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14. ABSTRACT This project centers on the NF1/neurofibromin tumor suppressor, which was best known as a GTPase Activating Protein (GAP) that repress Ras activity. The parent DoD award has successfully defined a new and GAP-independent activity that NF1 is also a transcriptional co-repressor for estrogen receptor α (ER) in ER^+ breast cancer. While the parent DoD award focused on endocrine therapy resistance caused by NF1 loss, in this Expansion Award, the focus instead is on metastasis, for which currently has no cure. An important feature of ER^+ breast cancer metastasis is that greater than 70% of the metastasis is in the bone. We **hypothesized** that the transcriptional co-repressor role of NF1 is also responsible for driving bone metastasis in ER^+ breast cancer. Therefore, the **objective** of this Expansion Award is to assess NF1's role in metastasis in order to establish a strategy to stop it. We have made progress in accomplish Task1/Aim 1 to fully define NF1-controlled genes that can impact bone metastasis. This was a key part of the data that was just published in the high impact journal *Cancer Cell*. This award has also supported the launching a Phase-II clinical trial to treat ER+ NF1-depleted breast cancer, and the awards of a SPORE and another DoD Level-2 project. However, in Aim 2 (Tasks 2 and 30) we are dependent on the use of animals to study how NF1-depleted cancer cells interact with the bone, but this line of study has been severely and negatively impacted by COVID-19. We discuss how we plan to overcome this problem in the future.

15. SUBJECT TERMS

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INTRODUCTION

This project centers on the NF1/neurofibromin tumor suppressor, which was best known as a GTPase Activating Protein (GAP) that represses Ras activity. The parent DoD award has successfully defined a new and GAP-independent activity that NF1 is also a transcriptional co-repressor for estrogen receptor α (ER) in ER^+ breast cancer. While the parent DoD award focused on endocrine therapy resistance caused by NF1 loss, in this Expansion Award, the focus instead is on metastasis, for which currently has no cure. An important feature of ER^+ breast cancer metastasis is that greater than 70% of the metastasis is in the bone. We **hypothesized** that the transcriptional co-repressor role of NF1 is also responsible for driving bone metastasis in ER^+ breast cancer. Therefore, the **objective** of this Expansion Award is to assess NF1's role in metastasis in order to establish a strategy to stop it. The specific aims are:

AIM 1: To define the full range of NF1 transcriptional activity in ER^+ breast cancer cells by identifying key metastasis-driving genes that are directly regulated by NF1.

AIM 2: To assess the impact of NF1 depletion on bone metastasis using BICA (bone in culture assay) *in vitro* and IIA (intra iliac artery) injection *in vivo,* and how to block these activities in order to reduce metastasis in ER^+ $NF1^-$ cancer.

KEYWORDS

4-OHT, 4-hydroxytamoxifen
AI, Aromatase inhibitor
BICA, bone in culture assay
ChIP, Chromatin immunoprecipitation
DOX, doxycycline.
E2, estrogen/estradiol
EMT, epithelial to mesenchymal transition
ER, estrogen receptor-α
EREs, Estrogen Response Elements
FS, frameshift
GAP, GTPase Activating Protein
HR, hazard ratio HR
IHC, immunohistochemistry
IIA, Intra-iliac artery
KI, knock-in
KM, Kaplan-Meier
KO, knock-out
MS, missense
NF1, Neurofibromatosis type 1
NS, nonsense
PDX, patient-derived xenograft
SERD, Selective ER Degrader
SERM, Selective ER modulator
TCGA, The Cancer Genome Atlas

ACCOMPLISHMENTS

Major task 1: Identify gene expression directly control by NF1.

NF1 is an ER co-repressor for transcription regulation; thus, we sought gene expression changes after

Enriched functional categories	Examples	p-value	FDR
Ossification	ATP6V0A4, BMP8A, BMP8B, CDK6, CLEC3A, COL5A2, EGR2, ENPP1, FBN2, FHL2, GLI1, IARS1, ID3, IGF2, JUNB, KREMEN1, MEF2D, MGP, NPNT, OSTF1, PBX1, PHEX, PKDCC, PTH1R, PTK2B, SLC26A2, SLC8A1, SMPD3, SNX10, SOX9, TMEM64, WNT3, XYLT1	4.9×10^{-18}	3.6×10^{-15}
Bone remodeling	CLEC3A, GHR, IGF2, MGP, NPR3, PTH1R, SOX9, TNFRSF11B	2.1×10^{-4}	3.7×10^{-3}
EMT	ANPEP, AREG, BASP1, BGN, CAP2, COL5A2, FBN2, GEM, GJA1, ID2, IGFBP4, MGP, TGM2, TNFRSF11B, CLDN4/9*, SNAI1*	2.1×10^{-7}	1.7×10^{-6}

Table 1. NF1-mediated gene expression that is potentially critical for treatment resistance and/or metastasis. Gene set enrichment analysis using indicated functional categories was performed as described in "Statistical plan." *These genes are obviously relevant for EMT but were not selected by the gene set, so they were included in this table manually.

NF1-depletion. This line of research has mostly been published in a paper of ours in the high impact journal *Cancer Cell* (Zheng et al 2020, see "Products" below). In this project period, we have conducted deeper gene signature analysis. As shown in Table 1, in addition to EMT, which is generally associated with metastasis, *NF1*-silencing most significantly affected "bone remodeling" and "ossification" (of the bone), which supports the hypothesis that NF1 loss can mediate bone metastasis by altering bone remodeling, which is usually dependent on a balanced activities between (bone-forming) osteoblasts and (bone-reducing) osteoclasts.

To show NF1 is directly involved in the regulation of these genes, chromatin immunoprecipitation (ChIP) was performed using an NF1 antibody and NF1-associated DNA sequences can be identified by qPCR or by deep sequencing. In the paper that was recently published with the support of this grant (Zheng et al *Cancer Cell* 2020), we demonstrated that NF1 was recruited to several promoter regions in the presence of tamoxifen by ChIP-qPCR. In this project period, we have begun to perform ChIP-seq; that is, all the associated DNA sequences were identified by sequencing to determine in a genome-wide fashion where NF1 binds. This is a challenging experiment because NF1 binds DNA indirectly by binding via ER so the sequencing signals were weak. However, as a proof of principle, we were able to observe NF1 recruitment to regions of chromatin enriched with estrogen responsive elements (ERE, Figure 2), DNA sequences located in the promoter regions of many estrogen-responsive genes. We are in the process of repeating this experiment and increasing the binding efficiencies.

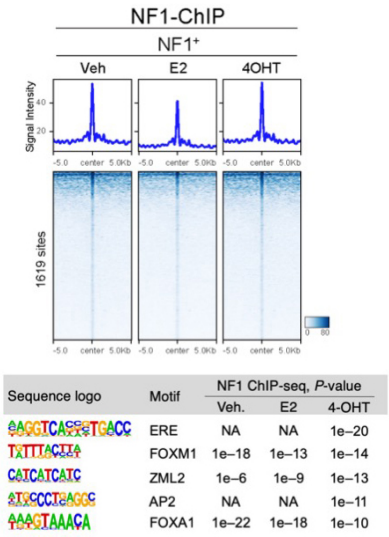

Figure 2. NF1 ChIP-seq showing NF1 recruitment to the chromatin in a ligand dependent manner. Top, presence of NF1 on the chromatin in response to different ligands and agreeing its role as a co-repressor, this recruitment is stimulated by tamoxifen. Bottom, DNA sequencing revealed that the NF1-bound sequences are enriched with ERE motif.

Major task 2: Assess NF1's role in bone metastasis *in vitro* using BICA.

Our key approach to study the interaction between the bone and ER+ cancer cells is to inject cancer cells tagged by a luciferase reporter via the intra-iliac artery (IIA) to be delivered to the hind leg bone in mice. The injected bones are harvested the next day, fragmented, and randomized to be seeded in 96 -well plates. Cell growth is then measured by luciferase activity. In preliminary study submitted previously with the proposal, we demonstrated that *NF1*-depleted MCF-7 cells grew more efficiently in the bone than the $NF1^+$ counterparts. In this project period, we repeated the experiment and obtained the same results (Figure 3A). However, due to COVID-19, we have had very limited access to the lab. Furthermore, mouse colonies were made to be greatly reduced, and no mouse surgery was allowed for a substantial period of time. We therefore have not been able to conduct any further BICA experiments.

To address the limitations placed upon us due to COVID-19, we turned to *in vitro* co-culture experiments to study whether *NF1*-silencing in ER^+ breast cancer cells can alter the activities of cells in the bone. Since the BICA data showed that *NF1*-silenced ER+ breast cancer cells can grow more efficiently in the bone than the NF1+ counterparts, we co-cultured these breast cancer cells with several cell types found in the bone in 3D (low attachment plates) and measure cell growth by measuring luciferase activity. As shown in Figure 3B, *NF1*-silenced breast cancer cells grow much better than NF1+ cells when co-cultured with nearly all the cells commonly found in the bone, but the growth-stimulatory effects by osteoblasts (FOB1.19) stood out.

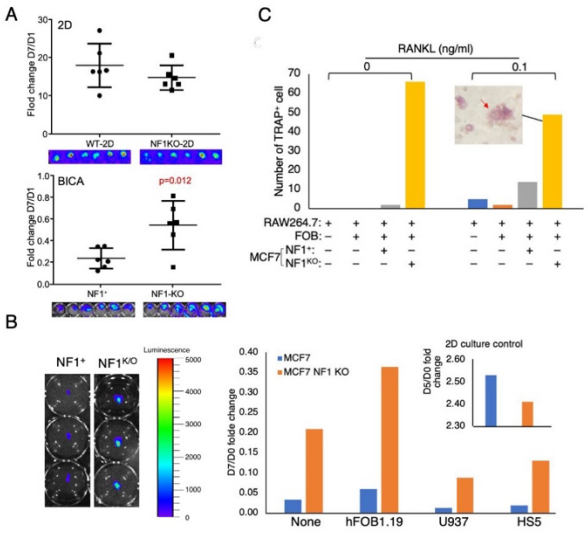

Fig. 4. NF1-depleted ER$^+$ breast cancer cells grow more efficiently in the bone and promote osteoclast formation. (A) Top: 2D NF1 knockout (KO) ER$^+$ breast cancer MCF7 cells tagged by luciferase were examined as control to show that these cells generally grow slightly more slowly than the parent NF1$^+$ cells (see Ref. #3). Bottom: Indicated MCF-7 cells were first introduced into the bone by intra-iliac artery injection and the bone fragments were later cultured *in vitro.* The cell numbers on day-7 relative to day-1 were measured by an IVIS instrument. **(B)** MCF-7 cells alone or together with indicated cells found in the bone micro-environment (see text) were seeded in low attachment plates to allow for interaction in 3D. Luciferase activities were measured in Day-0 vs Day-7, and the ratios quantified on the right. An example of MCF7+FOB cells in culture were shown on the left. Inset shows a control experiment that the NF1$^{K/O}$ cells grew more slowly when attached to plastic in 2D culture. **(C)** TRAP is an osteoclast marker phosphatase whose activity can be measured calorimetrically to produce a pink color in the cell. *In vitro* osteoclasts can be efficiently induced when their precursor cells (RAW264.7) are co-cultured with the osteoblast cells (hFOB1.19) in the presence of high RANKL concentration (>10 ng/ml). When NF1^{KO} MCF-7 cells were present, efficient TRAP+ cells can be seen at very low concentrations of RANKL. The inset is an example showing TRAP$^+$ cells displaying feature of a mature osteoclasts: multi-nucleation. Note The expression of *RANKL* is regulated by *PTH1R,* an NF1-repressible gene.

This suggests that the osteoblast is the key cell type that is responsible for stimulate the growth of *NF1*-silenced cells as seen in the BICA experiments.

Bone metastasis from ER+ breast cancer cells is characterized by pronounced bone loss, which is usually caused by osteoclasts. Bone loss often releases growth factors to stimulate the growth of cancer cells, and cancer cells may in turn promote more osteoclast activity to create a vicious cycle. As discussed above, bone remodeling gene is a key gene function category regulated by NF1. We therefore performed another set of co-culture experiments to investigate whether *NF1*-depleted ER+ breast cancer cells can promote osteoclast formation. This system consists of osteoblasts (FOB1.19) and an osteoclast precursor cell line (RAW264.7). Osteoclast formation, as detected by a colorimetric assay measuring the activity of an osteoclast marker enzyme, tartrate-resistant acid phosphatase (TRAP), is usually induced by the RANKL ligand. However, in the presence of *NF1*-silenced ER+ breast cancer cells, osteoclast formation was detected even in the absence of RANKL (Figure 3C). All together these data support the model that NF1-depletion can induce gene expression changes in ER+ breast cancer cells to allow these cells to grow more efficiently in the bone and promote osteoclast formation.

Major task 3: Assess NF1's role in bone metastasis *in vivo* using IIA injection in mice. This line of experiment was not performed due to limited access to the animals during the COVID-19 shut down in our institution. We have also realized that we did not complete the approval of our animal protocol. Both of these issues have been fully addressed, however, so we expect to pick up the pace of animal work in the next project period.

IMPACT

Germline loss of the *NF1* gene is responsible for neurofibromatosis type 1, the world's most common genetic disorder, occurring in 1 out of 3,000 cases, and NF1 is also somatically lost in a wide range of tumors, including breast cancer. NF1 is best known as a GAP (GTPase Activating Protein) to repress Ras signaling. However, at the end of the parent grant, we have made a surprising discovery that NF1 has a GAP-independent activity by also acting as a co-repressor for the estrogen receptor (ER) transcription activity. This discovery has two important impacts on our clinical practice. First, NF1 loss causes hyperactivation of ER which leads to resistance to tamoxifen and aromatase inhibitors. Thus, it is critical not to treat these ER+ but NF1–tumors with these agents. Second, we have demonstrated that in these tumors the ER activity can be more

efficiently inhibited by fulvestrant which induces ER turnover and is already FDA-approved to treat metastatic breast cancer, while the Ras pathway can be inhibited by binimetinib (which inhibits MEK, a Ras effector kinase) to stop the tumors from developing fulvestrant resistance. These findings have been translated into a Phase-II clinical trial to treat metastatic ER^+ NF1-delpeted patients with the support by the NCI ComboMATCH program.

In this Expansion Award, we are expanding our study to better understand how NF1-loss can impact metastasis, which occurs shortly after treatment resistance and currently has no cure. A major feature of metastasis in ER+ breast cancer is that greater than 70% of the distant metastasis go to the bone. Our results support the model that NF1 loss is a key driver for bone metastasis by reprograming cancer cells to equip with the ability to interfere with the normal bone remodeling processes. It is possible that NF1 loss can be established as a biomarker to mark tumors most susceptible to bone metastasis, and targeting pathways activated by NF1 loss may help prevent or treat bone metastasis.

CHANGES/PROBLEMS

A serious problem we have been facing has COVID-19, which has led to greatly limited lab access, and animal work was nearly shut down completely. In the next project period, we believe these limitations will be mostly behind us and will allow us to more efficiently conduction experiments requiring animals (e.g., task 3). NF1 ChIP-seq is a challenging experiment due to the fact that NF1's binding to the DNA is through the binding to ER, which directly binds DNA. In addition, we are limited by the tool set of ligands that promote NF1 binding to the DNA. Tamoxifen works, but its creation was optimized for treating ER+ breast cancer, but not for inducing the strongest binding of co-repressor to the DNA. These issues greatly reduce the sequencing signals in the study of any co-repressor, not just NF1. We are in the process of optimizing the cross-linking protocol to increase/stabilize the portion of DNA bound by NF1 to improve the sequencing performance.

PRODUCTS

Clinical Trial: Based on our compelling pre-clinical data, we designed a two-cohort phase II study of fulvestrant-binimetinib combination in patients with metastatic ER^+ breast cancers in collaboration with the NCI Combo MATCH program (Protocol #EAY191-N2). This trial design is approved by NCI Cancer Therapy Evaluation Program (CTEP) as one of ComboMATCH therapeutic arms with a projected initiation date in Q1 2022. ComboMATCH is a highly competitive program designed to study combinatorial therapeutics in advanced cancers rationally designed based on robust predictive biomarkers-guided selection of target population.

Grant awarded with support by this grant:

Our parent grant's partnering PI (Ellis) has submitted an Expansion award to better study other aspects of NF1's properties and got funded. The PI on this grant (Chang) is a co-PI on that grant:

Title: Optimizing Treatment for NF1-Deficient Metastatic ER+ Breast Cancers (Expansion Award)

Major Goals: The major goal is to further interrogate approaches to diagnose NF1 loss in clinical samples in order to expand eligibility criteria for trials targeting ER+ breast cancer with NF1 loss.

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PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Name	Project role	ORCID ID	Person Mon worked	Project contribution	Funding support
Eric Chang	PI	0000-0002-1375-5088	3.49	Design and execute all the studies in this project, and will write the pa-per.	This grant.
Zeyi Zheng	Staff Scientist	0000-0001-6536-4874	3.04	Assist Dr. Chang in the design and execution of all the studies in this project, and supervise Ms. Kenney	This grant.
Hilda Kennedy	Tech	NA	3.04	Provide technical support on all projects.	This grant
Zhao, Zifan	Graduate Student	0000-0002-1901-2752	12.00	A new graduate student who is responsible for Aim 1.	This grant
Matthew Baik	Student helper	NA	3.0	A student helper to assist in in vitro co-culture experiments.	This grant.