Calcium Channel TRPV6 as a Potential Therapeutic Target in Estrogen Receptor–Negative Breast Cancer


Abstract

Calcium signaling is a critical regulator of cell proliferation. Elevated expression of calcium channels and pumps is a characteristic of some cancers, including breast cancer. We show that the plasma membrane calcium channel TRPV6, which is highly selective for Ca$^{2+}$, is overexpressed in some breast cancer cell lines. Silencing of TRPV6 expression in a breast cancer cell line with increased endogenous TRPV6 expression leads to a reduction in basal calcium influx and cellular proliferation associated with a reduction in DNA synthesis. TRPV6 gene amplification was identified as one mechanism of TRPV6 overexpression in a subset of breast cancer cell lines and breast tumor samples. Analysis of two independent microarray expression datasets from breast tumor samples showed that increased TRPV6 expression is a feature of estrogen receptor (ER)-negative breast tumors encompassing the basal-like molecular subtype, as well as HER2-positive tumors. Breast cancer patients with high TRPV6 levels had decreased survival compared with patients with low or intermediate TRPV6 expression. Our findings suggest that inhibitors of TRPV6 may offer a novel therapeutic strategy for the treatment of ER-negative breast cancers. Mol Cancer Ther; 11(10); 2158–68. ©2012 AACR.

Introduction

Calcium homeostasis is tightly controlled by various calcium-transporting proteins, including calcium-permeable ion channels (1). Increasing evidence suggests that some cancers may be associated with perturbed regulation of intracellular free Ca$^{2+}$ via deregulation of the expression of specific calcium-transporting proteins (2, 3). Altered calcium signaling through such changes in expression may contribute to tumorigenesis via calcium-sensitive pathways such as apoptosis, migration, and proliferation (2–4). Examples of calcium channels contributing to altered calcium signaling in cancer cells include the store-operated calcium channel ORAI1 and many members of the transient receptor potential (TRP) channel family (5–10).

A TRP channel family member that has particularly high selectivity for Ca$^{2+}$ and has constitutive activity is TRPV6 (11). TRPV6 plays a critical role in calcium absorption by the intestine (12), with TRPV6 knockout mice exhibiting signs of calcium deficiency, such as decreased bone mineral density, defective intestinal calcium absorption, and reduced fertility (13). Expression of TRPV6 mRNA and protein has been detected in placenta, pancreas, intestine, prostate, and mammary gland (14–16). TRPV6 expression is elevated in prostate cancer compared with normal prostate and benign prostate hyperplasia and is associated with prostate cancer progression (17–19). Studies have also implicated TRPV6 as a regulator of proliferation; stable expression of TRPV6 in HEK-293 cells increases cell proliferation (20), whereas knockdown of TRPV6 in the LNCaP prostate cancer cell line decreases proliferation and induces apoptosis (21).

Several studies have assessed TRPV6 in the context of breast cancer. Zhuang and colleagues (15) showed increased TRPV6 protein expression in 2 breast cancer tissue samples compared with normal breast. More recently and consistent with findings in prostate cancer (21), knockdown of TRPV6 decreases proliferation in T-47D breast cancer cells (7). This study also identified elevated mRNA levels (2- to 15-fold) of TRPV6 in 7 of 12 breast tumor samples (7). A very recent study has shown increased levels of TRPV6 in more invasive breast cancers (22). However, differences in TRPV6 have not been correlated with pathologic (e.g., histologic type) or molecular [e.g., estrogen receptor (ER) status or molecular subtypes] parameters or prognosis, as has recently been shown for regulators of store-operated calcium entry (9). In this study, we assessed the consequences of TRPV6 siRNA–mediated downregulation on calcium homeostasis, the cell cycle, and migration in breast cancer cells. We
also explored TRPV6 gene copy number as a possible mechanism of regulating TRPV6-altered expression in breast cancer cell lines and in clinical breast cancer samples. Finally, we assessed whether TRPV6 expression is correlated with a specific subtype of breast cancer and to breast cancer survival.

Materials and Methods

Cell culture

The MDA-MB-231 and MCF-7 cell lines were obtained directly from American Type Culture Collection. MDA-MB-468 (23), T-47D, BT-483, ZR-71-5, SK-BR-3, 184A1, and 184BS (24) cell lines were obtained as previously described. Upon receipt of each cell line, the cells were expanded and primary, secondary, tertiary, and working stocks were stored in liquid nitrogen. Cell lines were cultured for less than 10 passages (5-6 weeks), and separate media was used for each cell line. Cell lines were tested 6 monthly for Mycoplasma contamination using the MycoAlert Mycoplasma Detection Assay (Lonza. Inc.; most recent test was September 2011) and monitored for morphologic characteristics. Where appropriate, cell lines were characterized by assessment of the levels of HER2, ER, vimentin, and E-cadherin using real-time reverse transcriptase PCR (RT-PCR) and/or immunohistochemistry as previously described (23). For studies that assessed the effect of TRPV6 downregulation in the T-47D breast cancer cell line, the cells were maintained in Dulbecco’s Modified Eagle’s Medium, (high glucose; DMEM; Invitrogen) supplemented with 10% FBS (Sigma Aldrich) and 4 mmol/L L-Glutamine (Invitrogen) at 37°C with 5% CO2.

Real-time RT-PCR

RNA for the analysis of TRPV6 in the cohort of cell lines and to confirm knockdown was isolated as previously described (24). RNA was reverse transcribed using the Omniscript RT Kit (Qiagen) followed by real-time RT-PCR using TaqMan Universal PCR Master Mix and TaqMan gene expression assays. TRPV6 was amplified using the TaqMan Gene Expression Assay Hs00367960_m1. Thermal cycling was carried out using a StepOnePlus Thermal cycling was carried out using a StepOnePlus Real-Time PCR System (Applied Biosystems) and universal cycling conditions. The mRNA levels were normalized to 18S rRNA and are presented relative to the appropriate control (as described in the figure legend). Relative expression levels were calculated using the comparative Ct method (25, 26).

siRNA transfection

ON-TARGETplus siRNA SMARTpool siRNA reagents (consisting of 4 rationally designed siRNAs) were purchased from Dharmacon (Millennium Science). To reduce off-target effects, these siRNAs have dual strand modification (27), and their design uses an algorithm to minimize seed region matches (28). General off-target effects of TRPV6 siRNA were assessed through assessment of changes in TRPV1, cyclophilin B, and E-cadherin mRNA, no changes for these targets were detected in T-47D cells (data not shown). Nontargeting (NT) siRNA had no effect on cell viability compared with mock (Dharmafect 4 without siRNA) treated T-47D cells (data not shown). T-47D cells were seeded at 5 x 10^3 or 7.5 x 10^3 cells per well in 96-well plates and transfected with TRPV6 siRNA (100, 25 nmol/L of each siRNA; L-003607-00-005) or NT siRNA (100, 25 nmol/L of each siRNA; D-001810-10-05) using 0.1 μL/well DharmaFECT 4 according to the manufacturer’s instructions. Knockdown of TRPV6 mRNA was assessed by real-time RT-PCR 24 hours post-transfection.

Measurement of intracellular-free Ca^{2+} [Ca^{2+}]

T-47D cells were seeded at 7.5 x 10^4 cells per well in a 96-well CellBIND plates (Corning) and transfected with the appropriate siRNA as described above. After 72 hours, [Ca^{2+}]i was measured using the BD PBX Calcium Assay Kit (BD Biosciences; ref. 29), as previously described with minor modifications (9). Briefly, cells were loaded with the Calcium Indicator and 500 μmol/L probenecid in physical salt solution (PSS; 140 mmol/L NaCl, 11.5 mmol/L glucose, 10 mmol/L HEPES, 5.9 mmol/L KCl, 1.4 mmol/L MgCl2, 1.2 mmol/L NaH2PO4, 5 mmol/L NaHCO3, 1.8 mmol/L CaCl2, pH 7.3) for 1 hour at 37°C. Cells were incubated for 15 minutes at room temperature. Loading solution was replaced with PSS and 500 μmol/L probenecid in PSS with nominal Ca^{2+}. To measure Ca^{2+} influx (7, 21), 500 μmol/L CaCl2 (0.6 or 2 mmol/L) were added to the cells sequentially and fluorescence was measured at 470 to 495 nm excitation and 515 to 575 nm emission using the fluorescence imaging plate reader FLIPr TETRA (Molecular Devices). Response over baseline was assessed as a relative measure of [Ca^{2+}]i; ref. 9, and rate of Ca^{2+} influx was measured between 51 and 151 seconds after Ca^{2+} addition as an assessment of TRPV6-mediated Ca^{2+} influx (7, 21).

Cell number, cell cycle, and EdU incorporation

Cell number, percentage of cells in S-phase, and cell-cycle distribution were assessed in T-47D cells 120 hours after siRNA treatment. T-47D cells were plated at 5 x 10^3 cells per well in 96-well BD Falcon microplates (BD Biosciences) and transfected with the appropriate siRNA. After 24 hours, transfection medium was replaced with serum-free DMEM for 48 to 72 hours. Cells were then incubated with 100 μmol/L EdU for 1 hour and then fixed with 3.7% (v/v) formaldehyde in PBS, washed with 3% (v/v) bovine serum albumin in PBS, and permeabilized with 0.5% Triton X-100 (v/v). Cells were then incubated with the Click-IT reaction cocktail (Alexa Fluor 555; Invitrogen), followed by DAPI (4′,6-diamidino-2-phenylindole; 400 nmol/L). Cells were then incubated with the Click-IT reaction cocktail (Alexa Fluor 555; Invitrogen), followed by DAPI (4′,6-diamidino-2-phenylindole; 400 nmol/L). Plates were scanned with the ImageXpress Micro (Molecular Devices) automated epifluorescent microscope. Images were acquired with x10 objective, DAPI- and EdU-positive cells were detected using DAPI-5060B and Cy3-4040B.
filter sets, respectively. For cell number and cell-cycle analysis, the DAPI integrated intensity was assessed using the cell-cycle application module (MetaXpress). The percentage of EdU-positive cells was calculated using the multiwavelength cell scoring application module (MetaXpress).

Migration assay

Cell migration assays were carried out using BD FluoroBlok 8-µm cell culture inserts for 24-well plates (BD Biosciences). T-47D cells were seeded at 7.5 × 10^4 cells per well in a 96-well plates and transfected with the appropriate siRNA. After 72 hours, the cells were trypsinized and seeded into the insert in serum-free DMEM (5 × 10^4 cells/350 µL). DMEM media supplemented with 1% FBS was used as the chemoattractant. After 24 hours, the cells were labeled with 4 µg/mL Calcein AM (Invitrogen) for 1 hour and fluorescence was detected at 485 nm excitation and 520 nm emission using the NOVOstar fluorescent plate reader (BMG Labtech).

Gene copy number assay

TRPV6 gene copy number was assessed using a real-time TaqMan Copy Number Assay (Applied Biosystems). Briefly, genomic DNA (gDNA) was isolated from breast cancer cell lines using the QIAamp DNA Mini Kit (Qiagen), and the DNA concentration was quantified using UV absorbance (A_{260}/A_{280}). A 20 µL PCR reaction was prepared according to the Applied Biosystems TaqMan Copy Number Assays instructions using 10 ng gDNA, TaqMan Copy Number Reference Assay RNase P, and a custom TaqMan Assay (TRPV6_cn_for, GCTTCACTGATGTGCCATCT; TRPV6_cn_rev, GGCCTGTCGCGTTATTGG; TRPV6_cn_pr_FAM, and 6-FAM-CCCCCTTCAAGCCAGG). All assays were carried out in quadruplicate using the StepOnePlus Real-Time PCR System (Applied Biosystems). Data were analyzed using CopyCaller v1.0 software (Applied Biosystems) by carrying out a comparative (∆∆Ct) relative quantitation analysis. The ∆Ct was determined by calculating the difference between the Ct values for TRPV6 and the reference assay, RNase P. The ∆∆Ct was then measured by comparing the ∆Ct for TRPV6 and the control gDNA (Applied Biosystems), which was assigned diploid copy number.

Analysis of publically available microarray data

The TRPV6 gene resides at chromosome 7: 142,279,082-142,293,599 (hg18 assembly). Two cohorts of samples with whole-genome DNA copy number data from different microarray platforms were analyzed to determine whether somatic alterations in DNA copy number occur in the TRPV6 gene region in breast tumors. The first cohort consisted of 35 invasive ductal carcinomas (IDC), of which 22 were ER-positive and 13 were ER-negative, analyzed by 4.7k BAC microarray comparative genomic hybridization (aCGH; ref. 30). The second cohort was derived from The Cancer Genome Atlas (TCGA) and consisted of 247 samples analyzed using the Affymetrix Genome-Wide Human SNP Array 6.0. SNP6.0 CEL files for each sample consisting of level 3 preprocessed data of segmental values were downloaded from the TCGA Data Portal (31). The data was processed in R version 2.13.0 to score DNA copy number alterations, as described by others (32). Briefly the thresholds less than −0.3 and more than 0.3 were assigned to call losses and gains, respectively, and the amplitude of gain at the region of the TRPV6 gene was assessed specifically by using thresholds of more than 0.3, 0.6, 0.8, and 1.0. To determine the status of TRPV6 gene copy number in different subtypes of breast cancer, this cohort was stratified using clinicopathologic data available from the TCGA according to histologic type [IDC or invasive lobular carcinoma (ILC)] and biomarker expression status [ER, progesterone receptor (PR), HER2] into the following groups: IDC, IDC ER+/HER2−, IDC ER+/HER2+, IDC ER−/HER2+, and IDC ER−/PR−/HER2−. This cohort was also stratified according to molecular subtype based on the gene expression profiling data from the same samples and using the Single Sample Predictor defined by Hu and colleagues (33).

To assess the gene expression patterns of TRPV6 in breast cancer, data were downloaded for the following 2 studies, the TCGA (see above) and the NKI-295 dataset (34). For the TCGA data, level 2 (log2 lowess normalized) gene expression data for 371 tumors was downloaded from the data portal. In the NKI-295 dataset, the TRPV6 gene was annotated under a previous name, ABP/ZF. The samples were stratified, as described above, using clinicopathologic data according to ER status, molecular subtype, and histologic type plus ER/PR/HER2 biomarker expression (TCGA data only).

Statistical analysis

Statistical significance was assessed as described in individual figure legends. Data analysis was carried out using Prism version 5.02 for Windows (GraphPad Inc.).

Results

TRPV6 is overexpressed in a subset of breast cancer cell lines

Increased TRPV6 levels are a feature of T-47D breast cancer cells compared with MDA-MB-231 and MCF-7 breast cancer cells (35). TRPV6 expression is also elevated (2- to 15-fold) in 7 of 12 clinical breast tumors (7). To begin to assess whether TRPV6 overexpression may be a feature of specific breast cancer subtypes, we assessed TRPV6 levels in a bank of nonmalignant and malignant breast cancer cell lines varying in ER, PR, and HER2 status and molecular subtype. TRPV6 levels were elevated in 4 cell lines, ZR-75-1, T-47D, SK-BR-3, and MDA-MB-468, compared with the nonmalignant breast cancer cell lines 184A1 and 184B5 (Fig. 1A). Levels in these 4 breast cancer cell lines were greater than 60-fold higher relative to 184A1 cells (Fig. 1A), whereas TRPV6 levels in MDA-MB-231, MCF-7, and BT-483 were similar (<7-fold higher to those observed in 184A1 cells; Fig. 1A).
Knockdown of TRPV6 reduces the rate of calcium influx

TRPV6 basal Ca\(^{2+}\) influx has previously been assessed as a measure of TRPV6 function in HEK293 cells transiently overexpressing TRPV6 (36), in LNCaP prostate cancer cells (21) and in T-47D breast cancer cells, in which a functional consequence of TRPV6 siRNA was shown by a 38% decrease T-47D migration; instead a modest increase was observed (Fig. 2F).

Effect of TRPV6 knockdown on viable cell number, cell cycle, and migration

We investigated potential effect of TRPV6 downregulation (Fig. 2A) on the cell cycle and cell migration in T-47D breast cancer cells. TRPV6 siRNA significantly reduced the number of viable cells at 24 and 48 hours (35% and 40% reduction, respectively) in the presence of serum (Fig. 2B). Assessment of proliferating cells using EdU staining showed that the percentage of cells in S-phase significantly decreased (~20%) with TRPV6 knockdown (Fig. 2C). DNA content histogram analysis showed that TRPV6 knockdown caused cells to accumulate in the G1-phase at 24 hours with serum (Fig. 2D), but not at 48 hours with serum (Fig. 2E). Knockdown of TRPV6 did not decrease T-47D migration; instead a modest increase was observed (Fig. 2F).

TRPV6 gene amplification in a bank of breast cancer cell lines

To assess whether gene amplification could be a mechanism for TRPV6 mRNA overexpression in some breast cancer cell lines, gene copy number was assessed using a real-time assay. TRPV6 copy number was greater than 6 in T-47D, SK-BR-3, ZR-75-1, BT-483, and MCF-7 cells (Fig. 3A). The highest level was detected in BT-483 cells (9 copies; Fig. 3A). Overexpression of TRPV6 mRNA in ZR-75-1, T-47D, and SK-BR-3 cells may be due, at least in part, to increased gene copy number (Fig. 3B). However, an association between gene copy number and mRNA levels for TRPV6 was not observed for MDA-MB-231, MCF-7, BT-483, or MDA-MB-468 cells (Fig. 3B).
2 cohorts of invasive carcinomas derived from BAC aCGH (cohort 1; ref. 30) and SNP-CGH (TCGA–cohort 2) platforms. The genomic region harboring the TRPV6 gene was subjected to both gains and deletions. The frequency of these gains and deletions differed across different tumor subtypes (Table 1). For instance, both cohorts showed a significant increase in the frequency of TRPV6 gene copy number gains in ER-negative tumors relative to ER-positive tumors ($P < 0.01$). This finding was also observed when triple-negative tumors (ER, PR, and HER2-negative) were compared with ER-positive or HER2-positive tumors and when basal-like tumors were compared with luminal A or HER2 tumor types ($P < 0.01$; Table 1).

The amplitude of gain was further measured using different thresholds for calling gains to determine the number of tumors with higher level gains (amplifications) in the region of TRPV6 (Fig. 4A and B) and determine, according to the copy number profile, whether there was a specific amplification in this region or whether the region was gained as part of an increase copy number covering a large region of the chromosome. Figure 4A shows a peak in gains very close to, but not encompassing the TRPV6 gene locus, highlighted by the red line. We identified 6 tumors overall (4 from cohort 1 and 2 from cohort 2) that had specific amplification in the region of the TRPV6 gene (Fig. 4A and B); the increase in copy number for the remaining samples were reflected by increased DNA copy number of chromosome 7q not specifically related to TRPV6 gene region. The gene region was lost most frequently in HER2 tumors when compared with ER-positive or triple-negative tumors and in comparison with basal-like tumors ($P < 0.01$; Table 1). The correlation between TRPV6 gene copy number and TRPV6 mRNA levels was strongest for the basal-like subtype compared with other molecular subtypes (Fig. 4C).

**TRPV6 gene expression in clinical breast tumors and association with survival**

Given the evidence from this study and others that TRPV6 overexpression is a feature of some specific breast
Characterization of TRPV6 in Breast Cancer

Elevated TRPV6 mRNA levels have previously been observed in 7 of 12 clinical breast cancer samples and in the T-47D breast cancer cell line compared with the MDA-MB-231 and MCF-7 cell lines (7). Elevated TRPV6 was identified in 4 of 7 breast cancer cell lines assessed in this study, with no obvious correlation between TRPV6 expression levels and molecular subtype, but this may simply be due to the small number of cell lines examined. Analysis of 2 independent cohorts of clinical samples, however, identified a correlation between TRPV6 mRNA expression levels and (i) immunophenotype and (ii) molecular subtypes. These data highlight a potential biologic role for TRPV6 in specific breast cancer subtypes. For instance, the highest TRPV6 mRNA levels were consistently associated with IDCs with an ER-negative phenotype, whether they were classified simply as ER-negative, as triple negative (ER-, PR-, and HER2-negative) or as basal-like (gene expression profiling subtype). The correlation we observed between elevated TRPV6 expression and a significantly decreased survival may reflect the association between TRPV6 expression and the ER-negative subgroup, which are among those breast cancers with the worst prognosis (37, 38).

Figure 3. Characterization of TRPV6 copy number in breast cell lines. A, TRPV6 gene copy number in control gDNA and malignant MDA-MB-231, MCF-7, BT-483, ZR-75-1, T-47D, SK-BR-3, and MDA-MB-468 breast cancer cell lines. The data are mean ± SD (N = 8) and are from 2 independent experiments carried out in quadruplicate. B, comparison of mRNA expression relative to 184A1 and gene copy number in breast cancer cell lines.

Discussion

TRPV6 is an essential calcium influx channel, which has been implicated in prostate cancer progression (18, 19). Elevated TRPV6 expression is seen in breast tumors (7); however, the mechanism of overexpression has not been addressed and a correlation with tumor subtype or prognosis also not determined. Our studies identify TRPV6 gene amplification as a possible mechanism for TRPV6 overexpression in some breast tumors. We provide further evidence for a role for TRPV6 in tumor progression with siRNA-mediated inhibition of TRPV6 reducing basal Ca\(^{2+}\) influx and breast cancer cell proliferation. Increased TRPV6 expression was a feature of ER-negative, HER2 and basal-like breast tumors, and increased TRPV6 levels were associated with reduced survival in breast cancer patients.

Elevated TRPV6 mRNA levels have previously been observed in 7 of 12 clinical breast cancer samples and in the T-47D breast cancer cell line compared with the MDA-MB-231 and MCF-7 cell lines (7). Elevated TRPV6 was identified in 4 of 7 breast cancer cell lines assessed in this study, with no obvious correlation between TRPV6 expression levels and molecular subtype, but this may simply be due to the small number of cell lines examined. Analysis of 2 independent cohorts of clinical samples, however, identified a correlation between TRPV6 mRNA expression levels and (i) immunophenotype and (ii) molecular subtypes. These data highlight a potential biologic role for TRPV6 in specific breast cancer subtypes. For instance, the highest TRPV6 mRNA levels were consistently associated with IDCs with an ER-negative phenotype, whether they were classified simply as ER-negative, as triple negative (ER-, PR-, and HER2-negative) or as basal-like (gene expression profiling subtype). The correlation we observed between elevated TRPV6 expression and a significantly decreased survival may reflect the association between TRPV6 expression and the ER-negative subgroup, which are among those breast cancers with the worst prognosis (37, 38).

Genome-wide, high-resolution studies of breast cancer samples show that between 10.5% to 12% of overexpressed genes are associated with gene amplification (39, 40). We explored whether increased gene copy number was a potential driver of enhanced expression levels seen in some breast cancers. We found an association between increased TRPV6 mRNA expression and increased TRPV6 gene copy number in 3 breast cancer cell lines, ZR-75-1, T-47D, and SK-BR-3. Analysis of clinical breast cancer samples showed that although TRPV6 gene does not reside within a region of the genome that is frequently amplified in breast cancer (such as, 8p12, 8q24, 11q13, and 17q12; ref. 41), the gene was subjected to frequent somatic alteration and that elevated copy
number was most likely to occur in ER-negative, triple-negative, or basal-like compared with other subtypes. We tested whether there was a correlation between TRPV6 gene expression levels and gene copy number data in the TCGA cohort of clinical samples, in which both data were available. The highest correlation was found in the basal-like subgroup in which a proportion of tumors exhibited both an elevated DNA copy number and an increased mRNA level. The data suggest gene amplification as a possible mechanism for driving TRPV6 overexpression in these cell lines and tumors. In contrast, TRPV6 expression did not correlate with gene copy number in 4 breast cancer cell lines and in many tumors, suggesting that there is an alternative mechanism driving the high levels of gene transcription. This scenario is not unexpected in breast cancer, as mRNA levels are not always linearly related to gene copy number and in which numerous alternative mechanisms for regulating transcription exist, including altered miRNA regulation, DNA methylation, or transcriptional activation (42). Solvnang and colleagues (42) showed that a quadratic relationship for copy number and mRNA expression was a better fit than a linear relationship for more than 80% of the breast tumors assessed. Furthermore, a gene may exhibit 2 expression subsets in a breast cancer cohort, such as amplification without a corresponding increase in expression and increased expression without amplification (42). Such a scenario may exist for TRPV6 in breast cancer cell lines and tumors.

Table 1. DNA copy number status of TRPV6 gene region in breast cancer subtypes

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NOTE: The values in parenthesis are given in percent. Statistical analysis was carried out using Fisher exact test, two tailed, and only significant (<0.05) P values are displayed.

Abbreviations: +, positive; −, negative; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; ER, estrogen receptor; PR, progesterone receptor.

| cohort 1 IDC ER+ vs. ER- .
| cohort 2 IDC ER+ vs. ER- .
| ID/ER (PR+/HER2- ) vs. IDC (ER+/HER2- )
| ID/ER (PR+/HER2- ) vs. IDC (HER2+)
| ID/HER2- ) vs. IDC (PR+/HER2- )
| Basal-like vs. luminal A.
| Basal-like vs. HER2.
| luminal B vs. basal-like.
| HER2 vs. basal-like.

We tested whether there was a correlation between TRPV6 gene expression levels and gene copy number data in the TCGA cohort of clinical samples, in which both data were available. The highest correlation was found in the basal-like subgroup in which a proportion of tumors exhibited both an elevated DNA copy number and an increased mRNA level. The data suggest gene amplification as a possible mechanism for driving TRPV6 overexpression in these cell lines and tumors. In contrast, TRPV6 expression did not correlate with gene copy number in 4 breast cancer cell lines and in many tumors, suggesting that there is an alternative mechanism driving the high levels of gene transcription. This scenario is not unexpected in breast cancer, as mRNA levels are not always linearly related to gene copy number and in which numerous alternative mechanisms for regulating transcription exist, including altered miRNA regulation, DNA methylation, or transcriptional activation (42). Solvnang and colleagues (42) showed that a quadratic relationship for copy number and mRNA expression was a better fit than a linear relationship for more than 80% of the breast tumors assessed. Furthermore, a gene may exhibit 2 expression subsets in a breast cancer cohort, such as amplification without a corresponding increase in expression and increased expression without amplification (42). Such a scenario may exist for TRPV6 in breast cancer cell lines and tumors.

The functional role for TRPV6 was investigated in the T-47D breast cancer cell line, which has elevated TRPV6 mRNA expression and gene copy number. Knockdown of TRPV6 using siRNA significantly decreased basal Ca2+ influx with the addition of physiologic levels of Ca2+, consistent with the one previous study of TRPV6 activity in a breast cancer cell line (7). Our current findings and those of Bolanz and colleagues (7) also identify a reduction in viable cell number with TRPV6 siRNA treatment in T-47D cells. Although Bolanz and colleagues (7) suggested an increase in the number of...
cells undergoing apoptosis, our data also suggest that TRPV6 siRNA reduces the percentage of cells in S-phase and increases the percentage of cells in the G1-phase. Ca\textsuperscript{2+} influx is a well known mechanism to promote the G1 to S-phase transition (43); therefore, the accumulation of cells in G1-phase is likely to be associated with the attenuation of calcium influx associated with TRPV6 inhibition. LNCaP prostate cancer cells also have a reduction in cells in S-phase, but no effect on the percentage of cells in the G1-phase with TRPV6 knockdown (21). This indicates some potential divergent roles for TRPV6 on cell cycle in breast and prostate cancer cells. Although, TRPV6 downregulation decreases breast cancer cell proliferation, knockdown of TRPV6 expression actually increased T-47D breast cancer cell migration, even though viable cell number was reduced. This may suggest that the influence TRPV6 has on outcome or prognosis is via mechanisms other than tumor cell migration.

However, this is in contrast to a very recent study in MDA-MB-231 and MCF-7 breast cancer cell lines, in which TRPV6 silencing reduced migration (22). Further studies assessing the role of TRPV6 in the migration of other breast cancer cell lines are now required.

The potential requirement for TRPV6 function specifically in tumor cell proliferation, through calcium influx, may highlight the underlying association between elevated TRPV6, an ER-negative tumor phenotype and poor outcome, as ER-negative tumors are most often of high grade and are highly proliferative tumors. It is unclear from this study whether TRPV6 overexpression drives or simply facilitates this enhanced proliferation in this aggressive breast tumor subtype. Nevertheless, targeted knockdown of the gene in cells with elevated expression leads to reduced growth and stalling of the cell cycle, suggesting that targeting the TRPV6 calcium channel maybe a useful therapeutic mechanism.

Figure 4. TRPV6 gene copy number in breast tumors. A, chromosome 7 frequency plots of SNP-CGH data derived from samples of invasive breast tumors (from TCGA) classified as either HER2 or basal-like molecular subtype. X-axis, chromosome location (in base pairs); y-axis, proportion of cohort with gain along this chromosome. TRPV6 gene region is highlighted by a red line. B, a profile of chromosome 7 copy number (from BAC aCGH) from an IDC. X-axis, chromosome location; y-axis, normalized, scaled log\textsubscript{2} ratios of individual BAC clones. The arrow indicates a specific amplification of the region harboring the TRPV6 gene. C, correlation between TRPV6 gene copy number and mRNA levels in the TCGA cohort of human breast tumors. Data were stratified according to molecular subtype. X-axis, DNA copy number at TRPV6 gene region according to segmental DNA values (level 3 data); y-axis, log\textsubscript{2} lowest normalized values for TRPV6 mRNA expression levels. A Pearson correlation coefficient (R values) was calculated to measure the relationship between gene copy number and its mRNA expression profile.
There is a requirement for targeted therapeutics in ER-negative, non-HER2–positive tumors owing to the lack of clinical benefit derived from giving these patients current targeted therapy (ref. 44; Herceptin or endocrine-based treatment). Chemotherapeutics such as taxanes do provide clinical benefit and the use of PARP inhibitors (PARPi) show promise by targeting apparent defects in DNA repair mechanisms in this category of breast tumors (45, 46). Much still needs to be resolved with regard to the role of PARPi in treating ER-negative tumors, and recent phase III clinical data for PARPi are somewhat disappointing (47), indicating that alternative therapeutic options such as targeting calcium signaling are warranted.

Inhibition of the calcium channel TRPV6 may not be associated with the predicted systemic side effects that would result from inhibition of some other calcium channels, as TRPV6 knockout mice are viable and exhibit traits that may be well tolerated compared with current therapies (e.g., decreased bone mineral density and defective intestinal calcium absorption; ref. 13). Our studies indicate that TRPV6 inhibitors are potential therapeutics for the treatment of ER-negative breast cancers that exhibit elevated TRPV6 expression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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