FINAL REPORT
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Co-applicant name (if applicable):
Grant title: Targeting IGFBP3 signaling as a novel therapy for ESR1-Y537S mutant driven bone metastasis
Date of Award: 8/18/2023
RFP name of award: METAvivor Translational Research Award
Date of submitting the report: 6/15/2026

Summary of the project goal (1 paragraph)

Developing more effective targeted treatment for metastatic breast cancer is a critical need since majority of breast cancer patients succumb to distant metastases developed in the bone, lungs, liver and brain. More than two-thirds of breast cancers are positive for estrogen receptor-α (ER), which is the primary target of endocrine therapies, but approximately 40% of ER-positive breast cancer patients who initially responded develop acquired resistance. One mechanism of acquired resistance that has been recently identified is the hotspot mutations in the ligand binding domain (LBD) of ER (encoded by *ESR1*), resulting in constitutively active ER mutants. Those mutations are almost exclusively found in metastatic lesions, arising in patients who received aromatase inhibitors. Among those hotspot mutations, Y537S mutation showed the most prevalent and the most resistance to existing anti-estrogen therapies, with shortest progression free survival in patients. Effective treatment targeting the most dominant and resistant *ESR1-Y537S* for metastatic breast cancer is still lacking. Our previously work on circulating tumor cells (CTCs) isolated and expanded in culture from metastatic ER+ breast cancer patients identified a CTC line BRx68 that carries the most common *ESR1-Y537S* mutation, and forms a high prevalence of bone metastases in metastatic assay in mice. To identify the *ESR1-Y573S* mutation specific effect, we analyzed ChIP-seq data from several datasets including our own and identified an enhancer region that is uniquely bound by Y573S-ER. Further analysis led to the identification of Insulin Like Growth Factor Binding Protein 3 (IGFBP3) as a potential downstream target of this enhancer. Our hypothesis for this project is that Y537S-ER binds to a unique enhancer region to upregulate IGFBP3, which then further enhances the activity of ER mutant to promote cell survival and proliferation in the bone metastasis. To address this hypothesis, we propose to evaluate the effect of targeting IGFBP3 signaling on *ESR1-Y537S* mutation driven bone metastasis, and investigate the regulatory network of Y537S-ER and IGFBP3 in promoting bone metastasis.

Specific Aims
Please paste over each aim and sub-aim in sequential order, including the approach proposed to achieve each aim as is from the contracted research proposal.

Aim 1. Evaluate the effect of targeting IGFBP3 signaling on *ESR1-Y537S* mutation driven bone metastasis.
1A. We will delete or suppress IGFBP3 and evaluate the effect *in vitro*. We will design sgRNA targeting the first exon of IGFBP3 gene and clone it into the PX458 lentiviral construct that carries the CRISPR-Cas9 system as well as GFP. GFP-positive cells will be FACS sorted into single wells of 96-well plate to generate clones and western blot used to confirm the knockout (KO) of IGFBP3. The EV and IGFBP3-KO clones will be utilized to perform cell proliferation analysis using CellTiter-Glo assay.

1B. We will evaluate the effects of suppressing IGFBP3 on bone metastasis. We will utilize a bone-in culture assay to evaluate the effect of IGFBP3 loss on the cell colonization in the bone marrow environment.

1C. We will evaluate the effect of an inhibitor against IGFBP3 downstream kinase on bone metastasis. We will test the efficacy of SKI-178, a SphK1 inhibitor, for its effect on inhibiting Y537S-ER formed bone metastasis *in vivo*.

Aim 2. Investigate the regulatory network of Y537S-ER and IGFBP3 in promoting bone metastasis.

2A. We will examine whether overexpression of IGFBP3 in the DPY enhancer KO cells can rescue the effect on cell proliferation and fulvestrant sensitivity. We will introduce the lentivirus that carries the open reading frame of IGFBP3 into the MCF7-Y537S DPY KO cells, perform cell proliferation analysis using CellTiter-Glo, evaluate the sensitivity to different concentrations of fulvestrant, and compare the results with EV control.

2B. We will validate the looping of DPY enhancer to the promoter of IGFBP3. We will perform 3C assay to evaluate the looping of the enhancer with the promoter of IGFBP3.

2C. We will investigate whether IGFBP3 facilitate Y537S-ER binding to DPY enhancer as a positive feedback loop.

Progress made so far

Previously, we have reported that IGFBP3 could be a potential downstream target of the DPY enhancer region that was preferably bound by Y537S ER mutant. We have performed CRISPR-Cas9 knock out of IGFBP3 and showed that loss of IGFBP3 recapitulated some phenotypes of loss of DPY enhancer region, such as cell proliferation, but not others like sensitivity to fulvastrant treatment. These data suggest that IGFBP3 is more likely a potential downstream target of the DPY19L1P1 enhancer. To evaluate this finding in patient data, we analyzed RNA-seq data from MET500 metastatic breast cancer cohort where there are 12 samples with ESR1 mutation compared to 34 wild type ER. There is a statistically significant higher IGFBP3 level in mutant compared to WT ER samples (Figure 1A), corroborating the result found in cell lines.

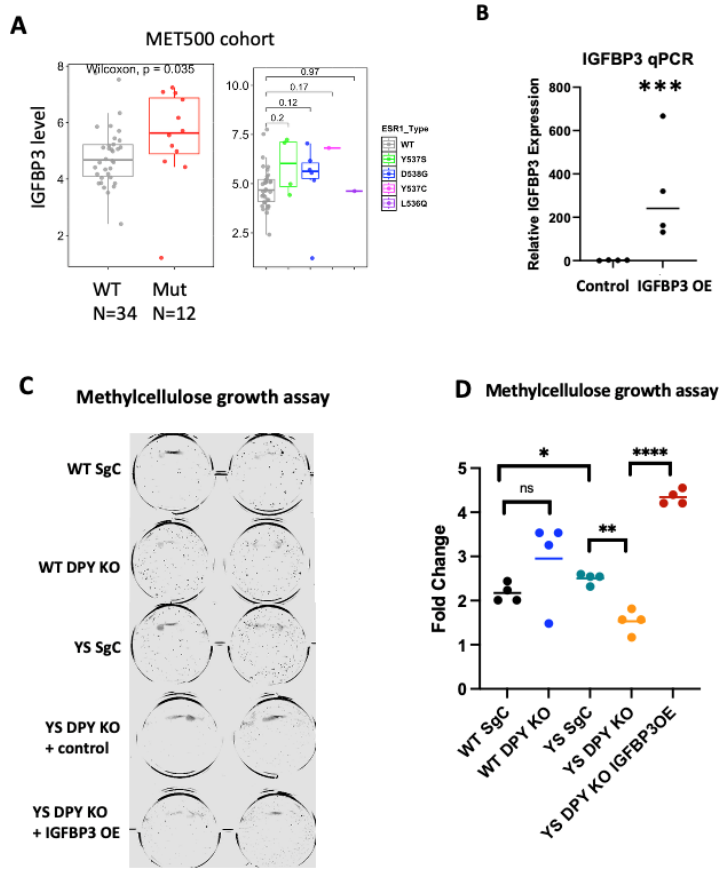

Figure 1. IGFBP3 level in breast cancer patients and its overexpression rescued the reduced anchorage-independent growth upon loss of DPY enhancer in Y537S ER mutant cells. (A) Boxplot comparing IGFBP3 expression between ESR1 wild-type (WT; n = 34) and ESR1-mutant (n = 12) tumors in the MET500 metastatic breast cancer cohort. Statistical significance was assessed using a two-tailed Wilcoxon rank-sum test. (B) qPCR analysis of relative RNA expression level of IGFBP3 Y537S cells with DPY19L1P1 KO cells that transduced with empty vector control or overexpression of IGFBP3. *** $P < 0.001$, N=4. (C) Representative images of the methylcellulose colony growth assay (2 wells each as technical replicates in one biological experiment) at day 7. GFP signal has been changed to black color for quantification. (D) Graph showing relative colony growth compared to day 1 in methylcellulose growth assay in WT SgC, WT DPY KO, YS SgC, YS DPY KO with empty control plasmid or with IGFBP3 overexpression (OE). * $P < 0.05$, **$P<0.01$, **** $P < 0.001$. N=4. Data was analyzed with Welch's T test.

Since the ER mutations are almost exclusively detected in metastatic breast cancers, we evaluated the effect of loss of DPY19L1P1 enhancer in cells growing in suspension to mimic the loss of anchorage effect during metastatic process (Figure 1B-D). Quantification of the growth of cell colonies after 7 days in methylcellulose culture showed that Y537S SgC cells grow better than WT SgC. Interestingly, loss of DPY enhancer in WT did not lead to statistical significant change, but trending towards increasing in growth. In contrast, loss of the DPY enhancer in Y537S cells suppressed the growth, whereas overexpress IGFBP3 rescue such effect in DPY KO Y537S cells (Figure 1B-D), supporting an important role of IGFBP3 downstream of DPY enhancer in anchorage-independent growth of Y537S cells.

Publications/Presentations and or Patent application

Amzaleg Y, Mecenas D, Flowers E, Thomas A, Li L, Thomas A, Hendrick H, Noh H, Moriarty A, Wang YJ, Li Z, Zhang J, Oesterreich S, **Yu M**. Identification and validation of an ESR1 Y537S mutant enriched enhancer and its downstream target IGFBP3. *Cancer Research Communication (in revision)*

****Required for METAvivor's board: Lay Description of Important Outcomes**

NOTE: this section may be shared on METAvivor's website and/or in donor publications as it is important for us for donor transparency – thank you!

Please briefly summarize the main project achievements or outcomes so far in lay terms. Please emphasize any clinical trials and/or important findings with potential to impact metastatic breast cancer treatment (now or in the future) stemming from the funded work.

You may use bullets to quickly highlight main points, for example:

- Submitted/in preparation/published publications if any

Amzaleg Y, Mecenas D, Flowers E, Thomas A, Li L, Thomas A, Hendrick H, Noh H, Moriarty A, Wang YJ, Li Z, Zhang J, Oesterreich S, **Yu M**. Identification and validation of an ESR1 Y537S mutant enriched enhancer and its downstream target IGFBP3. *Cancer Research Communication (in revision)*

- Funding support obtained with METAvivor data if any, and goal of the support

N/A

- Summary of important findings

About 75% of breast cancers rely on estrogen to grow, so they are known as estrogen receptor (ER)-positive cancers. The most common treatment for these breast cancers is the endocrine therapy that suppresses the ER function. However, one major mechanism of the developed resistance recently identified is the frequent mutations in the gene encoding ER (gene name ESR1), resulting in this receptor to remain active without estrogen, and rendering the cells resistance to the traditional endocrine therapies. Importantly, these mutations are almost exclusively found in metastatic lesions, pointing to the importance of this finding for metastatic diseases. Among those mutations, the most prominent one, Y537S mutation, has shown the most resistance to existing treatment. We identified a circulating tumor cell (CTC) line derived from a metastatic breast cancer

patient that carries the most common ESR1-Y537S mutation, and showed that it forms a high prevalence of bone metastases in metastatic assay in mice, recapitulating the bone metastatic status of the corresponding patient. To find the mechanism underlying this mutant ER driven bone metastasis, we analyzed where does the mutant ER bind in the genome and compared to the normal ER conditions. We identified a unique genomic region that specifically bound by the mutant ER which actives a gene called Insulin Like Growth Factor Binding Protein 3 (IGFBP3) that has been previously shown to play a role in breast cancer progression and bone metastasis. With the funding, in this proposal, we aim to understand the role of IGFBP3 in the mutant ER derived bone metastasis and evaluate available inhibitors as a treatment. In the past year of funding, we have used genetic method to delete the IGFBP3 gene in the breast cancer cells that carry the Y537S ER mutant and validated its role in cell proliferation. In addition, we have further validated the genomic region that regulate this gene for its role in tumor growth at the estrogen-low environment, mimicking the clinical setting.

- Clinical relevance of findings i.e., upcoming/in progress trials, impact on MBC treatment (now or future)

Bone metastasis is the most prominent, and often the first site of, metastasis in breast cancer patients. Patients with bone metastasis suffer from high morbidity and mortality. However, there are not enough effective bone metastasis specific targeted therapies, besides the bone symptom relieving drug, bisphosphonates. The proposed study has the potential to significantly advance the development of effective means to target mutant ER induced bone metastasis, and will lead to improved treatment strategies to benefit metastatic breast cancer patients with these mutations in general.