The role of TRPV6 in breast carcinogenesis

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Abstract

TRPV6 is an endothelial calcium entry channel that is strongly expressed in breast adenocarcinoma tissue. In this study, we further confirmed this observation by analysis of breast cancer tissues, which indicated that TRPV6 mRNA expression was up-regulated between 2-fold and 15-fold compared with the average in normal breast tissue. Whereas TRPV6 is expressed in the cancer tissue, its role as a calcium channel in breast carcinogenesis is poorly understood. Therefore, we investigated how TRPV6 affects the viability, apoptosis, and calcium transport in the breast cancer cell line T47D. Hormones can also affect the tumor development; hence, we determined the effects of estradiol, progesterone, and 1,25-vitamin D on TRPV6 transcription. Interestingly, the estrogen receptor antagonist tamoxifen reduced expression of TRPV6 and is able to inhibit its calcium transport activity (IC_{50}, 7.5 μmol/L). The *in vitro* model showed that TRPV6 can be regulated by estrogen, progesterone, tamoxifen, and 1,25-vitamin D and has a large influence on breast cancer cell proliferation. Moreover, the effect of tamoxifen on cell viability was enhanced when TRPV6 expression was silenced with small interfering RNA. TRPV6 may be a novel target for the development of calcium channel inhibitors to treat breast adenocarcinoma expressing TRPV6. [Mol Cancer Ther 2008;7(2):271–9]

Introduction

Ionized calcium (Ca^{2+}) is a central signaling ion that is critical for controlling growth, proliferation, and survival of normal and malignant cells. In cellular processes, calcium is a key modulator of numerous enzymes located in the cytosol, organelles, and nucleus. Ca^{2+} cannot be degraded like other second messenger signals. Therefore, the intracellular free calcium levels are tightly regulated on multiple levels by plasma membrane ion channels, ion exchangers, pumps, and Ca^{2+} binding proteins, as well as by the release of calcium from the endoplasmic reticulum and nuclear envelope (1, 2).

One such plasma membrane channel is the TRPV6 calcium entry channel. TRPV6 is a Ca^{2+} permeable ion channel that was identified using expression cloning by Hediger and colleagues in 1999 (3). TRPV6 plays a central role in total body calcium homeostasis, and its regulation directly affects intestinal calcium absorption, renal calcium excretion, and bone metabolism (4). TRPV6 seems to play a major role in facilitating the entry of Ca^{2+} into absorptive epithelial cells. This is evident in TRPV6 knockout mice, which exhibit defective intestinal Ca^{2+} absorption, increased urinary Ca^{2+} excretion, and decreased bone mineral density. Regulation of TRPV6 is controlled by 1,25-vitamin D, estrogen, and dihydrotestosterone (5–7).

Whereas TRPV6 functions to maintain normal calcium homeostatsis, it also seems to play a role in tumor development and progression. TRPV6 was observed to be up-regulated in tissue samples originating from prostate, breast, thyroid, colon, ovary, and pancreatic tumors (8). The channel has been localized to the apical membrane where it delivers calcium into the cells. Furthermore, it has been shown that TRPV6 is most strongly expressed in advanced stages of prostate cancer, whereas there is little to no expression evident in healthy tissue and benign prostate hyperplasia (7, 9). The transcript levels in both studies correlated positively with tumor progression and aggressiveness as indicated by the pathologic stage and Gleason scores of the prostate tumors. Endogenous store-operated channels play important roles in the apoptosis of LNCaP prostate cancer cells (10, 11). Numerous studies have linked enhanced endoplasmic reticulum Ca^{2+} accumulation to proliferation and/or apoptosis in prostate cancer (12–14). A study by Schwarz et al. (2006) showed that TRPV6 clearly increases the rate of Ca^{2+}-dependent cell proliferation in HEK cells (15).

In mammary adenocarcinoma tissue, immunohistologic analysis showed a clear enhancement in TRPV6 expression over normal tissue, suggesting that it may play some role in the tumor development (8). Data from epidemiologic studies suggest that higher intake of dietary calcium, which lowers 1,25-vitamin D levels in the blood, reduces the breast cancer risk in premenopausal women (16, 17). Calcium also seems important to breast cancer progression because bone metastases occur in up to 70% of patients with advanced breast cancer (18). The metastatic cells at the bone are thus exposed to increased levels of free extracellular Ca^{2+} released from the mineralized bone matrix.

The most common hormonal treatment for hormone receptor–positive breast adenocarcinoma is the selective

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estrogen receptor modulator tamoxifen. It blocks estrogens from signaling through the estrogen receptor and reduces the growth signals to the cells. The activity of tamoxifen can be observed best in breast tumors that express estrogen receptor and/or progesterone receptor. The highest response rates are observed in tumors expressing both estrogen receptor and progesterone receptor (70%), with lower response rates in estrogen receptor–negative/progesterone receptor–positive tumors (45%) and estrogen receptor–positive/progesterone receptor–negative tumors (34%; refs. 19, 20). The lowest tamoxifen response rates are found in estrogen receptor–negative/progesterone receptor–negative tumors (<10%). However, a portion of the supposed nonresponders may actually be attributable to false-negative assay data (21, 22).

Little is known about the calcium entry mechanisms by which intracellular calcium is regulated in breast cancer cells. Therefore, in this study, we investigated how TRPV6 affects proliferation, apoptosis, and calcium transport in the breast cancer cell line T47D. Additionally, the hormonal effects of estradiol, progesterone, and 1,25-vitamin D on TRPV6 expression were determined in the breast cancer cells. The apparent importance of calcium to breast tumor survival may lead to a therapeutic approach particularly apt for the treatment of breast tumors that are independent of estrogen and progesterone. These late stage tumors are highly metastatic and lethal, and there is currently no effective treatment strategy. Understanding the relationship between calcium uptake induced through TRPV6 and the progression of breast tumor types might unveil TRPV6 as a novel target for anticancer drug development.

Materials and Methods

Materials

T47D cells were obtained from NIH Culture Collection. Plastic six-well culture dishes, 96-well culture dishes, and T75 cultivation flasks were obtained from BD Falcon. RPMI 1640 cell culture medium, fetal bovine serum, trypsin, and penicillin/streptomycin were from Life Technologies. Trizol reagent for RNA isolation was purchased from Invitrogen. TaqMan Universal Master Mix for real-time PCR was from Applied Biosystems. Small interfering RNAs (siRNA) and HiPerFect transfection reagent were obtained from Qiagen. 1α,25-Dihydroxyvitamin D3, β-estradiol, and progesterone were purchased from Sigma. Tamoxifen was obtained from Acros Organics. Human Breast Cancer Rapid-Scan Gene cDNA Panel was purchased from Origene.

Cell Culture

A human breast cancer cell line T47D was used in this study. Tumor cells were grown in T75 culture flasks in RPMI medium supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) in a humidified atmosphere with 5% CO_2. Cells used in these studies were between passages 30 and 40. The cells were split twice a week.

siRNA Treatment

For TRPV6 knockdown, the following siRNAs were used:

siRNA1 (target sequence): CTG CAT GTC AGA GCA CTT TAA
siRNA2 (target sequence): AAC CTG CTG CAG CAG AAG AGG
siRNA control: AAT CAT CTA AGC TGG CTT TGC.

The cells were seeded in 2 mL of culture medium at 400,000 cells per well in six-well plates. After 24 h, siRNA was diluted in phenol red–free medium without fetal bovine serum to give a final siRNA concentration of 5 nmol/L (20 μmol/L stock). Then, HiPerFect was added according to the manufacturer's protocol, and the mixture was incubated for 10 min at room temperature and added drop-wise onto cells. Cells were incubated with siRNAs for 72 h before isolating RNA using the Trizol RNA isolation method according to manufacturer's protocol.

Hormonal Treatments

For hormone treatment, T47D cells were seeded at 400,000 cells per well in six-well dishes and grown for 48 h. Cells were serum-starved for 24 h before treatment. Then, cells were treated in the above-mentioned culture medium without fetal bovine serum for the duration of the studies. After pilot studies to determine appropriate concentrations, 100 nmol/L 1,25-dihydroxyvitamin D (100 μmol/L stock), 10 nmol/L estradiol (10 μmol/L stock), 100 nmol/L progesterone (100 μmol/L stock), and 1 μmol/L tamoxifen (1 mmol/L stock) were all dissolved in ethanol and applied to the cells along with ethanol-only controls for 24 to 72 h. Dose-response studies during a 24-h treatment time were done with estradiol and 1,25-dihydroxyvitamin D. Total RNA was harvested after 24, 48, and 72 h of treatment. Three independent experiments were done with every treatment in triplicate. TRPV6 mRNA expression was determined by real-time PCR.

Real-time PCR

cDNA was prepared for every sample by reverse transcription of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's manual. For all experiments, mRNA expression was measured by real-time PCR using an Applied Biosystems 7500 Real-Time PCR System. Reactions consisted of 1× Mastermix, 0.9 μmol/L forward and reverse primers, and 0.2 μmol/L dual-labeled fluorescent probes each for TRPV6 and β-actin. The sequences of the forward and reverse primers for TRPV6 were 5-GGT TCC TGC GGG TGG AA-3 and 5-CCT GTG CGT AGC GTT GGA T-3 respectively, with the resulting amplicon being 62 bp with a T_m of 60°C. The sequence of the probe for TRPV6 was 5-ACA GGC AAG ATC TCA ACC GGC AGC-3. The sequences of the forward and reverse primers for β-actin were 5-CCT GGC ACC CAG CAC AAT-3 and 5-GCC GAT CCA CAC GGA ATA CT-3, respectively, with the resulting amplicon being 69 bp with a T_m of 60°C. The sequence of the probe for β-actin was 5-ATC AAG ATC ATT GCT CCT CCT GAG CGC-3. The specificity of all primers was

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confirmed by BLAST search. Primer Express (Applied Biosystems) was used for designing primers for TRPV6 and β-actin. All primers were designed to cross exon-exon boundaries of the coding sequence. Primers were optimized and validated for the comparative C_t method, as described in the manufacturer's manual. ABI Prism SDS software version 1 was used for the analysis of the amplification plots. The TRPV6 expression values were normalized to β-actin and then compared. The results are shown as fold change ± SD.

Cell Viability and Apoptosis

Cell viability experiments were carried out in 96-well culture plates with an initial cell number of 2,500 cells per well to determine the influence of different hormones on cell growth. To determine the effect of siRNA on cell viability, the cells were seeded at 5,000 cells per well. After the cells were plated for 24 h, siRNA treatment was done for 72 h with 5 nmol/L siRNA. Before absorbance measurements, plates were kept continuously in cling film during incubation and the wells on the outer edge were not used for measurements. After incubation time, the number of living cells was determined by the Cell Proliferation Kit II (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt; Roche) following the manufacturer's instructions. Briefly, 50 μL of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt labeling mixture (including the electron-coupling reagent *N*-methyl dibenzopyrazine methyl sulfate) were added to each well, and plates were incubated for 4 h. The absorbance was measured by an ELISA reader (V_{max} microplate reader; Molecular Devices) at 450 nm with a reference wavelength at 650 nm.

Apo-one homogenous caspase-3/caspase-7 assay (Promega) was done according to manufacture's protocol. Cells and siRNAs were prepared as described above with the viability assay. The profluorescent substrate was added to the cells for 3 h before measuring the caspase activity on the Flexstation II (485 nm excitation and 527 nm emission). The percentage of apoptotic cells was calculated relative to the untreated control cells.

Ca^{2+} Uptake Activity Measurement

Five thousand cells per well were seeded in a black 96-well plate with 50 μL culture medium per well. After cells have been plated for 24 h, siRNA treatment was done with 5 nmol/L siRNA as described previously. Subsequently, after 72 h, the calcium uptake activity was observed in real time using the FLIPR calcium 3 assay kit (Molecular Devices) according to the manufacturer's manual. Briefly, 50 μL of calcium-free loading buffer were added to the cell medium. Then 100 μL of calcium 3 dye were added and incubated with the cells for 1 h at 37°C. The plate was put into the Flexstation II, EGTA (final, 5 mmol/L) was added to the cells to deplete intracellular calcium stores, and finally, calcium solution (10 mmol/L final) was added to the cells to observe calcium entry. Fluorescence units per second was calculated from the slope of the uptake curve to show the calcium uptake activity after siRNA treatment. Calcium uptake activity mediated by TRPV6 was also done in *Xenopus* oocytes using $^{45}Ca^{2+}$, as done previously (3).

Reverse Transcription – PCR

The Promega Access Reverse Transcription–PCR System was used according to the manufacturer's instructions for detection of calcium channel expression in T47D cells. After an initial heating step at 45°C for 45 min, the reactions were heated for 2 min at 94°C as a denaturing step. Subsequently, the cycle included a 30-s denaturing step, annealing at 60°C for 15 s, and extension reaction for 30 s at 68°C. Twenty-four cycles resulted in optimal linear amplification of products. A final extension was done for 5 min at 68°C. Samples were stored at −20°C before electrophoresis on a 1% agarose gel.

The following primers were used:

TRPC1A:
forward 5-TCTGCCCAAAGGCCATTG-3
reverse5-GGGTATACTACTCTCCTCCATATTTTC-TTC-3
TRPC3:
forward 5-CGGCCGCACGACTATTTC-3
reverse 5-CCAGCCCCTTGTAGGCATT-3
TRPC4:
forward 5-TGGCATGAAATATGGCTCAGTT-3
reverse 5-CGAGAGTTCTGATTCTGCTCTTACTATC-3
TRPC5:
forward 5-TGAGTTCAAGGCCGAGTATGAG-3
reverse 5-TCTCGATGGTTGAGGATGATCTC-3
TRPC6:
forward 5-TTCTCCCATGATGTGACTCCAA-3
reverse 5-GAGGCCGTTCAATCCTAGCA-3.
TRPC7:
forward 5-GGGCATGCTGAATTCCAAA-3
reverse 5-TCTGGTGGGCTTGCTCAAAG-3
TRPV5:
forward 5-GCATTGTCAACTTCGCCTTC-3
reverse 5-GATCATTGTGGTTCTCAACC-3
TRPV6:
forward 5-CCTGTGCGTAGCGTTGGAT-3
reverse 5-GGTTCCTGCGGGTGGAA-3.

Statistical Data Analysis

Statistical significances between treatments and controls were analyzed with Student's *t* test using GraphPad Prism 3.0. Differences in the comparison tests lower than $P < 0.01$ were considered significant.

Results

In vitro TRPV6 knockdown

The effect of TRPV6 knockdown on TRPV6 mRNA expression in T47D breast cancer cells was determined via real-time PCR (Fig. 1A). The TRPV6 mRNA level was significantly reduced in T47D cells after 72 h by two different TRPV6-specific siRNA molecules. The most effective siRNA molecule was siRNA2, which reduced the TRPV6 message by 47%. The siRNA1 had nearly the same effect on TRPV6 expression, subsequently reducing

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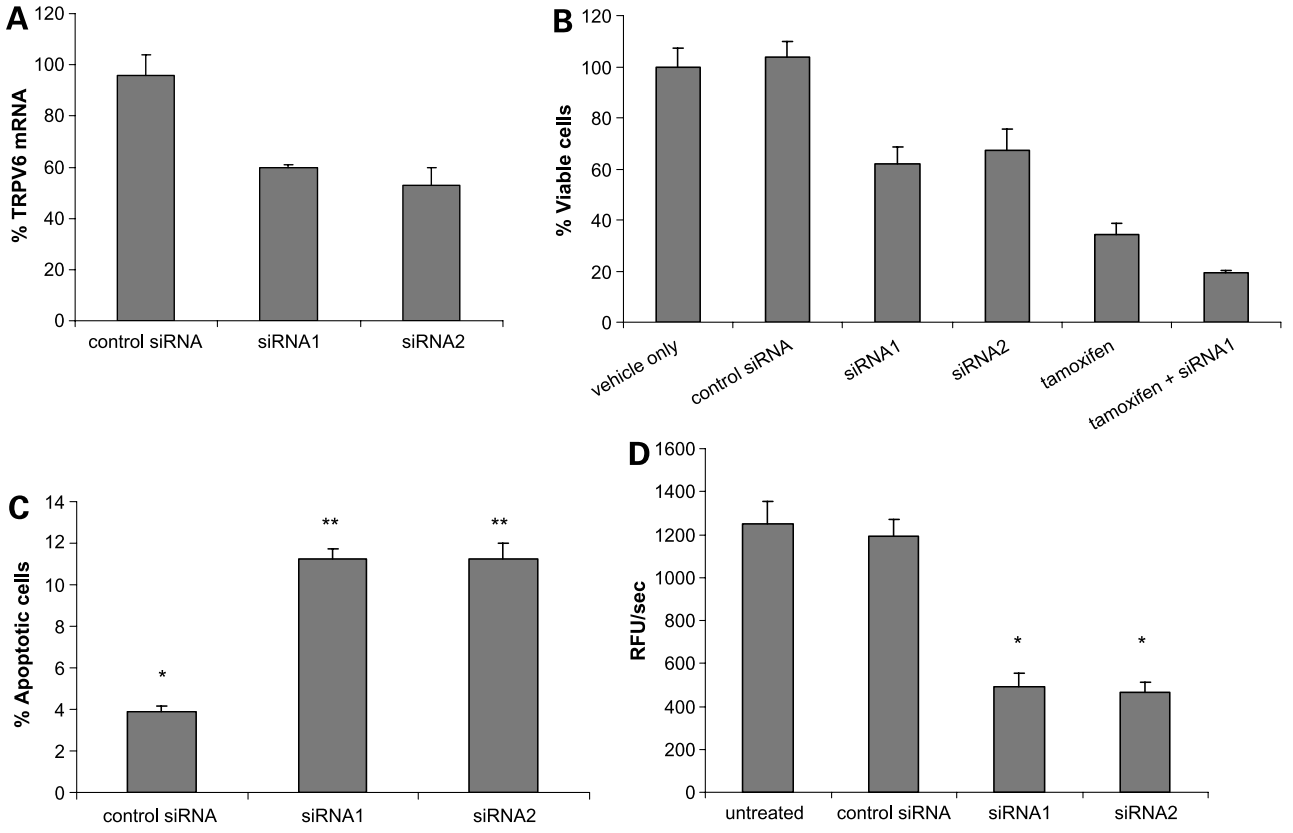

Figure 1. **A,** TRPV6 expression knockdown in T47D breast cancer cells after 72-h treatment with two specific siRNAs (5 nmol/L) and one siRNA control (5 nmol/L) compared with one untreated control. TRPV6 was measured by real-time PCR and standardized via β-actin. **B,** TRPV6 expression knockdown effect on T47D breast cancer cell viability. Determination was done with 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt viability assay 72 h after siRNA (5 nmol/L), tamoxifen (10 μmol/L), or a combination of siRNA (5 nmol/L) and tamoxifen (10 μmol/L) treatment compared with siRNA control or vehicle-only control. All treatments were done in the presence of serum. **C,** apoptosis assay after treatment of T47D breast cancer cells with siRNAs (5 nmol/L) for 72 h. Apoptosis was measured by caspase-3/caspase-7 assay. *, $P < 0.01$ significant difference between untreated cells and control siRNA; **, $P < 0.01$ significant difference between control siRNA and TRPV6 siRNAs (two-tailed *t* test). **D,** calcium uptake activity measured by fluorescence-based FLIPR calcium 3 assay after 72 h of TRPV6 knockdown with siRNAs (5 nmol/L). Calcium uptake activity is shown in fluorescent units per second measured as the slope of the uptake rates. *, $P < 0.01$ significant difference between control siRNA and TRPV6 siRNAs (two-tailed *t* test). *Columns,* mean; *bars,* SD ($n = 3$).

the mRNA by 40%. The control siRNA did not significantly change TRPV6 expression and was comparable with untreated controls.

After the treatment of T47D cells for 72 h in 96-well plates with both siRNAs, the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt viability assay was done. Figure 1B shows the percentage of growth reduction, measured as viable cells, of siRNA-treated cells compared with control-treated ones. The knockdown of TRPV6 led to a decrease in cell proliferation compared with siRNA-treated controls to 62% and 67%, respectively. There was no effect of the control siRNA treatment on the growth of the T47D cells. The antiestrogen tamoxifen (10 μmol/L) comparatively reduced the number of viable cells to 34% compared with vehicle-only treated cells. Dosing the cells with a combination of tamoxifen (10 μmol/L) and siRNA1 (5 nmol/L), in the presence of serum, diminished the number of viable cells even further (19%), demonstrating more effectiveness than either treatment alone.

The treatment of T47D cells with siRNA did not induce a large amount of apoptosis, as indicated by caspase-3/caspase-7 assay (Fig. 1C). Knocking down TRPV6 expression slightly increased the amount of apoptotic cells by 7% compared with control siRNA. Both siRNAs had similar effects on apoptosis. The treatment of cells with control siRNA produced a minimal increase in apoptotic cells, 4% more than the untreated control.

The reduced calcium channel activity after TRPV6 knockdown shows that siRNA treatment affects the function of the channel and illustrates the importance of the channel in this cell line. The knockdown led to a decrease in calcium uptake activity to 47% for siRNA1 and 48% for siRNA2 compared with control. The uptake activity was measured in real-time and displayed in fluorescent units per second (Fig. 1D).

TRPV6 Expression in Breast Cancer

To investigate the levels of TRPV6 expression in breast cancer tissues, a commercial cDNA panel was used to

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quantitate expression by real-time PCR. Twelve cDNA samples from breast cancer tissue and 11 samples from normal mammary tissue were included in the analysis. The majority of samples originated from invasive ductal carcinoma tissue (n = 9), whereas sample 3 (invasive lobular carcinoma), sample 5 (invasive mixed tubular carcinoma), and sample 9 (adenoid cystic carcinoma) were the only exceptions. The TRPV6 expression from the 11 normal mammary tissues was averaged and used to compare expression from individual carcinoma tissue samples. Thus, the data in Fig. 2 is represented as fold difference over normal. From this analysis, TRPV6 mRNA expression in 7 of 12 patients was detected to be up-regulated between 2-fold and 15-fold over the average in normal breast tissue (Fig. 2). In eight samples, TRPV6 transcript levels were up-regulated on average at 4.6-fold compared with the normal tissue average. Furthermore, the coefficient of variance of TRPV6 expression in normal breast tissue was 8% (n = 11). TRPV6 expression in the breast cancer samples was over twice as variable as in normal tissue, showing a coefficient of variance of 17% (n = 12).

In vitro Hormone Effects on TRPV6 Expression

The most common treatments for breast tumors are hormonal treatments that act on the estrogen receptor to control breast cancer cell growth. Therefore, we investigated whether estrogen and progesterone could regulate the expression of TRPV6. The influence of these two hormones and controls on TRPV6 mRNA expression was determined via real-time PCR. T47D cells were treated with different concentrations of estradiol, progesterone, and 1α,25-dihydroxyvitamin D3. Additionally, tamoxifen, an estradiol receptor antagonist which is used in breast cancer therapy, and 5-fluorouracil, a compound used in several cancer therapies, were assessed for their affects on TRPV6 transcription regulation.

Dose and time experiments were done to determine adequate concentration and treatment times. Figure 3 shows the TRPV6 mRNA expression compared with vehicle-treated controls after 24-h, 48-h, and 72-h treatments with estradiol (10 nmol/L), progesterone (100 nmol/L), a combination of both estradiol and progesterone, 1α,25-dihydroxyvitamin D (100 nmol/L), tamoxifen (1 μmol/L), and 5-fluorouracil (1 μmol/L). After 24 h, the effect of progesterone on TRPV6 expression was only slightly different compared with vehicle-treated control. A time-dependent increase in expression was seen with estradiol after 24 h, wherein, after 72 h, the TRPV6 mRNA increased 69%. Treatment with progesterone also had a mild effect on increasing TRPV6 expression up to 56% after 72 h. Both hormones applied together stimulated induction of TRPV6 levels 96% over control when cells were treated for 72 h.

The estrogen receptor antagonist tamoxifen inversely affected TRPV6 expression at all time points tested. Tamoxifen moderately reduced the TRPV6 mRNA by 31% at 24 h and was slightly less effective after 48 and 72 h when used at 1 μmol/L. A log dose-response study was done with 24 h of treatment time from which it was evident that reducing the tamoxifen concentration lessens the effect on TRPV6 mRNA (Fig. 4A). Dosing the cells with 10 μmol/L tamoxifen reduced TRPV6 mRNA by 35%. The highest dose tried with tamoxifen was 50 μmol/L; however, the cells became apoptotic. At 100 nmol/L, tamoxifen only slightly reduced (12%) the TRPV6 expression level.

The hormone 1α,25-dihydroxyvitamin D (100 nmol/L) was on its own far more potent at stimulating TRPV6 mRNA expression (114%) when the T47D cells were treated for 24 h (Fig. 4B). However, the stimulatory effect seen reduced over time and lead to only 39% induction after a 72-h treatment. A dose-dependent relationship can be seen during a 24-h treatment as seen in Fig. 4B. With log reductions of 1α,25-dihydroxyvitamin D concentration, the stimulation effect nearly disappears at 100 pmol/L (14%).

As a control, 5-fluorouracil was used as another anticancer compound with an estrogen receptor–independent mechanism (Fig. 3). After 24 h with 5-fluorouracil (1 μmol/L), the treatment had no effect on TRPV6 mRNA level. There was a slight up-regulation, 14% and 21%, after 48 and 72 h.

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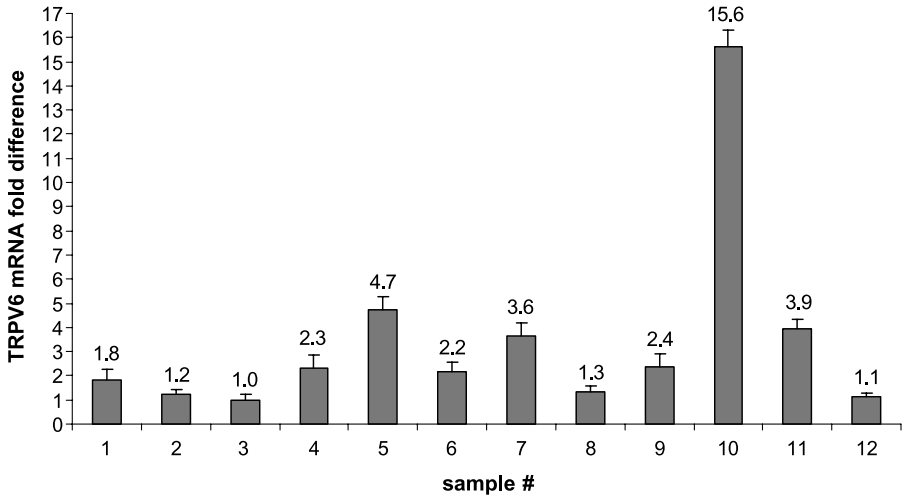

Figure 2. TRPV6 mRNA expression in 12 breast cancer tissue samples compared with the average of 11 normal samples based on real-time PCR. TRPV6 was measured by real-time PCR and standardized via β-actin. The data is represented as fold difference compared with the average expression from 11 normal breast tissue samples. *Columns,* mean; *bars,* SD (n = 6).

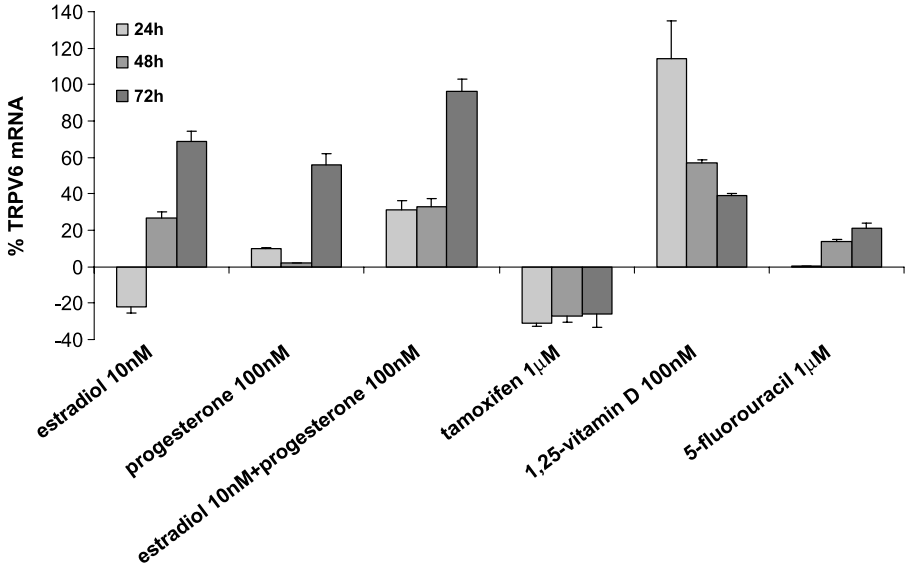

Figure 3. TRPV6 mRNA expression changes after treatment with estradiol (10 nmol/L), progesterone (100 nmol/L), both estradiol and progesterone, 1α,25 dihydroxyvitamin D (100 nmol/L), tamoxifen (1 μmol/L), and 5-flurouracil for 24, 48, and 72 h as determined by real-time PCR. Cells were starved of serum 24 h before treatment and kept without serum for the duration of the study. Data is percentage change compared with vehicle only–treated cells. *Columns,* mean; *bars,* SD (n = 3).

Tamoxifen Effect on TRPV6 Calcium Transport Activity

TRPV6-expressing oocytes were used to probe the inhibition effect of tamoxifen on calcium transport. The drug showed a dose-responsive blocking ability on TRPV6 mediated $^{45}Ca^{2+}$ uptake into oocytes (Fig. 5). Tamoxifen inhibits the channel with an IC_{50} value of 7.5 μmol/L, which suggests that it may have an additionally beneficial mechanism to slow the growth of breast cancer cells. The IC_{50} value was determined using nonlinear data fitting (GraphPad Prism version 3.0).

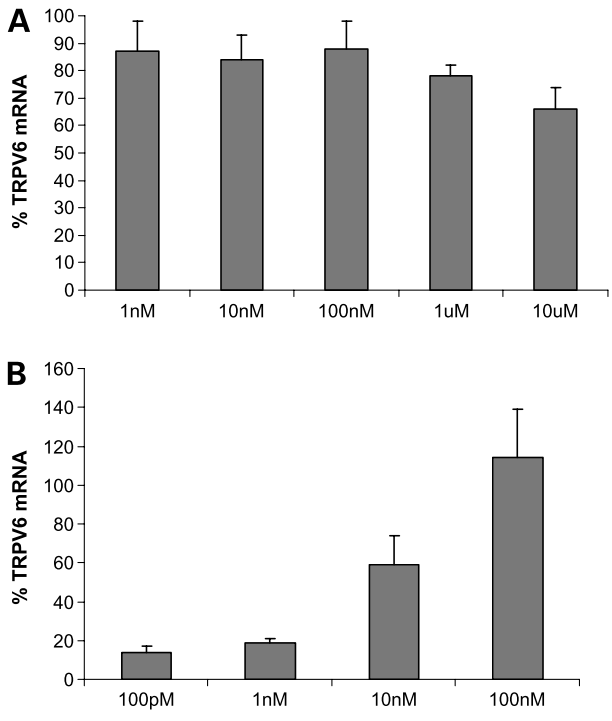

Figure 4. **A,** dose-dependent effect of 1α,25 dihydroxyvitamin D (100 nmol/L, 10 nmol/L, 1 nmol/L, and 100 pmol/L) on TRPV6 mRNA expression in T47D cells after 24 h determined via real-time PCR. Percentage change in TRPV6 mRNA compared with vehicle-only control. **B,** dose-dependent effect of tamoxifen (100 nmol/L, 10 nmol/L, 1 nmol/L, and 100 pmol/L) on TRPV6 mRNA expression in T47D cells after 24 h determined via real-time PCR. *Columns,* mean; *bars,* SD (n = 3).

Calcium Channel Expression in T47D Cells

To investigate the mRNA predominance of TRPV6 in T47D breast cancer cells, the expression of it and expression of other calcium channels in this cell line were determined with reverse transcription–PCR and gel electrophoresis. Figure 6 shows the relative expression levels of the calcium channels: TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, TRPC7, TRPV5, and TRPV6 in T47D cells. From this analysis, TRPC4, TRPC6, and TRPV6 are the most predominately expressed calcium channels on the RNA level.

Discussion

The TRPV6 calcium entry channel has previously been shown to be linked to prostate tumor progression and

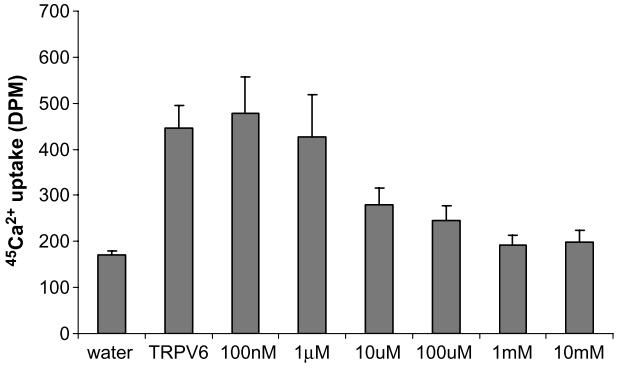

Figure 5. Tamoxifen inhibits TRPV6 calcium transport activity of $^{45}Ca^{2+}$ into *Xenopus* oocytes expressing TRPV6. Log dilutions of tamoxifen were used to inhibit TRPV6-mediated calcium uptake into oocytes. *Columns,* mean; *bars,* SD (n = 6).

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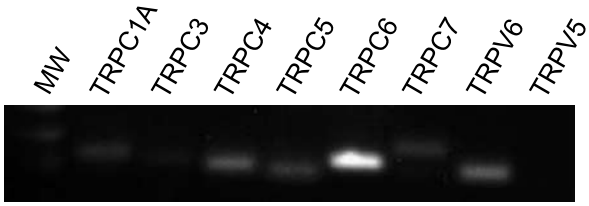

Figure 6. Relative RNA expression levels of calcium channels as determined by semiquantitative reverse transcription–PCR. MW (*lane 1*), TRPC1A (*lane 2*), TRPC3 (*lane 3*), TRPC4 (*lane 4*), TRPC5 (*lane 5*), TRPC6 (*lane 6*), TRPC7 (*lane 7*), TRPV6 (*lane 8*), and TRPV5 (*lane 9*) in T47D cells. Amplicons range in size from 100 to 150 bp.

development. The role of TRPV6 in the progression of tumor cells is not completely clear but seems to involve accumulation of cellular calcium to promote cancer cell growth or survival.

Previously, it has been shown that TRPV6 seemed to be up-regulated on the protein level in breast adenocarcinoma tissue (8). In this report, we further investigated the expression level of TRPV6 in breast cancer tissue from patients compared with normal mammary tissue. In the 12-patient samples analyzed by real-time PCR, eight samples had TRPV6 transcript levels that were up-regulated on average of 4.6-fold compared with the normal tissue average. This further strengthens our earlier observation where we see up-regulation on the protein level (8). There are associated estrogen receptor and progesterone receptor values for each cancer tissue sample (data not shown), but we were unable to find any clear, statistically significant correlation between these values and TRPV6 expression.

We investigated this relationship under more controlled conditions to see how estrogen receptor may regulate TRPV6 in breast cancer cells *in vitro*. When stimulated with estrogen for 24 h, the RNA transcript of TRPV6 was slightly reduced compared with vehicle only–treated cells. Likewise, when prostate cancer cells were treated with dihydrotestosterone for a similar period of time, the transcription of TRPV6 also decreased (7). In contrast, when activated longer with estrogen from 48 to 72 h, the cells increased the transcription of TRPV6 up to 69%. It has long been known that estrogen promotes T47D cell proliferation (23, 24). Long-term up-regulation of TRPV6 seems to be part of this cell growth mechanism. The antiestrogen tamoxifen showed interesting activity upon TRPV6 expression at all time points tested. Blocking the estrogen receptor with tamoxifen caused the TRPV6 transcript levels to reduce. The limited signaling that can occur from estrogen receptor seems to lead to lower TRPV6 expression. And with increasing concentrations of tamoxifen, this effect becomes even more pronounced. These observations suggest that the estrogen receptor is able to regulate TRPV6 expression to possibly promote increased calcium entry into the cells. When used at low concentrations (0.1–1 μmol/L), tamoxifen has been shown to induce G_1 cell cycle arrest in T47D cells, thereby stopping cell proliferation (23, 25). Correspondingly, the T74D cells reduced TRPV6 expression, which acts negatively to inhibit cell growth. Larger doses of tamoxifen (>5 μmol/L) have been reported to induce apoptosis in T47D cells (26). In our studies, we have observed that the activity of tamoxifen was enhanced by knocking down TRPV6 expression. We would expect a stronger enhancement if the expression was further knocked down. However, affecting the channel activity may be done more effectively through designing TRPV6-specific chemical inhibitors. These studies show that a combination therapy using tamoxifen and a TRPV6 inhibitor would be a beneficial approach to treating breast cancer cells.

Estrogen is known to induce increased calcium absorption via up-regulating TRPV6 in female mouse intestine via a vitamin D–independent mechanism (6). During pregnancy, the increased calcium absorption is needed to compensate for increased nutritional demands. Likewise, 1,25-vitamin D is also a known TRPV6 stimulator, as shown in this study and previous studies (27). At low concentrations (0.05–0.8 nmol/L), vitamin D promotes growth (28), whereas increasingly higher concentrations cause cell cycle block in G_1 phase and subsequent apoptosis (29, 30). In our experiments, we see that TRPV6 expression reduces significantly, after 24 h after 1,25-vitamin D (100 nmol/L) treatment. Thus, this effect seems to be in coordination with the T47D cell reducing its proliferation.

Additionally, we observed that tamoxifen cannot only down-regulate TRPV6 mRNA expression, but it can directly inhibit the calcium channel activity. We observed this effect when we expressed TRPV6 in *Xenopus* oocytes, giving an IC_{50} value of nearly 7.5 μmol/L. This is intriguing, given the fact that it is an estrogen receptor inhibitor. However, it does share several structural similarities with known T-type calcium channel inhibitors, such as a tertiary nitrogen, aromatic groups, and an ether bond. In support of this, it can be found in the literature that both tamoxifen and raloxifene are able to affect calcium channel activity. In whole-cell patch clamp studies in vascular smooth muscle cells, tamoxifen reduced the current through L-type calcium channels with an ID_{50} of 2 μmol/L and through T-type channels with 10 μmol/L (31). The antiestrogen SERM, raloxifene, is also known to affect voltage sensitive L-type calcium channel activity (32–34).

Tamoxifen is also known to be effective in ~10% estrogen receptor–negative and progesterone receptor–negative patients (19, 20). This might suggest that tamoxifen is directly acting on the TRPV6 calcium channel in those patients. Therapeutic concentration of tamoxifen in the breast cancer tissue can reach low micromolar values (35). Therefore, IC_{50} concentration for TRPV6 inhibition measured in oocytes is in a similar range to what has been seen in tumor tissues and, thus, may be clinically relevant in the affect of the drug. Regardless, it would be interesting to collect data on TRPV6 expression along with receptor values when assessing the effectiveness of tamoxifen in patients. More specific TRPV6 inhibitors could be used to regulate the growth of breast tumor cells expressing TRPV6.

In the T47D breast cancer cells, TRPV6 is one of several potential calcium entry channels expressed, but TRPV6

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is the only calcium-specific entry channel present. The importance of TRPV6 could be seen after siRNA knockdown studies, because the proliferation rate of the cancer cells was reduced by 60% and calcium transport was reduced by 50%. Importantly, the siRNA knockdown of TRPV6 was not complete in these experiments so the full influence in these cells could not be fully assessed. There are two other potential calcium permeable channels, TRPC4 and TRPC6, which could also be contributing to the calcium uptake into these cells. However, these channels are receptor-operated, nonselective cation channels (36). Specifically, TRPC6 behaves as a nonselective cation channel that is activated by diacylglycerol in a membrane-delimited fashion, independently of protein kinase C (37). The TRPC6 channel is not considered to be store-operated and only has limited Ca^{2+} permeability relative to monovalent cation permeability (38). We cannot rule out their contributions at this time, but our results indicate that TRPV6 has a major influence on cellular calcium entry.

The breast cancer cells used in this study were slowed by reduced calcium channel expression and lower calcium uptake potential. The stable expression of TRPV6 in the kidney cell line HEK-293 promoted increased cellular proliferation and calcium accumulation (15). Clearly, the calcium state inside cells can influence their proliferation rate. Furthermore, previous studies in prostate cancer cell lines indicate that TRPV6 plays a significant role in prostate cancer calcium influx (39, 40).

In T47D cancer cells, TRPV6 also contributes a rather significant portion of the calcium entry activity and leads to a large reduction (~60%) in the proliferative ability of the cells. Furthermore, it was found that treatment with TRPV6-specific siRNAs produced a small increase (~7%) in apoptosis in the breast cancer cells, as indicated by a caspase-3/caspase-7 assay. Therefore, the net result indicates that the cancer cells are growing less rapidly rather than dying from reduced calcium uptake. Silencing TRPV6 in the breast cancer cells may be causing cell cycle arrest, which may explain the less rapid cell proliferation. We have not provided direct evidence for this, but it has been shown that TRPV6 silencing in the prostate cancer cell line LNCaP results in a reduced number of cells entering S phase (39). Similar to our study, the LNCaP cells show a higher rate (~15%) of apoptosis after specific TRPV6 siRNA knockdown (39). From their study in LNCaP, it was suggested that calcium entry via TRPV6 not only maintains an increased proliferation rate but increases cellular survival and provides resistance to apoptosis. Here, we see similar results; however, in T47D cells, TRPV6 seems to provide less resistance to apoptosis. This may be due to the presence of TRPC4, TRPC6, or other entry channels permeable to calcium or insufficient TRPV6 knockdown. Therapeutic treatments aimed at inhibiting the TRPV6 channel would thus keep the cancer cells from rapidly growing. For instance, it has been shown that calcium channel inhibitors that likely block TRPV6 are able to restrict the growth of prostate cancer cells in a cytostatic manner and, when given to mice, show no toxicity (41).

In this study, we have shown that TRPV6 is expressed at elevated messenger levels in breast cancer patient samples. In our *in vitro* model, estrogen, progesterone, and 1,25-vitamin D were all able to regulate TRPV6 transcription. Interestingly, tamoxifen acts negatively on two levels to reduce both the expression and activity of TRPV6. This suggests that TRPV6 might be involved in the antiproliferative activity of the widely used breast cancer treatment tamoxifen. However, further clinical data would be needed to address this hypothesis. Regardless, TRPV6 seems be able to control the proliferative ability of T47D breast adenocarcinoma cells. The activity of tamoxifen was enhanced by combination treatment with TRPV6 silencing siRNA. Our studies indicate that TRPV6 may be suitable target for development of specific chemical inhibitors. Furthermore, when hormonal therapies are no longer effective, inhibitors for TRPV6 may be of increased value, particularly when the tumors become estrogen receptor–negative or progesterone receptor–negative.

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References

1. Clapham DE. Calcium signaling. Cell 1995;80:259 – 68.
2. Berridge MJ, Bootman MD, Lipp P. Calcium-a life and death signal. Nature 1998;395:645 – 8.
3. Peng JB, Chen XZ, Berger UV, et al. Molecular cloning and characterization of a channel-like transporter mediating intestinal calcium absorption. J Biol Chem 1999;274:22739 – 46.
4. Bianco SD, Peng JB, Takanaga H, et al. Marked disturbance of calcium homeostasis in mice with targeted disruption of the Trpv6 calcium channel gene. J Bone Miner Res 2007;22:274 – 85.
5. Song Y, Peng X, Porta A, et al. Calcium transporter 1 and epithelial calcium channel messenger ribonucleic acid are differentially regulated by 1,25 dihydroxyvitamin D3 in the intestine and kidney of mice. Endocrinology 2003;144:3885 – 94.
6. Van Cromphaut SJ, Rummens K, Stockmans I, et al. Intestinal calcium transporter genes are upregulated by estrogens and the reproductive cycle through vitamin D receptor-independent mechanisms. J Bone Miner Res 2003;18:1725 – 36.
7. Peng JB, Zhuang L, Berger UV, et al. CaT1 expression correlates with tumor grade in prostate cancer. Biochem Biophys Res Commun 2001;282:729 – 34.
8. Zhuang L, Peng JB, Tou L, et al. Calcium-selective ion channel, CaT1, is apically localized in gastrointestinal tract epithelia and is aberrantly expressed in human malignancies. Lab Invest 2002;82:1755 – 64.
9. Fixemer T, Wissenbach U, Flockerzi V, Bonkhoff H. Expression of the Ca2+-selective cation channel TRPV6 in human prostate cancer: a novel prognostic marker for tumor progression. Oncogene 2003;22:7858 – 61.
10. Vanden Abeele F, Skryma R, Shuba Y, et al. Bcl-2-dependent modulation of Ca(2+) homeostasis and store-operated channels in prostate cancer cells. Cancer Cell 2002;1:169 – 79.
11. Skryma R, Mariot P, Bourhis XL, et al. Store depletion and store-operated Ca2+ current in human prostate cancer LNCaP cells: involvement in apoptosis. J Physiol 2000;527 Pt 1:71 – 83.
12. Legrand G, Humez S, Slomianny C, et al. Ca2+ pools and cell growth. Evidence for sarcoendoplasmic Ca2+-ATPases 2B involvement in human prostate cancer cell growth control. J Biol Chem 2001;276:47608 – 14.
13. Tombal B, Weeraratna AT, Denmeade SR, Isaacs JT. Thapsigargin induces a calmodulin/calcineurin-dependent apoptotic cascade responsible for the death of prostatic cancer cells. Prostate 2000;43:303 – 17.
14. Wertz IE, Dixit VM. Characterization of calcium release-activated apoptosis of LNCaP prostate cancer cells. J Biol Chem 2000;275:11470 – 7.
15. Schwarz EC, Wissenbach U, Niemeyer BA, et al. TRPV6 potentiates calcium-dependent cell proliferation. Cell Calcium 2006;39:163 – 73.
16. Shin MH, Holmes MD, Hankinson SE, Wu K, Colditz GA, Willett WC. Intake of dairy products, calcium, and vitamin d and risk of breast cancer. J Natl Cancer Inst 2002;94:1301 – 11.

17. Berube S, Diorio C, Masse B, et al. Vitamin D and calcium intakes from food or supplements and mammographic breast density. Cancer Epidemiol Biomarkers Prev 2005;14:1653 – 9.

18. Coleman RE, Rubens RD. The clinical course of bone metastases from breast cancer. Br J Cancer 1987;55:61 – 6.

19. McGuire WL, Clark GM. Prognostic factors for recurrence and survival in axillary node-negative breast cancer. J Steroid Biochem 1989;34: 145 – 8.

20. Clarke R, Leonessa F, Welch JN, Skaar TC. Cellular and molecular pharmacology of antiestrogen action and resistance. Pharmacol Rev 2001; 53:25 – 71.

21. Fisher B, Jeong JH, Dignam J, et al. Findings from recent National Surgical Adjuvant Breast and Bowel Project adjuvant studies in stage I breast cancer. J Natl Cancer Inst Monogr 2001;30:62 – 6.

22. Rhodes DJ, Hartmann LC, Perez EA. Breast cancer prevention trials. Curr Oncol Rep 2000;2:558 – 65.

23. Chalbos D, Vignon F, Keydar I, Rochefort H. Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). J Clin Endocrinol Metab 1982;55:276 – 83.

24. Papendorp JT, Schatz RW, Soto AM, Sonnenschein C. On the role of 17 α-estradiol and 17 β-estradiol in the proliferation of MCF7 and T47D-A11 human breast tumor cells. J Cell Physiol 1985;125:591 – 5.

25. Jones JL, Daley BJ, Enderson BL, Zhou JR, Karlstad MD. Genistein inhibits tamoxifen effects on cell proliferation and cell cycle arrest in T47D breast cancer cells. Am Surg 2002;68:575 – 7; discussion 7 – 8.

26. Zheng A, Kallio A, Harkonen P. Tamoxifen-induced rapid death of MCF-7 breast cancer cells is mediated via extracellularly signal-regulated kinase signaling and can be abrogated by estrogen. Endocrinology 2007; 148:2764 – 77.

27. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, et al. Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. Proc Natl Acad Sci U S A 2001;98:13324 – 9.

28. Freake HC, Marcocci C, Iwasaki J, MacIntyre I. 1,25-dihydroxyvitamin D3 specifically binds to a human breast cancer cell line (T47D) and stimulates growth. Biochem Biophys Res Commun 1981;101:1131 – 8.

29. Eisman JA, Sutherland RL, McMenemy ML, Fragonas JC, Musgrove EA, Pang GY. Effects of 1,25-dihydroxyvitamin D3 on cell-cycle kinetics of T 47D human breast cancer cells. J Cell Physiol 1989;138:611 – 6.

30. Mathiasen IS, Lademann U, Jaattela M. Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. Cancer Res 1999;59:4848 – 56.

31. Song J, Standley PR, Zhang F, et al. Tamoxifen (estrogen antagonist) inhibits voltage-gated calcium current and contractility in vascular smooth muscle from rats. J Pharmacol Exp Ther 1996;277:1444 – 53.

32. Tsang SY, Yao X, Essin K, et al. Raloxifene relaxes rat cerebral arteries *in vitro* and inhibits L-type voltage-sensitive Ca2+ channels. Stroke 2004;35:1709 – 14.

33. Chan YC, Leung FP, Yao X, Lau CW, Vanhoutte PM, Huang Y. Raloxifene relaxes rat pulmonary arteries and veins: roles of gender, endothelium, and antagonism of Ca2 + influx. J Pharmacol Exp Ther 2005;312:1266 – 71.

34. Liew R, Stagg MA, MacLeod KT, Collins P. Raloxifene acutely suppresses ventricular myocyte contractility through inhibition of the L-type calcium current. Br J Pharmacol 2004;142:89 – 96.

35. Kisanga ER, Gjerde J, Guerrieri-Gonzaga A, et al. Tamoxifen and metabolite concentrations in serum and breast cancer tissue during three dose regimens in a randomized preoperative trial. Clin Cancer Res 2004;10:2336 – 43.

36. Bodding M. TRP proteins and cancer. Cell Signal 2007;19:617 – 24.

37. Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G. Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature 1999;397:259 – 63.

38. Estacion M, Sinkins WG, Jones SW, Applegate MA, Schilling WP. Human TRPC6 expressed in HEK 293 cells forms non-selective cation channels with limited Ca2 + permeability. J Physiol 2006;572:359 – 77.

39. Lehen'kyi V, Flourakis M, Skryma R, Prevarskaya N. TRPV6 channel controls prostate cancer cell proliferation via Ca(2 +)/NFAT-dependent pathways. Oncogene 2007;26:7380 – 5.

40. Kahr H, Schindl R, Fritsch R, et al. CaT1 knock-down strategies fail to affect CRAC channels in mucosal-type mast cells. J Physiol 2004;557: 121 – 32.

41. Haverstick DM, Heady TN, Macdonald TL, Gray LS. Inhibition of human prostate cancer proliferation *in vitro* and in a mouse model by a compound synthesized to block Ca2 + entry. Cancer Res 2000;60:1002 – 8.

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