 9). This **subtask 3** clearly indicates that the novel combination therapy using hemin and metformin significantly reduced breast tumor growth without overall toxicity in a mouse model.

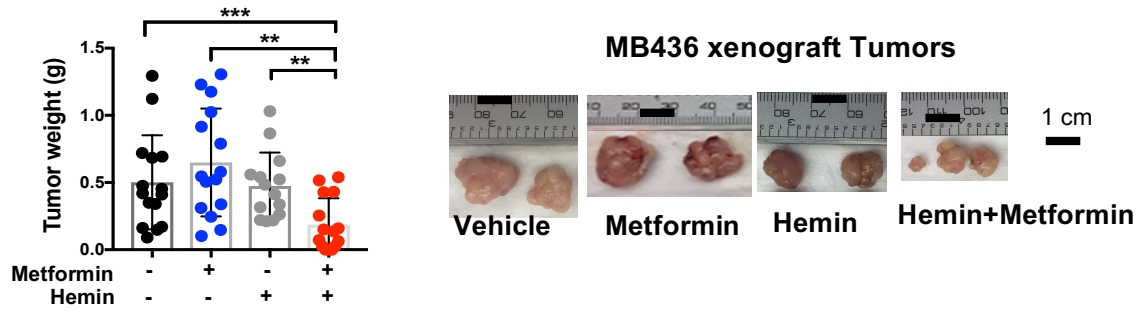

Figure 8. Tumor weight collected and measured at the end of the treatment using hemin and metformin of MB436-xenograft mice (left). Representative tumor images from each treatment group of MB436-xenograft mice are shown (bar = 1 cm).

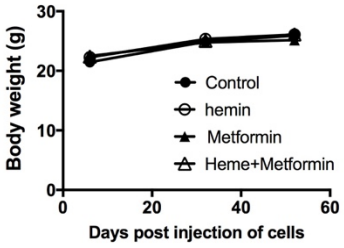

Figure 9. Plots of body weights (grams) of mice monitored before and after treatment of hemin and metformin. Arrow indicates treatment initiation of hemin (H) or metformin (M). Values are shown as mean ± s.e.m.

-End of Accomplishment in Year 1.

What opportunities for training and professional development has the project provided?

- I attended **workshop/coursework for Scientific Writing Retreat 2018** (November 15-19, 2017, Cold Spring Harbor, NY, US) provided by Cold Spring Harbor laboratory during my grant years (**Year 1**). This is only one-time workshop/coursework that provides junior faculties to improve clarity and effectiveness of professional communication skills which will beneficial for PI to successfully publish research projects.

How were the results disseminated to communities of interest?

- "Nothing to report" during this reporting period.

What do you plan to do during the next reporting period to accomplish the goals?

- As I proposed in SOW, I will follow the proposed work flow with time lines. First, I will continue to define molecular mechanism by which BACH1 regulates mitochondrial genes using luciferase assays (Major Task 2-1. Subtask 2, **Month 14-15**), siRNA to silence mitochondrial genes such as COX15 and UQCRC1 in shBACH1 cells to identify whether these genes play critical role for mitochondrial Oxphos when combined with metformin treatment (Major Task 2-1, Subtask 3-4, **Month 16-21**).

- To establish whether combination therapy using hemin and metformin is effective for breast cancer treatment in immune competent mouse model, I proposed to use M6C cells for C3(1)-TAg mouse model. However, previous study as shown in **Figure 6** in this report, cells are already highly sensitive to metformin alone, thus I will omit this cell line for the future experiment. Instead, I will be focused alternative syngeneic mammary cancer model using Balb/c and 4T1.2 mouse TNBC cells as proposed in **SOW**. As proposed, I will continue to inject cancer cells into mouse fat pad and test combination treatment using metformin and hemin for 6-8 weeks (Major Task 3-2, Subtask 1-2, **Month 14-20**).

- Moreover, I will elucidate metabolic flow using labeled substrate such as 13C-glucose and 13C-glutamine in BACH1-depleted cells (Major Task 1-2, Subtask 1**, Month 22-24**).

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

This proposal has impact on development of novel combination therapy to enhance efficacy of metformin, a drug, that is most prescribed for diabetic patients to treat high-risk breast cancer patients. Our preliminary data indicated that targeting BACH1 will generate a synthetic lethality strategy for cancers that are not responsive to metformin treatment through driving metabolic pathway changes in cancer to sensitize to metformin treatment.

Our data that I obtained in **Year 1** showed that BACH1 regulates mitochondrial metabolic genes such as electron transport chain (ETC) through direct binding on promoter regions as a transcriptional regulator in breast cancer cells. Thus, BACH1 depletion using shRNA increased both mRNA and protein levels of mitochondrial gene expression. Upon depletion using shRNA in TNBC cells, metabolic phenotypes such as oxygen consumption rates (OCR) representing mitochondrial respiration and extracellular acidification rates (ECAR) representing glycolysis were changed. Subsequently, metabolic cofactors such as NAD+, ATP, and ROS were also monitored to be altered by BACH1 depletion. Taken together, these data indicate that BACH1 is a metabolic regulator in breast cancer. BACH1 depletion changed metabolic phenotypes depending on mitochondrial respiration. Thus targeting BACH1 either using shRNA or drug will drive metabolism changes in breast cancer. Since the BACH1 targeting drug (panhematin or hemin) is already approved by U S Food and Drug Administration, the combinational therapy we propose here (**Year 2** and **Year 3**) has an immediate impact on clinical therapeutics, although thorough investigation about toxicity of using panhematin with metformin in preclinical setting should be performed.

What was the impact on other disciplines?

"Nothing to Report" during this reporting period.

What was the impact on technology transfer?

This novel combination therapy has commercial technology or public uses to treat breast cancer patients that might be useful to initiate a start-up company. And this idea would be applied to other types of cancers expressing BACH1.

What was the impact on society beyond science and technology?

"Nothing to Report" during this reporting period.

CHANGES/PROBLEMS
"Nothing to Report" during this reporting period.

PRODUCTS
Poster presentations at Conferences

1. **Keystone Tumor Metabolism Meeting**, at Snowbird, UT (Jan. 21-25, 2018)
   Title: Effective combination therapy for breast cancer targeting BACH1 and mitochondrial oxidative phosphorylation.
2. **American Association for Cancer Research (AACR) Annual Meeting**, at Chicago, IL (Apr. 14-18, 2018)
   Title: Effective combination therapy for breast cancer targeting BACH1 and mitochondrial metabolism.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

Name:	Jiyoung Lee
Project Role:	"no change"
Research Identifier	"no change"
Nearest person month worked:	"no change"
Contribution to project	"no change"
Funding Support:	"no change"

Has there been a change in the active other support of the PI(s) or senior/key personnel since the last reporting period?
"Nothing to Report" during this reporting period.

What other organizations were involved as partners?
"Nothing to Report" during this reporting period.

SPECIAL REPORTING REQUIREMENTS
"Nothing to Report" during this reporting period.

APPENDICES
"Nothing to Report" during this reporting period.