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14. ABSTRACT

The goals of this project were to test whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers would 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 the 3 years plus one no-cost extension, we made progress on achieving all of the tasks outlined in our original SOW. In addition, we initiated new work on how the CaSR and TGF-beta interacted to regulate PTHrP. However, due to a series of technical issues, personnel changes and the ongoing COVID pandemic, we are still working on this project and will carry it forward using discretionary funds in the PI's laboratory. The full details of our efforts are outlined in the attached report.

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 was 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 previously 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 was 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 was 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 were 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*. The original 3 specific aims were as follows:

Aim 1 - to determine whether genetic knockdown of the CaSR or the GABAB R1 would inhibit PTHrP production, reduce proliferation and increase 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 would 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 could inhibit the growth of osteolytic bone metastases in mouse models.

Progress towards accomplishing Goals

It should be noted that the onset of the COVID epidemic in the last year of this award significantly impaired out ability to finish the originally proposed research. Yale University shut

down all bench research for 3 months at the onset of the pandemic in March 2020 and restrictions on personnel density have persisted, resulting in an ongoing reduction of research output of about 50%. For this reason, all work has slowed significantly and we were never able to complete all the tasks noted in the Statement of Work. Nevertheless, we made significant progress on each Specific Aim and we will report our progress and accomplishments as organized in the Statement of Work.

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

We had difficulty generating stable BT474 and 4T1 cell lines with significant knockdown of GABABR1, suggesting that loss of this protein was somehow toxic to these cells and/or put them at a competitive growth disadvantage. However, we did succeed with creating a stable knockdown cell line for MDA-MB231.1833 cells. We selected a cell line with about 55-60% reductions in GABABR1 expression to grow up and use in subsequent experiments (clone 17, see Fig. 1). However, over time, the knockdown of GABAB R1 appeared to become less efficient, again 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, we decided 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. Dr. Hens in the lab 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. Subsequently, she rederived the GABAB R1-shRNA expressing and the tTa-expressing constructs into lentiviral vectors. The plan was to use these lentiviral vectors to infect cell lines with both halves of the required tet-regulated shRNA system. However, these experiments were sidelined due to COVID-related slowdown in lab work and we have yet to create these cell lines. We still plan to develop and characterize these inducible knockdown cell lines for GABBR1, in order to proceed with the experiments in Aim 1, Task 3. If we are successful, any resulting publications will cite this award.

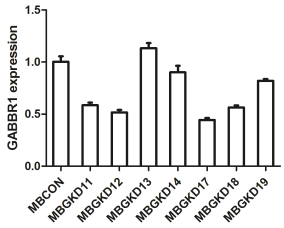

Fig. 1. Separate clones of MDA-MB231 cells with stable knockdown of GABBR1 expression. We chose to use clone 17, which had the greatest knockdown.

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.

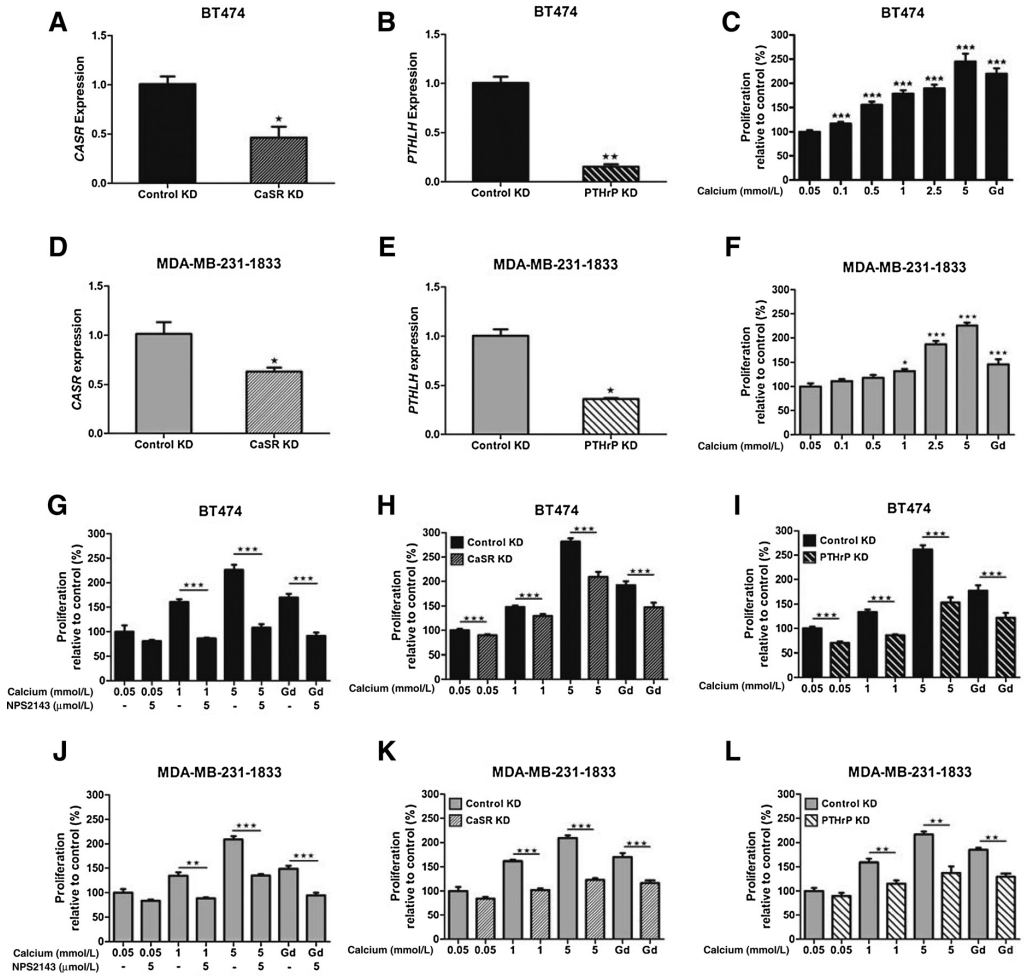

Figure 2

CASR and *PTHLH* mRNA levels in control and CaSRKD or PTHrPKD BT474 (**A** and **B**) or MDA-MB-231.1833 (**D** and **E**) cells. **C** and **F,** BrdUrd incorporation in response to increasing calcium or gadolinium (100 μmol/L) in control BT474 (**C**) and MDA-MB-231.1833 (**F**) cells. **G** and **J,** BrdUrd incorporation in control BT474 (**G**) or MDA-MB-231.1833 (**J**) cells ± treatment with NPS2143. **H** and **K,** BrdUrd incorporation in control vs. CaSRKD BT474 (**H**) or MDA-MB-231.1833 (**K**) cells. **I** and **L,** BrdUrd incorporation in control versus PTHrPKD BT474 (**I**) or MDA-MB-231.1833 (**L**) cells. Bars represent mean ± SEM, $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

We had been working on these experiments while the grant was under review and they were finished and published in *Cancer Research* just before the start of the current grant. We found that knocking down CaSR expression or inhibiting CaSR function with the calcilytic compound, NPS-2146, in breast cancer cells resulted in a reduction in the cAMP and PTHrP responses to high extracellular calcium levels. It also blunted proliferation and increased calcium-induced apoptosis. These changes in cell turnover were accompanied by an increase in p27 levels and an increase in nuclear AIF levels (see Fig. 2 and Kim et al 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 once we have generated Tet-regulated expression of shRNA to knock down 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

As reported previously (Cancer Res 76:5348, 2016), initial experiments by Dr. Kim had suggested that combining MNNG treatment with CaSR-knockdown synergized to augment cell death. However, when we tried to expand on these experiments, 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.

Milestone to determine whether genetic inhibition of CaSR/GABAB R1 heterodimers sensitizes breast cancer cells to MNNG or radiation was initiated but only partially achieved. We plan to attempt these experiments using discretionary funds available to the PI. If these experiments result in a publication, then the work will be attributed to this grant.

Aim 2, Task 2: Examine whether treatment with NPS2143 sensitizes breast cancer cells to MNNG or radiation.

As above, the difficulties with MNNG complicated these experiments and we have not done any experiments with radiation of the cells yet. We decided to wait until we had the genetic knockdown cells so that we could do pharmacologic and genetic KD in parallel.

Milestone to determine whether pharmacologic inhibition of CaSR/GABAB R1 heterodimers sensitizes breast cancer cells to MNNG or radiation was not initiated. We plan to attempt these experiments using discretionary funds available to the PI. If these experiments result in a publication, then the work will be attributed to this grant.

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. We are waiting to do the same with GABAB R1 knockdown cells once we have crated the Dox-inducible cells described above.

As expected, control MB231.1833 cells formed osteolytic lesions and knocking down PTHrP production reduced the total osteolytic lesion area (Fig 3). 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 3). 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*. In order to do this, we will need to repeat these experiments. However, this was complicated by the onset of the COVID pandemic as well as the fact that Dr. Guise recently moved to Baylor College of Medicine in Houston and is still setting up her new laboratory there.

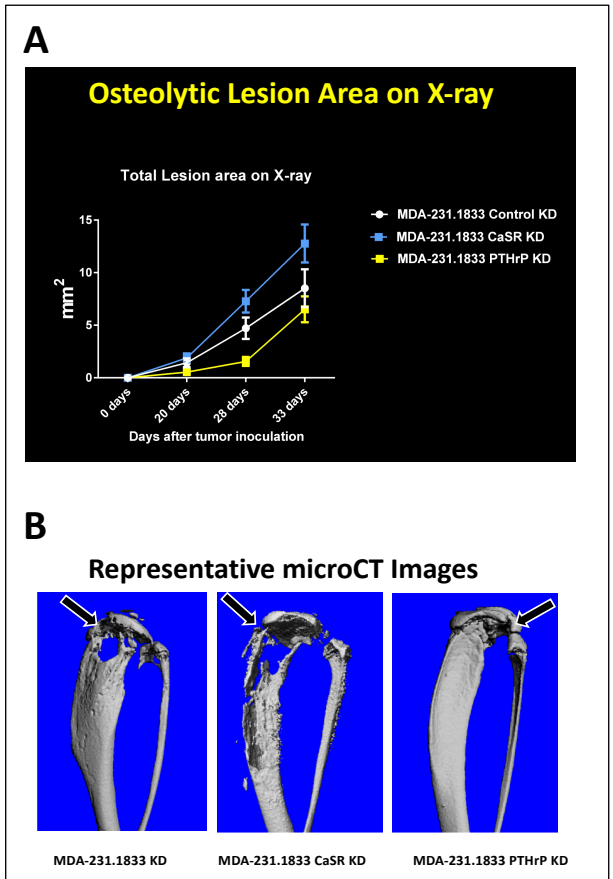

Figure 3. A) Total Osteolytic lesion by X-Ray analysis of mice injected with control MDA-231.1833 cells (white), CaSR KD MDA-231.1833 cells (blue), or PTHrP-KD MDA-231.1833 cells (yellow). B) Typical osteolytic lesions from sample mice described in A as assessed by micro-CT analysis.

If PTHrP levels are not appropriately reduced, then one possibility is that loss of the CaSR is still not enough to reduce PTHrP in the metastatic environment. Dr. Guise's laboratory has previously shown that TGFβ from the bone matrix can also upregulate PTHrP production, Therefore, we also began examining how the CaSR and TGFβ interact in regulating PTHrP expression in metastatic lesions. As expected from prior publications from our laboratory and from the Guise laboratory, treatment of MDA-MB231 cells with either high calcium or 5 or 10 ng/ml of TGFβ alone increased PTHrP mRNA expression. However, the effects of treating with the cells both high calcium and TGFβ were negligible as compared to TGFβ alone. However, in contrast, pretreatment of these cells with TGFβ clearly augmented PTHrP secretion into conditioned media of the cells (Fig. 4A). This suggests that any synergy between these pathways occurs at a translational level as these cell do not typically store peptides in secretory granules. This is an interesting result that may have clinical significance to the pathophysiology of bone metastases. We plan to follow up and investigate how these signaling pathways interact to increase PTHrP protein production.

We are also examining the interactions of CaSR and TGFβ signaling with respect to proliferation and apoptosis in MDA-MB231 cells. As shown in Fig. 4B, as we had previously reported (Kim et al Cancer Res 76:5348, 2016), increased extracellular calcium stimulated proliferation of these cells, but the addition of TGFβ did not augment the effects of calcium. We are now in the process of examining how interactions between CaSR and TGFβ signaling may affect apoptosis in these cells as well. These experiments are ongoing.

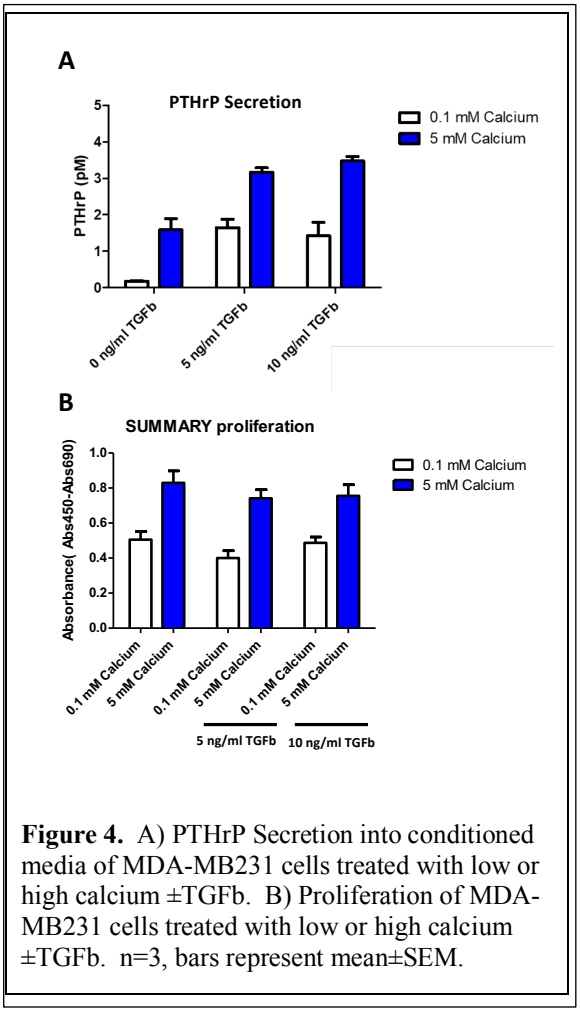

Figure 4. A) PTHrP Secretion into conditioned media of MDA-MB231 cells treated with low or high calcium ±TGFb. B) Proliferation of MDA-MB231 cells treated with low or high calcium ±TGFb. n=3, bars represent mean±SEM.

In addition to completing the experiments examining interactions between calcium and TGFβ, we also plan to repeat the bone metastasis 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. It will also allow us to examine how removal of CaSR signaling may interact with TGFβ inhibitors in bone metastases in vivo.

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. In addition, new studies examining how CaSR signaling interacts with TGFβ have been initiated.

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.

Nothing to Report

4. Impact

Impact on the principal discipline.

Our initial Cancer Research paper has stimulated other work in the field of CaSR and bone metastases from breast cancers. These include the following papers:

Das et al. *Front Oncol* Feb, 2020 https://doi.org/10.3389/fonc.2020.0069
Boudot et al. Oncotarget 8:56460, 2017 doi:10.18632/oncotarget.16999

Once we generate further publications, this project will impact the wider breast cancer research community.

Impact on other disciplines.

Nothing to report

Impact on Technology Transfer.

Nothing to report

Impact on Society.

Nothing to report

5. Changes/Problems

Nothing to report.

6. Products

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7. Participants and Other Collaborating Organizations.

Individual that have worked on this project

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

Name: Julie Hens, PhD
Project Role: Associate Research Scientist
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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.

Name: Wonnam Kim, PhD
Project Role: Post-doctoral Associate
Research Identifier:
Nearest person month worked:
Contribution to Project: Performed experiments described in this report.