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14. ABSTRACT

The goal of this project are to test whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can antagonize the growth and/or survival of breast cancer cells exposed to high extracellular calcium *in vitro* or grown in animal models of bone metastases *in vivo*. Over this past 3rd year of the project, we continued to make progress on generating tetracycline-regulated knockdown of the CaSR and GABAB R1 in breast cancer cells. We also performed initial experiments examining how genetic knockdown of PTHrP and the CaSR affected the formation and growth of osteolytic bone metastases in MDA-MB-231 breast cancer cells in nude mice. As expected knocking down PTHrP expression reduced the size of osteolytic metastases but, surprisingly, knocking down CaSR expression actually increased the size of osteolytic lesions. We are continuing to validate and better understand these results while waiting for the new regulated knockdown cell lines for the CaSR and GABAB R1. We received a no-cost extension to finish up these experiments but they were further delayed by the COVID situation.

15. SUBJECT TERMS. Calcium-sensing receptor, Gaba B receptors, breast cancer, osteolytic bone metastases, parathyroid hormone-related protein, G-protein-coupled receptors

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Progress Report

1. Introduction.

The purpose of this project is to study whether the formation of CaSR/GABABR1 heterodimers allows breast cancer cells to upregulate PTHrP production in response to high extracellular calcium concentrations instead of the usual downregulation of PTHrP by CaSR homodimers in normal mammary epithelial cells. We have shown that PTHrP acts in the nucleus to stimulate the proliferation of tumor cells and to protect them from the toxic effects of high extracellular calcium. Therefore, our hypothesis is that this pathway is critical for tumor cells to thrive in the bone microenvironment and that inhibiting CaSR/GABABR1 heterodimers might kill breast cancer cells in osteolytic bone metastases. The purpose of the project is to test this hypothesis by examining whether genetic or pharmacologic inhibition of the CaSR and/or GABABR1 would sensitize breast cancer cells to DNA-damaging agents *in vitro* and in bone metastases *in vivo*.

2. Keywords

Calcium-sensing receptor, Gaba B receptors, breast cancer, osteolytic bone metastases, parathyroid hormone-related protein, G-protein-coupled receptors

3. Accomplishments

Major Goals of the Project

The goals of this project remained the same as the original proposal: to test whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can antagonize the growth and/or survival of breast cancer cells exposed to high extracellular calcium *in vitro* or grown in animal models of bone metastases *in vivo*. We continued to work towards the original 3 specific aims:

Aim 1 - to determine whether genetic knockdown of the CaSR or the GABAB R1 inhibits PTHrP production, reduces proliferation and increases apoptosis of breast cancer cell lines exposed to high extracellular calcium.

Aim 2 – to determine whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can synergize with radiation or PARP activation to kill breast cancer cells at high extracellular calcium.

Aim 3 – to determine whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can inhibit the growth of osteolytic bone metastases in mouse models.

Progress towards accomplishing Goals

We will report our progress and accomplishments as organized in the Statement of Work.

Dr. Julie Hens, who is an Associate Research Scientist, has been performing the experiments after replacing Dr. Wonnam Kim who is now in a faculty position in South Korea.

Aim 1, Task 1: Create stable GABAB R1 knockdown cell lines in BT474, 4T1 and MDA-MB231.1833 breast cancer cells.

Dr. Hens noted that, over time, the knockdown of GABAB R1 appeared to become less efficient, suggesting that there might be some growth advantage to the cells with more GABAB R1 expression. Given that our experiments required stable knockdown of CaSR and GABAB R1 expression, she has been working to generate regulated knockdown cell lines using a tetracycline-regulated system hoping to be able to study the effects of acute loss of GABAB R1 on the behavior of breast cancer cells and CaSR signaling. She decided to use the T-Rex™ System with the pcDNA™6TR plasmid from Thermo-Fischer Scientific to generate the tetracycline-regulated plasmids to express shRNA against GABAB R1. However, her initial attempts to generate cell lines that expressed both constructs were not successful. Therefore, she rederived the GABAB R1-shRNA expressing and the tTa-expressing constructs into lentiviral vectors. She has been using these constructs to create the needed cell lines. Once these are in hand, she will validate that treatment with doxycycline properly results in inhibition of CaSR or GABAB R1 mRNA levels. We will concentrate our studies on MDA-MB231.1833 and 4T1 cells in order to examine the potential effects on bone metastases. Once we develop and characterize inducible knockdown cell lines for GABBR1, we will proceed with the experiments in Aim 1, Task 3.

Milestone of Creating GABAB R1-knockdown cells is in progress.

Aim 1, Task 2: Examine cAMP levels, PTHrP production, cell proliferation and cell death in 4T1 and MDA-MB231.1833 CaSR-knockdown and control breast cancer cells.

These experiments were completed and published in *Cancer Research* in year 1 (Cancer Res 76:5348, 2016).

Milestone of Measuring effects of CaSR knockdown on cell growth in breast cancer cells was achieved.

Aim 1, Task 3: Examine cAMP levels, PTHrP production, cell proliferation and cell death in BT474, 4T1 and MDA-MB231.1833 GABAB R1-knockdown and control breast cancer cells.

Although we had generated some preliminary data with the prior stable GABAB-R1 cells, we are now not sure about whether these cells had stably suppressed levels of GABAB R1 expression. Therefore, we will repeat these experiments using the new Tet-regulated acute KD of the CaSR and GABAB R.

Milestone of Measuring the effects of GABAB R1 knockdown on cell growth in BT474, 4T1 and MDA-MB231.1833 cells is only partly achieved.

Aim 2, Task 1: Examine whether CaSR-knockdown or GABAB R1-knockdown cells are more susceptible to cell death after treatment with MNNG or radiation
Aim 2, Task 2: Examine whether treatment with NPS2143 sensitizes breast cancer cells to MNNG or radiation

As reported previously, initial experiments by Dr. Kim had suggested that combining MNNG treatment with CaSR-knockdown synergized to augment cell death. Dr. Hens found that MNNG was very difficult to work with since it caused significant cytotoxicity by itself. She had planned to determine whether CaSR-knockdown synergized with radiation exposure or with other DNA damaging agents such as platinum-based chemotherapeutic agents. However, these experiments remain on hold until the proper Tet-regulated cell lines are available.

Milestones to determine if genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers sensitizes breast cancer cells to MNNG or radiation have been partially achieved.

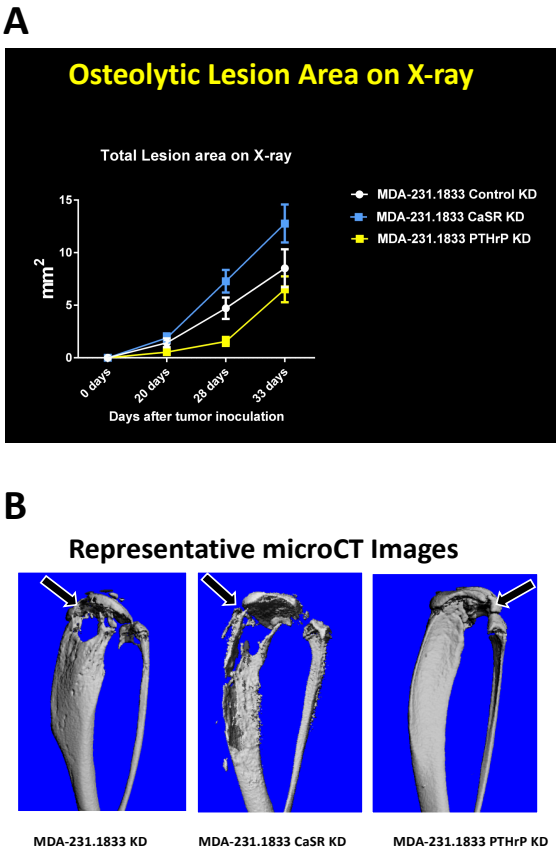

Figure 1. A) Total Osteolytic lesion by X-Ray analysis of mice injected with control MDA-231.1833 cells (white), CaSR KD MDA-231.1833 cells (blue), or PTHrP-KD MDA-231.1833 cells (yellow). B) Typical osteolytic lesions from sample mice described in A as assessed by micro-CT analysis.

Specific Aim 3 - To determine whether inhibition of CaSR/GABAB R1 heterodimers can inhibit the growth of osteolytic bone metastases in mouse models.

We initiated these experiments by comparing CaSR-knockdown and PTHrP-knockdown MDA-MB231.1833 cells injected into the left ventricle in collaboration with Drs. Theresa Guise and Khalid Mohammad at the University of Indiana. As expected control MB231.1833 cells formed osteolytic lesions and knocking down PTHrP production reduced the total osteolytic lesion area (Fig 1). What was unexpected was that knocking down the CaSR led to an apparent increase in osteolytic bone metastases as evidenced by a significant increase in total osteolytic area (Fig 1). It is not clear why knocking down PTHrP and CaSR expression would give divergent results in this assay given that knocking down the CaSR reduces PTHrP expression in MB231.1833 cells in vitro. We are examining whether

CaSR and/or PTHrP expression were reduced as predicted in the bone metastatic lesions in vivo. One possibility is that loss of the CaSR is still not enough to reduce PTHrP in the metastatic environment. This has led us to begin examining how the CaSR and TGF-beta interact in regulating PTHrP expression in metastatic lesions. In addition, we plan to repeat the same experiments using the tetracycline-regulated cells, once they are available. This will allow us to study the same cells ± dox treatment, which will avoid any potential alterations in cellular phenotype due to differences that occurred in the clone selection process.

Milestones to determine whether inhibition of CaSR/GABAB R1 heterodimers can inhibit the growth of osteolytic bone metastases in mouse models have been initiated and partly achieved.

Opportunities for training and professional development

Nothing to report.

Dissemination of Results to Communities of Interest

Nothing to report.

Plans to Accomplish Goals During Next Reporting Period.

Obviously, we will not be able to complete all the studies that were initially described. However, we have requested a no-cost extension of this project that would allow us to prioritize the following remaining tasks.

Aim 1, Task 1: Create stable GABAB R1 knockdown cell lines in BT474, 4T1 and MDA-MB231.1833 breast cancer cells

We will finish developing tetracycline-regulated GABBR1 knockdown cells as described.

Aim 1, Task 3: Examine cAMP levels, PTHrP production, cell proliferation and cell death in BT474, 4T1 and MDA-MB231.1833 GABAB R1-knockdown and control breast cancer cells

After developing tetracycline-regulated GABBR1 knockdown cells, we will proceed with the characterization of cAMP, PTHrP and proliferation assays.

Aim 2, Task 1: Examine whether CaSR-knockdown or GABABR1-knockdown cells are more susceptible to cell death after treatment with MNNG or radiation

After developing tetracycline-regulated CaSR-knockdown cells and GABABR1-knockdown cells, we will examine the effects of knocking down the CaSR and GABABR1 on cell viability and apoptosis after radiation-induced or platinum-based DNA damage in the presence of elevated calcium levels.

Aim 3, Task 1: Determine whether genetic knockdown of either CaSR or GABAB R1 expression inhibits the growth of osteolytic lesions

We will continue our analysis of tetracycline-regulated, CaSR-knockdown, PTHrP-knockdown and GABAB R1-knockdown MDA-MB-231.1833 cells into nude mice and examine whether how this alters the number and/or size of osteolytic bone metastases.

4. Impact

Impact on the principal discipline.

As we generate publications, this project will also impact the wider breast cancer research community.

Impact on other disciplines.

Nothing to report

Impact on Society.

Nothing to report

5. Changes/Problems

Nothing to report.

6. Products

Nothing to report.

7. Participants and Other Collaborating Organizations.

Individuals working on this project

Name: John Wysolmerski
Project Role: PI
Research Identifier:
Nearest person month worked: 1
Contribution to Project: Oversaw the entire project. Supervised Dr. Hens and Ms Dann.

Name: Julie Hens, PhD
Project Role: Associate Research Scientist
Research Identifier:
Nearest person month worked: 12
Contribution to Project: Performed experiments described in this report.

Name: Pamela Dann
Project Role: Senior Research Associate
Research Identifier:
Nearest person month worked: 2
Contribution to Project: Helped Dr. Hens with cell culture and routine proliferation and cell death assays.

Changes in Other Support for the PI

There has been a change in the PI's Other Support, although no changes that alter his effort on the current project.

The updated Other Support for Dr. Wysolmerski is contained in the following pages:

OTHER SUPPORT - WYSOLMERSKI, JOHN J

ACTIVE SUPPORT

1 R21 AR073146-01 Wysolmerski (PI) 03/01/2019 – 01/31/2021 0.6 calendar mos.
NIH/NIAMS $125,000 (direct costs/yr)

PTHrP and Cancer Cachexia
The major goals of this project are: 1) To determine whether overexpression of PTHrP in breast tumors induces systemic lipolysis, and 2) To determine whether hypercalcemia synergizes with PTHrP to induce lipolysis and cancer associated cachexia syndrome.
Role:PI
Funding Agency Contact: Thomas Cheever, Thomas.Cheever@nih.gov. Program Officer, National Institute of Arthritis and Musculoskeletal Disease
Overlap: None

1 R21 HD100751-01 Wysolmerski (co-PI) 04/01/2020 – 03/31/2022 0.6 calendar mos
NIH/NICHD $150,000 (direct costs year 1)

The Protective Effects of Lactation on Diabetes
The major goals of this project are: 1) To examine whether lactation mobilizes tissue triglycerides and improves insulin sensitivity in mice, and 2) To examine whether lactation improves insulin sensitivity and increases lipid turnover in women.
Role: Co-PI with Dr. Belfort DeAquiar
Funding Agency Contact: Andrew Bremer, andrew.bremer@nih.gov; Eunice Kennedy Shriver National Institute of Child Health and Human Development
Overlap: None

BC151665 Wysolmerski (PI) 09/01/16 – 9/30/2020 0.6 calendar months
DOD/BRCP $125,000 (direct costs/yr)

Targeting CaSR/GABAB R1 Heterodimers to Treat Bone Metastases in Breast Cancer
The major goals of this project will be 1) To determine whether genetic knockdown of the CaSR or the GABAB R1 inhibits PTHrP production, reduces proliferation and increases apoptosis in breast cancer cell lines exposed to high extracellular calcium; 2) To determine whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can synergize with radiation or PARP activation to kill breast cancer cells at high extracellular calcium; 3) To determine whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can inhibit the growth of osteolytic bone metastases in mouse models
Role: PI
Funding Agency Contact: Ms. Danielle Rickey (Danielle.l.reckley.civ@mail.mil) or Ms. Wendy Baker (wendy.a.baker.civ@mail.mil), DOD BCRP Grant Specialists
Overlap: None
On 1-year no-cost extension

PENDING SUPPORT

1 R01 HD100468-01A1 Wysolmerski (PI) 07/01/2020 – 06/30/2025 3.6 calendar months
NIH/NICHD $328,389.00 (direct costs/yr)

Heterodimerization of the Calcium-Sensing Receptor with the GabaB Receptors in the Breast

The major goals of this project are: 1) To examine how heterodimerization with GABBR1 or GABBR2 alters CaSR expression, signaling and PTHrP production in breast epithelial cells *in vitro*. 2) To use genetically modified mice to examine how loss of GABBR2 affects the ability of the CaSR to regulate PTHrP production and milk calcium transport in lactating mice *in vivo* and in 3-D cultures of MECs *in vitro*. 3) To use genetically modified mice to examine how loss of GABBR1 affects the ability of the CaSR to regulate PTHrP production and milk calcium transport in lactating mice *in vivo* and in 3-D cultures of MECs *in vitro*.
Role: PI
Overlap with current proposal: None
Received 6th percentile score. Awaiting funding decision

2 R01 HD076248-06 Wysolmerski (PI) 12/01/2020 – 11/30/2025 2.4 calendar mos
NIH/NICHD $371,562 (direct costs/yr)

PMCA2 regulates mammary gland involution and breast cancer

The major goals of this project are: 1) To examine the mechanisms whereby decreased PMCA2 levels and increased intracellular calcium concentrations activate lysosome biogenesis, STAT3 and LDCD; 2) test whether alterations in PMCA2 localization accelerate malignant transformation in cells overexpressing ErbB2/HER2; and 3) test whether further upregulation of interactions between PMCA2, NHERF1, Ezrin, Erbin, HSP90 and ErbB2 contributes to trastuzumab resistance.
Role: PI
Overlap: None

Team Challenge Grant (Wysolmerski co-PI) 07/01/2020 – 06/30/2020. 0.12 calendar mos
Yale Comprehensive Cancer Center $150,000 (direct costs)

Defining The Effects Of Dietary Fatty Acids On Breast And Pancreatic Cancer

The major goals of this project are: 1)To examine the effects of specific dietary fatty acids on glucose, lipid and energy metabolism in female mice; 2)To examine the effects of specific dietary fatty acids on tumor growth in PDX models of human breast cancer; and 3) To examine the effects of specific dietary fatty acids on tumor progression in transgenic models of pancreatic cancer.
Role: co-PI
Overlap: None

NIH R01 (Rodeheffer, PI) 07/01/2020–06/30/2025 0.3 cal mos
NIH $352,320 (direct costs)

The role of dietary fat in metabolic disease and obesity-associated breast cancer
Goal: To determine precisely how different dietary fats impact the onset and severity of metabolic diseaseand breast cancer in females.
Role: Collaborator
Overlap: None