ORAI-Mediated Calcium Influx in Lactation and in Breast Cancer

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Abstract

The entry of calcium into the mammary epithelial cell from the maternal plasma (i.e., calcium influx mechanisms) during lactation is poorly understood. As alterations in calcium channels and pumps are a key feature of some cancers, including breast cancer, understanding these calcium influx pathways may have significance beyond mammary biology. We show that the store-operated calcium influx protein, Orai1, is increased during lactation whereas the Orai1 activator Stim1, but not Stim2, is downregulated. Stim2 siRNA reduced basal calcium levels in a lactation model. Our results suggest that calcium influx is remodeled in mammary epithelial cells during lactation, with calcium influx increased through Orai1, activated by Stim2. Breast cancer cell lines had increased levels of ORAI1. ORAI1 siRNA in breast cancer cells reduced store-operated calcium entry and remodeled the calcium influx associated with invasive stimuli. Analysis of microarray data from 295 breast cancers showed that the transcriptional breast cancer subtype with the poorest prognosis (basal) was associated with an altered relationship between the ORAI1 regulators STIM1 and STIM2, and that women with breast cancers with STIM1 high/STIM2 low tumors had a significantly poorer prognosis. Our studies show that during lactation there is a remodeling in the nature of calcium influx and that alteration in the ORAI1 influx pathway may be a feature of some breast cancers, particularly those with the poorest prognosis. Our studies suggest that this pathway may be a novel therapeutic target for breast cancer treatment in these women.

Mol Cancer Ther; 10(3); 448–60. ©2011 AACR.

Introduction

Ca2+ has a vital role in regulating diverse processes, such as gene transcription, secretion, and contraction (1). The transport of calcium is also critical in lactation, where calcium is a key requirement for neonatal nutrition (2). Large quantities of calcium are transported from the maternal bloodstream into milk (3), which requires specialized transcellular pathways (2). Initially Ca2+ must cross the basolateral membrane of mammary gland epithelial cells, before being transported across the epithelial cell, and into the lumen of the mammary alveoli (2). Although over recent years our understanding of the specific calcium ATPase (calcium pump) isoforms involved in the secretory pathway and apical transport of Ca2+ during lactation has increased, little is known about the channels involved in the transport of Ca2+ into mammary epithelial cells during lactation. Regulation of these calcium channels is important as the epithelial cell must ensure that most of the Ca2+ entering via these channels is functionally inert (i.e., the calcium ions are passengers rather than signaling effectors), to maintain normal cellular homeostasis and avoid aberrant cell proliferation and/or cell death signaling. Transport of Ca2+ across the plasma membrane into sections of calcium stores close to the plasma membrane is one way this could be achieved.

Calcium channels expressed in mammary gland epithelial cells may have significance beyond calcium enrichment of milk, as loss of normal Ca2+ homeostasis can produce subtle alterations in cellular functions, which may contribute to diseases, including cancer (4, 5). Indeed, multiple Ca2+ channels are deregulated in various cancers, including breast cancer (4).

The recently identified ORAI family of calcium channels has properties that make this class of calcium channel a potential influx mechanism for calcium during lactation and in tumorigenic pathways in breast cancer cells (5, 6). There are 3 ORAI calcium channels in humans (ORAI1–3) (7, 8), with ORAI1 the most extensively characterized. The ORAI1 protein forms the pore subunit of the calcium influx pathway, which operates in response to endoplasmic reticulum Ca2+ depletion (9, 10), and this pathway is often referred to as store-operated calcium influx. The endoplasmic reticulum Ca2+ sensor, STIM1 is essential to the activation of this calcium influx pathway, as it responds to declines in endoplasmic reticulum store...
Ca\(^{2+}\) by aggregating and interacting with apposed plasma membrane ORAI proteins, causing ORAI1 to open (11).

The ORAI family of calcium channels, and their classic regulators STIM1 and STIM2 (12), have not been studied as potential mechanisms for calcium influx during lactation despite their association with the regulation of calcium stores. Recently, ORAI1-mediated calcium influx was linked to processes important in breast cancer metastasis (13). Although store-operated calcium entry is implicated in cancer cell proliferation and the regulation of calcium-regulated transcription factors, no studies have assessed components of store-operated calcium influx in the context of breast cancer molecular subtypes, or disease prognosis.

In this study, we addressed the hypothesis that components of the ORAI1-mediated calcium influx pathway are upregulated during lactation and contribute to calcium influx in mammary epithelial cells. We also addressed the hypothesis that specific components of the ORAI1-mediated calcium influx pathway are altered in some breast cancer cells and may be predictors of prognosis. Our data indicate that components of the ORAI1-mediated calcium influx pathway contribute to a novel mechanism of calcium influx in mammary epithelial cells during lactation and that this pathway is significant in breast cancer.

**Materials and Methods**

**Animals**

Mammary gland from nulliparous animals was obtained from CBA × C37Bl6 mice at 14 weeks, approximately day 10 of pregnancy, day 1 of lactation, and involution approximately day 2 postforced weaning (14).

**Cell culture**

MCF-7 and MDA-MB-231 cell lines were originally obtained from ATCC and were maintained DMEM (Sigma Aldrich) with Penicillin G (100 U/mL; Invitrogen), streptomycin sulfate (100 µg/mL; Invitrogen), 10% fetal bovine serum (FBS; Sigma Aldrich), and 4 mmol/L L-glutamine (Invitrogen). The HC11 mouse mammary cell line was obtained from Dr. Nancy Hynes, Friedrich Miescher Institute for Biomedical Research, Switzerland (16) and maintained in RPMI 1640 (Invitrogen) with Penicillin G (100 U/mL), streptomycin sulfate (100 µg/mL), 10% FBS, 4 mmol/L L-glutamine, and 5 µg/mL bovine insulin (Sigma Aldrich). HC11 cells were differentiated by plating cells at 1.5 × 10^5 cells/well in a 6-well plate in the presence of murine epidermal growth factor (10 ng/mL; EGF; Sigma Aldrich), then withdrawing EGF 48 hours postplating and adding dexamethasone (1 µmol/L; Sigma Aldrich) and ovine prolactin (5 µg/mL; Sigma Aldrich) 72 hours postplating. Cell lines were routinely (6 monthly) tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Assay (Lonza, Inc.) and monitored for morphological characteristics. Where appropriate cell line characterization was carried out through assessment of the levels of ERBB2, estrogen receptor, vimentin, and E-cadherin using real time RT-PCR and/or immunohistochemistry.

**RNA isolation and real-time PCR**

RNA was isolated from the mouse mammary glands as described in Faddy and colleagues (14) and from the cell lines using the RNeasy Plus Mini Kit (Qiagen). RNA for ORAI comparative studies had been isolated previously as described (15). RNA was reverse transcribed using the Omniscript RT Kit (Qiagen) followed by real-time PCR using TaqMan gene expression assays (Applied Biosystems) and TaqMan Universal PCR Master Mix. Assays included mouse Orai1, Orai2, and Orai3 (Mm00774349_m1, Mm01207170_m1, Mm01276333_m1), human ORAI1, ORAI2, and ORAI3 (Hs00385627_m1, Hs00259863_m1, Hs00743683_s1), mouse Trpc1 (Mm00441975_m1), mouse Stim1 and Stim2 (Mm00486423_m1, Mm01223102_m1) and human STIM1 and STIM2 (Hs00162394_m1, Mm01223102_m1), mouse ß-casein (Mm00839664_m1), and the endogenous control 18S rRNA. Thermal cycling was carried out using a ABI PRISM 7500 Sequence Detector, at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. For all experiments mRNA levels were normalized to 18S rRNA and analyzed relative to the appropriate control (as described in the figure legends). Relative expression levels were calculated using the ΔΔCt method (17).

**siRNA**

All siRNAs used were Dharmacon ON-TARGETplus SMARTpools, which consist of a pool of 4 rationally designed siRNA sequences for each target gene. To reduce off-target effects, these siRNAs have dual strand modification (18) and use an algorithm to minimize seed region matches (19). The Dharmafect 4 transfection reagent was used at final concentration of 0.1–0.2 µL per well (100 µL total volume) for MCF-7 and HC11 cells and 0.05 µL per well for MDA-MB-231 cells. The following pooled siRNAs were used at a final concentration of 100 nmol/L: human ORAI1 (L-014998–00), human STIM1 human (L-011785–00), human STIM2 (L-013166–01), mouse Stim2 (L-055069–01), and pooled nontargeting siRNA (D-001810–10-05). For mock transfection, an equivalent volume of siRNA buffer replaced the pooled siRNAs. For siRNA knockdown experiments in MCF-7 and MDA-MB-231 cells, cells were plated in antibiotic-free media at 2–9 × 10^6 and 1–3 × 10^6 cells/well, respectively, into 96-well plates and siRNA added 24 hours postplating, at which time, FBS concentration was changed from 10% to 1%. For HC11 cells, cells were plated in antibiotic-free media at 3.5 × 10^6 cells/well and siRNA added 48 hours postplating in the absence of insulin and EGF. At 54 hours postplating, dexamethasone, insulin, and prolactin were added to the cells. For experiments using siRNA treated cells, mRNA knockdown of the target was confirmed 48–72 hours posttransfection.

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www.aacrjournals.org Mol Cancer Ther; 10(3) March 2011 449

Published OnlineFirst January 11, 2011; DOI: 10.1158/1535-7163.MCT-10-0923

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Experiments were only carried out when greater than 80% knockdown of the target was achieved relative to the nontargeting siRNA control.

**MTS assay**

Cell viability was determined 96 hours after siRNA treatment or 72 hours after incubation in the presence of 2-aminoethoxydiphenyl borate (2-ABP; Sigma Aldrich) using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay; Promega; ref. 20). For viability assays, cells were plated at 1 x 10^4 and 5 x 10^3 cells/well for MCF-7 cells and MDA-MB-231 cells, respectively, in 96-well plates.

**EdU incorporation**

The percentage of cells in S-phase was determined in MDA-MB-231 cells 96 hours after siRNA treatment. MDA-MB-231 cells were plated at 2.5 x 10^3 cells/well in 96-well BD Falcon microplates (BD Biosciences) and transfected with the appropriate siRNA as described above with 0.1 μL Dharmacompe 4 per well. After 24 hours, transfection medium was replaced with serum-free DMEM for 48 hours. Cells were then treated with EdU (10 μM) supplemented with 8% serum for 16 hours or 20 hours. DMEM was replaced with serum-free transfection medium was added to the appropriate siRNA. After 72 hours, cells were loaded with FBS and L-glutamine and then transfected with the appropriate siRNA. 72 hours later, cells were incubated for 15 minutes at room temperature, and then the loading solution was removed and replaced with PSS containing nominal calcium with PBB Signal Enhancer solution. BAPTA (500 μmol/l; Invitrogen) was added followed by cyclopiazonic acid (10 μmol/l; CPA; Sigma Aldrich) and/or trypsin (100 nmol/l; Sigma Aldrich). After depletion of endoplasmic calcium, extracellular calcium was added. Fluorescence was determined at 470–495 excitation and 515–575 emission.

**STIM1 and STIM2 analysis in human breast tumors**

A gene expression dataset consisting of the microarray profiles of 295 primary human breast tumors (21) was obtained from Rosetta Inpharmatics. Tumors were assigned to transcriptional subtypes based on their gene expression profiles [luminal A (n = 88), luminal B (n = 81), normal-like (n = 31), basal-like (n = 46), and ERBB2+ (n = 49)] as described (22). One probe on the array (annotated as NM_003156) corresponded to STIM1 and 1 probe (annotated as KIAA1482) corresponded to STIM2. Tumors were divided into STIM1 or STIM2 high and low groups based on the median expression level of each gene in the cohort and chi-squared analysis was used to determine whether the balance of STIM1/2 differed significantly for any transcriptional subtype. Survival curves were computed using the method of Kaplan and Meier and statistical significance was evaluated using the log-rank test.

**Statistical analysis**

Statistical significance was assessed as described in the individual figure legends. Data analysis was done using Prism version 5.02 for Windows (Graphpad Inc).

**Results**

**Orai1 calcium channels and mammary gland development**

Mammary gland development during pregnancy, lactation, and involution is associated with alterations in the transcription of specific calcium ATPase isoforms, more specifically the pronounced upregulation of the secretory calcium ATPase isoform, Spca2, and the plasma membrane Ca^{2+}-ATPase isoform, PMCA2 (14, 23, 24). In this study, we assessed mRNA levels of calcium channels Orai1, Orai2, and Orai3 and Trpc1 during mammary gland development. We show that mouse mammary gland development is associated with a significant upregulation of Orai1 calcium channels during lactation, with this upregulation reversed during involution (Fig. 1A). In contrast, the related isoforms Orai2 and Orai3 were associated with a decline in levels during mammary gland development and their expression did not recover during the early stages of involution (Fig. 1B and C). Given the reports of members of the transient receptor

**Measurement of intracellular-free Ca^{2+} ([Ca^{2+}]_i)**

For calcium measurements, HC11 cells were plated at 3.5 x 10^3 cells per well in 96-well CellBIND plates (Corning), differentiated, and then at 120 hours basolateral calcium levels were measured using 4 μmol/l Fura-2 AM (Molecular Probes) in physiological salt solution [PSS; NaCl (140 mmol/l), glucose (11.5 mmol/l), CaCl_2 (1.8 mmol/L), HEPES (10 mmol/l), KCl (5.9 mmol/l), MgCl_2 (1.4mmol/L), NaH_2PO_4 (1.2 mmol/l), NaHCO_3 (5 mmol/l) pH 7.3]. Changes in fluorescence were measured using a NOVOstar fluorescent multiwell plate reader (BMG LABTECH), with 340 nm and 380 nm excitation and 510 nm emission.

Store-operated calcium entry was measured using a FLIPRTETRA (Molecular Devices). Briefly, MDA-MB-231 or MCF7 cells were seeded into 96-well plates (4,000 or 7,500 cells/well, respectively) in antibiotic-free DMEM with FBS and L-glutamine and then transfected with the appropriate siRNA. After 72 hours, cells were loaded with PSS with PBX Signal Enhancer (PBX Calcium Assay Kit; BD Biosciences), Fluo-4 AM (4 μmol/l; Molecular Probes, Invitrogen), and probenecid (1 mmol/l) for 1 hour. Cells were then incubated for 15 minutes at room temperature, and then the loading solution was removed and replaced with PSS containing nominal calcium with PBX Signal Enhancer solution. BAPTA (500 μmol/l; Invitrogen) was added followed by cyclopiazonic acid (10 μmol/l; CPA; Sigma Aldrich) and/or trypsin (100 nmol/l; Sigma Aldrich). After depletion of endoplasmic calcium, extracellular calcium (2 mmol/l) was added. Fluorescence was determined at 470–495 excitation and 515–575 emission.
potential cation (TRPC) channel family contributing to store-operated calcium entry (25, 26), we assessed Trpc1 levels during mammary gland development, and similarly to Orai2 and Orai3, no increase in Trpc1 was seen in lactation (Fig. 1D). There were lower levels of Trpc1 during lactation and the early stages of involution (Fig. 1D). Hence, a significant change in the transcription of calcium channels is associated with lactation, with pronounced upregulation of Orai1 in vivo. We also saw that in the HC11 mouse mammary epithelial cell in vitro model Orai1 levels modestly increased with culturing (Fig 1E).

**ORA11 but not ORAI2 and ORAI3 levels are altered in human breast cancer cell lines**

Given the association between proteins involved in mammary gland development and tumorigenesis pathways (15, 27), the link between store-operated calcium

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**Figure 1. Orai1, Orai2, Orai3, and Trpc1 mRNA expression in the mouse mammary gland during development.** Orai1 (A), Orai2 (B), Orai3 (C), and Trpc1 (D) mRNA levels during the 4 stages of mammary gland development. Ct values were normalized to 18S rRNA. Values are relative to the nulliparous sample. Each dot represents mean values from 1 mouse derived from 2 independent real time RT-PCRs, carried out in triplicate. *P < 0.05 compared with nulliparous gland using a 1-way ANOVA with a Tukey multiple comparison posttest. E, Orai1 mRNA levels in HC11 cells. Data are from 3 wells from 3 independent experiments 2 days and 8 days (differentiated) postplating (fold changes are relative to day 2 and are shown as geometric mean ± 95% confidence interval). *P< 0.05 Mann-Whitney test.
influx and proliferation signaling in cancer (5), and the role of ORAI1 in invasion pathways in MDA-MB-231 breast cancer cells (13), we assessed the levels of ORAI channel isoforms in a bank of human breast cell lines. Relative ORAI2 and ORAI3 levels were similar in breast cancer derived cell lines (T-47D, MDA-MB-231, ZR-75–1, MCF-7, BT-483 and SK-BR-3) and nonmalignant mammary epithelial cell lines (184B5 and 184A1; Fig. 2A). In contrast, levels of ORAI1 were elevated up to 21-fold in many of the breast cancer cell lines compared with the nonmalignant mammary epithelial cell lines, with only the SK-BR-3 cell line having a less than

Figure 2. The characterization of ORAI in breast cell lines. A, the expression of ORAI1, ORAI2, and ORAI3 mRNA in breast cell lines; nonmalignant 184A1 and 184B5 and malignant BT-483, MCF-7, ZR-75–1, MDA-MB-321, and T-47D. Data are fold change, normalized to 18S rRNA, and relative to 184A1. Quantitation was based on 2 independent real time RT-PCRs carried out in triplicate. B, inhibition of ORAI1 in MCF-7 and MDA-MB-231 breast cancer cell lines using siRNA compared with nontargeting siRNA (NT siRNA) and mock transfected cells (Mock). The data are mean ± SEM (N = 6) and are from 2 independent experiments. C and D, the effect of ORAI1 inhibition on store-operated calcium influx in MCF-7 (C) and MDA-MB-231 (D) cells. The left panel depicts results typical of 3 independent experiments in each cell line, where relative [Ca^{2+}] is represented as response over baseline. Data are from individual wells. The first arrow corresponds to the addition of the calcium chelator BAPTA (500 μmol/L), the second arrow (where relevant) refers to the addition of the SERCA inhibitor CPA (10 μmol/L) to deplete calcium stores, and the third arrow to the addition of extracellular Ca^{2+} (2 mmol/L) to assess store-operated calcium influx. The right panels represent pooled data for the peak ratio (mean ± SEM; N = 12) consisting of the maximal response over baseline value during the Ca^{2+} influx transient divided by the maximal value obtained during the CPA-induced Ca^{2+} transient. This more directly assesses store-operated calcium influx through correction of alterations in the degree of calcium store release. *P < 0.05 compared with NT siRNA using a 1-way ANOVA and a Dunnett’s multiple comparison post test. E, ORAI1 levels are higher in the basal breast cancer subtype (P = 0.00063, Student’s t-test) Oncomine [Version 3.6, Compendia Bioscience, Ann Arbor, MI; (55)].
5-fold increase in transcription compared with 184A1 cells (Fig. 2A). ORAI1 was the predominant isoform in MCF-7, ZR-75-1, MDA-MB-231, and T-47D cell lines. To probe the potential of modulating the ORAI1 pathway therapeutically, we assessed the consequences of ORAI1 knockdown in 2 breast cancer cell lines that had elevated levels of ORAI1.

**Knockdown of ORAI1 attenuates store-operated calcium entry, viable cell number, and the response to an invasive stimuli**

ORAI1 knockdown using siRNA was achieved without pronounced effects on the related isoforms ORAI2 and ORAI3 in MCF-7 (Fig. 2B) and MDA-MB-231 (Fig. 2B) breast cancer cells. To confirm functional knockdown was achieved, we assessed store-operated calcium influx in these cell lines. Store-operated calcium influx was induced through incubation of breast cancer cell lines with the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA) in the absence of extracellular calcium to deplete the endoplasmic reticulum calcium store (28, 29). Readdition of extracellular calcium was associated with a pronounced influx of calcium in store depleted (CPA treated) cells in both cell lines compared with cells not treated with CPA (Fig. 2C and D). ORAI1 inhibition does not completely eliminate calcium influx associated with store depletion (Fig. 2C and D). Our studies assessing calcium leak (calcium influx in the absence of a SERCA inhibitor) show that this remaining calcium influx is not due to enhanced non-CPA–mediated calcium influx (calcium leak) in breast cancer cell lines. The presence of ORAI2 and ORAI3 isoforms in breast cancer cell lines (Fig. 2A) would be expected to contribute to a proportion of store-operated calcium influx unaffected by ORAI1 inhibition. Correlative with the greater mRNA levels of ORAI1 in MDA-MB-231 cells, the effects of ORAI1 inhibition on store-operated Ca\(^{2+}\) entry were more pronounced in MDA-MB-231 cells (45%) than MCF-7 cells (36%), which have lower levels of ORAI1 mRNA. ORAI1 levels may also be dependent on breast cancer subtype, with elevated levels in the basal breast cancers compared with other subtypes in microarray studies (Fig 2E).

We then assessed the consequence of inhibition of ORAI1-mediated store-operated calcium influx. ORAI1 siRNA significantly affected MCF-7 and MDA-MB-231 breast cancer cell lines (Fig. 3A). Thus, although some non-ORAI1 store-operated calcium influx remains in ORAI1 siRNA treated cells (Fig. 2C and D), ORAI1 inhibition in breast cancer cells is still sufficient to significantly affect breast cancer cells (Fig. 3A). Pharmacological inhibition of store-operated calcium influx mediated by the nonspecific inhibitor 2-APB was associated with a similar result (Fig. 3B). To then determine in a pathophysiological context if ORAI1 inhibition could remodel the calcium signaling initiated by an invasive stimulus, MDA-MB-231 cells were treated with the PAR-2 receptor activator trypsin (30). PAR-2 promotes the invasiveness of MDA-MB-231 cells (31–34) and our studies show that ORAI1 inhibition reduces the calcium influx associated with this pathway (Fig. 3C and D). The precise mechanisms involved in these phenotypes may be complex; our studies show that ORAI1 inhibition does not affect entry into the S phase of the cell cycle (Fig 3E) implicating later phases and/or alterations in viability or cellular adhesion.

**Orai1 calcium channel modulators Stim1 and Stim2 during mammary gland development**

Given the increased levels of Orai1 during the lactation stage of mammary gland development, we assessed the endogenous regulators of Orai1, the calcium store sensor Stim1 and its isoform Stim2, in the context of lactation. Despite its well-characterized association with Orai1 in store-operated calcium influx, levels of the Orai1 activator Stim1 did not increase during lactation, instead a significant decline was observed during pregnancy, lactation, and early involution (Fig. 4A). Assessment of the related isoform Stim2 showed that this isoform did not decline during lactation, and instead a modest, but statistically significant, increase in levels was observed (Fig. 4B). This increase was reversed during early involution [as was the case for Orai1 during early mammary gland development (Fig. 1A)]. Few studies have addressed the role of Stim2 in the regulation of calcium influx. Recent work has identified Stim2 as a key determinant of basal calcium levels (35). Orai1-mediated Stim2-activated enhancement of basal calcium influx during lactation would be an effective way for mammary gland epithelial cells to ensure Ca\(^{2+}\) was available to enrich milk with Ca\(^{2+}\). To assess the potential role of Stim2 in mammary epithelial cells during lactation, we used the HC11 mouse mammary epithelial cell line model (36). Differentiation of HC11 cells using lactogenic hormones significantly induced the mRNA expression of the milk protein β-casein (data not shown), which was undetectable prior to differentiation (16, 37). Treatment of differentiated HC11 cells with Stim2 siRNA caused a significant decrease in basal [Ca\(^{2+}\)]\_i (Fig. 4C), suggesting that Stim2 is an activator of basal calcium influx in mammary epithelial cells.

**STIM1 and STIM2 and breast cancer**

In contrast to the elevated levels of ORAI1 in some of the breast cancer cell lines, there were no similar elevations in STIM1 or STIM2 mRNA relative to the nonmalignant mammary cell line 184A1 (Fig. 5A). Given the report of opposing effects of STIM1 and STIM2 on ORAI1-mediated calcium influx (38), the ratio of the expression of these 2 genes was compared in breast cancer cell lines. The comparison between the relative levels of STIM1 and STIM2 indicated that the relative levels of STIM1 to STIM2 were similar in the breast cancer cell lines, with the exception of the ERBB2 overexpressing cell line 5K-BR-3 (Fig. 5B). The altered relative levels of STIM1 and STIM2 in 5K-BR-3 cells did not seem to be a
consequence of ERBB2 activity or expression, as neither treatment with the ERBB2 inhibitor AG825 (10 μmol/L) in SK-BR-3 cells nor the activation of ERBB2 in MCF-7 cells with heregulin β1 (10 ng/mL) had a significant effect on the STIM1/STIM2 ratio (data not shown). Given the different roles and properties of STIM1 and STIM2 (35), and the report that overexpression of STIM2 may have inhibitory effects on the STIM1 activation of ORAI1 (38), and our own finding that some breast cancer cell lines are characterized by an alteration in the STIM1/STIM2 ratio, we explored further the significance of STIM1 and STIM2 expression levels in breast cancer.

We examined the distribution of STIM1 and STIM2 genes in the transcriptional profiles of 295 primary breast
tumors previously reported by van de Vijver et al. (21; Fig. 6A). In this dataset, comprised of tumors of several different transcriptional subtypes, there was no obvious correlation between STIM1 and STIM2 levels ($r^2 = 0.0045$). To determine whether an alteration in the balance between STIM1 and STIM2 might be indicative of 1 or more of the transcriptional classes of breast cancer (39), we assigned each tumor to the appropriate class based on its gene expression profiles (Fig. 6B). Although the ERBB2, normal-like and luminal A and B tumors had a considerable variation in their STIM1/STIM2 levels, a majority of the basal tumors clustered in the lower right quadrant of the graph (high STIM1; low STIM2), a distribution that was highly statistically significant (Chi-Square $P$ value = 0.00018). Tumors in this quadrant had significantly poorer outcomes than the other groups (Fig. 6C). Tumors in the upper quartile of STIM1 expression also had significantly worse outcomes compared with other groups, as did tumors in the lowest quartile for STIM2 expression although these relationships were not as significant as a high STIM1; low STIM2 (data not shown). Consistent with a potentially different role of STIM1 and STIM2 in breast cancer cells, STIM1 inhibition had a greater impact on store-operated Ca$^{2+}$ influx in MDA-MB-231 cells than STIM2 inhibition (Fig 6D).

Figure 4. Stim isoform expression in the mouse mammary gland during development and the effect of Stim2 inhibition on basal calcium levels in mammary epithelial cells. For Stim 1 (A) and Stim 2 (B) values are normalized to 18S rRNA and are relative to the nulliparous sample. Each dot represents mean values from 1 mouse derived from 2 independent real time RT-PCRs, carried out in triplicate. C, Stim2 siRNA reduced basal cytosolic calcium levels compared with the nontargeting control. Data are mean ± SEM from 4 independent experiments carried out in quadruplicate. *$P < 0.05$ compared with nulliparous gland using a 1-way ANOVA with Tukey posttest (Panel A and B) or using a paired $t$ test (Panel C).

Discussion

A key component of the nutritional benefit of milk is calcium. Over recent years our understanding of how calcium is transported into milk from the mammary epithelial cell has become clearer (2). Studies have highlighted the apical transport and the secretion of calcium during lactation (2, 14, 23, 24, 40, 41), however, how calcium enters the mammary epithelial cell is not well understood (2, 27, 40). No studies have identified a
calcium influx pathway that has a pronounced upregulation during lactation. From our studies it seems that ORAI1 and its calcium store sensing regulator STIM2 are important components in the remodeling of calcium influx that occurs to meet the calcium transport demands of lactation. We also identified alterations in the transcription of ORAI1 in some breast cancer cell lines, including the basal-like breast cancer cell line MDA-MB-231, and that ORAI1 siRNA-mediated inhibition causes a transformation in the nature of calcium influx. Alterations in the relationship between STIM1 and STIM2 transcription in basal-like breast cancers and the utility of this difference as a predictor of prognosis provide further evidence of the importance of store-operated calcium entry in breast cancer. These studies define a new physiological role for the ORAI1-mediated calcium influx pathway, and further highlight that calcium influx pathways are potential novel drug targets for breast cancer.

In the mammary gland during lactation there was a significant increase in ORAI1 transcription with a reduction in ORAI2 and ORAI3. The ORAI isoforms differ in their calcium selectivity, conductance, and activation kinetics (42, 43). TRPC1 is also implicated in store-operated calcium entry (25, 26), in some cases with ORAI1 (44, 45), so we assessed its potential role in lactation. However, there are contradictory reports of the role of TRPC1 in store-operated calcium entry (46, 47) and our studies show that Trpc1 is unlikely to be associated with the ORAI1-mediated calcium influx occurring during lactation, as there was a significant decline in its levels during lactation.

Despite the well-defined relationship between the endoplasmic reticulum calcium sensor STIM1, and ORAI1 activation (6, 7, 12), lactation was associated with a pronounced and significant decline in Stim1. However, considering the differences between STIM1 and the isoform STIM2, the apparent contradiction of the modest rise in Stim2 and a decline in Stim1, suggests that this is well suited to the calcium homeostasis changes that occur during lactation. Brandman et al. (35), proposed that Stim1 activates ORAI1 only after pronounced endoplasmic reticulum calcium store depletion, whereas STIM2 is a key regulator of basal calcium influx, and does not require the pronounced depletion of endoplasmic reticulum calcium stores to activate ORAI1. STIM2 is therefore responsible for the tight regulation of resting calcium levels in the cytosol and intracellular calcium stores. Lactation is not associated with a pronounced decline in endoplasmic reticulum calcium levels (48), and a sustained basal-like calcium influx, such as that regulated by STIM2, would be well suited to the supply of calcium for apical and secretory pathway transport. Indeed, basal calcium levels declined in the presence of Stim2 siRNA-mediated inhibition in HC11 mammary cells. A modest, rather than an order or magnitude increase, in STIM2 levels during lactation would avoid the potential inhibitory effects of STIM2 on ORAI1 activity (38), which occurs with sustained high STIM2 overexpression (35). It is likely that the relative levels of STIM1, STIM2, and ORAI1 are important in the context of the physiological environment, as the expression levels of STIM1 and ORAI1 dictate the nature of the channel current (49). The decline in STIM1 during lactation may avoid the possible detrimental effects of excessive activation of store-operated calcium entry in mammary gland epithelial cells during lactation. It is also possible that there may be other regulators of ORAI1 that act during lactation. The recent demonstration that the calcium pump SPCA2, which is also increased during lactation, can directly activate ORAI1 (50), is a further indication that there may be multiple mechanisms by which ORAI is activated independent of STIM1 during lactation and in disease future studies using combinations of ORAI1, STIM1, and STIM2 selective knockdown in mammary gland epithelial cells in vivo and the measurement of milk Ca2+ content are required to definitively define the significance of STIM2-mediated activation of ORAI1 in the enrichment of milk with calcium.

Our studies indicate that ORAI1 elevation is a feature of some breast cancer cell lines. Our work also shows that ORAI1-mediated calcium influx, facilitated by calcium store depletion is present in multiple breast cancer cell lines and calcium leak (calcium influx in the absence of
Figure 6. STIM1 and STIM2 in breast cancer. A, and B, the relationship between STIM1 and STIM2 expression levels in 5 transcriptional subtypes of breast cancer. The expression levels of STIM1 and STIM2 were median-normalized to around zero. All tumors are shown in A and each subtype is highlighted in red in individual graphs in B. Chi-Squared analysis was used to determine if tumors of a particular subtype have a nonrandom STIM1/STIM2 distribution (if STIM1 and STIM2 are unrelated to a particular subtype, the red dots would be expected to be evenly distributed to each of the 4 quadrants). Basal-like tumors predominantly cluster in the lower right quadrant indicating a STIM1high/STIM2low phenotype ($P = 0.00018$). STIM1/STIM2 levels were randomly distributed in other breast cancer subtypes ($P > 0.05$). C, the STIM1high/STIM2low phenotype is associated with reduced survival in breast cancer patients. Using Kaplan–Meier analysis, patient survival was compared between tumors from the lower right quadrant of panel A (STIM1high/STIM2low) and the other 3 quadrants (STIM1low/STIM2low, STIM1high/STIM2high, and STIM1low/STIM2high). Significance was evaluated using the log-rank test. D, the effect of STIM1 and STIM2 inhibition on store-operated calcium influx in MDA-MB-231 cells. Data shown are from 4 individual wells from a representative experiment. The first arrow corresponds to the addition of the calcium chelator BAPTA (500 μmol/L), the second arrow (where relevant) refers to the addition of the SERCA inhibitor CPA (10 μmol/L) to deplete calcium stores, and the third arrow to the addition of extracellular Ca$^{2+}$ (2 mmol/L) to assess store-operated calcium influx. The inset represents pooled data for the peak ratio (mean ± SEM; $N = 12$), consisting of the maximal response over baseline value during the Ca$^{2+}$ influx transient divided by the maximal value obtained during the CPA-induced Ca$^{2+}$ transient. *$P < 0.05$ compared with NT siRNA using a 1-way ANOVA and a Dunnett’s multiple comparison posttest.
store depletion) is modest. Despite the presence of other ORAI isoforms, selective inhibition of ORAI1 is sufficient to alter breast cancer cell properties and the nature of the calcium influx associated with growth factors and proinvasive stimuli associated with calcium store mobilization, such as seen in our studies with a PAR-2 activator.

PAR-2 is a G-protein coupled receptor activated by specific proteases including trypsin (30), and its activation mobilizes calcium from internal stores and promotes the proliferation and invasion of MDA-MB-231 breast cancer cells (31–34). The ability of ORAI1 inhibition to modify the nature of calcium responses elicited by growth factors and proinvasion factors points to the need for further exploration of mechanism and potential differences between stimuli, specifically the effects on gene transcription via NFAT, which seems particularly sensitive to changes in sustained calcium influx (5). The greater ability of ORAI1 to inhibit Ca\(^{2+}\) influx mediated by PAR-2 activation compared with CPA treatment may show that the consequences of ORAI1 inhibition may be context and stimuli dependent. The effect of ORAI1 inhibition may also be isoform and breast cancer subtype dependent given our results showing greater effects of ORAI1 inhibition on calcium influx in a basal breast cancer cell line, and the report of a greater role of ORAI3 in estrogen receptor positive breast cancer cells (51).

The STIM proteins have been linked with different tumor types. STIM2 is amplified at the gene level in human Glioblastoma multiforme tumors (52) whereas STIM1 is overexpressed in primary glioblastomas compared with nonneoplastic tissue (53). STIM1 was initially proposed as a potential tumor suppressor in melanoma and rhabdoid tumor cell lines (54). However, STIM1 siRNA prevents invasion and metastasis in a xenograft breast cancer model (13). Although the luminal-like cell line SK-BR-3 was associated with an alteration in the STIM1/STIM2 ratio, this was due to a lower level of STIM2, in fact the only basal breast cell line examined, MDA-MB-231, was associated with the highest levels of both STIM proteins. Our studies in clinical samples further highlight the cancer subtype specificity of the STIM isoforms, as a STIM1\(^{\text{high}}\)/STIM2\(^{\text{low}}\) phenotype was associated with the basal breast cancer molecular subtype and correlated with a poorer prognosis. Differences between STIM1 and STIM2 were also reflected in calcium assays in the basal-like breast cancer cell line MDA-MB-231, where STIM1 had a greater effect on calcium influx compared with STIM2, which seems to have more of a fine tuning role.

The identification of ORAI1- and STIM1-mediated calcium influx as a potential pathway for therapeutic targeting in breast cancer raises some issues, including the possible consequences of modulating this pathway on other physiological processes. Inhibition of the ORAI1/STIM1 pathway is likely to have effects on the immune system, given that ORAI1 is linked to a hereditary severe combined immunodeficiency (SCID) syndrome. T lymphocytes from the affected individuals have a defect in store-operated calcium entry due to a mutation in ORAI1 (6). Individuals heterozygous for the ORAI1 mutation associated with the SCID syndrome have no detectable phenotype (6), despite a subtle change in store-operated calcium signaling detected by limiting extracellular calcium during assessment of store-operated calcium entry (6). However, given the limited treatment options with a lack of molecular therapeutic targets for basal breast cancer, there is a need to further study this pathway.

Disclosure of Potential Conflicts of Interest

S.J. Roberts-Thomson, G.R. Monteith, and D. McAndrew are coinventors of a patent application that covers the use of ORAI1 as a novel target for breast cancer therapy/diagnosis. The patent application has been filed in the name of The University of Queensland.

Grant Support

This work was funded by the NHMRC (569645 and 631347) an APA to D. McAndrew and D.M. Grice and an NHMRC Biomedical Postgraduate Research Scholarship to E.M. Davis. PK was supported by Susan G. Komen for the Cure (KG100888).

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Received October 6, 2010; revised November 8, 2010; accepted December 5, 2010; published OnlineFirst January 11, 2011.

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Molecular Cancer Therapeutics

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