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14. ABSTRACT

Breast cancer is a devastating disease affecting both women and men, and despite many advancements it remains incurable when metastatic. Efficient novel treatments, or enhancements to current ones, are desperately needed to improve breast cancer therapy and extend patient lives. The overall goal of this proposal is to develop tumor-specific, safe and effective therapy for breast cancer. We will concentrate on HER2-overexpressing tumors, which are diagnosed in one in four patients. Despite enormous progress in developing targeted therapies, such as the anti-HER2 antibodies, cancer cells eventually acquire resistance which clinically is manifested by tumor growth/recurrence in spite of targeted therapy. Currently HER2 targeted therapies achieve highest response rates when combined with chemotherapy; but chemotherapy causes in undesirable side effects due to off-target effects on normal tissue which diminishes quality of life for the patient. A way to address this problem is to use a drug that either attacks only tumor cells or enhances the response of tumors (but not normal tissues) to current therapies. Such an approach enables the use of smaller doses to treat the tumor, resulting in fewer side effects. Our research shows that so called low voltage activated (LVA) calcium channels are aberrantly expressed in breast cancer cells, most notably in HER2-positive tumors. We propose to investigate how expression of LVA calcium channels may promote cancer cell growth and progression, and confer resistance to therapy. We will further investigate whether LVA channel inhibitors, mibefradil and similar drugs, can be employed to treat an advanced HER2-positive breast cancer. Our proposed research is expected to reveal an important mechanism(s) that support the progression of HER2+ breast cancer. The successful outcome of this project will advance the rational approach to develop a new
generation of anticancer agents for the treatment of patients suffering from advanced breast tumors. Since specific LVA calcium channel inhibitors are already available, relatively safe, and FDA-approved for hypertension and related conditions, we believe that our studies could progress quickly into clinical trials. Importantly, mibefradil a LVA channels inhibitor which will be used extensively in our studies, is already undergoing clinical trials in brain tumor patients, and is considered for treatment of other types of cancer. Successful accomplishment of the goals of this three year study, together with positive outcomes from the clinical trials mentioned above, could quickly (3 to 5 year time frame) lead to the breast cancer-specific clinical trials, and to the development of a novel, safe and efficient therapy.

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INTRODUCTION

Breast cancer is a devastating disease affecting both women and men, and despite many advancements it remains incurable when metastatic. Efficient novel treatments, or enhancements to current ones, are desperately needed to improve breast cancer therapy and extend patient lives. The overall goal of this proposal is to develop tumor-specific, safe and effective therapy for breast cancer. We will concentrate on HER2-overexpressing tumors, which are diagnosed in one in four patients. Despite enormous progress in developing targeted therapies, such as the anti-HER2 antibodies, cancer cells eventually acquire resistance which clinically is manifested by tumor growth/recurrence in spite of targeted therapy. Currently HER2 targeted therapies achieve highest response rates when combined with chemotherapy; but chemotherapy causes in undesirable side effects due to off-target effects on normal tissue which diminishes quality of life for the patient. A way to address this problem is to use a drug that either attacks only tumor cells or enhances the response of tumors (but not normal tissues) to current therapies. Such an approach enables the use of smaller doses to treat the tumor, resulting in fewer side effects. Our research shows that so called low voltage activated (LVA) calcium channels are aberrantly expressed in breast cancer cells, most notably in HER2-positive tumors. We propose to investigate how expression of LVA calcium channels may promote cancer cell growth and progression, and confer resistance to therapy. We will further investigate whether LVA channel inhibitors, mibefradil and similar drugs, can be employed to treat an advanced HER2-positive breast cancer. Our proposed research is expected to reveal an important mechanism(s) that support the progression of HER2+ breast cancer. The successful outcome of this project will advance the rational approach to develop a new generation of anticancer agents for the treatment of patients suffering from advanced breast tumors. Since specific LVA calcium channel inhibitors are already available, relatively safe, and FDA-approved for hypertension and related conditions, we believe that our studies could progress quickly into clinical trials. Importantly, mibefradil a LVA channels inhibitor which will be used extensively in our studies, is already undergoing clinical trials in brain tumor patients, and is considered for treatment of other types of cancer. Successful accomplishment of the goals of this three year study, together with positive outcomes from the clinical trials mentioned above, could quickly (3 to 5 year time frame) lead to the breast cancer-specific clinical trials, and to the development of a novel, safe and efficient therapy.

KEYWORDS

T type Ca2+ channels, breast cancer, p53, TP63, differentiation, calcium

ACCOMPLISHMENTS

Major Goals

Specific Aim 1: Determine the role of LVA channels in HER2+ breast cancer cells survival and resistance to therapy *in vitro*.	**Timeline**	**Site 1**
Major Task 1: Characterize expression of LVA channels in selected BC cell lines differing in HER2 expression and sensitivity to anti-HER2 therapy.	Months	
Subtask 1.1: RT-qPCR and WB analysis of LVA expression in HER2+ BC cell lines.	1-3	Dr. Dziegielewski
Subtask 1.2: Using si/shRNA and DNA plasmid constructs characterize the effects of down- and up-regulation of LVA channels on BC cells.	3-9	Dr. Dziegielewski
Milestone(s): Define the relationship between LVA expression and HER2+ breast cancer cells proliferation and viability.	9	
Major Task 2: Test the clinically available LVA channels inhibitor mibefradil on BC cells in combination with anti-HER2 treatments and/or chemotherapy.		
Subtask 2.1: Test the effects of mibefradil alone on HER2+ BC cells proliferation, cell cycle and viability.	10-12	Dr. Dziegielewski
Subtask 2.2: Test the effects of mibefradil in combination with anti-HER2 treatments and/or chemotherapy.	12-16	Dr. Dziegielewski
Subtask 2.3: Evaluate additional LVA inhibitors.	12-18	Dr. Dziegielewski
Milestone(s): Establish LVA inhibitor mibefradil as therapeutic and sensitizing agent in HER2+ BC cells in vitro.	18	
Specific Aim 2: Characterize the molecular pathway(s) linking the LVA channels with HER2-activated signaling network.	**Timeline**	**Site 1**
Major Task 3: Elucidate the molecular mechanism(s) of action of LVA channels inhibitors.		
Subtask 3.1: Characterize signaling events	6-24	Dr. Dziegielewski

leading from LVA channels inhibition to down regulation of PI3K/AKT/mTOR activit in HER2+ BC.		
Subtask 3.2: Characterize signaling events involved in cell death caused by LVA channels inhibition combined with therapy targeted against HER2.	12-24	Dr. Dziegielewski
Milestone(s): Define the molecular mechanism(s) involved in cancer cells susceptibility to LVA inhibition. Publication of 1-2 peer-reviewed manuscripts.	24	
Specific Aim 3: Characterize the role of LVA channels in HER2+ breast cancers *in vivo*, and validate the use of channels inhibitors to enhance current standard therapies for HER2+.	**Timeline**	**Site 1**
Major Task 4: Evaluate the expression of LVA channels in samples from HER2+ breast cancer patients		
Subtask 4.1: Assess anonymized archival samples of breast cancer and normal breast tissue for LVA expression.	12-36	Dr. Dillon
Milestone(s): Define the relationship between LVA expression and aggressiveness of HER2+ breast cancer, and the outcomes of therapy in BC patients.	36	
Major Task 5: Characterize the role of LVA channels in animal models of HER2+ BC.		
Subtask 5.1: Determine the role of LVA channels in development and progression of HER2+ tumors in PyMT mice model in vivo.	1-24	Dr. Bouton
Subtask 5.2: Evaluate the *in vivo* efficacy of mibefradil in combination with standard treatments in human HER2+ cancer xenografts.	24-36	Dr. Dziegielewski
Milestone(s): Validate LVA channels as novel molecular targets for HER2+ BC therapy. Publication of 1-2 peer-reviewed manuscripts. Initiate clinical trial in collaboration with Cavion LLC.	36	

Accomplishments

Please see Appendices page for manuscript describing accomplishments.

Training and Professional Development Opportunities:

Nothing to Report

How were the results disseminated to communities of interest:

Nothing to Report

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

Nothing to Report

IMPACT

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

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

CHANGES/PROBLEMS

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

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PRODUCTS

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- **Journal publications:** Nothing to Report
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- **Other publications, conference papers, and presentations.**
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- **Inventions, patent applications, and/or licenses:** Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Jaroslaw Dziegielewski – no change

Barbara Dziegielewski – no change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

SPECIAL REPORTING REQUIREMENTS

Nothing to Report

APPENDICES

T-type calcium channels support murine PyMT breast cancer progression by inhibition of p53/TP63 expression and suppression of differentiation

Barbara Dziegielewska[1], Kristen A. Atkins[2], William T. Warnock[1], Patrick Dillon[3], Amy H. Bouton[4], David L. Brautigan[4,5,] and Jaroslaw Dziegielewski[1,5,#]

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Running title: Role of T type Ca^{2+} channels in murine luminal breast cancer

Key words: T type Ca^{2+} channels, breast cancer, p53, TP63, differentiation, calcium

ABSTRACT

T-type calcium channels (TTCC) are aberrantly expressed in several epithelial cancers, including breast, ovarian and prostate, and play positive reinforcing role in cancer proliferation. To examine TTCC role in breast carcinoma we used a mouse model of breast cancer derived from mouse mammary tumor virus promoter driven polyoma middle T antigen (MMTV-PyMT). Using short hairpin RNA targeting CACNA1G gene, and a pharmacological inhibitor of TTCC, mibefradil, we provide the evidence for TTCC contribution to breast cancer progression and chemo-resistance. Downregulation of CACNA1G with shRNA in mouse breast cancer cells, Py230, increased expression of the markers of cell cycle arrest and tumor suppressors, p19Arf and p53. Furthermore, downregulation of CACNA1G gene expression reduced expression of PyMT transgene and HER2 protein, while increasing expression of basal cell markers, TP63, cytokeratin 14 and EpCAM, suggesting trans-differentiation into basal-like phenotype. Importantly, knock-down of CACNA1G or chemical inhibition with mibefradil, increased cells susceptibility to anticancer therapies used in breast tumor patients, including lapatinib, tamoxifen and camptothecin. Finally, treatment with TTCC antagonist mibefradil significantly lowered breast tumor stage in treated animals, decreasing percentage of invasive carcinomas as compared to hyperplasias and ductal *in situ* carcinomas. Our data present the evidence for TTCC contribution to breast cancer progression and chemo-resistance.

INTRODUCTION

Breast tumor is still one of the most common cancer and the leading cause of cancer-related death in women (Siegel, Miller, and Jemal 2019; DeSantis et al. 2019). Despite marked progress in treatment, especially recent development of targeted therapies, this disease remains difficult to cure due to its high heterogeneity (both inter- and intra-tumoral) (Zardavas et al. 2015; Roulot et al. 2016; Joseph et al. 2018). A plethora of common cellular processes is dependent on calcium signaling, such as differentiation, proliferation, motility and apoptosis in cancer, including breast cancer (So et al. 2018), therefore targeting such universal signaling mechanisms could provide effective therapy in case of heterogeneous tumors.

Recently, T-type calcium channels (TTCC) gained attention in cancer research as being targetable with drugs (Sallán et al. 2018), such as a re-purposed anti-hypertension agent, mibefradil (Mib) (Keir et al. 2013; Valerie et al. 2013; Dziegielewska et al. 2016). TTCC are expressed during embryonic development (Cribbs et al. 2001; Bernhardt et al. 2015), but also in cancer (Dziegielewska, Gray, and Dziegielewski 2014). Two of the three subunits of TTCC, CACNA1G (Cav3.1) and CACNA1H (Cav3.2), are expressed in breast tissues and breast cancer cells (Taylor et al. 2008; Pera et al. 2016). Increased CACNA1H gene expression is associated with worse survival prognosis of estrogen receptors positive (ER+) breast cancer patients, and was reported to be increased in trastuzumab-resistant breast cancer SKBR3 cells (HER2+) (Pera et al. 2016).

To investigate TTCC role in breast cancer we used mouse mammary tumor virus long terminal repeats-driven polyoma middle T antigen (MMTV-PyMT/C57Bl/6) model of breast cancer and PyMT/C57Bl/6 derived cell line, Py230 (Bao et al. 2015). Starting from week 5-7

of mouse age, MMTV-PyMT/C57Bl/6 female mice, develop early stages of breast cancer, such as multifocal hyperplasia, followed by Mammary Intraepithelial Neoplasia (MIN), Ductal In Situ Carcinoma (DISC), that progresses into adenoma and invasive breast cancer in week 10-12 of age. Finally, in weeks 12-21 of mouse age, the disease advances into invasive metastatic carcinoma and breast cancer outgrowth in lung (Guy, Cardiff, and Muller 1992; Lin et al. 2003).

Breast cancer is a heterogeneous disease comprising sub-population of cancer stem cells proficient in proliferation and differentiation. Stem cells plasticity of mouse luminal breast cancer cells Py230 was revealed in differentiation assays using specific culture conditions, such as challenging Py230 with retinoic acid, insulin and rosiglitazone to differentiate cells to adipocytes with increased expression of adipocyte specific genes, such as peroxisome proliferator-activated receptor gamma and delta (PPARγ/PPARδ) and fatty acid binding protein 4 (Fabp4). Upon treatment with lactogenic hormones, dexamethasone/prolactin, Py230 cells differentiated into domes with alveolar cells characteristic in increased expression of β-casein (Bao et al. 2015). Depending on the stressor in *in vivo* environment in ovariectomized mice, luminal ER+ Py230 breast cancer cells, could form basal, basal HER2+ or claudin-low type of tumor (Bao et al. 2015).

Proper epithelial differentiation is dependent upon expression of tumor protein 63 (TP63). TP63 is a marker for myoepithelial, basal and supra-basal epithelial stem cells and a member of p53 family (Senoo et al. 2007; Moll and Slade 2004)(Senoo et al. 2007; Moll and Slade 2004). Similarly to p53, TP63, drives expression of unique target genes, such as Wnt4 (wingless-like MMTV integration site 4), a secretory glycoprotein (Osada et al. 2006), and Cdh3 (P-cadherin), typically expressed in myoepithelial cells (G. L. Radice et al. 1997) and breast stem cells (Glenn L. Radice et al. 2003). When Wnt4 is expressed in human basal and/

or triple negative breast cancer, it stimulates tumor growth and aggressive phenotype (Schlange et al. 2007; Koval and Katanaev 2018).

Our results show that shRNA-mediated down-regulation or drug-induced inhibition of T-type Ca^{2+} channels in luminal PyMT breast cancer induces activation of tumor suppressor (p19Arf /p53), increases expression of basal cell markers (TP63/CK14/EpCAM), reduces PyMT oncogene-dependent signaling, and decreases activation of HER2/AKT pathway. As a result, such treatments increase breast cancer cells sensitivity to chemotherapy *in vitro*, and reduce tumor progression *in vivo*.

MATERIALS AND METHODS

Cell Lines, viral transfection, drugs and reagents

Mouse breast tumor derived Py230 breast cancer cell line was kindly provided by Dr. Lesley Ellies (University of California San Diego) and described previously (Bao et al. 2015). Py230 cells were cultured in F12K media (Invitrogen) supplemented with 5% fetal bovine serum (Fetal Clone II, Fisher Scientific), mouse epidermal growth factor, (1:1000, MITO, Corning), 50 μg/ml gentamycin (Life Technologies) and 2.5 μg/ml amphotericin B (Life Technologies) in humidified incubator supplemented with 5% CO_2. Lentivirus vector carrying short hairpin RNA (shRNA), with non-targeting (shNT, RHS4743, Dharmacon) or targeting CACNA1G gene (shA1G-6, CCAGTTGACACAGGTGGT, V3THS_391358; shA1G-4, TTAGCCTCCAGAAGGCCAG, V2THS_20825) sequence were purchased from Dharmacon. Virus packaging plasmids pMD2.G and psPAX2 were gift from Didier Trono (Addgene, cat. #12259 and #12260, respectively). Virus was transduced into mouse breast cancer Py230 cells in presence of 8 μg/ml polybrene (Sigma) as described (Wang, Brugge, and Janes 2011). Mibefradil was kindly provided by Cavion LLC; adriamycin and camptothecin were a generous gifts from Dr. Terry A. Beerman (Roswell Park Cancer Institute, Buffalo, NY); lapatinib and tamoxifen were from Selleckchem.

Cells viability

Py230 cells were seeded in 96 well plates at 4,000 cells/well 24h prior drug treatment. Next day, cells were treated in triplicates with increasing drug concentrations lapatinib (0.3-30 μM), tamoxifen (0.3-30 μM), camptothecin (0.03-3 μM) and adriamycin (0.1-10 μM), alone or in combination with mibefradil (10 μM). After continuous 4 days drug treatment AlamarBlue

(Invitrogen) was added to the media to asses cells viability. Fluorescence was measured at 540 nm / 620 nm (excitation/emission) and cells viability calculated based on fluorescence normalized to control (not-treated) cells signal. Cell growth inhibition was based on the four parameters semi-log curve fit using GraphPad 8.0 and IC_{50} values derived from fitted curve.

Protein expression by FACS analysis

Cells were fixed and stained with primary antibodies or isotype controls for 20 min at dark on ice: rat IGg2a kappa anti-MoCD326 (EpCAM)-APC (Invitrogen), anti-HER2 (MA5-13675, Thermo Fisher Scientific), anti-CACNA1G (6100, GeneTech), anti-cytokeratin 14 (MA5-11599, Thermo Fisher Scientific), anti-CD49f-BV421 (BioLegend) and anti-Wnt4 (AA240-290, Antibodies-OnLine). If using not-conjugated primary antibodies, cells were washed and stained with the following secondary antibodies on ice at dark for 20 min: goat anti-mouse AlexaFluor 568 (Invitrogen), donkey anti-rabbit PE (BioLegend), goat anti-mouse brilliant violet BV421 (BioLegend). Rat IgG2a kappa-APC (Invitrogen) and -BV421 (BioLegend) antibodies were isotype controls. Expression of proteins of interest in cells was detected using BDCalibur, with CellQuest software for data acquisition and FCS Express 6.0 for data analysis.

***In vivo* experiment, tissues processing and immunohistochemistry**

All procedures involving mice were conducted in accordance with University of Virginia (UVA) Institutional Animal Care and Use Committee (IACUC) regulations. Male PyMT mice CL57Bl/6:MMTV-PyMT background were bred with CL57Bl/6 females (wild type WT, Jackson lab) to obtain female mice heterozygous for the MMTV-PyMT transgene. At weaning time, female mice were genotyped using PCR (specific primers are listed in **Supplementary Table**

1). To monitor disease progression, PyMT females were sacrificed at appropriate age: 7, 9, 11, 13, 15, 17 or 19 weeks, and mammary glands number 3, 4 and 5 were excised, and used for epithelial cells isolation. As a control, WT females corresponding in age were sacrificed and mammary glands were harvested as described above.

Animals used in mibefradil (Mib)-treatment experiment were virgin females heterozygous in PyMT transgene. Starting with 5-6 week old PyMT females were randomly assigned into either control or mibefradil-containing diet (14 animals per treatment) with mibefradil concentration at 1.143 g/kg to deliver 160 mg/kg mibefradil per day (Dziegielewska et al. 2016). Mice were monitored weekly for changes in body mass and for tumor appearance, and were sacrificed when tumor size or the sum of all tumors reached and exceeded 1500 mm^3. Otherwise, animals were sacrificed at the 21 week of age and the mammary glands/tumors were removed.

Mammary gland number 1 was excised from sacrificed animals and fixed in 10% zinc-formalin (Sigma) at room temperature for 24h. Next day tissues were transferred to 70% ethanol, paraffin embedded and sectioned (5 μm) on slides. Mammary glands/tumor tissues were stained with hematoxylin and eosin (H&E).

Mammary glands number 5, 4 and 3 were excised from either wild type or tumors from MMTV-PyMT animals. Tissues were minced and digested in collagenase solution (DMEM/F12 (Life Technologies), 5% fetal bovine serum (FBS, Clontech), 2 mg/ml Collagenase IV (Sigma), 2% trypsin (Sigma), 5 μg/ml insulin (Sigma), 50 μg/ml gentamycin (Life Technologies) as described elsewhere (Nguyen-Ngoc et al. 2015). After 45 min incubation at 37^{o}C, epithelial mammary cells were collected by centrifugation, treated with 40-30 U of DNase1 (Worthington Bio) and separated from larger pieces using centrifugal elutriation. A 100 μm nylon strainer (Corning) was used to remove undigested parts of tumors at the last stage of

centrifugal elutriation. Cells were collected, washed with PBS, pellets stored in -80°C and used for either protein analysis or total RNA extraction and gene expression analysis.

Total RNA Isolation and gene expression analysis by RT-qPCR

Total RNA was isolated from epithelial cells using Trizol extraction. Total RNA was subjected to DNase 1 treatment (DNAfree DNA Removal Kit, Thermo Scientific), recovered by precipitation and RNA quality assessed by BioAnalyzer (Agilent). RNA with RNA Integrity Number (RIN) >7 was used for subsequent gene expression analysis. DNAse 1 treated RNA (2 µg/40 µl) was used in reverse transcriptase reaction (iScript, Bio-Rad) and subsequently 50 ng of cDNA was used for quantitative PCR (CFX96, Bio-Rad), in a total 10 µl reaction containing 1x Universal iTaq SYBR-Green Master Mix (Bio-Rad) and specific primers (0.4 µM/ reaction) as listed in **Supplementary Table 2** (Bao et al. 2015). Amplification was as follows: initial denaturation 95 °C 30 sec, followed by 40 cycles of 95 °C 5 sec and 60 °C for 30 sec. Gene expression was normalized to the average expression of peroxiredoxin 1 (Prdx1) and TATA-binding protein (TBP), two stable genes selected according to recommendations (van de Moosdijk and van Amerongen 2016).

Protein extraction and Western blot

Cell pellets were lysed in modified RIPA lysis buffer supplemented with proteases and phosphatases inhibitors (Dziegielewska et al. 2016). Equal amounts of proteins were resolved on gradient SDS-PAGE gels (Bio-Rad), transferred to nitrocellulose and probed with specific antibodies: mouse monoclonal anti-P-Thr308 AKT, mouse monoclonal anti-P-Ser473 AKT, rabbit monoclonal anti-AKT (#5106, #4051, #4691, Cell Signaling Tech), mouse monoclonal HER2 (MA5-13675, Thermo Fisher Scientific), mouse monoclonal anti-cytokeratin 14 (MA5-

11599, Thermo Fisher Scientific), mouse monoclonal alpha smooth muscle actin (α-SMA, 14-9760-82, eBioscience), mouse monoclonal anti-p53 (#2524, Cell Signaling Tech), rat monoclonal antibody anti-Arf/p19 (MA1-16664, Thermo Fisher Scientific), mouse monoclonal anti-GAPDH (sc-32233, Santa Cruz Biotech). Fluorescent labeled with either IR-680 or IR-800, secondary antibodies were from Li-Cor (Oddyssey, Li-Cor Biotechnology). Membranes were scanned using Oddyssey CLx Imager and signal from specific protein band quantified.

Data graphing and statistical analysis

GraphPad v. 8.0 was used for all data plotting except for flow cytometry data analyzed by FCS Express 6.0. Unpaired student t-test was used for data comparison and to derive statistical significance. Where applicable, one-way ANOVA was used for multiple comparison followed by either Tukey or Dunnett *post hoc* test.

RESULTS

T-type Ca^{2+} channels are expressed in normal mouse mammary epithelium, breast tumors in PyMT, and in PyMT tumor-derived cell line.

To determine TTCC genes expression in mouse mammary epithelium and cancer cells, total RNA was isolated from mammary epithelial cells of either MMTV-PyMT (PyMT) or C57BL/6 wild type (WT) female mice at 13-15 week of age, or from Py230 cancer cell line, and RT-qPCR performed, as described in Materials and Methods. Expression of CACNA1G and CANA1H genes was detected in all samples, WT and PyMT mouse mammary epithelium, and Py230 breast cancer cells (**Fig 1A**), while CACNA1I subunit was mostly undetectable. In addition, TTCC expression was quantified in different mouse tissues, revealing similar pattern with higher expression of CACNA1G/A1H, except for the brain and cerebellum tissues with relatively high expression of CACNA1I (**Supplementary Fig 1**). Because in mouse mammary tumors expression of CACNA1G was higher than CACNA1H, this gene was selected for downregulation experiments.

To elucidate the role of TTCC in breast cancer cells the CACNA1G gene expression was down regulated by transfection with lentiviral vectors expressing short hairpin RNA (shRNA) targeting CACNA1G gene. Two shRNA sequences targeting different parts of the gene were used (shA1G-4 and shA1G-6, Materials and Methods), while scrambled, non-targeting sequence was used as control (shNT). Stable transfectant were selected with puromycin, establishing PyshNT, PyshA1G-4 and PyshA1G-6 cell lines, and tested for downregulation of CACNA1G gene expression using RT-qPCR and immunostaining followed by fluorescence activated cell sorting (FACS) analysis. As shown in **Fig 1B**, shRNA decreased expression of CACNA1G gene at mRNA level by 60% and 80% for shA1G-4 and

shA1G-6, respectively. Downregulation of CACNA1G gene expression at protein level was confirmed with specific antibody staining and FACS (**Fig 1C**).

CACNA1G gene knock-down increases expression of tumor suppressors Arf/p53 and basal markers TP63, Cdh-3 and Wnt4.

Suppression of CACNA1G gene with small interfering RNA in colon cancer cells was previously associated with strong p53-dependent cell cycle arrest and apoptotic response (Dziegielewska et al. 2014), therefore the expression of p53 and related genes was tested in Py230 cells expressing shRNA. Upon CACNA1G gene downregulation, PyshA1G-6 expressed high level of tumor suppressors p53 and p19Arf (**Fig 2A**). Interestingly, expression of p53-dependent genes regulating cell death and cell cycle, such as NOXA, BBC3 and p21, was not significantly different although slightly elevated in PyshA1G-4 and PyshA1G-6, as compared to PyshNT control cells (**Fig 2B**). Consequently, decrease in CACNA1G gene expression in Py230 cells despite increased p53 expression/stabilization, did not induce cell cycle arrest or p53 dependent apoptosis.

TP63, a p53 family member and p53 transcriptional target, was also significantly elevated in knock-down cells as compared to PyshNT cells, at both protein (**Fig 2A**) and mRNA levels (**Fig 2C**). The overexpression of both TP63 full length and ΔNTP63 in CACNA1G knock-down cells was confirmed using specific primers (**Supplementary Fig 2**). TP63 upregulation resulted in a significantly higher expression of P-cadherin (Cdh3) and Wnt4 genes (**Fig 2C**), genes typical for basal progenitors, myoepithelial cells, or basal cancer cells (Osada et al. 2006; van Amerongen, Bowman, and Nusse 2012). Stem like phenotype of PyshA1G-4 and PyshA1G-6 cells was not confirmed with additional gene expression profiling, such as SH2-containing domain 5'-inositol phosphatase (S-SHIP), a gene characteristic for

mammary stem cells (Bai and Rohrschneider 2010). Thus, knock-down of CACNA1G elevated p53, p19Arf and TP63 genes/proteins expression, and TP63-dependent P-cadherin and Wnt4 gene expression, suggesting trans-differentiation into basal-like state.

Suppression of CACNA1G in Py230 cells reduces HER2 and induces EpCAM and cytokeratin 14 expression.

To further characterize cellular phenotype caused by decreased CACNA1G gene expression, PyshA1G-6 and control PyshNT cells were stained for presence of luminal or basal epithelium markers, such as epithelial adhesion molecule EpCAM, human epidermal growth factor receptor 2 (HER-2), cytokeratin 14 (CK14), and stem cells markers integrin 6α (CD49f) and Wnt4 (Ma et al. 2012; Zeng and Nusse 2010).

Downregulation of CACNA1G was accompanied by reduction of HER2 expression (**Fig 3A**, additionally confirmed by Western blotting, **Fig 4C**). Moreover, TTCC channels knock-down induced luminal to basal-like transition with increased level of CK14 and EpCAM (with CK14 gain from ~18% to ~30%, and EpCAM increase by ~20%, **Fig 3B**). However distinct basal phenotype of PyshA1G-4 and PyshA1G-6 cells characterized by $CK14^{high}$/$HER2^{low}$/ $EpCAM^{high}$ was α-smooth muscle actin (αSMA) negative (**Supplementary Fig 3**), suggesting that basal phenotype was not differentiated into myoepithelial type of basal cells. While stem cells marker such as Wnt4 increased from 11% in PyshNT to ~28% in PyshA1G-6 (**Fig 3D**), a slight decrease in integrin CD49f, by ~10% was observed in PyshA1G-6 cells as compared to PyshNT cells (**Fig 3C**). These changes could indicate loss of stem cells population, consistent with differentiation. In conclusion, CACNA1G gene knock-down induced trans-differentiation of breast cancer Py230 cells from luminal to basal-like phenotype with loss of HER2 and CD49f, and gain of basal markers CK14, EpCAM and Wnt4.

Downregulation of CACNA1G decreases expression of PyMT and HER2, and reduces activation of their down-stream targets

To explore consequences of CACNA1G gene knock-down at the level of intracellular signaling, cells were probed for expression of PyMT oncogene and HER2 growth factor receptor, and for activation of AKT. Reduction of CACNA1G gene expression lowered PyMT mRNA and protein levels (**Fig 4A/B**), and reduced HER2 protein expression level (**Fig 4C**, HER2 panel). Furthermore, CACNA1G knock-down cells showed diminished activation of AKT measured as phosphorylation of Ser473 and Thr308 (**Fig 4C**), either under standard cell culture conditions, or following serum starvation and stimulation with mouse epidermal growth factor (mEGF). Upon mEGF stimulation, decrease in total HER2 protein level and in AKT phosphorylation was apparent in PyshA1G-6 as compared to PyshNT cells, indicating an inhibition of pro-survival signaling. Thus, downregulation of CACNA1G resulted in reduced expression of PyMT and HER2, and consequently in decreased phosphorylation/activation of AKT.

Downregulation or inhibition of TTCC reduces chemoresistance in mouse breast cancer cells

Trans-differentiation could offer growth advantage in driving aggressive tumor phenotype, invasion, metastasis and chemoresistance. To characterize consequences of TTCC gene downregulation on Py230 breast cancer, PyshA1G-4 and PyshA1G-6 cells were challenged with lapatinib (LAP), a bi-specific small tyrosine kinase inhibitor of EGFR and HER2, and an estrogen receptor antagonist 4-hydroxy-tamoxifen (TAM), often used as anti-estrogen therapy. In addition, standard DNA targeting anticancer chemotherapeutics,

camptothecin (CPT) and adriamycin (ADA) were tested. These treatments were selected because Py230 cells are derived from luminal A PyMT/Bl6 breast cancer expressing ER, and PyMT tumors express HER2 during disease progression (Lin et al. 2003). LAP and TAM are considered targeted therapies, whereas both CPT and ADA kill proliferating cancer cells by poisoning topoisomerase I or topoisomerase II, respectively.

PyshA1G-4 or PyshA1G-6 (downregulated CACNA1G gene), and parental Py230 cells were exposed to the drugs for 4 days, while Py230 parental cells were treated with mibefradil, a relatively specific antagonist of TTCC, concomitantly with chemotherapeutics. TTCC inhibition with mibefradil decreased IC_{50} of lapatinib in Py230 cells from 2.7 ±0.49 to 0.8 ±0.10 μM (**Fig 5A**). Sensitization to lapatinib was also observed in CACNA1G knock-down cells, with IC_{50} reduction from 2.8 ±0.50 μM in control PyshNT cells to 1.2 ±0.5 μM in PyshA1G cells (**Fig 5B**). Treatment of Py230 with mibefradil also decreased cells sensitivity to anti-estrogen therapy, tamoxifen, with IC_{50} shift from 3.1 ±0.60 to 1.7 ±0.50 μM (**Fig 5A**). Consistently, CACNA1G gene knockdown sensitized cells to tamoxifen, with IC_{50} reduction from 11 ±3.4 μM in control PyshNT cells to 4.8 ±0.60 μM in PyshA1G cells (**Fig 5B**).

In addition, chemical inhibition of TTCC increased cells sensitivity to CPT and ADA, with IC_{50} shift from 0.3 ±0.11 to 0.1 ±0.01 μM for CPT, and from 2.4 ±0.50 to 1.5 ±0.57 μM for ADA (**Fig 5A**, and **Table 1**). Similarly to TTCC antagonist, CACNA1G gene knock-down significantly sensitized both cell lines tested PyshA1G-4 and PyshA1G-6, to CPT (**Fig 5B**), as compared to PyshNT cells, with IC_{50} decreased from 0.3 ±0.1 to 0.07 ±0.02 μM (**Table 1**). Based on these results we conclude that TTCC contribute to breast cancer chemo-resistance and could be targeted to increase therapeutic responses.

TTCC antagonist mibefradil decreased breast cancer progression from locally advanced disease to invasive carcinoma in MMTV-PyMT mouse model

To determine TTCC role in breast cancer progression *in vivo*, MMTV-PyMT animals were subjected to TTCC inhibitor, mibefradil (Mib), treatment starting from age of puberty (5-6 week), and continued until 20-21 week of mouse age, or until humane endpoint was met. At the end of experiment, mammary gland/tumor isolated from either Mib or Control animals was fixed and paraffin embedded. The disease stage was determined based on the microscopic analysis of H&E stained tissues. With distinguishing 4 stages of breast cancer progression: 1- normal looking ducts and mulifocal hyperplasia, 2- adenoma and locally advanced disease, with increased in size ducts filled with luminal cells and uninterrupted basal membrane, ductal in situ carcinoma (DCIS) and mammary intraepithelial neoplasia, 3- locally advanced invasive carcinoma with interrupted basal membrane, well defined stroma surrounding locally advanced disease, 4- invasive breast carcinoma with advanced disease stage, well defined stroma, lacking defined basal membrane and loss of mammary ducts, with a mass formed by luminal cells, and ongoing process of metastasis.

As shown in **Fig 6A/B**, treatment with TTCC antagonist reduced progression of breast tumor, preserving hyperplastic multifocal lumens, DCIS (indicated by arrows on 20x magnification pictures) and fat cells. Based on the data analysis we conclude that inhibition of T-type Ca^{2+} channels decreased significantly stage of breast tumor development and tumor progression in MMTV-PyMT mice.

DISCUSSION

Here, we demonstrate that downregulation of T-type calcium channels, CACNA1G, in mouse luminal breast cancer cells induces expression of p53 and p19Arf, that is co-incidental with accumulation, or increased expression of TP63 and its transcriptional targets P-cadherin (Cdh3) and Wnt4. Knock-down of CACNA1G resulted in enhanced expression of cytokeratin 14 (CK14) and epithelial adhesion molecule EpCAM, while decreasing expression of PyMT, HER2 and CD49f. Taken together, such changes in epithelial cells markers suggest trans-differentiation from luminal to basal-like phenotype. Furthermore, the activation of pro-survival/anti-cell death PI3K/mTOR/AKT pathway was reduced in breast cancer cells with down-regulated CACNA1G, resulting in increased sensitivity to a dual EGFR/HER2 inhibitor lapatinib, ER modulator/antagonist tamoxifen, and chemotherapeutic camptothecin. Consistent with chemosensitivity of TTCC knockdown breast cancer cells, pharmacological inhibitor of TTCC, mibefradil, produced similar sensitizing effect in Py230 cell line *in vitro*. TTCC inhibitor, mibefradil treated MMTV-PyMT mice during the breast cancer development significantly decreased the number of invasive disease in mammary glands, as compared to mock-treated control animals indicating TTCC supporting role in *in vivo* breast cancer progression.

Increased expression of well-defined Ca^{2+} dependent signaling components during cancer formation was observed in both, SV40 T/t antigen and MMTV-PyMT, mouse models of breast cancer (Desai et al. 2002), yet significance of intracellular Ca^{2+} signaling in breast cancer formation and progression remains mostly unclear.

TTCC, low voltage activated calcium channels, have been reported to be expressed in human breast tumors (Pera et al. 2016), in breast cancer xenografts, and in breast cancer derived cell lines (Taylor et al. 2008; Pottle et al. 2013; Pera et al. 2016) with pre-dominant

CACNA1H subunit expressed in human luminal, ER positive breast cancer (Pera et al. 2016). Here we focused on CACNA1G (Cav3.1) subunit as one of predominantly expressed TTCC in mouse mammary epithelium and in MMTV-PyMT mammary tumors.

Our previous work demonstrated CACNA1G (Cav3.1) supportive role for activity of PI3K/mTOR/AKT intracellular signaling in tumor cells (Valerie et al. 2013), pathway that is crucial for breast cancer re-programming and invasive phenotype (Van Keymeulen et al. 2015; Koren et al. 2015). We hypothesized that TTCC crosstalk with HER2/PI3K/AKT pathway thus providing breast cancer extra survival advantage. In addition to attenuation PI3K/AKT pathway, inhibition of TTCC with transient downregulation of CACNA1G gene in HCT116 cancer cell lines, induced p53 dependent growth arrest (Dziegielewska et al. 2014). In contrast to our previous observations, stable CACNA1G gene knockdown in luminal murine ER+ breast cancer cells induced breast cancer trans-differentiation from luminal to basal-like phenotype with co-expression of both tumor suppressors p19Arf/p53 and basal-stem cells - like markers TP63/Wnt4/Cdh3.

Trans-differentiation is a process connected to breast cancer cells plasticity. Since parental Py230 cells are derived from luminal A tumor (Bao et al. 2015) and knowing that luminal tumors originate from the common luminal/basal cells progenitor, decrease in CACNA1G gene expression induced stress that conferred phenotypic switch from luminal to basal-like status. Interestingly oncogenes, such as: PyMT and HER2 decreased in Py230 CACNA1G knockdown cells, what in turn attenuated activation of pro-survival/anti-cell death AKT kinase.

During breast cancer formation oncogene PyMT expression, similarly to oncogenic $Kras^{G12V}$, induces p19Arf and p53 tumor suppressors pathway that when activated could lead to irreversible cell cycle arrest and senescence (Serrano et al. 1997; Lomax and Fried 2001;

Moule et al. 2004). Oncogenic Ca^{2+} flux through TTCC is likely suppressing p19Arf/p53 pathway up-regulation and play a positive role in maintaining PyMT oncogenic signaling. Consistent with our in vitro observation, a significant decrease in a number of invasive disease in mammary glands isolated from TTCC inhibitor, mibefradil, treated MMTV-PyMT mice during tumor development, was observed. In mammary glands/tumors isolated from mibefradil treated mice, a decreased number of invasive breast cancer and increased number of hyperplasia and locally advanced disease was statistically significant. This provides additional evidence for TTCC supportive oncogenic role in epithelial breast cancer formation and progression in vivo.

Often, trans-differentiation and de-differentiation is coincident with invasion and chemo-resistance (Gupta et al. 2019). Recent study by Granados et al., (Granados et al. 2020) using de-differentiated melanoma cells resistant to MAPK inhibitors (MAPKi) provides evidence for TTCC inhibitors-induced cancer cells differentiation and increased sensitivity to MAPKi. Similarly, in our study luminal breast cancer cells with down-regulation of CACNA1G gene expression simultaneously induced trans-differentiation and increased breast cancer cells sensitivity to EGFR/HER2 inhibitor, lapatinib, anti-estrogen therapy, tamoxifen and standard chemotherapy, such as camptothecin. While lapatinib sensitivity could be explained by decreased HER2 protein level in CACNA1G gene knockdown PyshA1G cells, sensitivity to Tamoxifen or to camptothecin indicates that TTCC provide tumor cells chemo-resistant advantage. Consistently, a pharmacological inhibitor of TTCC, mibefradil, produced similar sensitizing effect in Py230 breast cancer cell line *in vitro*. Thus, it appears that basal trans-differentiation of CACNA1G knock-down Py230 cells does not completely mirror de-differentiated, chemo-resistant basal type of breast cancer, but rather induces cancer cells differentiation. Transition of Py230 breast cancer luminal A type into either basal, or stem-like

tumor was previously reported with serial passaging of Py230 cells through castrated animals (Bao et al. 2015). It is interesting whether down-regulation of TTCC gene expression would prevent from luminal breast cancer cells growth and progression in vivo or allow for basal like breast tumor formation. Our data from PyMT mice treated with mibefradil suggest a decreased potential for disease progression when TTCC genes are suppressed in luminal A breast cancer.

Together, our data support TTCC and Ca^{2+} accessory role in breast cancer progression and chemo-resistance. Although more research needs to be done to determine how TTCC inhibition alters breast cancer development, progression and metastatic potential, TTCC could provide an attractive target in therapy-resistant disease.

CONFLICT OF INTEREST

The authors express no conflict of interest.

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TABLES

Drug IC_{50} (µM)					
Drug (µM)	**Py230**	**Py230+Mib**	**PyshNT**	**PyshA1G-4**	**PyshA1G-6**
Lapatinib	2.7 +/-0.49	0.8 +/-0.1	2.8 +/-0.5	2.2 +/-0.6	1.2 +/-0.2
Tamoxifen	3.1 +/-0.6	1.7 +/-0.5	11.0 +/-3.4	4.8 +/-0.6	5.9 +/-1.6
CPT	0.26 +/-0.11	0.1 +/- 0.01	0.26 +/- 0.09	0.07 +/-0.02	0.09 +/-0.04
Adriamycin	2.4 +/-0.5	1.5 +/-0.57	ND	0.7 +/- 0.28	0.6 +/- 0.1

TABLE 1. Growth Inhibitory concentrations by 50% (IC_{50}) calculated for lapatinib, tamoxifen, camptothecin and adriamycin in Py230 and shRNA-transduced cell lines.

Values represent IC_{50} concentrations from growth curves determined by AlamarBlue assay as described in Materials and Methods and derived from fitted curves to data from at least three independent experiments ± SD.

FIGURES and LEGENDS

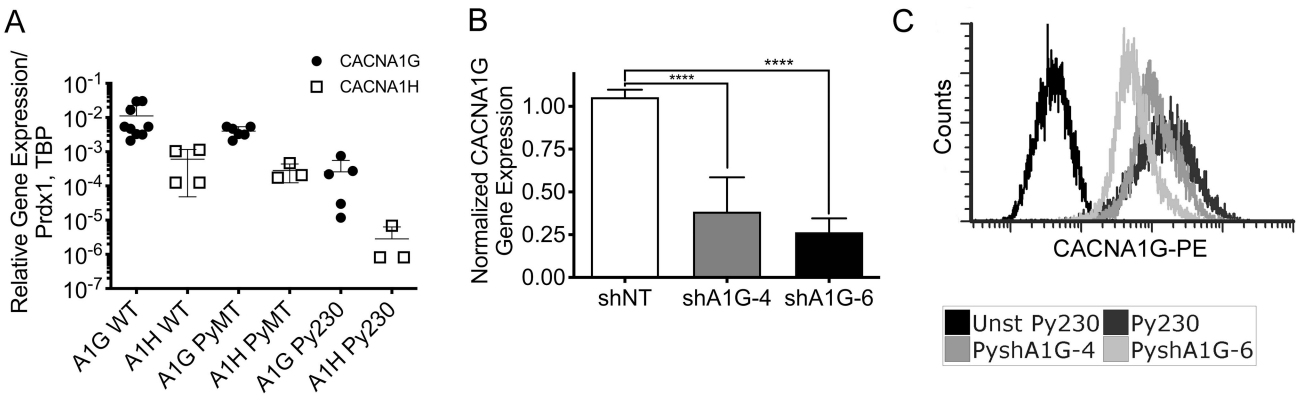


Figure 1. Knockdown of T-type Ca^{2+} channel (TTCC) CACNA1G gene expression in breast cancer cells, Py230. A- basal expression of CACNA1G and CACNA1H gene expression in mammary glands of WT or MMTV-PyMT breast tumors and tumor derived cells, Py230, as determined by RT-qPCR. Single points correspond to mice or to independent experiment performed on Py230 cells ±SEM. **B**- Downregulation of CACNA1G gene expression in Py230 breast cancer cells with specific shRNA (shA1G-4, shA1G-6) at RNA level was determined using RT-qPCR. **C**- Downregulation of CACNA1G gene expression in Py230 cells as measured by flow cytometry, using specific antibody to CACNA1G and fluorescently labelled secondary antibody. Unpaired t-test was used for statistical analysis, **** $p<0.0001$.

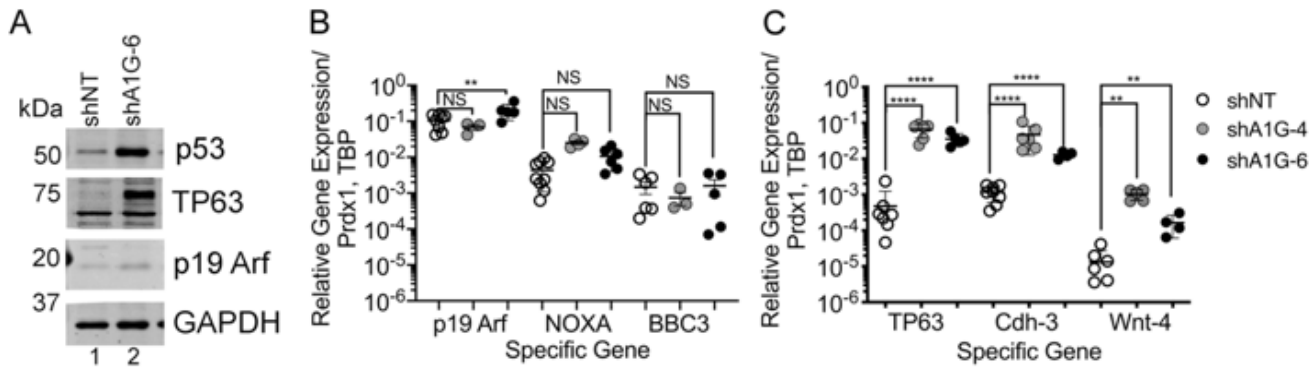


Figure 2. Downregulation of T-type Ca^{2+} channels increased expression of p53/TP63 and TP63 target genes products with appearance of trans-differentiation (basal) markers. A- Western Blot analysis of whole cell extracts isolated from PyshNT or PyshA1G-6 cells. GAPDH was used as a loading control. **B**- p53, and **C**- TP63 dependent gene expression measured by RT-qPCR and specific primers (listed in Supplementary Table 2) to indicated genes using total RNA isolated from PyshNT, PyshA1G-4 or PyshA1G-6 cells. Data were analyzed using unpaired t-test or one way ANOVA (**p<0.001, **** p<0.0001).

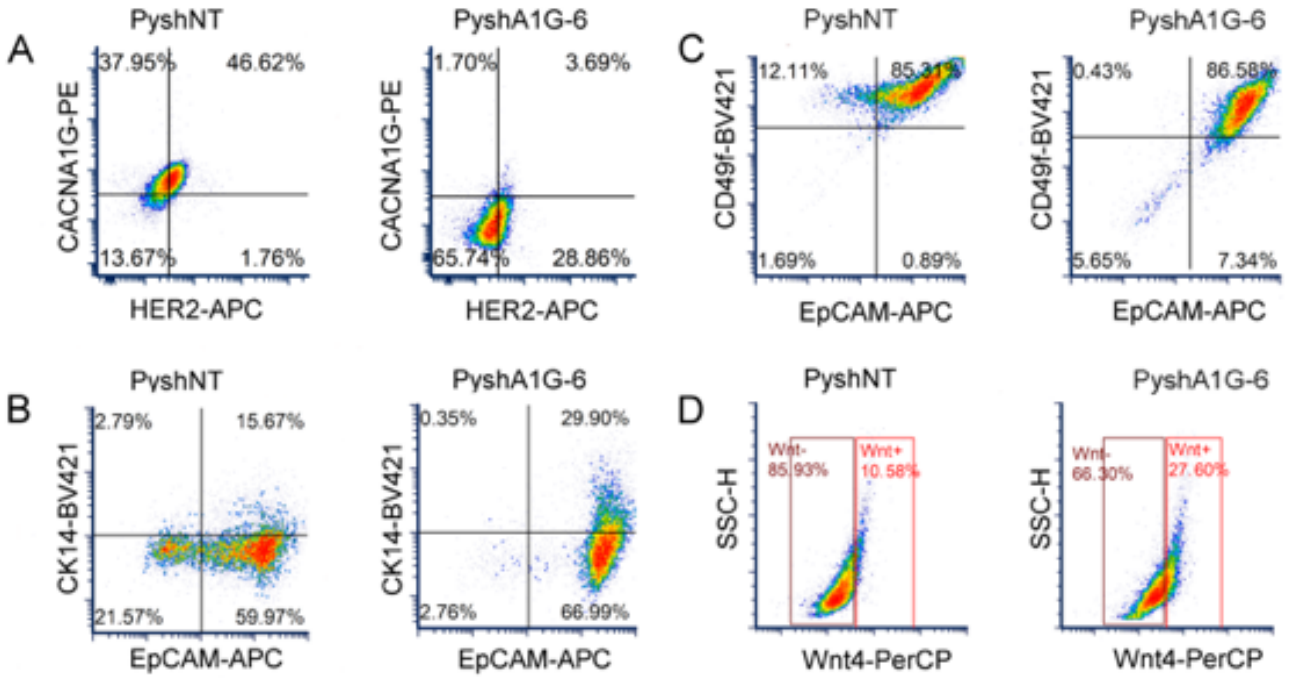


Figure 3. Decreased CACNA1G gene expression is associated with loss of HER2 and CD49f, and higher expression of EpCAM and basal cells markers, CK14 and Wnt4. Flow cytometry analysis of PyshNT versus PyshA1G-6 cells stained with specific antibodies: **A**- Double staining with CACNA1G-PE and HER2-APC or **B**- Double staining for Cytokeratin 14, CK14-BV421, and EpCAM-APC was done as described in Materials and Methods and analyzed by BD FACSCalibur and FCS Express 6.0 software. **C**- Double staining for EpCAM-APC and CD49f-BV421 was done as described in Materials and Methods and analyzed using FACS. **D**-Single staining of Wnt4-PerCP in PyshNT and shA1G-6 cells as measured by flow cytometry. Graphs represent data from at least 3 independent experiments.

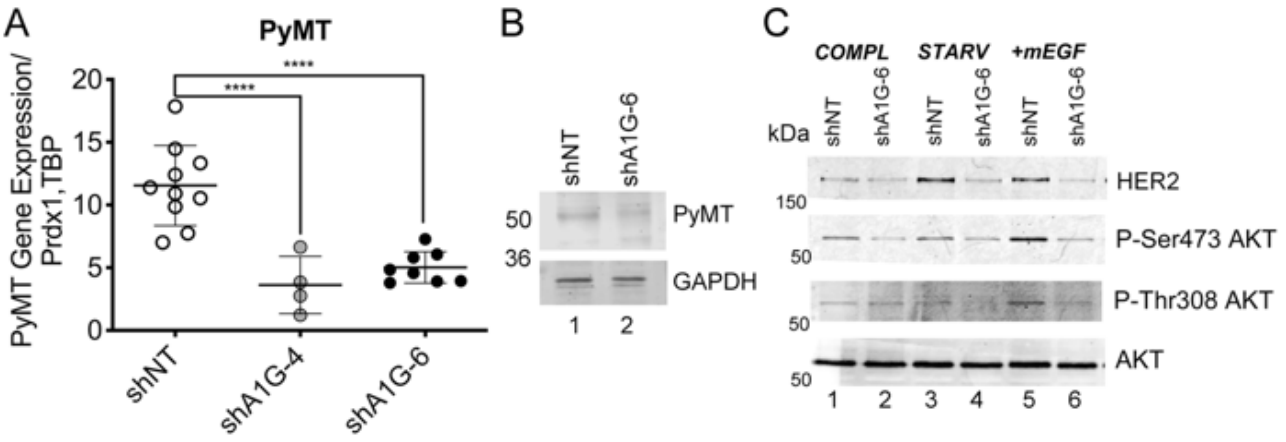


Figure 4. Decrease in CACNA1G lowers PyMT protein level and Her2 dependent signaling in Py230 breast cancer cells. A- PyMT gene expression measured using RT-qPCR in control PyshNT and CACNA1G gene-down regulated PyshA1G-4 and PyshA1G-6 cells. **B**- PyMT protein expression using Western blot and specific antibody, GAPDH was used as a loading control. **C**- Western Blot analysis of HER2 protein level and AKT activation in PyshNT versus PyshA1G-6 cells. Cells were starved for 6 h prior activation of signal transduction AKT pathway with mouse EGF (mEGF). Blots represent three independent experiments.

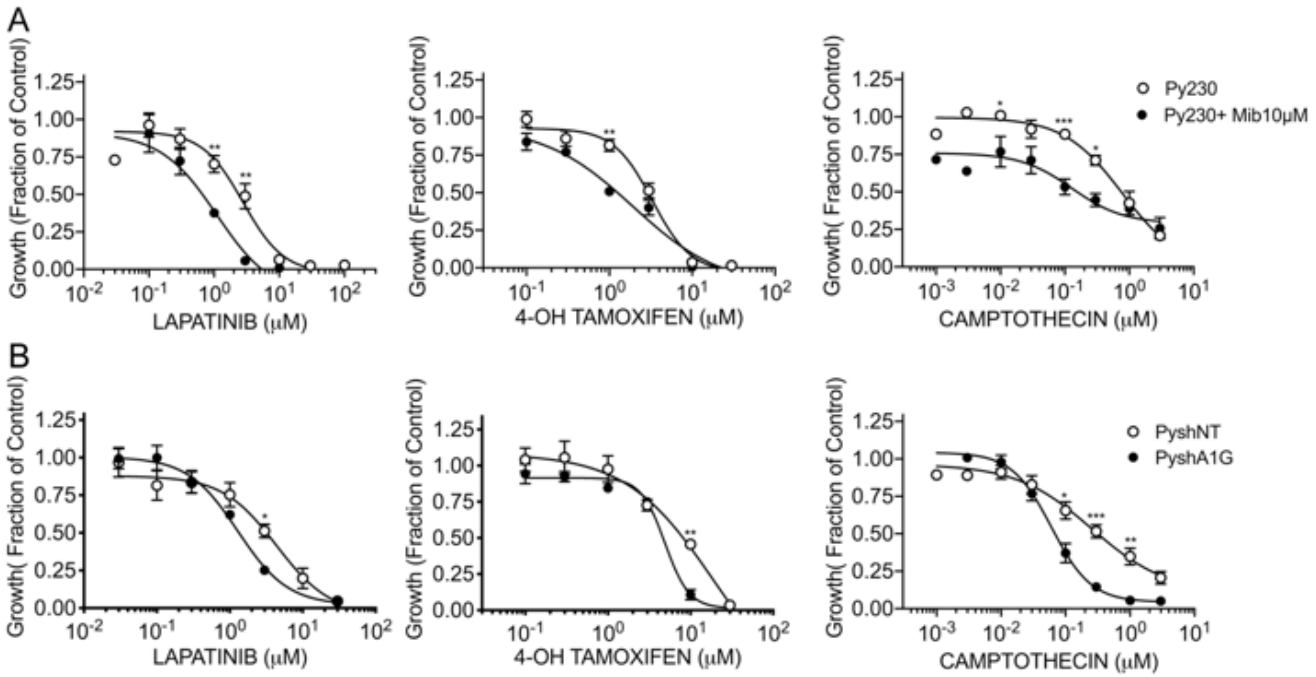


Figure 5. T-type Ca^{2+} channels increases resistance of breast cancer cells to dual EGFR/HER2 inhibitor lapatinib, estrogen therapy 4-hydroxytamoxifen, and to chemotherapeutic camptothecin. Growth inhibition curves of Py230 cells with either **A**- combined treatment with Mibefradil (10μM) or **B**- with Py230 derived cell lines stably transfected with shRNA non-targeting (PyshNT) or targeting CACNA1G gene (PyshA1G). Cells were treated with increasing drug concentrations diluted in media and treated continuously for 4 days, after what time alamarBlue was used to determine growth. Graphs represent data from at least 3 independent experiments ±SEM. Multiple t-test was performed to derive statistical significance between the curves using GraphPad Prizm v 8.0, *p<0.05, **p<0.01, ***p<0.001.

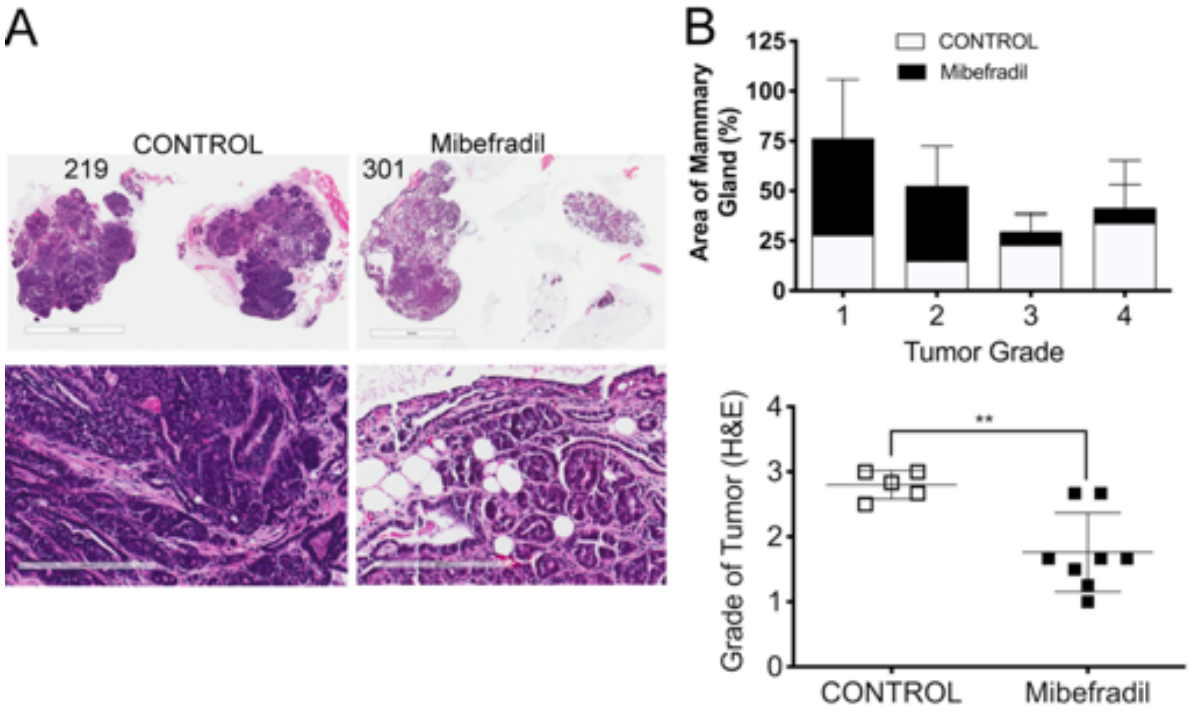


Figure 6. TTCC inhibitor, mibefradil treatment of PyMT mice suppressed breast cancer progression, as indicated by a lower disease stage and higher number of healthy lumens, hyperplasia and early stage of breast cancer. A- H&E staining of mammary glands isolated from mice non drug treated or treated with mibefradil. Upper panel magnification 2x, lower panel magnification 20x. Scale bars are 3-4 μm, and 200 nm, respectively. **B**- tumor grade analysis in mammary gland was done by selecting from each slide 6 random fields of tissue under the magnification of 20x, scoring them and averaging according to the description in Results. Each point represents the average score from individual mouse mammary gland.

Supplementary Tables and Figures.

Supplementary Table 1. List of primer pairs used for genotyping:

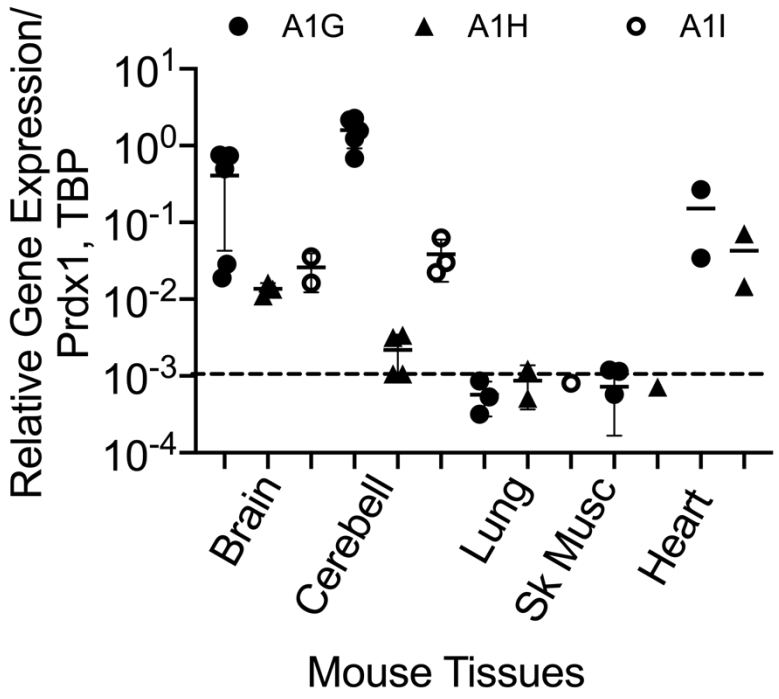
Gene	Primer Sequence 5'→3'	PCR Product (bp)	Accession Number
PyMT	F: CAGAATAGGTCGGGTTGCTC R: TGTGCACAGCGTGTATAATCC	265	AF442959.1
Pyk2	F: CCTGCTGGCAGCCTAACCACAT R: GGAGGTCTATGAAGGTGTCTACACGAAC	372	Gil372099096

Supplementary Table 2. RT-qPCR primers sequences.

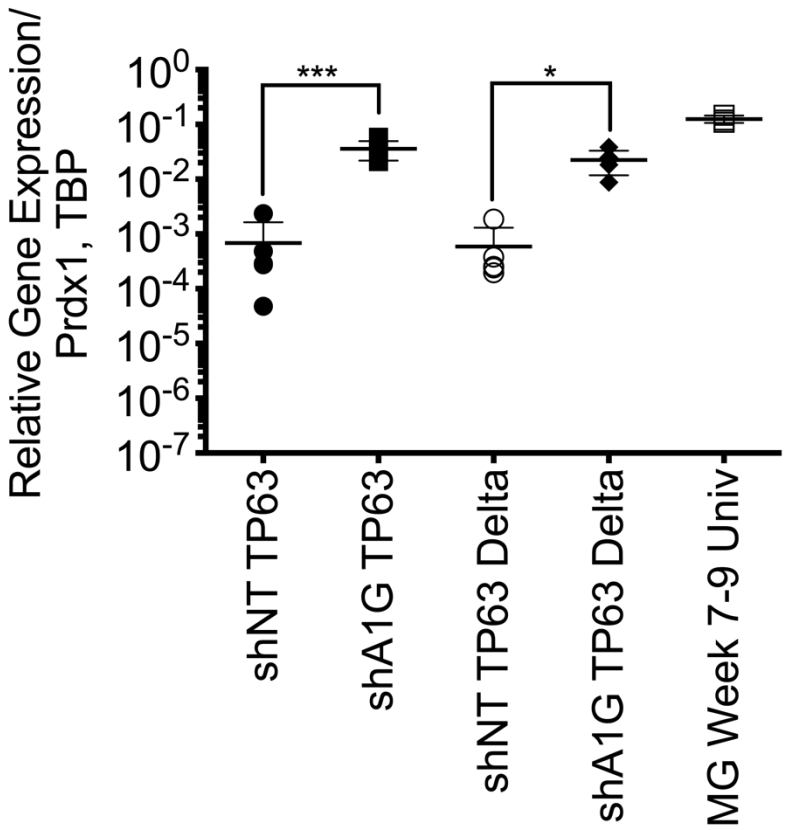
Gene Name	**Accession Number**	**Primer Sequence 5'→ 3'**	**Product (bp)**
Prdx1*	NM_011034	F: AATGCAAAAATTGGGTATCCTGC R: CGTGGGACACACAAAAGTAAAGT	150
Tbp*	NM_013684	F: GTCATTTTCTCCGCAGTGCC R: GCTGTTGTTCTGGTCCATGAT	151
CACNA1G	NM_009783.3	F: AGGAGCTGTTGGGAGAAAGC R: GCCGCCGTTGGGTTGTAATA	271
CACNA1H	NM_021415.4	F: GGTCACCTCTGCTCACTCG R: GCAGAGTATCCGTGAGAGGC	151
CACNA1I	NM_001044308.2	F: GAGCCGGGAATCACTGAGC R: GGTTGGTTCCATCCAATGGC	85
P19Arf	NM_009877.2	F: CTTGGTGAAGTTCGTGCGAT R: AGAAGGTAGTGGGGTCCTCG	259
APAF1	NM_001042558	F: GCAGCTAACACAGACTTGC R: CGCAGCTAACACAGACTTGC	200
BBC3	NM_133234.2	F: CAACTAGGTGCCTACACCCG R: CACCATGAGTCCTTCAGCCC	259
PMAIP1	NM_021451.2	F: CTCGCTTGCTTTTGGTTCCC	162

		R: ACTTCCCTAGCTCCACGACT	
CDKN1a	NM_001111099.2	F: CTTGTCGCTGTCTTGCACTC R: ACGCCTATGGAATGGCTTGG	227
Wnt4*	NM_009523	F: ACTGGACTCCCTCCCTGTCT R: TGCCCTTGTCACTGCAAA	109
TP63 Univ	NM_001127259	F: ACACAGACCACGCACAGAAT R: ACTGCTGGAAGGACACATCG	147
ΔNTP63	NM_001127259	F: AAGAGGAGAGCAGCCTTGAC	304
Cdh3	NM_001037809.5	F: CACTCACCCAGAGACCAACC R: CAAACACAGGGGCTTCGTTG	182
S-SHIP	NM_010566.3	F: TCCAAGAATGGTCCTGGCAC R: CAAACCGTACCACCAGCTCT	190

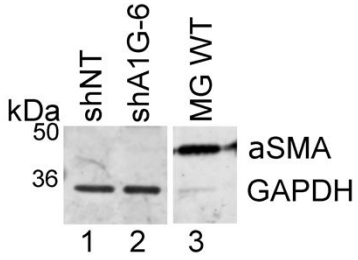
*- primers sequences from (van de Moosdijk and van Amerongen 2016)
**-primers sequences from (Bao et al. 2015)



Supplementary Figure 1. TTCC distribution in different mouse tissues. TTCC specific gene expression was measured using RT-qPCR and specific primers to CACNA1G, CACNA1H or CACNA1I subunit. Mouse tissues were harvested from virgin female WT animals, total RNA isolated, used for cDNA synthesis and in subsequent qPCR reaction. Each point corresponds to RNA preparation from a single mouse.



Supplementary Figure 2. Comparison of TP63 (universal) and ΔNTP63 gene expression in Py230 cells transfected with control shNT and shA1G-6. Total RNA was isolated from proliferating cells and subjected to RT-qPCR. Gene expression of TP63 Univ (TP63) or Delta was compared to mammary gland mouse epithelium isolated from virgin female mouse at 7-9 weeks of age.



Supplementary Figure 3. Py230 cells shNT and shA1G-6 lack expression of alpha Smooth Muscle Actin (αSMA), marker of myoepithelial cells. Cell extracts was analyzed using SDS-PAGE and Western Blotting using specific antibody as described in Materials and Methods. As a positive control normal mammary epithelial cells isolated from 21 week old WT female mouse were used on the same blot (lane 3).