CALCIUM AT THE PUMP:
REGULATION OF INTRACELLULAR CALCIUM BY SECRETORY PATHWAY
CALCIUM ATPASE ISOFORM 2 (SPCA2) IN MODELS OF LACTATION AND
BREAST CANCER

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Abstract

Secretory Pathways Calcium ATPase 2 (SPCA2) is a non-redundant isoform of SPCA expressed in highly secretory cells. In this study we aimed to investigate physiological regulation of SPCA2 as a possible regulator of calcium during lactation and its interaction with the canonical, store-dependent calcium channel, Orai1. Furthermore, we asked if there were other calcium channels which showed positive interactions with SPCA2. We found that TRPC6 also interacts with SPCA2 during lactation, but is, however reciprocally regulated by SPCA2 expression. Ultimately, we learned that differential regulation of calcium transporters we entitled CALTRANS, had specific expression patterns in the progression of cancer, as well. Furthermore, we show explicit calcium signaling modes for a tumorigenic and metastatic cell model of cancer. The addition of SPCA2 to a migratory cell line showed promise in increasing adhesion, while overexpression of SPCA2 in a tumorigenic cell model was attenuated with natural phytochemicals.
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Chapter 1

Calcium in Lactation and Breast Cancer

This chapter is being prepared for submission as a review article.
Summary

Breast cancer is the second most common form of cancer diagnosed in women, after skin cancer. It is predicted that the disease will cause nearly 40,000 deaths and almost 2,000 new cases will be diagnosed this year alone in the U.S. One of the most defining characteristics of breast cancer is the radiographic presence of microcalcifications. These palpable mineral precipitates are commonly found in the breast after the formation of a tumor. Since free Ca\textsuperscript{2+} plays a crucial role as a second messenger inside cells, we hypothesize that these chelated precipitates may be a result of dysregulated Ca\textsuperscript{2+} secretion associated with tumorigenesis. Transient and sustained elevations of Ca\textsuperscript{2+} are prerequisite for controlling cell proliferation, apoptosis and cell migration, and offer numerous therapeutic possibilities in controlling tumor growth and metastasis. During lactation, a developmentally determined program of gene expression controls the massive transcellular mobilization of Ca\textsuperscript{2+} from the blood into milk by the coordinated action of \textbf{Calcium Transporters}, including pumps, channels, sensors and buffers, in a functional module that we term CALTRANS. We assess the evidence implicating genes that regulate free and buffered Ca\textsuperscript{2+} in normal breast epithelial and cancer cells, and discuss mechanisms that are likely to contribute to the pathological characteristics of breast cancer.
Regulation of Cytosolic Calcium by CALTRANS: a Calcium Transporting Module

Ionized Ca\(^{2+}\) is a ubiquitous second messenger, regulating a wide swathe of cellular events ranging in time scale from nanoseconds (in vesicle fusion) to hours (in cell proliferation) (1). Unlike other second messengers, however, ionic Ca\(^{2+}\) can neither be synthesized nor degraded. Hence, regulation of cytosolic Ca\(^{2+}\) occurs by dynamic compartmentalization and buffering.

An array of membrane embedded transport proteins move Ca\(^{2+}\) ions into and out of the cytoplasm. Active transport mechanisms drive Ca\(^{2+}\) ions uphill, generating large transmembrane Ca\(^{2+}\) gradients of up to 10,000-fold. These include primary active transporters, or calcium pumps, that directly harness energy from ATP, and secondary active transporters that couple uphill movement of Ca\(^{2+}\) to the energetically favorable transport of Na\(^{+}\) or H\(^{+}\) down their electrochemical gradients. Although Na\(^{+}\)/Ca\(^{2+}\) exchangers play an active role in Ca\(^{2+}\) extrusion in some tissues, including plasma membrane of cardiomyocytes, evidence for the role of secondary transporters in mammary epithelia is lacking. Considerable attention has focused on the expression and regulation of Ca\(^{2+}\)-ATPases distributed in the membranes of the ER, secretory pathway and plasma membrane. They belong to the superfamily of P-type ATPases, so-named after a conserved mechanism involving a catalytic phosphorylated intermediate (2). Together, they establish pools of stored Ca\(^{2+}\) within cellular organelles and actively efflux Ca\(^{2+}\) out of the cell, maintaining baseline levels in the low submicromolar range. These external and organelar reservoirs drive cytoplasmic influx of Ca\(^{2+}\) through multiple classes of ion channels, activated by voltage, ligands or store depletion. The resultant temporal and spatial elevations in Ca\(^{2+}_{\text{cyt}}\) activate a set of effectors that include
calmodulin, Ca\textsuperscript{2+} dependent kinases and phosphatases that decode the signal and in turn, 
act on secondary effectors such as transcription factors that mediate gene regulation. The 
master regulator of systemic Ca\textsuperscript{2+} is the Calcium Sensing Receptor, CaSR, a member of 
the large family of G protein coupled receptors (Class C GPCR) that monitors 
extracellular free Ca\textsuperscript{2+} in the circulation and is the principal regulator of parathyroid 
hormone, PTH, and the parathyroid hormone related protein, PTHrP (3).

In the breast, an exquisitely orchestrated program of changes in the activity and 
expression of Ca\textsuperscript{2+} transporters and modulators commences during pregnancy, culminates 
with the initiation of lactation at parturition and concludes with the process of involution 
(4). Activation of a so-called “alveolar switch” induces proliferation and differentiation 
of the secretory alveolar compartment (5). Many of the proteins in the secretory 
pathway, including milk proteins, are transcriptionally upregulated prior to parturition (4- 
6). Key members of the transcellular calcium transport system that we name 
CALTRANS for Calcium Transport module (Figure 1-1) are upregulated during 
alveolar differentiation and have been identified in microarray studies of expressed 
transcripts during pregnancy, lactation and involution (7-8). Conversely, abnormal 
expression of several Ca\textsuperscript{2+} transporters and ion channels has been documented in breast 
cancer, resulting in oncogenic Ca\textsuperscript{2+}signaling that drives tumorigenesis. Therefore, we 
propose that a transcellular Ca\textsuperscript{2+}transport module (CALTRANS) is coordinately 
regulated in normal breast development and lactation, and that components of the same 
module are dysregulated upon transformation of breast cancer cells to the malignant 
phenotype. Further, we propose that CALTRANS module interactions with the altered 
signaling environment in transformed cells leads to localized, inappropriate secretion of
calcium in the absence of calcium buffers, resulting in microcalcifications which are diagnostic of breast cancer. Here, we will discuss the background and evidence that supports this hypothesis and consider how these changes may be exploited in cancer therapy.

**Mobilization of Calcium in Breast Development and Lactation**

Calcium is a key nutrient in milk, essential for the development of teeth and bone in the neonate. Total milk calcium in mammals can be as high as 60 mM in mice, although it is typically 10 mM in humans (9). Much of this calcium is bound, either to milk protein (casein) or to anionic carriers such as phosphate, citrate and bicarbonate. Free Ca$^{2+}$ levels are not much above 3 mM in milk, limiting the osmotic stress on mammary epithelial cells. To cope with this extra demand for calcium secretion into milk, Ca$^{2+}$ absorption from the intestine is stimulated, the kidneys retain Ca$^{2+}$ and bone Ca$^{2+}$ is mobilized to the extent that bone density declines by ~5% over the course of nursing. Efficient delivery into milk must be accompanied by synthesis, transport and secretion of milk proteins, triglycerides, phosphates and other anions that complex with Ca$^{2+}$.

During lactation the secretory pathway is mobilized to deliver Golgi-derived vesicles to the apical side of mammary epithelia, excreting calcium and protein into the milk (Fig. 1-1). Secretory pathway calcium ATPases show coordinated increased expression during lactation for this purpose (10). Recently, we detected a physical interaction between SPCA2 and Orai1 in lactating tissue and used a cell culture model to study the role of the SPCA2 in lactation. In mouse mammary epithelial cells, SPCA2
regulated trafficking of Orai1 to the plasma membrane (11) and was linked to calcium storage, store-independent calcium influx and surprisingly, store-operated calcium influx. As we will see in Chapter 2, genetic ablation of SPCA2 expression resulted in retention of Orai1 in perinuclear compartments, inhibition of development in response to lactogenic hormones (prolactin) and inhibition of store-operated calcium signaling, similar to those effects seen in cells which lack Orai1, altogether.

**Figure 1-1: Coordinated calcium influx by CALTRANS module mobilization during lactation.** In lactating mammary epithelia, intracellular Ca\(^{2+}\) pumps such as SERCA, SPCA2 and PMCA2 increase in expression along with Ca\(^{2+}\) channels such as Orai1 and TRPC6. Orai1 interacts with STIM and SPCA2 at the basolateral membrane to promote the elevated, transcellular Ca\(^{2+}\) influx required for lactation.
Plasma Membrane Ca\(^{2+}\) ATPases (PMCA\(_{s}\)) have long been the focus of multiple studies of Ca\(^{2+}\) homeostasis in the breast, including development, lactation and the onset of cancer. PMCA1 is the ubiquitous isoform and is considered to have a housekeeping function in cytoplasmic calcium homeostasis while other PMCA isoforms show limited tissue distribution and specialized functions in lactation and tumorigenesis. PMCA2 has the highest affinity for Ca\(^{2+}\) and is upregulated during lactation when it has been shown to reside on the apical membrane of lactating cells (12). Mice completely lacking the PMCA2 isoform have a 60% reduction in the amount of Ca\(^{2+}\) in their milk, when compared to their heterozygous littermates (13) During lactation, PMCA2 extrudes Ca\(^{2+}\) directly into the milk duct in its ionic form rather than bound to phosphates or milk caseins.

The Calcium Sensing Receptor, CaSR is expressed in the basolateral membrane of the lactating alveolus, and regulates PTHrP secretion (14). In a haploinsufficiency model of CaSR (CaSR\(^{+/−}\)), an increase in PTHrP and decrease in calcium transport into milk was observed, confirming the important role of CaSR in lactation (15). Mammary-specific knockdown of CaSR was recently achieved by mating CaSR\(^{\text{floxed/floxed}}\) mice to mice expressing Cre driven by the β-lactoglobulin gene, which leads to CaSR knockdown in late pregnancy and during lactation (3). These studies demonstrated that CaSR is not required for mammary cell differentiation or lactation, but is involved in regulation of systemic calcium homeostasis during lactation, i.e., by regulating PTHrP content in milk and maternal circulation, and by modulating maternal bone mineralization and calcium excretion. Mammary-specific CaSR knockout during lactation also significantly decreased the calcium content in milk without reducing PMCA2 expression, leading to
attenuated calcium accrual in the pups. CaSR in the parathyroid gland and kidneys compensated for loss of mammary CaSR, since global knockout of CaSR in CaSR\(^{-/-}\) x PTH\(^{-/-}\) mice caused persistent hypercalcemia throughout lactation due to reduced calcium excretion. Overall, the study suggests that CaSR in breast coordinates maternal and fetal calcium metabolism by regulating both calcium and PTHrP levels.

**Microcalcifications in breast cancer: diagnostic evidence of dysregulated calcium transport**

Microcalcifications have been extensively used to diagnose and characterize breast cancers although the mechanism(s) producing microcalcifications remain largely undefined (16). Two types of microcalcifications—calcium oxalate and calcium apatite—can be distinguished by their radiographic “signatures” on mammograms, with the latter type correlating more closely to malignant transformation (17-20). We propose that microcalcifications result from abnormal expression of bone matrix proteins (osteonectin, osteopontin, bone sialoprotein, bone matrix proteins) in breast cancer cells (21-22) coupled with an inappropriate upregulation of calcium transport (23-24) in the absence of caseins and other calcium buffers (25). A mechanistic understanding of the source(s) of calcium and implications of distinct microcalcification “signatures” would improve diagnosis and stratification of breast cancer metastatic risk.
Figure 1-2: Distinct Calcium Modes in CALTRANS Dysregulation

A.) Tumorigenic cells overexpress intracellular and plasma membrane Ca$^{2+}$ pumps and channels, as in lactation, relying on store-independent Ca$^{2+}$ signaling to fill their stores and hoard Ca$^{2+}$ for use in cell division. B.) Migratory cells increase expression of IP3R, releasing the ER Ca$^{2+}$ stores under stimulation by increased GPCR signaling at the plasma membrane to which are used seek out Ca$^{2+}$ to fill depleted stores.
Dysregulation of CALTRANS in Breast Cancer

$\text{Ca}^{2+}$ is a double-edged sword, promoting both cell growth and cell death, and hence $\text{Ca}^{2+}_{\text{cyt}}$ levels must be tightly regulated and finely tuned. This explains the complex modulation of $\text{Ca}^{2+}$ transporters accompanying oncogenic transformation and the seemingly contradictory effects of inactivation and activation of specific CALTRANS members in protecting against or promoting tumorigenesis (Fig. 1-2).

Calcium Pumps

In general, elevation of $\text{Ca}^{2+}$-ATPases serves to remove $\text{Ca}^{2+}$ ions from the cytoplasm, clamping $\text{Ca}^{2+}_{\text{cyt}}$ to resting levels. However, at least one isoform, SPCA2 also elevates $\text{Ca}^{2+}$ entry via a novel interaction with plasma membrane $\text{Ca}^{2+}$ channels. In cancer, abnormal expression of several $\text{Ca}^{2+}$ pumps, including SPCA2, results in dysregulated $\text{Ca}^{2+}$ homeostasis that drives tumor growth and migration.

(i) Secretory Pathway $\text{Ca}^{2+}$-ATPases (SPCA): Homozygous deletion of the $\text{ATP2C1}$ gene encoding the ubiquitously expressed, housekeeping isoform SPCA1 is not viable in mouse models, but the aged heterozygotes exhibit squamous tumors in keratinized epithelia and esophagus cells (26). Although the same genotype causes the ulcerative skin disorder Hailey-Hailey disease in humans, it is interesting that some studies show an association with preneoplastic lesions and malignancy (27). SPCA1 levels were found to be elevated in basal-like breast cancers, when compared to luminal types, and knockdown of SPCA1 in MDA-MB-231 cells delayed the Golgi processing of pro-IGF1 receptor, whose expression corresponds with poor prognosis of breast cancer outcome (28).
A second isoform SPCA2 was highly expressed in luminal-type breast cancer lines, with highest levels in ERBB2+ breast tumors (29) Knock down of SPCA2 in MCF7 cells attenuated cell proliferation, colony formation in soft agar and tumor formation in nude mice. Ectopic expression of SPCA2 in the non-tumorigenic cell line MCF10A conferred increased cell proliferation and soft agar colony formation, which could be abrogated by knockdown of the Orai1 channel. SPCA2 was partially localized to vesicles near the plasma membrane where it interacted with and elicited the opening of Orai1 Ca\(^{2+}\) channels, resulting in the elevation of Ca\(^{2+}\)\(_{cyt}\) and activation of ERK1/2 signaling pathways. Thus, inappropriate expression of SPCA2 in non-lactating mammary epithelial cells is sufficient to confer oncogenic potential via Store Independent Ca\(^{2+}\) Entry (SICE) and activation of cell proliferation. Since activation of Ca\(^{2+}\) influx by SPCA2 was independent of its Ca\(^{2+}\)-ATPase and transport function, active site inhibitors of the pump would not be effective as a cancer therapeutic. However, a minimal 40 amino acid fragment of the N-terminal domain of SPCA2 functioned as a dominant negative blocker of SPCA2-Orai1 interaction, suggesting a peptide-based approach may be an option. Of note, SPCA2 was not expressed in the highly metastatic cell line, MDA-MB-231, where STIM1/Orai1 mediated SOCE was required for cell migration. Thus, understanding the specific molecular signature of dysregulated CALTRANS components would be important in directing treatment approaches.

(ii) Plasma Membrane Ca\(^{2+}\)-ATPases (PMCA): Isoform-specific upregulation of PMCA has been investigated in a variety of breast cancer tissues and cell lines, with up to 100-fold elevation of PMCA2, relative to non-transformed cells. Knockdown of PMCA2 in MCF7 cells slows progression of cell cycle through G2/M phase (30). In comparison,
PMCA1 levels were only modestly increased, whereas there was a small decrease in the PMCA4 isoform. The latter was confirmed in colon cancer cells, where PMCA4 is upregulated post-confluence, accompanying differentiation, but downregulated in proliferating cancer cells (31). It has been suggested that PMCA remodeling in cancer recapitulates the changes in lactation to counter Ca\(^{2+}\)-mediated apoptotic pathways (32). These observations suggest that isoform-specific inhibitors may be useful in cancer therapy. In one approach, a random peptide phage display library was screened against an extracellular domain of PMCA4 to generate a series of specific inhibitors with micromolar affinity (33).

(iii) Sarco-Endoplasmic Reticulum Ca\(^{2+}\)-ATPases (SERCA): A highly specific non-competitive inhibitor of SERCA pumps is thapsigargin, a sesquiterpene lactone from the roots of Thapsia garganica. Thapsigargin was initially recognized as a tumor promoter in a mouse model of skin cancer (34). A null mutation to one copy of the SERCA2 gene leads to squamous cell carcinomas in the murine model (35). This phenotype was demonstrated in cells which had no ras or p53 mutations and show that haploinsufficiency of SERCA2 can predispose the cell to a cancer phenotype even if the tumor-suppressing genes have a normal genotype. However, thapsigargin has been shown to inhibit cancer cell proliferation and migration in multiple cell types (36-37). Because thapsigargin is a nonselective cytotoxin, therapeutic efforts via clinical trials (NCT01056029 and NCT01734681) are focused on a prodrug form coupled to prostate specific antigen (PSA) that targets cytotoxicity to prostate cancer cells (38). While SERCA has not been specifically evaluated in breast cancer tumors or cell culture
models, it is reasonable to conclude that the effect of thapsigargin may also implicate SERCA's in breast cancer.

**Calcium Channels**

Cytosolic Ca\(^{2+}\) elevations in mammary epithelia derive from two sources: extracellular (from a variety of ion channels) and intracellular, primarily from the ER and secretory pathway via the IP3-gated receptor channel (IP3R).

(i) **IP3 Receptor Channel**: The inositol 1,4,5-trisphosphate receptor (IP3R) is the main Ca\(^{2+}\)-release channel for the ER. There are three isoforms of IP3R known and two have been shown to be present in abundance in the breast cancer cell line, T47D. IP3R1 and IP3R3 were shown to be upregulated and have high amounts of phosphorylation in T47D cells (39). In this study it was shown that IP3R1 and IP3R3 functionally interacted with cy/cdk complexes (cyclin/cyclin-dependent kinases) in T47D cells. The data suggest that cy/cdk complexes not only regulate cell proliferation, but also cellular Ca\(^{2+}\).

(ii) **TRP Channels**: Transient receptor potential (TRP) mediated channels are a large superfamily of plasma localized Ca\(^{2+}\)Ca\(^{2+}\) channels that are expressed in many cell types. This family includes TRPC, TRPV and TRPM subtypes. Several isoforms of the canonical, non-selective and lipid-regulated TRPC family have been implicated in tumor formation, breast cancer cell proliferation and migration. In MCF7 cells, activation of CaSR by extracellular Ca\(^{2+}\) turned on PLC/PKC signaling pathways to elicit Ca\(^{2+}\)-influx via TRPC1. Silencing of TRPC1 blocked downstream phosphorylation of ERK1/2 and proliferation of MCF7 cells. Furthermore, TRPC1 expression itself was elevated by ERK1/2 phosphorylation in MCF7 cells (40). TRPC6 was not detected in normal breast
tissue (41), but was found to be consistently elevated in tissue biopsies of breast tumors, albeit without correlation with tumor grade, ER expression and lymph node metastasis (42). Whereas TRPC3 and TRPC6 isoforms were upregulated in MCF-7 cells, in the highly metastatic breast cancer cell line, MDA-MB-231, both isoforms showed intracellular localization. Surprisingly, hyperforin, a specific activator of TRPC6 and active component of St. Johns Wort, inhibited proliferation of these cancer cell lines but not in the control MCF10A cells, and silencing of TRPC6 reduced cell proliferation without any effect on cell viability (41). We will see in Chapter 3, the reciprocal regulation of TRPC6 and SPCA2 by prolactin and how co-expression of TRPC6 and SPCA2 is permissive to raising intracellular calcium levels.

The TRPM (melastatin-related TRP) family represents cold-activated TRP channels that are Ca$$^{2+}$$ and Mg$$^{2+}$$ permeable. They appear to play a significant role in cancers involving the reproductive organs. Specifically, TRPM7 was expressed at high levels in grade III breast tumors and plays a role in sequestration of intracellular Ca$$^{2+}$$ into breast cancer cells (43) and TRPM8 expression was shown to be regulated by the estrogen receptor ER alpha and correlated with the ER$$^{+}$$ state of the tumors (44). TRPV (vanilloid-activated) channels are typically expressed in epithelial cells, and comprise six isoforms. TRPV1 has not been implicated in breast cancer, but menstrual breast pain, raising the possibility that pain associated with breast cancer may be mediated by TRPV1 nociception (45). Not much is known about the localization and specificity of the TRPV channels in mammary tissue, except in the case of TRPV6. TRPV6 has been shown to be upregulated at the mRNA level in T47D cells and can be regulated through Vitamin D and female hormones. Furthermore, TRPV6 mediates the action of tamoxifen on breast
cancer cells (46-47), suggesting that the therapeutic effect of tamoxifen and protein kinase C inhibitors used in breast cancer therapy might involve TRPV6-mediated calcium entry.

**(iii) Orai Channels:** The Store-Operated Ca\(^{2+}\) Entry (SOCE) pathway is regulated by the ER/plasma membrane localized single-pass protein, STIM1. When ER stores are low, STIM1 forms puncta at the ER membrane. These puncta are formed near plasma membrane junctions to open the Ca\(^{2+}\)-selective, plasma membrane channel, Orai1. The coupling of STIM1 with Orai1 is dependent upon the absence of Ca\(^{2+}\) from the EF-hand of the luminal portion of STIM1, which signals a reduction or loss of Ca\(^{2+}\) in the ER. It is thought that the refilling of the ER store through the ER pump, SERCA, causes a reloading of Ca\(^{2+}\) in the EF-hand domain of STIM1, resulting in a conformational change which then uncouples STIM1 from Orai1. In the breast, it is thought that ER/Golgi complexes are increased to afford the cell enough Ca\(^{2+}\) for milk via the stabilization of high amounts of vesicular trafficking to the apical membrane. However, in breast cancer, this system has been shown to be dysregulated by SPCA2 and thus, the Orai channel is allowed to open independent of the ER store Ca\(^{2+}\) concentrations (29).

During lactation, there is a significant increase in the amount of Orai1, but not Orai 2 or 3 (48). This suggests that Orai1 may be the source of Ca\(^{2+}\) entry in the mammary gland during lactation, perhaps also accounting for the enlargement of the ER/Golgi complex during the process. On the pathological end of the spectrum, Orai1 has been shown to have dramatic increases in basal-type patient tumor samples mammary tumor cell lines, MCF-7 and MDA-MB-231.
In another study, it was shown that estrogen receptor positive mammary cancer cells (ER$^+$) use the Orai1/STIM1 pathways while estrogen receptor negative (ER$^-$) mammary cancer cell lines may use the elusive Orai3 coupling mechanism, thought to have the same or a similar mechanism as the canonical Orai1/STIM1 pathway (49). The specific STIM isoform partner for Orai3 and its direct mechanism of coupling, however, remain to be identified. Another study showed that siRNA inhibition of Orai3 in MCF-7 cells arrested their cell cycle at the G1 phase, thereby inhibiting cell proliferation (50). Further analysis of this data revealed that the silencing of Orai3 also reduced the cyclin-dependent kinases (CDKs 2/4), cyclin E and cyclin D1 together with the accumulation of p21 and p53. In the normal, immortalized cell line, MCF-10A, the silencing of Orai3 had no significant effect on cell proliferation, viability or [Ca$^{2+}$]. This more recent data suggests that the role of Ca$^{2+}$ in breast cancer changes to optimize uncontrolled proliferation and apoptosis resistance which are hallmarks of the cancerous state.

**Ca$^{2+}$ Sensing Receptor (CaSR)**

CaSR is expressed in normal, fibrocystic and cancerous human breast epithelium (51). Given the roles of CaSR in normal mammary gland/breast, it is not surprising that CaSR is also expressed in many breast cancer cell lines, where it regulates parathyroid hormone-related peptide (PTHrP) secretion (52). Studies of CaSR signaling in mammary epithelium have focused primarily on alterations associated with breast cancer. In MCF-7 cells, activation of CaSR by either extracellular calcium or neomycin, an allosteric activator of CaSR, led to increases in intracellular calcium, and increased colocalization of Calbindin-D$_{28k}$ and CaSR, although the mechanism and significance of the association remains to be determined (53). The estrogen receptor expression status of breast cancers
has implications for the likelihood of bone metastases and treatment outcomes. MCF-7 cells treated with elevated extracellular calcium (up to 20 mM, indicative of the bone resorptive niche) or CaSR allosteric agonists show a decrease in estrogen receptor protein but increase in transcriptional activation (54). If these results are verified in vivo, they may suggest that CaSR serves as a link between estrogen receptor status and bone metastases. CaSR activation by extracellular calcium triggers breast cancer cell migration (55), providing independent evidence for an additional role for CaSR in bone targeting. CaSR stimulation also leads to increased proliferation of MCF-7 cells, through pathway(s) involving extracellular signal-regulated kinases 1 and 2, and transient receptor potential channel TRPC1 (56-57). CaSR undergoes unique alterations in signaling preference in malignant breast cells. CaSR heterotrimeric G protein preference shifts from G_{q11}/G_{i}/G_{12/13} in normal breast epithelium to G_{o} in malignant cells [Manillapalli 2008]. This shift is significant because the normal down-regulation of PTHrP secretion upon CaSR activation is converted increased secretion, contributing to hypercalcemia of malignancy (58). In breast cancer cell lines (MDA-MB-231 or MCF-7), CaSR-activates choline kinase through a G and Rho-dependent pathway, while non-malignant breast cell lines (Hs 578Bst or MCF-10A), this pathway is not activated (59). The mechanism(s) contributing to these shifts in CaSR signaling preference are not known, but likely include a combination of altered expression and/or subcellular targeting of CaSR and interacting proteins.

Differences in CaSR expression and/or signaling have been explored as potential targets in treatment of breast cancer and/or attenuation of bone targeting. Several studies
argue that activation of CaSR reduces breast cancer cell proliferation, decreases expression of survivin, and sensitizes cells to the cytotoxic agent paclitaxel, suggesting CaSR acts as a tumor suppressor in breast epithelium (60-61). BRCA1 mutations which inactivate the protein represent a major risk factor for breast cancer. BRCA1 activity is required for CaSR expression, which leads to suppression of survivin expression (61). Ectopic expression of CaSR in BRCA1 defective cells restores survivin suppression and attenuates proliferation. Our understanding of the contributions of CaSR to normal physiology and its derangements in cancer is at present rudimentary, although the weight of evidence argues that targeting CaSR in combinatorial cancer therapies may prove beneficial (62-64).

In summary, calcium homeostasis in the breast is both complex and dynamic, with critical changes occurring during lactation to facilitate the massive secretion of calcium into milk. In contrast to the coordinated induction of a calcium transporting module in lactation, breast cancer is associated with a dysregulation of calcium transporters, channels and sensors, leading to tumor proliferation and migration. This thesis will cover both physiological and pathophysiological aspects of calcium homeostasis, and identify key components in these processes.
References


cell proliferation and TRPC1 cation channel over-expression potentially through EGFR pathways. *Arch Biochem Biophys.* 486: 58-63.


transcriptional activity through calcium-sensing receptor in breast cancer cells.


Chapter 2:

SPCA2 Regulates Orai1 Trafficking and Store Independent Ca\textsuperscript{2+} Entry in a Model of Lactation

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Summary

An unconventional interaction between SPCA2, an isoform of the Golgi secretory pathway Ca\(^{2+}\)-ATPase, and the Ca\(^{2+}\)-influx channel Orai1 has previously been shown to contribute to elevated Ca\(^{2+}\)-influx in breast cancer derived cells. In order to investigate the physiological role of this interaction, we examined expression and localization of SPCA2 and Orai1 in mouse lactating mammary glands. We observed co-induction and co-immunoprecipitation of both proteins, and isoform-specific differences in the localization of SPCA1 and SPCA2. Three-dimensional cultures of normal mouse mammary epithelial cells were established using lactogenic hormones and basement membrane. The mammospheres displayed elevated Ca\(^{2+}\)-influx by store independent mechanisms, consistent with upregulation of both SPCA2 and Orai1. Knockdown of either SPCA2 or Orai1 severely depleted Ca\(^{2+}\)-influx and interfered with mammosphere differentiation. We show that SPCA2 is required for plasma membrane trafficking of Orai1 in mouse mammary epithelial cells and that this function can be replaced, at least in part, by a membrane anchored C-terminal domain of SPCA2. These findings clearly show that SPCA2 and Orai1 function together to regulate Store-independent Ca\(^{2+}\)-entry (SICE), which mediates the massive basolateral Ca\(^{2+}\)-influx into mammary epithelia to support the large calcium transport requirements for milk secretion.
**Introduction**

Secretory pathway Ca\(^{2+}\)-ATPases (SPCA) are important in sequestering Ca\(^{2+}\) and Mn\(^{2+}\) from the cytoplasm into the Golgi and post-Golgi vesicles where they are important for post-translational modification, sorting and quality control of cargo proteins (1). The two isoforms, SPCA1 (*ATP2C1*) and SPCA2 (*ATP2C2*) share significant sequence similarity, but have distinct distribution and function (2,3). SPCA1 is ubiquitously expressed in mammalian tissues where it serves an essential housekeeping function, as evidenced by embryonic lethality in the homozygous knockout mouse (4). In contrast, expression of SPCA2 is confined to highly secretory or absorptive epithelia, including mammary, testis, salivary glands, intestinal tract and lung (2). Because it shares similar transport characteristics as SPCA1, SPCA2 appears redundant at first glance. Recently, we showed pathologically elevated expression of SPCA2 in breast cancer derived tissue and cell culture models, leading to an investigation of its role in tumorigenicity (5).

We showed that SPCA2, but not SPCA1, traffics to the plasma membrane in the breast cancer derived MCF7 cell line, where it interacts with the store-operated Ca\(^{2+}\) channel Orai1 to elicit constitutive Ca\(^{2+}\)-influx. This signaling function was independent of Golgi and ER Ca\(^{2+}\) stores, and did not require ATPase or transport function of SPCA2. Constitutive elevation of Ca\(^{2+}\)-influx in these cells activated MAP kinase signaling pathways and promoted tumor proliferation. Knockdown of either SPCA2 or Orai1 expression was found to attenuate Ca\(^{2+}\)-signaling and inhibit tumor growth in mouse xenografts. Analysis of mutants and chimeras revealed that the membrane-anchored C-terminal tail of SPCA2 was sufficient for interaction with Orai1 to elicit Ca\(^{2+}\)-influx (5). Interestingly, an alternative splice variant of *ATP2C2*, encoding a ~20 kDa membrane-
anchored C-terminal domain is expressed in several secretory tissues under control of the helix-loop-helix transcription factor MIST1 (6) suggesting a physiological role for Orai1-SPCA2 interaction. However, the functional significance of this interaction remains to be elucidated. Selective and prominent lactation-induced expression of SPCA2 in mammary epithelium (7,8) provides a first insight into function.

Total calcium concentration in milk, including ionized Ca$^{2+}$ and bound calcium, ranges between 40-80 mM in various mammalian species (9). Multiple Ca$^{2+}$ transporters, regulators and binding proteins must be upregulated to drive transeucytosis of calcium while maintaining submicromolar cytoplasmic Ca$^{2+}$ concentrations to avoid Ca$^{2+}$ mediated toxicity and cell death. Transport and sequestration of Ca$^{2+}$ is achieved by coordinated increase in the expression of Ca$^{2+}$ pumps, and channels (10). Current models suggest that polarized mammary secretory cells take up Ca$^{2+}$ at the basolateral membrane via yet to be described Ca$^{2+}$ channel(s) (9). Ca$^{2+}$ entering the mammary secretory cells then travels one of 2 routes to secretion into milk. In the first transport route (accounting for ~40% of calcium in milk) Ca$^{2+}$ is likely rapidly pumped in Golgi/secretory stores via SPCA1 and/or SPCA2 where it is bound to casein to facilitate casein micelle formation, packaged in secretory vesicles and secreted into milk primarily as beta-casein (7,8,11). The second transcellular route (accounting for ~60% of calcium in milk) cell Ca$^{2+}$ is pumped directly across the mammary secretory cells apical membrane into milk by PMCA2bw (12,13). The proteins involved in sequestering cell Ca$^{2+}$ while it is shuttled to PMCA2bw are unknown but calbindin-9k is a candidate calcium binding protein that could provide this function (14). An alternate but yet unproven role for PMCA2bw is that PMCA2bw may also pump Ca$^{2+}$ directly into the secretory vesicles while it is trafficked to the apical
membrane (12).

The least understood part of mammary calcium transport into milk is the mechanism by which calcium enters the basolateral membrane of the lactating mammary secretory cell. Based on our recent findings in mammary tumor cells, we evaluated a potential role for SPCA2 in eliciting Ca\textsuperscript{2+} entry into the lactating mammary secretory cells, by interaction with Orai1 channels (5). In this study, we examine the interaction of SPCA2 with Orai1 channels throughout lactation, both in native mouse tissue and in a three-dimensional cell culture model derived from mammary epithelium (“mammospheres”). We show that SPCA2 and Orai1 are simultaneously induced early in lactation, colocalize, and are required for Ca\textsuperscript{2+} influx into mammary epithelial cells. Our observations point to a new role for store-independent Ca\textsuperscript{2+} influx (SICE) in the sequestration of Ca\textsuperscript{2+} from the blood for transport to milk.

Results

Coordinated Induction of a Calcium Transporting Module in Lactation

Lactation is characterized by massive transcellular flux of calcium, from the basolateral side of mammary alveolar epithelium (blood) into lumen (milk). This involves coordinated induction of a host of Ca\textsuperscript{2+} channels, transporters, buffering proteins and regulators, and must be tightly modulated to avoid cytoplasmic calcium toxicity. Previously, analysis of transcripts from mouse mammary gland tissue starting at Day 10 before parturition revealed induction of all three classes of Ca\textsuperscript{2+}-ATPases, including isoforms of SERCA, PMCA and SPCA pumps (18). Of note, while PMCA2b isoform
showed the largest transcriptional induction of ~100-fold, the secretory pathway
$\text{Ca}^{2+}$-ATPase was induced early and significantly prior to parturition. Subsequently,
isoform-specific differences in mRNA were noted, with SPCA2 showing higher
transcriptional elevation relative to SPCA1 in lactating mouse mammary gland (7,8).
Here, we validate and quantify isoform-specific differences in SPCA proteins by Western
analysis of mouse lactating mammary tissue. While the housekeeping isoform SPCA1
shows a substantive increase of ~10-fold upon parturition (Figure 2-1A), SPCA2 protein
is elevated by more than 100-fold (Figure 2-1B). Both ATPases remain elevated through
lactation. Next, we evaluated transcriptional induction of Orai1 (19), an SPCA2-activated
$\text{Ca}^{2+}$ channel previously described to elicit $\text{Ca}^{2+}$ influx in breast derived tumor cells (5).
Semi-quantitative PCR analysis showed early induction of Orai1, matching that of both
SPCA2 and PMCA2 (Figure 2-1C). Two other isoforms, Orai2 and Orai3 were also
transcriptionally induced, but at a later stage concordant with SPCA1 induction. STIM1
and STIM2, the calcium sensors in the ER, showed little relative change through the
lactation cycle (Figure 2-1C). Previously, we had shown that SPCA2 interacts with Orai1
by co-immunoprecipitation from breast cancer derived MCF-7 cells and in HEK293 cells
expressing a variety of chimeric and tagged proteins (5). Here, we extended these
observations to endogenous proteins expressed in native mammary tissue where such
interactions may be physiologically relevant to lactation. Immunoprecipitation of SPCA2
from lactating (Day 5) mouse mammary tissue confirmed a physical association with
Orai1 (Figure 2-1D).

**Distinct Localization of Two SPCA Isoforms in Lactating Mouse Mammary Gland**
Previously, Faddy et al. (7) showed that whereas SPCA1 could be stained in all mammary gland cell types, including stromal and myoepithelial cells, expression of SPCA2 was restricted to the luminal epithelium, with no detectable staining outside the acini. Here, we show a further distinction between the two isoforms in subcellular localization as seen in Figure 2-2. Immunostaining of SPCA1 was discretely localized to compartments apical to the nucleus (Figure 2-2A), overlapping largely with the Golgi marker GM130 (Figure 2-2A; merge). In contrast, SPCA2 displayed a broader, punctate distribution with little colocalization with GM130 (Figure 2-2B). Thus, we conclude that in lactating mammary tissue SPCA1 has a conventional Golgi distribution, but SPCA2 is largely found in extra-Golgi vesicles. Secondary antibody controls and pre-block with antigenic peptides are shown in Supplementary Figure S1. We also examined the distribution of Orai1 and STIM1 proteins. As expected for its known ER localization, STIM1 had a diffuse reticular distribution, whereas Orai1 was restricted to basolateral domains of the plasma membrane (Figure 2-2C). Both Orai1 and STIM1 stained myoepithelial cells, seen as patches in the tissue section. We conclude that SPCA2 and Orai1 are co-expressed in luminal epithelial cells of lactating mammary glands where their interaction may be important in mediating transepithelial Ca\textsuperscript{2+} flux.

**Expression and Localization of SPCA2 and Orai1 in Mammospheres**

In order to evaluate potential functional roles in Ca\textsuperscript{2+} handling for SPCA2 and Orai1 in a lactation model, we turned to a three-dimensional culture that mimicked some aspects of native lactating tissue while still retaining the advantages of *in vitro* cell culture. The mouse mammary epithelial line SCp2 responds to basement membrane (Matrigel) and lactogenic hormone (prolactin) by differentiating into alveolus-like
structures characterized by induction and secretion of milk protein, β-casein (15). Formation of mammospheres with distinct lumen and tight junctions occurred over 10 days (Figure 2-3A-B). Transcriptional analysis revealed induction of β-casein in the mammospheres, confirming lactation-induced differentiation. We show increase of SPCA2, PMCA2 and Orai1 expression (Figure 2-3C), consistent with initiation of a lactogenic program for Ca\(^{2+}\) transport as seen in native tissue. Other Orai and STIM isoforms also showed varying levels of transcriptional induction (Figure 2-3C).

Immunofluorescence staining and confocal microscope imaging of mammospheres revealed punctate distribution of SPCA2 throughout the cell, reminiscent of mammary gland staining, with some concentration of puncta near the cell membranes. A merge with the basolateral marker E-cadherin showed apparent colocalization, although more careful evaluation of transverse sections suggests a juxtaposition of SPCA2 puncta immediately under the cell membrane (Figure 2-3D; Supplementary Movie 1). Orai1 localization was enriched at the outer basal membrane of the mammosphere (Figure 2-3E) and a top view of the mammosphere showed a close juxtaposition of SPCA2 with Orai1 (Figure 2-3F; Supplementary Movies 2 and 3). Secondary antibody controls showed no specific staining (Figure 2-3G). Taken together, these observations place a portion of SPCA2 at or near the basal membranes of mammospheres where it may be in position to functionally interact with Orai1 to regulate Ca\(^{2+}\) influx.

**SPCA2 and Orai1 are Critical for Ca\(^{2+}\) entry in Mammary Epithelial Cells**
To investigate the potential contribution of SPCA2 and Orai1 in Ca\(^{2+}\) entry, we used shRNA constructs packaged in lentiviral vectors to knockdown their expression in SCp2 cells. Figure 4A is a Western analysis of cultured SCp2 cells showing significant reduction in expression of both proteins following transfection and selection of shRNA viral constructs. Examination of transcripts by semi-quantitative RT-PCR confirmed knockdown of SPCA2 and all three Orai isoforms (Figure 2-4B). We also noted small, potentially significant changes in transcript levels of SERCA2b (decreased) and SPCA1 (increased) in response to the knockdowns. SCp2 cells with either Orai or SPCA2 knockdown formed normal monolayers and grew at similar rates to control (scrambled shRNA), as seen in Supplementary Figure S2A-B. Although Orai knockdown cells were able to polarize and form tight junctions as seen by the staining with E-cadherin (Supplementary Figure S3A), mammosphere production was nearly absent, and was also noticeably decreased in shSPCA2 treated cells, with concomitant increase in number of small clumps of cells (spheroids; Figure 2-4C-D).

We examined the effect of SPCA2 and Orai1 knockdown on Ca\(^{2+}\) signaling pathways in monolayer SCp2 cells. Resting Ca\(^{2+}\) levels were significantly lowered in both SPCA2 and Orai1 knockdown cells (Figure 2-4E inset), consistent with our previous observation in HEK293 and tumor-derived MCF7 cells (5). In control SCp2 cells, addition of thapsigargin blocks the SERCA2 Ca\(^{2+}\)-ATPase resulting in passive release of SERCA2-filled stores, followed by store-operated Ca\(^{2+}\) entry (SOCE) upon reintroduction of extracellular Ca\(^{2+}\) Figure 2-4E). However, thapsigargin-induced Ca\(^{2+}\) release and subsequent Ca\(^{2+}\) entry were both largely diminished upon SPCA2 and Orai1 knockdown (Figure 2-4E). One interpretation was that ER stores and SOCE were both severely
depleted in these knockdowns, however their normal growth and appearance was not consistent with ER stress or subsequent cell death (Supplemental Figure 2). Indeed, total stored Ca\(^{2+}\) released by ionomycin was similar in control and knockdown cells (Figure 2-4F). Alternatively, a decrease in thapsigargin-releasable Ca\(^{2+}\) can be explained by a shift to thapsigargin-insensitive stores consistent with the transcriptional changes shown in Figure 2-4B. Previously, we showed that overexpression of the thapsigargin resistant SPCA1 pump in HEK293 cells blocked release of stored Ca\(^{2+}\) by thapsigargin and STIM1-mediated SOCE in response to thapsigargin (5). These findings provide functional evidence for a major role in Ca\(^{2+}\) handling for SPCA pumps and Orai channels in a mammary epithelium.

**SPCA2 is required for Cell Surface Trafficking of Orai1 in SCp2 Cells**

Given the interaction between SPCA2 and Orai1, we considered the effect of gene knockdowns on their biogenesis and trafficking in mammary epithelial cells. In control SCp2 cells grown as monolayer, SPCA2 had punctate, perinuclear distribution whereas Orai1 showed both intracellular and plasma membrane staining (Figure 2-5 A, C). There was minor colocalization of the two at the plasma membrane, as detected by cell surface biotinylation (not shown). Upon knockdown of Orai1, SPCA2 localization appeared more restricted to the perinuclear region, with less punctae near the plasma membrane (Figure 2-5B). Strikingly, distribution of Orai1 largely shifted to the same perinuclear localization upon SPCA2 knockdown (Figure 2-5D). Consecutive confocal sections through individual cells showed that Orai1 localized around, but not in the nucleus in shSPCA2 knockdown cells (Supplemental Figure 3B-D). Given that expression of the major Golgi Ca\(^{2+}\)-ATPase SPCA1 was retained (Figure 2-4B), the results suggest that
specific interaction with SPCA2 may be important for cell surface trafficking of Orai1 in mammary epithelium cells.

**C-terminal Domain of SPCA2 Partially Rescues Orai1 Trafficking and Restores Ca\textsuperscript{2+} Influx**

To confirm the role of SPCA2 in cell surface localization of Orai1 in mouse SCp2 cells, we reintroduced full-length, GST-tagged human SPCA2 into shSPCA2 treated cells using virally packaged vectors for efficient transfection. We also introduced the membrane-anchored C-terminal domain of hSPCA2 (hSPCA2C) previously shown to be necessary and sufficient to interact with Orai1 and elicit Ca\textsuperscript{2+} influx (5). hSPCA2C was at least partially effective in redistributing Orai1 out of the perinuclear region to a more punctate location (Figure 2-6A). Full-length hSPCA2 fully reversed the effect of the knockdown, resulting in plasma membrane trafficking of Orai1 (Figure 2-6A).

shSPCA2 knockdown and re-transfected SCp2 cells were loaded with Fura-2 for Ca\textsuperscript{2+} imaging. Neither full-length or C-terminal domain of SPCA2 fully restored thapsigargin-sensitive stores (Figure 2-6B), again consistent with a shift to thapsigargin-insensitive stores. Strikingly, upon addition of extracellular Ca\textsuperscript{2+}, Ca\textsuperscript{2+} influx was elevated and sustained with both constructs, to levels even higher than control (empty vector, EV-GST).

**Store-Independent Calcium Entry (SICE) Requires SPCA2 and Orai1, and is Elevated in Mammospheres**

Recently, we obtained evidence for a novel mode of store independent Ca\textsuperscript{2+} entry (SICE) elicited by SPCA2 via interaction with Orai1 (5). Following brief exposure of
SCp2 monolayer cells to nominally Ca^{2+} free medium, reintroduction of extracellular Ca^{2+} was accompanied by rapid and transient influx that was abolished by knockdown of either SPCA2 or Orai channels (Figure 2-7A). Addition of thapsigargin to SCp2 at 5-30 second intervals following transfer to Ca^{2+}free medium demonstrated that the stores were largely unchanged (Supplemental Figure S4A) as seen by the rate of Ca^{2+} release and peak height. Therefore, the Ca^{2+} influx observed in Figure 7A is not likely to be SOCE. Furthermore, we evaluated maximal SOCE in these cells by comparing thapsigargin-elicited elevation of Ca^{2+} levels in the presence or absence of extracellular Ca^{2+} (2 mM), shown in Supplemental Figure S4B. Maximal SOCE estimated by this method was significantly smaller than that observed in Figure 2-7A. Therefore we conclude that SPCA2 and Orai1 contribute to a store-independent mechanism of Ca^{2+} entry (SICE) that is unmasked upon brief removal of extracellular Ca^{2+}. As additional demonstration of this mechanism of Ca^{2+} entry, we evaluated SICE in shSPCA2 treated cells that were transfected with full-length or C-terminal domain of hSPCA2. Both constructs were able to confer elevated and sustained Ca^{2+} entry to the SPCA2 knockdown cells, consistent with plasma membrane delivery and functional rescue of Orai1 (Figure 2-7B). These results also suggest that a portion of the Ca^{2+} entry observed after thapsigargin addition, as seen in Figure 2-6B, was due to SICE.

Finally, we investigated whether this store-independent mode of Ca^{2+} entry occurred in mammospheres, where SPCA2 and Orai1 are induced upon differentiation. Monolayer and mammosphere cells (Figure 2-7C) were loaded with Fura2-AM, washed and imaged. In differentiated mammospheres, SICE occurred with slower kinetics relative to monolayer cells, and remained elevated and sustained (Figure 2-7D), slowly
returning to baseline (not shown). Basal Ca\textsuperscript{2+} concentrations were similar in mammospheres and monolayer SCp2 cells (Figure 2-7E). It is possible that the slow rise in Ca\textsuperscript{2+} upon induction of SICE is due to slower recruitment of SPCA2-containing vesicles to the mammosphere basal membrane, relative to STIM1, as well as active Ca\textsuperscript{2+} sequestration consistent with transcriptional induction of SPCA pumps in mammospheres. Thapsigargin elicited a smaller release of Ca\textsuperscript{2+} in mammospheres (Figure 2-7F), again consistent with induction of thapsigargin-insensitive SPCA pumps, but Ca\textsuperscript{2+} entry was significantly larger. Taken together, we propose a model in which the induction and interaction of Orai1 and SPCA2 upon lactogenic differentiation results in increased store-independent Ca\textsuperscript{2+} influx.

Discussion

Orai1 Mediates Basolateral Ca\textsuperscript{2+} Influx in Mammary Epithelium

Polarized localization of Orai1 in secretory epithelium has previously been investigated only in pancreatic acinar and salivary gland cells. In pancreatic acinar cells, where secretagogue-induced Ca\textsuperscript{2+} signaling is accompanied by robust Ca\textsuperscript{2+} extrusion and refilling of stores, Orai1 co-localized with STIM1 in puncta along the basolateral membrane (20), consistent with a role in store-operated Ca\textsuperscript{2+} entry. Paradoxically, however, the bulk of Orai1 was seen in the apical membrane where it co-localized and interacted with IP3R but not STIM1. Thus, additional roles for Orai1 separate from SOCE, are likely and remain to be elucidated. An independent study by Hong et al. (21) localized Orai1 to both apical and lateral membranes of pancreatic acinar cells. These
studies highlight the possibility of store-independent roles for this so-called “store-operated” Ca\(^{2+}\) influx channel.

The molecular identity of the calcium influx pathways in mammary epithelial cells has been a mystery. A genome-wide screen revealed several voltage-gated and TRP channels that were expressed but not increased dramatically during Day 1 of lactation (13). McAndrew et al. (22) reported an increase in Orai1, but not Orai2 or Orai3 transcript in lactating mice. Here we show early induction of Orai1, concomitant with calcium pump isoforms SPCA2 and PMCA2, whereas Orai2-3 isoforms are elevated later in lactation (Day 5, post parturition), as seen for SPCA1. Thus, the Orai channels are good candidates for Ca\(^{2+}\) entry in mammary epithelial cells. This was substantiated by a distinct localization of endogenous Orai1 to the basolateral membrane of lactating mammary epithelia and in differentiated mammospheres cultured in vitro. Finally, knockdown of all three Orai channel isoforms abolished store-independent Ca\(^{2+}\) entry in SCp2 cells, implicating a prominent role for these channels in basolateral influx.

**SPCA2 interacts with Orai1 to mediate Store-Independent Ca\(^{2+}\) Entry in Mammary Epithelium**

Previously, we had demonstrated an unexpected moonlighting function of SPCA2, independent of pumping activity, in which the N- and C- termini interacted with Orai1 to elicit Ca\(^{2+}\) entry into cells. A consequence of this signaling activity was the oncogenic role of SPCA2 in breast cancer cells, where dysregulation of expression led to constitutive Ca\(^{2+}\) influx, activation of MAP kinase pathways, high rates of proliferation and tumorigenesis. Furthermore, SPCA2-mediated activation of Ca\(^{2+}\) entry appeared to be
independent of both Golgi and ER stores, and of the STIM sensors (5). Thus, in non-polarized cells, this unusual property would result in a futile cycle of energy independent activation of Ca\(^{2+}\) entry, via Orai1, and energy (ATP)-requiring Ca\(^{2+}\) efflux via active sequestration of Ca\(^{2+}\) into the secretory pathway. However, SPCA2 is restricted to polarized cells of secretory or absorptive epithelia (intestinal, lung, mammary and other glands 2). Therefore, we hypothesized that induction of SPCA2 expression in polarized cells may allow activation of Ca\(^{2+}\) influx channels at one membrane domain, and active transport of Ca\(^{2+}\) at the other membrane domain, to facilitate transepithelial transport of Ca\(^{2+}\) across the epithelium. Here, we provide evidence for a robust physiological role of SPCA2 in promoting SICE in a model of lactating mammary epithelia.

Although both SPCA isoforms are upregulated during lactation, we found that SPCA2 was elevated earlier and to significantly higher levels, compared to SPCA2. Whereas SPCA1 showed discrete localization to Golgi compartments, SPCA2 was predominantly localized to a vesicular compartment, closely associated with both apical and basolateral membranes. These differences are consistent with non-redundant roles for two isoforms of the secretory pathway pumps in mammary epithelium and suggested an isoform-specific critical function for SPCA2 in lactation. We began by showing co-expression, partial colocalization and co-immunoprecipitation of endogenous SPCA2 with Orai1 in lactating mouse mammary epithelium and in differentiated mammospheres. Next, we showed significant induction of SICE concomitant with induction of Orai1 and SPCA2 upon lactogenic differentiation of SCp2 cells into mammospheres. Finally, knockdown of either SPCA2 or Orai channels virtually abolished SICE in mammospheres. Taken together, these data provide evidence for a physiologically
relevant function for SPCA2 in the activation of Orai1. Further investigation should reveal whether SPCA2 also interacts with other Orai isoforms in lactation.

We note that a C-terminal transcript of SPCA2 is expressed under control of MIST1, a beta helix-loop-helix transcription factor, in pancreatic acinar cells (6). This ~20 kDa fragment lacks ATP and Ca$^{2+}$ binding sites, and therefore, has no transport function. However, we found that a minimal membrane-anchored C-terminal domain is sufficient to activate Orai1 and elicit Ca$^{2+}$ influx. It remains to be determined whether native expression of this membrane-embedded C-terminal domain also mediates SICE in various secretory epithelia by interaction with basolateral Orai1.

**Interaction between SPCA2 and Orai1 is required for Orai1 trafficking and SOCE in Mammary Epithelium**

The severe reduction in thapsigargin-sensitive but not ionomycin-sensitive stores upon knockdown of Orai channels suggested a shift in expression and/or localization of thapsigargin-insensitive SPCA pumps. We show a redistribution of SPCA2 to a more perinuclear location, continuous with the nuclear membrane, in the absence of Orai channels. More dramatically, distribution of endogenous Orai1 was limited to a similar perinuclear reticular compartment in shSPCA2 cells, suggesting an arrest in trafficking out of the endoplasmic reticulum. Distinct from conventional endoplasmic reticulum, this specialized sub-compartment remains to be identified. The biogenesis defect was effectively reversed by introducing hSPCA2 into the knockdown cells, ruling out any non-specific effects of the shRNA reagent, with concomitant functional restoration of Ca$^{2+}$ influx. Given the expression of SPCA1 in these cells, we reasoned that Orai1
trafficking did not require secretory pathway/Golgi Ca\(^{2+}\) stores. Indeed, the ability of the 
C-terminal domain of SPCA2 to partially mediate Orai1 exit from this perinuclear 
compartment suggests that Orai1 trafficking requires a chaperone-like interaction with 
SPCA2 protein. Further studies using longer fragments, or chimeric constructs with both 
N- and C-termini implicated in Orai1 binding (5), may narrow down the precise domains 
of SPCA2 that mediate this function. It also remains to be seen whether SPCA2 
facilitates Orai1 trafficking in other secretory epithelia where it is expressed.

In conclusion, we demonstrate an isoform-specific physiological role for SPCA2 
in lactating mammary epithelium. This role is mediated in large part by interaction with 
Orai1, although additional Ca\(^{2+}\) entry channels may also be involved. These studies lay a 
framework for the investigation of SPCA2 function in other secretory or absorptive 
epithelia, where high expression levels have been documented. Prior to this study nothing 
was known about the mechanisms that mediate basolateral calcium entry into the 
mammary secretory cell to support calcium needs for milk production. The data 
presented here demonstrate that SPCA2 and Orai1 function together to regulate SICE, 
which mediates the massive basolateral Ca\(^{2+}\) influx into mammary epithelia to support the 
large calcium transport requirements of lactation.

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Experimental Procedures:

Animals.

All animal work was conducted according to relevant national and international guidelines. The National Animal Disease Center’s Animal Care and Use Committee approved all animal procedures. Pregnant or lactating 129/SV mice were housed individually, in hanging basket cages on sawdust bedding. All mice were equalized to 6 pups per mouse mother on day one of lactation. Mice were killed at times indicated. Mice were anesthetized with a 50:50 mix of CO$_2$:O$_2$ followed by decapitation. Mammary tissue was removed and fixed as described below or flash frozen in liquid N$_2$, and stored at −70 °C until processed as described.

Cell Culture

SCp2 cells (15; gift of Andrew Ewald, Johns Hopkins University) were cultured in monolayer media-DMEM/F-12 (1:1) with 5% HI-FBS and insulin (5 μg/ml) at 37°C in a 5% CO$_2$ incubator with ample humidity. Upon 80% confluence, they were trypsinized in 0.25% Trypsin with EDTA (Invitrogen) and centrifuged at 8 rpm for 2 minutes to remove excess trypsin. Cell growth was monitored using Cell Titer 96 AQ from Promega according to manufacturer’s instructions. For mammospheres, trypsinized cells were then mixed with a solution containing 50% Matrigel (Sigma) and 50% differentiation media (DMEM/F-12 (1:1), hydrocortisone (1 μg/ml), insulin (5 μg/ml) and recombinant mouse prolactin (3 μg/ml). Cells were plated at 1x10$^3$ on a 100% Matrigel matrix on 25 mm round coverslips. Matrix was again allowed to solidify prior to the addition of differentiation media to cover the layered coverslip. Mammosphere culture was allowed
to differentiate for 10 days, changing the media every second day, prior to assays for differentiation.

_Mammosphere Culture_

SCp2 cells were plated on 25 mm coverslips for three-dimensional culture at $10^3$ cells per coverslip. These coverslips were placed in a standard cell culture incubator and allowed to differentiate. Differentiation liquid media was changed every two days and replaced with fresh media. Cellular clusters (consisting of dense groups of >4 cells each) were counted and categorized into one of three categories: 1) undifferentiated clusters—cells which maintained characteristic of monolayer culture but were in close proximity to one another (single cells); 2) spheroids—partially differentiated cell clusters with characteristics which showed lack of tight junction formation and lumen; 3) mature mammospheres—cells which grouped into a differentiated mass with tight junctions and a lumen. All three classifications were considered clusters (belonging to group 1) but the proportion of spheroids to mature mammospheres and the percentage of cells, which went through the entire development program, were noted.

_RNA collection and RT-PCR_

$10^6$ cells were washed with sterile Hank’s Balanced Salt Solution (HBBS; Invitrogen) prior to RNA extraction. An RNAeasy kit was used to collect the total RNA via centrifugation (Qiagen) per the manufacturer’s instructions and quantified by absorption at 260 nm by spectrometry. RNA was converted to cDNA by reverse transcription using iScript (Bio-Rad Laboratories). Equal amounts of cDNA was subjected to PCR amplification for specific transcripts using the following primers:
Orai: 5’-ACCCACGAGCGCATGCATC-3’ (forward) and 5’-GCTTGGTGGGGGCTTGGCTGT-3’ (reverse); Orai2: 5’-CTGAGGTGGTGCTCTGCTCTC-3’ (forward) and 5’-GCTAAGGTGGATGGTGAAG-3’ (reverse); Orai3: 5’-CATCCACACATCTCACTCTG-3’ (forward) and 5’-GACCTGCTGCTGCTGACGG-3’ (reverse); SPCA1: 5’-CCAGTGTTGCGGCTTGGCTGGAC-3’ (forward) and 5’-TCAGCCTGGAGGACGGCTGCAA-3’ (reverse); SPCA2: 5’-GACCTGCTGCTGCTGACGGG-3’ (forward) and 5’-CAGGCCAGAGGCACCCAAAGC-3’ (reverse); PMCA2: 5’-CAGGGCTCGACCCCTCGGAG-3’ (forward) and 5’-CATGGGTCGGGACAGCTCCCCTA-3’ (reverse); SERCA2B: 5’-ACTTCTTGATCCCTCTACGTG-3’ (forward) and 5’-AGACCAGAACATATCGCTAAA3’ (reverse); actin: 5’-GCAGCTCCTTCTGCTTGCCGGT-3’ (forward) and 5’-TACAGCCGGGGAGCATGTT-3’ (reverse). The number of amplification cycles was adjusted to ensure that generation of products was not saturated. Loading control was mActin.

Membrane Preparation and Western Blotting

Mammary tissue microsomes were prepared as previously described (12). Briefly, tissue was homogenized in 10 volumes of Buffer A containing Tris–HCl (10 mM), MgCl₂ (2 mM), sucrose (300 mM) and a complete protease inhibitor cocktail (Boehringer
Mannheim Indianapolis, IN) at pH 7.5. The homogenate was mixed with an equal volume of Buffer B (Buffer A plus 0.3 M KCl) and centrifuged at 4000g for 10 min. The supernatant was collected, adjusted to 0.7 M KCl, and centrifuged at 100,000g for 1 h. The supernatant was discarded and the pellets were resuspended in Buffer C (Buffer A plus 0.15 M KCl).

Microsomes from mammary tissue were incubated for 15 min at room temperature in a modified Laemmli buffer containing 150 mg/ml urea and 65 mM DTT. Samples were then electrophoresed for 50 minutes at 200 volts in a 4-12% Novex NuPAGE® Bis-Tris Gel using MOPS SDS running buffer (Life Technologies, Grand Island, NY). Proteins were transferred to nitrocellulose membranes using the iBlot Dry Blotting System (Life Technologies, Grand Island, NY) at 23 volts for 7 minutes. The blots were blocked with StartingBlock T20 (Thermo Fisher Scientific Inc). SPCA1 (RS-1 #227) or SPCA2 (orange #2) antibodies were diluted 1/2000 in StartingBlock T20 and the blots were incubated over night at 4°C. After washing they were incubated 1 hr at RT with 1/50000 HRP goat anti-rabbit #31460 (Thermo Fisher Scientific Inc) and washed. Blots were developed using Pierce’s Supersignal (Pierce Products, Rockford IL) using the protocol provided by the manufacturer. Developed film was imaged and bands quantitated with a Gel Doc EZ imager (BioRad, Hercules, CA).

1×10⁶ cells from cell culture were washed three times with sterile HBBS (Invitrogen) then immediately lysed in buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 5 mM Na₃P₂O₇, 1 mM Na₃VO₄, 10 mM NaF, pH
7.4 with 1% SDS in the presence of protease inhibitor cocktail (Roche). Protein concentrations were assayed using a bicinchoninic acid assay kit (Pierce). Approximately 50 μg of protein was loaded onto a NuPAGE gel for SDS-PAGE Analysis and Western blotting. Immunoblots were made on 0.45μm nitrocellulose membrane (Bio-Rad). Antibodies for mouse SPCA2, Orai1 and GAPDH were incubated with the membrane overnight at 1:1000 dilution in 1xPBST (PBS plus 0.2% Tween). Membranes were washed five times in PBST then mouse or rabbit secondary antibody conjugate to HRP was added (GE Healthcare UK Limited) at 1:2000 for one hour at room temperature on a rotator and the membranes were washed three times with PBST then four times with normal PBS to remove Tween residue. Blots were visualized using ECL kit (Pierce) on an Intelligent Dark Box Imager (Fuji Film).

Immunofluorescence Microscopy

Mammary tissue was fixed in Telleyesniczky's fixative (70% ethanol, formalin, glacial acetic acid, 20:2:1) for 5 h at room temperature (16) and then stored in 70% ethanol prior to paraffin embedding. Paraffin-embedded sections were cleared in xylene and rehydrated through an alcohol series. Tissue sections were immersed in antigen unmasking solution H-3300 (Vector Laboratories; Burlingame, CA) in a preheated pressure cooker. Sections remained in the pressure cooker for 20 minutes after the pressure maximized. The sections were then allowed to cool, wash 3 times with distilled, deionized water followed by PBS for 5 minutes. After permeabilization/blocking of the sections with PBS containing 0.5% Triton X-100, .01 g sodium azide and 50 mg/ml BSA, the primary antibodies were applied and incubated at 4°C overnight. Primary antibodies used were 1/50 Orai1 # 4281 (ProSci Incorporated, Poway, CA), 1/50 Stim1 # 610954
Monolayer cultures were washed three times with 1xPBS prior to fixation in 4% paraformaldehyde and subsequent permeation in blocking buffer (1% BSA and 0.5% Triton-X in 1xPBS). Cells were then blocked and permeabilized in 0.1% Triton and 1% BSA in 1xPBS and washed three times in 1xPBS. Cells were incubated with antibodies to SPCA2 (5), Orai1 (Sigma) and E-cadherin overnight at 4°C. The next day cells were washed again (three times in 1xPBS) and incubated with conjugated anti-rabbit and anti-mouse (AlexaFluor 288 and 388, respectively) for one hour at room temperature. Finally, cells were washed in 1xPBS three times, washed briefly in DAPI and in sterile water then mounted onto slides with DAKO Fluorescent Mounting Medium (DAKO).

Mammospheres were fixed in ice-cold methanol and acetone (−20°C; 1:1) for five minutes on ice (7). Subsequently, the mammospheres were permeabilized and blocked in 0.1% Triton and 1% BSA in 1xPBS for 1 hour at RT. They were then washed in 1xPBS
for 5 minutes three times. Primary antibodies against SPCA2, Orai1 and E-cadherin were
diluted in 0.2% BSA in 1xPBS. Mammospheres were allowed to incubate in primary
antibodies at 1:250 dilutions overnight at 4°C. The next day, the mammospheres were
washed gently in 1xPBS three times for 5 minutes at RT. Next, rabbit and mouse
conjugated AlexaFluor secondaries (AlexaFluor 288 and 388, respectively) were
incubated at a 1:1000 dilution at RT for 1 hour in the dark, with slow agitation. Cells
were washed with 1x PBS three times for 10 minutes with slow agitation in the dark for 5
minutes per wash. Cells were DAPI stained by brief submersion, washed in distilled,
deonized water for 1 minute then the coverslips were permanently mounted to slides
with fluorescent mounting medium (DAKO) and allowed to dry overnight in the dark.

Lentiviral Production and Transfection

pLK0.1 plasmids were obtained from Sigma (Mission shRNA constructs) for
SPCA2 and Orai1. HEK293T cells were inoculated with 1µg plasmid DNA for each
accessory packaging protein for lentiviral packaging. Media was removed from the cells
after 72 hours and virus was purified using Lenti-X (Sigma) per the manufacturer’s
instructions. Approximately 10µg of virus was added to each well of a 6 well plate
containing SCp2 cells at 50-70% confluence and allowed to incubate for 48 hours. The
virus was then removed and cells were washed then inoculated with new media
containing 500µg/ml of puromycin for selection over 3 days. Once the cells were
selected, they were grown in the selection media for two weeks then discarded. Each
experiment was done within 5 passages of the cells to ensure complete RNA inhibition.

Calcium Imaging
Cells were cultured as a monolayers or mammospheres on 25 mm circular coverslips. Briefly, cells were washed in sterile HBBS (Invitrogen) for five minutes at a time, three times. After washing, FURA2-AM (Invitrogen) was added at a final concentration of 1 μg/ml in imaging buffer (20 mM Heps, 126 mM NaCl, 4.5 mM KCl, 2 mM MgCl₂, 10 mM glucose at pH 7.4) containing 2 mM CaCl₂. After incubation at room temperature for 20 minutes, cells were washed briefly in imaging buffer without calcium and 0.15% EGTA. Cells were then washed twice in imaging buffer without calcium to wash away residual EGTA and Ca²⁺.

For mammosphere imaging, protocols were kept the same, however, after FURA2 loading, two 2 mM Ca²⁺ washes were added with slow agitation to remove FURA2 from the Matrigel, prior to washing in imaging buffer without calcium. Store dependent (SOCE) and independent (SICE) Ca²⁺ entry was measured as described (5). Cells were excited at 340 nm and 380 nm, and Fura emission was monitored at 505 nm. For SOCE, cells were switched from 2 mM Ca²⁺ to nominally Ca²⁺ free recording buffer. Thapsigargin (2 μM) or ionomycin (2 μM) was added where indicated, and followed by readdition of 2 mM Ca²⁺ to measure store-dependent Ca²⁺ influx. For SICE, fluorescence was recorded from cells placed in nominally Ca²⁺ free recording buffer, followed by addition 2 mM Ca²⁺, activating store-independent Ca²⁺ influx.
Figure 2-1: Expression profiles of calcium transporters in lactation. Mouse mammary tissue, starting from day 10 prior to parturition, was evaluated for the expression of (A) SPCA1, and (B) SPCA2 by Western blotting. Loading was normalized relative to tissue DNA concentrations, and expressed relative to starting levels at Day -10. C) RT-PCR of mRNA for isoforms of SPCA, Orai, PMCA and STIM proteins at distinct time points of mammary development, including pre-pregnancy (virgin), parturition (Day 0), lactation (Day 5) and involution (Day 5 after removal of pups). Transcripts are grouped as early, mid and unchanged, according to their time of induction following lactation on Day 0. D) SPCA2 was immunoprecipitated from lactating mouse mammary tissue using polyclonal rabbit anti-hSPCA2 peptide antibody or rabbit IgG as control. Orai1 was detected as a co-immunoprecipitate by immunoblotting (IB).
Figure 2-2: **Immunofluorescence microscopy of SPCA1, SPCA2, Orai1 and STIM1 in lactating mouse tissue.** Confocal microscopy imaging of sections treated as described under Experimental procedures. A) SPCA1 co-localizes with the Golgi marker, GM130. B) SPCA2 has a diffuse distribution, with little co-localization with Golgi marker, GM130. C) Orai1 (left panel) and STIM1 (right panel) show basolateral and reticular localization, respectively. Scale bar: 100 μm.
Figure 2-3: Expression of SPCA2 and Orai1 in mammospheres. A) Schema for induction of SCp2 cells into mammospheres. B) Cells in monolayer migrate and assemble into mammospheres following induction. C) Semi-quantitative RT-PCR showing that SPCA2, Orai1-3 and STIM1-2 are induced along with known markers β-casein and PMCA2 in the transition of SCp2 cell into differentiated mammospheres. D-F) Confocal sections of mammosphere showing indirect immunofluorescence of the indicated proteins; asterisks mark some areas of co-localization. Orthogonal sections are shown with the merged image. The cartoons indicate approximate location of optical plane through the mammosphere. D) Confocal section of a single layered mammosphere shows vesicular distribution of SPCA2 with some colocalization in the vicinity of E-cadherin as seen in the orthogonal view. E) Differentiated mammosphere showing Orai1 limited to the basal membrane with some SPCA2 punctae just below the plasma membrane, seen in orthogonal view. F) Top view of mammosphere shown in (E). Colocalized SPCA2 (green) and Orai1 (red) appear yellow. G) Secondary anti-mouse or anti-rabbit antibody alone shows no staining of mammosphere.
Figure 2-4: Effect of SPCA2 and Orai1 knockdown on mammosphere formation and Ca\textsuperscript{2+} influx. A) Western blot of SCp2 lysates derived from cells expressing shSc (scrambled shRNA), shSPCA2 and shOrai1 showing effectiveness of knockdown. B) RT-PCR of SPCA, Orai, SERCA2b and STIM1 isoforms in SCp2 cells following treatment with shRNA as indicated. Note that all three Orai isoforms were depleted in cells treated with shOrai1 virus. C) Comparison of small, aborted spheroids with mammospheres in SCp2 cells. D) Quantification of spheroids and mammosphere types in shRNA treated SCp2 cells ten days after lactogenic induction (* p\textless 0.05; ** p\textless 0.005). E) SOCE is drastically reduced in SCp2 monolayer cells treated with shSPCA2 and shOrai1 constructs (results averaged from n=76 cells for shEV; n=98 for shSPCA2; n=123 for shOrai1). Fold change is the normalized change in fluorescence ratio (340/380 nm) of Fura-2. Inset: Fura2 fluorescence ratios showing baseline levels of free Ca\textsuperscript{2+} are lower in knockdown.
**Figure 2.5: Depletion of SPCA2 blocks cell surface trafficking of Orai1. A-B**)

Immunofluorescence labeling of mSPCA2 shows less vesicular distribution of SPCA2 in cells treated with shOrai1. **C-D**.) Immunofluorescence labeling of mOrai1 in SPCA2 knockdown cells shows retention to the perinuclear region only. SPCA2 and Orai1 signals from conjugated anti-rabbit and anti-mouse (AlexaFluor 288 and 388, respectively) secondary antibody were pseudocolored for ease of comparison.
Figure 2-6: Ectopic expression of hSPCA2 constructs restores Ca\textsuperscript{2+} influx and Orai1 trafficking. A) Immunofluorescence staining of mOrai1 shows trafficking defect in shSPCA2 cells (top panel). When the C-terminal end of hSPCA2, hSPCA2-C is ectopically expressed mOrai1 staining appears more vesicular (middle panel). Full-length hSPCA2 fully rescues mOrai1 trafficking, showing both plasma membrane and vesicular localization seen (bottom panel). B) SOCE is restored for shSPCA2 treated cells ectopically expressing either full-length hSPCA2 or C-terminal domain, hSPCA2C (results averaged from n=111 cells for EV/GST; n=63 for shSPCA2/CMV-EV; n=67 for shSPCA2/CMV-hSPCA2C and n=75 for shSPCA2/CMV-shSPCA2). Fold change is the normalized change in fluorescence ratio (340/380 nm) of Fura-2. Error bars reflect standard deviation from the mean for each measurement and time point using a Student’s t test.
Figure 2-7: Store Independent Ca$^{2+}$ Entry requires SPCA2 and Orai1.

A) SICE is virtually abolished in monolayer cells expressing shRNA constructs for SPCA2 and Orai1 constructs (results averaged from n=91 cells for shSc; n=82 for shSPCA2; n=92 for shOrai1). Error bars reflect standard deviation from the mean for each measurement at each time point. B) SICE is restored and elevated in shSPCA2 treated cells expressing either hSPCA2 or hSPCA2C (results averaged from n=94 cells for EV/GST; n=74 for shSPCA2/CMV-EV; n=91 for shSPCA2/CMV-hSPCA2C and n=104 for shSPCA2/CMV-shSPCA2). C) Fluorescence image of Fura-2AM loaded in monolayer cells, and mammospheres. (D) Store independent Ca$^{2+}$ influx in monolayer cells and differentiated mammospheres. After a brief (~20 s) incubation in nominally Ca$^{2+}$ free extracellular medium, readdition of Ca$^{2+}$ (2 mM) elicits Ca$^{2+}$ entry that is transient in monolayer cells but elevated and sustained in mammospheres (n=129 and n=84 for monolayer and mammosphere cells, also respectively). E) Basal Ca$^{2+}$ levels are similar in mammospheres, relative to monolayer cells as seen by Fura-2 fluorescence ratio. F) Store-dependent Ca$^{2+}$ influx in monolayer cells and differentiated mammospheres. Addition of thapsigargin (Tg) empties the internal (ER) stores in nominally Ca$^{2+}$-free medium. Upon readdition of extracellular Ca$^{2+}$ (2 mM), Ca$^{2+}$ influx is slightly higher in mammospheres (n=89 and n=67 for monolayer and mammosphere cells, respectively). Fold change is the normalized change in fluorescence ratio (340/380 nm) of Fura-2.
Supplemental Figures
Supplemental Figure 2-1: Control experiments for immunostaining of mammary gland. Sections of lactating mouse mammary tissue were treated with a mixture of anti-SPCA1 or anti-SPCA2 antibody either with or without preincubated with the immunogenic peptide as indicated. Secondary antibody controls used in the absence of SPCA antibodies resulted in no specific signal, as shown. Nuclei are detected by DAPI staining as described in Methods.
Supplemental Figure 2-2: Morphology and Growth of SCp2 cells after transfection with shRNA constructs. A) Growth of SCp2 cells after transfection with lentivirus carrying empty vector or shRNA against SPCA2 or Orai1 was monitored using MTT assay as described in Methods. No significant differences were observed following knockdown. B) Morphology of SCp2 cells following knockdown of SPCA2 and Orai1 is similar to that of control cells.
Supplemental Figure 2-3: Confocal sections of Immunofluorescence staining in Orai1 (A) and SPCA2 (B, C) knock down SCp2 cells. A) Consecutive optical sections, starting from the bottom, of SCp2 cells knocked down for Orai channels stained with E-cadherin (red), SPCA2 (green) and DAPI (blue). Note the formation of tight junctions indicated by E-cadherin stain and normal shape of the cells. B) Consecutive optical sections, starting from the bottom, of SCp2 cells knocked down for SPCA2 stained with Orai1 (green) and DAPI (blue). Note the separation of Orai1 stain from the nucleus at the bottom and top sections. C) Confluent SCp2 cells treated with shEV (top) or shSPCA2 (bottom) stained for Orai1 (green) and DAPI (blue). Note the change in Orai1 localization from the cell boundaries (top) to circumnuclear (bottom).
Supplemental Figure 2-4: Estimation of Stored Ca$^{2+}$ in SCp2 cells. A) SCp2 cells were transferred to nominally Ca$^{2+}$ free medium at time 0 and thapsigargin was added between 5-30 seconds as indicated. Ca$^{2+}$ release was monitored by Fura2 (340/380 ratio). B) SCp2 cells were transferred to nominally Ca$^{2+}$ free medium or not, at time 0 as indicated. Baseline Ca$^{2+}$ before addition of thapsigargin was unchanged (inset i) Thapsigargin was added and Ca$^{2+}$ release was monitored as fold-change relative to starting 340/380 ratios. The difference between the traces, indicated by gray shading, was plotted in inset ii. This indicates maximal SOCE resulting from thapsigargin mediated store release. Note that it is smaller than Ca$^{2+}$ influx observed in Figure 6B.
References


Chapter 3

SPCA2 Interacts with TRPC6 Channels during Mammary Development
Introduction

The Secretory Pathway Calcium ATPases (SPCAs) are highly conserved Ca\textsuperscript{2+}/Mn\textsuperscript{2+} pumps, localizing to the Golgi and Golgi-derived vesicles. Whereas SPCA1 is expressed ubiquitously, SPCA2 has a more specific expression in secretory cells (1) such as brain, salivary glands, testis, and mammary tissue. Recently, our lab has shown that SPCA2 opens Orai1 Ca\textsuperscript{2+} channels at the plasma membrane, independent of its ATPase function (2). Here, we evaluated interaction of SPCA2 with Transient Receptor Potential cation channel, subfamily C (TRPC) channels, in particular, TRPC6.

TRPC channels are Na\textsuperscript{+}/Ca\textsuperscript{2+} permeable ion channels with a preference for Ca\textsuperscript{2+}. They have been shown to be upregulated in multiple cancer subsets (3), including breast cancer (4-6), as described in Chapter 1. We sought to examine the whether SPCA2 interacted promiscuously with other channels, including TRPC type. Unpublished work from a former graduate student indicated a physical interaction between TRPC6 and SPCA2 when expressed in HEK293 cells. Therefore, we sought to determine if these interactions occurred in native tissue. Here, we present preliminary evidence for both physical and transcriptional coupling between SPCA2 and TRPC6.

Results

Reciprocal alterations in SPCA2 and TRPC6 expression

Lactation is a normal physiological process wherein Ca\textsuperscript{2+} is sequestered into the mammary cells from the blood pool (2mM Ca\textsuperscript{2+}) and secreted at a much higher concentration, into the milk (40-80mM Ca\textsuperscript{2+}) (7). In this process, Ca\textsuperscript{2+} must be mobilized in vast amounts for storage and secretion. An ordered series of transcriptional changes
bring about biochemical and structural transitions of the breast tissue through the stages of breast development and recession (involution) (8). It has been reported that this process induces plasticity in the polarized epithelia of mammary glands, which may be preventative for breast cancer (8, 10).

We used normal mouse mammary tissue to look at the expression of SPCA2 and TRPC6 during lactation (Fig. 3-1A). As reported previously, we observed early elevation of SPCA2 during parturition as well as a biphasic upregulation of TRPC6 (8-9). SPCA1 increased in expression with the continuation of lactation, whereas TRPC6 levels fell during the mid-phase of lactation before increasing during involution. Other calcium transporters showed distinct patterns of expression: some were elevated early (PMCA2), or mid-lactation (SPCA1), others remained unchanged (PMCA1) (Fig. 3-1a; Pumps). Calcium channels (Fig. 3-1a; Channels) increased relatively later in lactation, around day 5 with the phospholipases (Fig. 3-1c; Regulators) with the exception of TRPV6, which showed a decrease in expression upon progression of lactation and, conversely, Orai1, which shows increased expression. Notice that the increase to SPCA2 in early lactation is reciprocal to that of TRPC6 transcript. Surprisingly, we also obtained evidence that these two proteins physically interacted with each other in vivo. Thus, when we pulled-down SPCA2 from lactating mouse mammary tissue, we observed TRPC6 in the immunoprecipitate (Fig. 3-1B). The functional effect of this physical interaction remains to be determined and is suggestive of a dual regulation of TRPC6 by SPCA2.

**Alteration in SPCA2 and TRPC6 in the prolactin-inducible cell line, Scp2**
To further explore the reciprocal transcriptional expression of SPCA2 and TRPC6, we used the prolactin-inducible, mouse mammary cell line, Scp2. We collected RNA from the undifferentiated/monolayer cells and the differentiated/3-D cells and compared the expression of SPCA2 and TRPC6, using the elevation of the milk protein, β-casein as a control for differentiation and GAPDH as a loading control (Fig. 4-1D). We saw a significant decrease in the mRNA transcript for TRPC6 upon differentiation of the Scp2 cells. Upon prolactin exposure we knew that SPCA2 increased dramatically, both at the mRNA and protein level (previously shown; Chapter 2).

We wondered if the effects we saw in lactation were dependent on the expression of molecular SPCA2 or induced by the lactogenic hormone cascade. Thus, we sought to inhibit the expression of SPCA2 by shRNA expression. We examined the effect of SPCA2 knockdown in monolayer Scp2 cell cultures with shSPCA2 (Fig. 4-1E). Again, we obtained evidence for reciprocal transcription of SPCA2 and TRPC6. We show that TRPC6 mRNA levels increased in cells treated with shSPCA2. However, no change to TRPC6 transcript occurred for the shOrai1 transfected cells. This suggests a molecular specificity for regulation of TRPC6 under the control of SPCA2 expression.

**SPCA2 and TRPC6 colocalize in Scp2 cell model of lactation**

Since both native tissue and prolactin-induced cultures showed significant, albeit, reciprocal expression of SPCA2 and TRPC6, and they could be complexed together, we next evaluated colocalization. We labeled SPCA2 and TRPC6 via immunofluorescence in the monolayer cultures (Fig. 3-2A) and following differentiation into the 3D cell culture model of lactation (Fig. 3-2B). In both cases, we observed partial co-localization
of SPCA2 and TRPC6. Further work is needed to quantify and evaluate the functional effects of this colocalization.

**OAG-inducible calcium influx reflects reciprocity of SPCA2 and TRPC6 expression**

A subset of the TRPC family, including TRPC3, TRPC6 and TRPC7, can be activated by diacyl glycerol and its synthetic analog, OAG. We used OAG-elicited Ca\(^{2+}\) fluxes, measured by Fura2 fluorescence, to evaluate the activity of TRPC channels in monolayer and differentiated Scp2 cells. Indeed, we saw specific OAG-activated calcium fluxes for monolayer (Fig. 3-3A) and differentiated cells (Fig. 4-3B). Furthermore, when we probed shSPCA2-treated Scp2 cells for OAG-inducible calcium flux (Fig. 4-3C), we noticed a significant increase to the peak height of the recording, indicating a higher rise in cytoplasmic calcium than monolayer cells with SPCA2 present. When we used IMF to localize TRPC6 in shSPCA2 and control cells (shSc), we had great differences in fluorescence emission (Fig. 3-4A). We used confocal microscopy techniques to quantify this increase which was apparent in the micrographs (Fig. 4-4B).

To investigate whether artificial induction of SPCA2 via retroviral vector (pLXRN-CMV), under the control of a high transcription, CMV promoter would decrease the amount of TRPC6 expressed in breast epithelia (Fig. 3-4-C). We noticed a significant decrease in TRPC6 expression for C-terminal truncated SPCA2 overexpression (CMV-SPCA2C) and the catalytically dead mutant, SPCA2-D379N (CMV-D379N). Control (CMV-EV) cells and those transfected with full-length SPCA2 (CMV-SPCA2), which, surprisingly, showed the highest expression of TRPC6 mRNA. FURA-2AM (Fig. 3-4D) imaging in 2mM Ca\(^{2+}\) showed that 1-Oleoyl-2-acetyl-sn-
glycerol (OAG; 100µM) stimulation of calcium influx was significantly reduced in CMV-SPCA2C and CMV-D379N cells, while CMV-EV and CMV-SPCA2 cells showed little significant difference in calcium influx upon stimulation via OAG.

**Discussion**

Evidence remains inconclusive for the regulation of TRPC6 by SPCA2 expression. In the lactogenic cycle we noticed the same biphasic regulation as previously described. However we were able to show that should SPCA2 be prevented from expression, TRPC6 seems to play a compensatory role, with high functional expression levels in the Scp2 model of lactation. Interestingly, the knockdown of SPCA2 by an artificial plasmid would inhibit the expression of TRPC6, while the overexpression of SPCA2 would have a slight increase to the levels of TRPC6 in the same cell line. Additionally the changes to the calcium signatures of overexpressing cells seems to suggest that there is a likelihood of a complex change to intracellular calcium upon overexpression of these varying forms of SPCA2. The increase to calcium fluxes in the store-operated calcium influx pathways affirms our hypothesis of cross-talk between the secretory pathway and canonical store-operated calcium pathways. The increases to intracellular calcium, suggest that SPCA2 plays a variety of roles increasing the basal concentration of calcium through eliciting other pathways. Since we see no significant change in store-independent calcium signaling, it would be interesting to probe further into the role of the secretory pathway calcium and ions role in filling intracellular stores.

**Materials and Methods**
Cell Lines and Cell Culture

Scp2 Normal Mouse Mammary Epithelia 2D Culture:

Scp2 cell lines were a generous gift from the laboratory of Dr. Andrew Ewald (Johns Hopkins University- School of Medicine, Department of Cell Biology). The Scp2 cells were cultured in monolayer in DMEM-F12 1:1 media with HI- FBS (5%) and insulin (5µg/ml). Cells were split upon reaching 80% confluency then split for monolayer stock and 3-D culture. Cells used in biochemical assays (Fura2) were used at 80% confluency for the undifferentiated, monolayer control.

Scp2 Normal Mouse Mammary Epithelia 3D Culture:

3-D culture was carried out by resuspending trypsinized cells in differentiation media (DMEM-F12 with insulin (5µg/ml), hydrocortisone (1µg/ml) and prolactin (3µg/ml). Cells were pipetted onto Matrigel™ coated coverslips (5mg/ml diluted in differentiation media; 25mm round flint glass coverslips). Cells were allowed to adhere to the Matrigel™ for 10 minutes then Matrigel™ was added to the top of the cells in a drop wise manner to a final concentration of 50% Matrigel™ (2.5 mg/ml). Differentiation media was changed every 2 days and the cells were allowed to differentiate for 10 days prior to use.

RT-PCR

Mouse mammary cDNA was synthesized from RNA extracted using RNeasy extraction kit (Qiagen) from either ScP2 cells (10^6 cells) or from mouse tissue (100µg). mRNA was reverse transcribed using iScript™ cDNA synthesis kit (Biorad). cDNAs were
diluted 1:10 in nuclease-free water (Sigma) and Taq amplification of SPCA2 and TRPC6 was performed using the following mouse specific primers:

mTRPC6: forward 5’-CTTCCACTGTGGCGGACGGGAGGGACGG-3’ and reverse 5’-CCTGCACCCCTGCTTTCCGTG-3’

mSPCA2: forward 5’-GACCTGCTGCTGACGGGAGGGACGG-3’ and reverse 5’-CAGGCCAGGAGGCACCCAAGC-3’.

Amplified products were run on a 2% agarose gel containing ethidium bromide and visualized by UV transillumination.

**Fura-2 Ca^{2+} Imaging:**

Cells were harvested at 80% confluency (monolayer/undifferentiated Scp2, HEK293) or at 10 days (Scp 3D cultures) and washed with Imaging Buffer (IB: 121 mM NaCl, 6 mM NaHCO3, 5.4 mM KCl, 5.5 mM D-glucose, 0.8 mM MgCl2, 25 mM HEPES, 1.8 mM CaCl2, pH 7.4) three times for 5 minutes per wash. Cultures were then incubated with Fura-2 dye (2μM) in IB for 30 minutes and subsequently washed three times for 5 minutes per wash. Cells were then subjected to a brief EGTA wash (0.1mM), washed in Ca^{2+}-free IB, prior to being imaged on an Olympus inverted microscope. Thapsigargin or OAG was added to cells as the baseline normalized (t=100sec.) in Ca^{2+}-free IB. Ca^{2+} was added back to the cells in IB once Thapsigargin or OAG effect went back to baseline (t=500 sec.). Images were taken at 5 second intervals and recorded via the ratio of fluorescence of 340 to 380nm (340nm/380nm= ΔR). Results were subjected to a student’s t-test to establish statistical relevance (error bars represent the standard deviation from the mean).
**Mouse Mammary Tissue Immunoprecipitation**

For co-immunoprecipitation, we used 200 μg (wet weight) lactating mammary tissue from 129/SV mice was pulverized with a mortar and pestle in dry ice. Protein extraction was acquired with 0.5 mL MOPS buffer with 1% Triton-X and protease inhibitor cocktail (Roche). Lysates were clarified by 3 subsequent rounds of 13,200 rpm centrifugation at 4°C followed by sonication. Gammabind™ Plus Sepharose™ (GE Healthcare) beads were pre-clarified with lysates while another set of beads were incubated with either 5 μg SPCA2 antibody or 5 μg rabbit IgG antibody. Pre-cleared lysates divided and then incubated overnight with the beads containing antibodies. Beads were washed three times with 1xPBS + 1% Triton X-100 then 1/5th of each aliquot was ran on a 5-12% SDS-PAGE gradient gel. Gels were transferred to nitrocellulose blots and probed with either SPCA2 antibody or TRPC6 antibody. These blots were then probed with a secondary antibody for non-denatured IgG and imaged with horseradish peroxidase chemiluminescence (as described previously in Cross, et al., 2013)

**Immunofluorescence of 3D Mammospheres**

Once 3D cultures were 10 days old, they were washed 3 times with 1xPBS then fixed in ice cold methanol/acetone solution (v/v; 1:1). Mammospheres were then blocked in IMF wash buffer (0.1% BSA, 0.2% TritonX-100, 0.05% Tween, 10% goat serum) for one hour. Primary antibodies were added at 1:1000 in IMF buffer and allowed to incubate overnight at 4°C. The following day, the slides were prepared for secondary antibody by washing in IMF wash buffer three times for 5 minutes each and gently shaking.
Secondary antibodies used were: Alexa Fluor® 488 (goat anti-rabbit) and Alexa Fluor® 568 (goat anti-mouse) at 1:1000 dilutions in IMF wash buffer for 1 hour. The embedded cells were then washed, as mentioned previously; DAPI stained, washed in sterile water and mounted in Dako fluorescent mounting media and allowed to dry overnight.
Figure 3-1: Expression Profiles Of CALTRANS Components In Breast Tissues And Cell Culture Model Of Lactation. A) mRNA samples were taken from lactating mouse tissue before (Virg), at the onset (Lac 0), during (Lac 5) and after (Inv. D2) the lactation cycle. mActin was used as a loading control. C) TRPC6 immunoprecipitates with SPCA2 from lactating tissue at the onset of lactation (Lac 0). D) The Scp2 cell line recapitulates lactating cell physiology, showing a decrease in TRPC6 during that time. E) Knockdown of SPCA2 in this cell line increases the expression of TRPC6.
Figure 3-2: Immunofluorescence of A) monolayer Scp2 cells before and B) after differentiation (Day 10 in Matrigel), treated with prolactin. Consecutive 0.5um series image sections were photographed on Zeiss Axio Scope fluorescence microscope (Carl Zeiss, Germany).
Figure 3-3: FURA-2 AM imaging of calcium influxes induced in A) monolayer, B) Mammosphere (Day 10) and C) shSPCA2 cells compared to a scrambled shRNA control (shSc). ML-9 is an inhibitor of TRPC6 and abolishes Ca^{2+} influx when used at 10 μM.
Figure 3-4: The effect of SPCA2 knockdown and overexpression on TRPC6 in Scp2 cells. Although, A) fluorescence intensity data and B) representative images for TRPC6 expression in shSc (control) and shSPCA2 Scp2 cells in monolayer culture show an increase in TRPC6 upon knockdown of SPCA2; Overexpression of a CMV promoter version(C) of SPCA2 as a C-terminal truncation (SPCA2C), an ATP deficient mutant (D379N) or in its full form (SPCA2) has differential effects on TRPC6 expression and OAG (D) elicited calcium influx.
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Chapter 4

SPCA2 Plays a Key Role In Calcium Dysregulation in Breast Cancer Cells
Introduction

Throughout the lactation cycle we see a coordinated regulation of calcium transporter expression and interaction (1). In the study we call this module of calcium transporters CALTRANS as a whole entity. We aimed to establish the differences between calcium transporter regulation in cancer cell lines versus nontransformed cell lines. Calcium dysregulation has been reported for several cancer cell types and CALTRANS members have been detected at abnormally high levels in breast cancer. CaSR overexpression in cancer cell lines can be stimulated by PTHrP hormone, inducing them to migrate and possibly participate in bone reabsorption and aid bone metastasis (2-3).

SPCA2 is highly overexpressed in breast cancer cell lines and tissues (4). Furthermore, knockdown of SPCA2 blocks proliferation of MCF7 cells in vitro, while SPCA2 overexpression in non-tumorigenic MCF-10A cells conferred tumorigenic properties (5). Additionally, the role of SPCA2 in breast cancer is thought to induce a constitutive Ca\(^{2+}\) signaling by overexpression, leading to unregulated cell division and tumor growth. PMCA2 and PMCA4 have both been investigated for their possible role in breast cancer via breast cancer cell lines (6). In the study, there was evidence that PMCA2 is highly elevated at the mRNA level in several breast cancer cell lines while no pattern of expression was found for PMCA4 in the same cell lines. Although PMCA4 may play a role in breast cancer, no evidence was found for the mechanism existing through transcriptional regulation. Several groups have reported the upregulation of TRP channel expression in breast cancer cell lines. TRPCs (canonical), TRPV (vanilloid) and
TRPM (melastatin); over expression of several TRP channels in each of these families has been correlated with tumorigenicity and cancer progression (7-8).

In recent studies, it has been determined that interactions of calcium-specific entities, such as the Orai/STIM canonical SOCE pathway may be altered in breast cancers (9). It has been determined that in estrogen receptor positive (ER+) breast cancers, the canonical Orai1/STIM pathway is mediated to Orai3/STIM whereas, in estrogen negative breast cancers (ER-), cellular calcium seems to regulated by the classic Orai1/STIM pathway. (10); this kind of molecular isoform swapping is the first to be seen in the SOCE pathway.

Migration of MDA-MB-231 is dependent upon the interaction of Orai1 and STIM1 (11). Migratory behavior has also been linked to CaSR expression in MDA-MB-231. CASR knockdown was found to reduce migratory behavior and extracellular Ca$^{2+}$-induced cell migration. Phospholipase Cβ signaling is also highly dependent on CaSR expression (12). MDA-MB 231 cells have shown high amounts of serotonin biosynthesis (TPH1) and receptors (5HT7) in metastasized tumors, with suppression in early stage, primary tumors.

**Results**

**Nontransformed (MCF10A) and cancer cell lines (MCF7 and MDA-MB 231) show distinct CALTRANS signatures in their mRNA profiles**

Semi-quantitative RT-PCR was used to determine the relative levels of CALTRANS components in three breast cancer lines (Figure 4-1). For intracellular calcium pumps, Orai1 and TRPC6 we saw definitive increases for MCF7 cells but similar
levels in MCF10A and MDA-MB 231 cells. However, for the PMCAs the only increase we saw in mRNA levels is for PMCA1A. For other isoforms of PMCA, the mRNA levels were either reduced in the cancer cell lines or maintained the same level as in the MCF10A cells. MDA-MB 231 cells show a preference for increasing, not transporters but calcium regulatory proteins such as buffers, GPCRs, phospholipases and other molecular regulators of calcium. In addition, we saw no change in STIM levels, which correlated with data from the study of Orai1/3 switching in tumorigenic cell lines. From this information we were able to elucidate a possible, independent CALTRANS mechanisms of the contribution of calcium to tumorigenicity versus migration.

We hypothesize that the tumorigenic cancer cells engage in “calcium hoarding” throughout their life cycle in order to contribute to the unrestricted division of cancer cells within the tumor microenvironment. With exceeding growth, the tumor microenvironment would limit extracellular calcium availability in the absence of angiogenesis. We believe that this event causes a switching to “calcium seeking” behaviors associated with migration and excessive calcium signaling in the presence of Ca$^{2+}$. Using our mRNA data, we were able to select specific molecules for genetic mediation. In this study we look at the determinants of cancer cell state and progression through calcium mediating molecules and the potential to use popular natural chemicals to change the molecular composition of these cancer cell lines.

**Overexpression of SPCA2 in MDA-MB 231 cell line confers rapid growth.**

MDA-MB 231 cells were retrovirally transfected with GST-tagged, CMV promoted SPCA2 or SPCA2C. SPCA2C vector comprised of the last transmembrane
helix and the tail while SPCA vector expressed the full length protein. Expression
profiles were confirmed by Western and RT-PCR. Cellular growth is shown here as the
OD$_{490}$ recorded via Cell Titer™ assay. We noticed that the cells overexpressing SPCA2
C-terminal fragment (Fig 4-3A) exhibited a much higher growth rate than the control.
Additionally, overexpression of the entire SPCA2 pump conferred a much higher rate of
growth than the C-terminal or control cells (Fig 4-3B).

**SPCA2 Induction Confers Tumorigenic Characteristics**

To investigate whether artificial induction of SPCA2 via retroviral vector
(pLXRN-CMV), under the control of a high transcription, CMV promoter would increase
the tumorigenicity of MDA-MB 231 cells we compared the ability of these transfected
cells on 50% Matrigel basement membrane to form adherent colonies (Fig. 4-3C). We
noticed that transfected cells showed tumorigenic properties, forming many small to
medium sized colonies on the matrix.

To investigate whether calcium influx changed in the SPCA2 overexpressing
cells, we used a variety of FURA2-based Ca$^{2+}$ imaging protocols. Overall, transfected
cells showed (Fig. 4-4A) an increase in calcium stores and subsequent decrease in store-
dependent calcium influx. Additionally, full-length CMV-SPCA2 transfection showed a
significant increase to the total intracellular calcium (Fig. 4-4B). Interestingly, we saw
very little change in store-independent calcium influx (Fig. 4-4C). This data further
suggests a fundamental role for SPCA2 in store-dependent calcium influx, as previously
shown by our lab.
Phytochemical Attenuation of the Calcium Dysregulation in Breast Cancer Cell Line MCF7

Phytochemicals have recently been found to promising candidates for treatment of cancer. Recent studies have shown that cannabidiol (often referred to as CBD), a modest constituent of the *Cannabis sativa* plant, a fragrant shrub, closely related to hops (*Humulus lupulus*); confers anti-tumorigenic properties (13-17) as well as cancer preventative characteristics (18). A study (19) showed that CBD acts by releasing intracellular stores from the mitochondria, coupled with the increase of nitric oxide. In breast cancer cells this release was thought to cause a Caspase3 induced apoptotic program (20-22).

We wanted to know if store-independent calcium or store-operated calcium influx played a role in the uptake of calcium upon treatment with CBD (Fig. 4A). We added either vehicle (VO; EtOH) or 1mM CBD stock (final concentration 10 μM) to MCF7 cells in nominal calcium buffer (~0mM) and allowed the release of intracellular calcium back to baseline (Fig. 4B). Once baseline was reached, CaCl₂ was added to reach a final concentration of 2mM. We recorded the peak calcium influx and compared the difference in influx maximum peak heights (Fig. 4C).

We wanted to know whether we could attribute these calcium changes to reduced tumoriginecity in the MCF7 cell line. We seeded MCF7 cells at 10⁵ cells in 0.60% soft agar and 500μl normal media either containing 10μM CBD or the appropriate amount of Vehicle (VO) which was 100% ethanol. We noticed the cells grown in the presence of CBD exhibited reduced density (Fig. 5A). We attributed this decreased density to
problems with calcium regulation and thus, decided to see if SPCA2 was involved or TRPV1 (which is known to bind members of the cannabinoid family). Although we saw a reduction in both proteins after 10μM CBD treatment (Fig. 4B), the difference we recorded may have been affected by the extreme proportionality of cell density proportion and thus must be repeated. Next, we sought to compare the cellular growth rate between thapsigargin (Thap), which is a known anti-proliferative agent and CBD. We seeded MCF7 cells at 10^3 per well in 96 well plates and recorded their growth over five days. By Day 3, CBD is shows a significant decrease in cell proliferation when compared to vehicle-only, control cells or their thapsigargin counter-parts.

We wanted to know whether e-cadherin expression increased in the presence of CBD, stopping spread of the tumor, as well as proliferation. We stained non-treated and CBD treated cells (Fig. 4-6 A-B) and took serial sections to see if e-cadherin and CB2 receptor. We noticed the co-localization of these two markers was increased with CBD treatment. We now know that there is a significant amount of CB2 receptor in the MCF7 cell line and it co-localizes with e-cadherin at the plasma membrane. With striking differences in size, we wanted to measure the colonies and count the colony number for the treated and non treated MCF7 cells. We seeded matrigel with MCF7 cells and grew them for 48 hours in media with either vehicle-only (Vo) or cannabidiol (CBD) we noticed a decrease in colony number and size (Fig.4-6 A-D).
Discussion

This data suggests that there is a clear evidence for unique calcium signatures from cancer cells undergoing EMT. One of the characteristics we notice is the clear evidence for constitutive calcium influx and storage in the proliferative stage of breast cancers, similar to the early lactogenic cycle discussed by several groups. There is also clear evidence for a calcium-depleted store in the metastatic stage and more evidence for calcium-seeking behavior by these migrating cells. Taken together, the difference in the CALTRANS module offers several pathways of inhibition for specific breast cancer types. Although, the MCF7 line is ER+, the reality is that ER+ breast cancers can be as deleterious as ER- or progress towards ER- breast cancer without proper treatment. Our data offer a insight into early and late stage progression of breast cancer.

CBD may exert some of its anti-proliferative effect via manipulation of the plasma membrane regulated calcium influx to destroy breast cells which are in the proliferative state, such as tumorigenic breast cancers. This data adds to a significant amount of evidence suggesting that Cannabis is a safe and effective treatment (and some suggest is preventive for) a variety of cancers and immunological conditions. The data suggest that the pathways which converge for the destruction of cancer cells, may aid in the well-being of normal cells. Also, Cannabis is a relatively low-maintenance weed which can be grown almost anywhere at high density for less than corn per gram and has high potential for bioremediation and fiber production. Cannabis and cannabinoids are
relatively nontoxic, in fact. The only reported cases of cannabinoid reaction were attributed to synthetic substitutions.

Taken together, these data support the hypothesis that calcium module dysregulation is a key factor in the progression of breast cancer. These changes may include calcium hoarding as a proliferative tactic and calcium starvation as a reason for migration. With further study we may be able to elude why natural products are able to attenuate cancer cells while keeping healthy cells intact. Additionally, phytochemicals offer a plethora of constituents which would make affordable, non-toxic cancer treatment and prevention feasible in the complex area of clinical biology.

Materials and Methods

Cell Culture

All cell lines were cultured at 37°C in and aseptic, humidified incubator with a 5% CO2 atmosphere and grown to at least 80% confluence prior to assay or split. MCF 10A cells were cultured in 1:1 (DMEM:F12) media (Invitrogen) with 5% horse serum (Invitrogen), 100ng/ml cholera toxin (CalBiochem), 10μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma) and 20ng/ml EGF (Upstate). MCF7 and MDA-MB 231 cell lines were cultured in DMEM, high glucose and supplemented with 10% FBS, 1% penicillin/streptomycin. For Matrigel™ assays, the media content was kept the same, however the cells were seeded onto approximately 25μm thick basement matrix consisting of 50% Matrigel™ and 50% cell media.
RT-PCR

mRNA was collected form $10^7$ cells using the RNeasy kit (Invitrogen), per the manufacturer’s instructions, for each of the cancer cell line. Cellular mRNA was normalized to 1μg/ml and reverse transcribed with iScript™ reverse transcription kit (Biorad). 1:10 dilutions of Taq amplified products were ran on 2% agarose gels and imaged via transillumination. Custom primers were generated for the following genes(IDT Technologies):

hSPCA1: *forward* 5’-GGGCTTCTCTTCTTGGCTCT-3’ and *reverse* 5’-ACGTGCAACCTTCATTTTCC-3’

hSPCA: *forward* 5’-GTTCCGAAGAGTTAAGT-3’ and *reverse* 5’-ACCAGCCAATGAGCATGA-3’

hPMCA1b: *forward* 5’-CCCTGGCTCTGGCAACGGAA-3’ and *reverse* 5’-TGCATTCACTCGAATCTGTT-3’

hPMCA2: *forward* 5’-TGGACGCCACGGAGCAAA-3’ and *reverse* 5’-TTCTGTCGTACGCCTGC-3’

hPMCA3: *forward* 5’-AGTTCAGGGGTGCTGTGCGCC-3’ and *reverse* 5’-GGCGCTCCTCTCTCTCGCC-3’

hPMCA4: *forward* 5’-CCCCCCACCACCTTCTTCTGC-3’ and *reverse* 5’-TGTTGGGTTGGGTGATGCA-3’

hOrai1: *forward* 5’-AGCATGGAGGGAAGAGGATT-3’ and *reverse* 5’-GACCTGGAGCTGGAAGAACA-3’
hPLCβ: *forward* 5’-CTACCATGGCTGGACCGGAC-3’ and *reverse* 5’-ACGCCCTTGCCATGTGACGCT-3’

hBetaCasien: *forward* 5’-TCTGCTCCTCTGTGCTCCAGGCT-3’ and *reverse* 5’-GGCACCACCTTGCTGGGGGAT-3’

h5-HT7: *forward* 5’-GCGCTGCGCCGCACCTTC-3’ and *reverse* 5’-TCTTCTGGCCAGCCTTGTAAATCT-3’

hCASR: *forward* 5’-CTGCTTTGAGGTGTGAGGTGTG-3’ and *reverse* 5’-GGTTTCATTGGACGAGATCAT-3’

hTPH1: *forward* 5’-AGGTCATGTCGCTTGGGCT-3’ and *reverse* 5’-ACAGCCTCCTCTGAGCCGC-3’

hBetaActin: *forward* 5’-TTGAGACCTTCAACACCC-3’ and *reverse* 5’-TAGCACAGCGCTGGATGCAA-3’

**Western Blot**

1x10^6 cells from cell culture were washed three times with sterile HBBS (Invitrogen) then immediately lysed in buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 5 mM Na3P2O7, 1 mM Na3VO4, 10 mM NaF, pH 7.4 with 1% SDS in the presence of protease inhibitor cocktail (Roche). Protein concentrations were assayed using a bicinchoninic acid assay kit (Pierce). For each well of a 10 well (15mm) Nupage™ gel, ~50 μg of protein was loaded for SDS-PAGE Analysis and Western blotting. We used transferred gels to blot at 100mA overnight to 0.45 μm nitrocellulose membrane (Bio-Rad). Antibodies for mouse SPCA2, TRPC6 and
IP3R were incubated with the membrane overnight at 1:500 dilution in 1× PBST. Membranes were washed three times in PBST then mouse or rabbit secondary antibody conjugate to HRP was added (GE Healthcare UK Limited) at 1:2000 for one hour at room temperature on a rotator and the membranes were washed twice in PBST then three times in normal PBS to remove Tween residue. Blots were visualized using ECL kit (Pierce) on an Intelligent Dark Box Imager (Fuji Film).

Measurement of Cell Proliferation

We monitored cell proliferation CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega, Madison, WI, USA) according to manufacturer's instructions. We seeded 1× 10⁴ cells into a 96-well plate. We obtained absorbance readings at 490nm every 24 hours for 3 days (72 hours). We used 20 μl/well of CellTiter 96 Aqueous One Solution and incubated at 37°C for 2 hours, prior to noting the absorbance on a SpectraMax plate reader.

Colony Formation on Matrigel™

Colony formation in Matrigel was performed by seeding 1x10⁷ cells onto 0.5 μm layers of 50% Matrigel on 25mm coverslips (VWR) in CoStar 6-well dishes (CoStar Corning, NY). 1 × 10⁷ cells were resuspended in DMEM containing 10% and pipetted onto a base layer of solidified 50% agar and 5% FBS. Cells were allowed to adhere and grow on the matrix for 48 hours prior to live imaging on an inverted microscope. Multiple images were taken and cell colon number and size were noted and averaged using a two-rialed ANOVA student’s t-test.
FURA2-AM Imaging

FURA2-AM imaging was performed on 60% confluent cells, grown on 25mm (1 oz.) coverslips using an inverted fluorescence recording microscope. Briefly, cells were washed with 1xPBS and incubated in FURA2-AM, 2mM calcium imaging buffer (20 mM Hepes, 126 mM NaCl, 4.5 mM KCl, 2 mM MgCl2, 10 mM glucose containing 2 mM CaCl2) for 20 minutes at room temperature. Cells undergoing analysis in 2mM calcium imaging buffer were washed in the buffer twice before imaging. Cells undergoing analysis in nominal (0mM Ca2+) buffer were washed once in 2mM calcium imaging buffer then quickly in 0.1% EGTA calcium buffer and subsequently, nominal calcium FURA2-AM imaging buffer (20 mM Hepes, 126 mM NaCl, 4.5 mM KCl, 2 mM MgCl2, 10 mM glucose at pH 7.4) was simultaneously added to the chamber as imaging assays began.

Cells were excited at 340 and 380 nm simultaneously and the ratio and 505nm emission was recorded for both excitation wavelengths and plotted against time (seconds). Thapsigargin (2 μm) or ionomycin (2 μm) was added where indicated, and followed by readdition of 2 mM Ca2+ to measure store-dependent Ca2+ influx. For SICE, fluorescence was recorded from cells placed in nominally Ca2+ free recording buffer, followed by addition of 1M CaCl2 to 2 mM Ca2+, activating store-independent Ca2+ influx.

Confocal Imaging

Cells were fixed in 3.7% paraformaldehyde (Fisher) in IMF Buffer (0.1% BSA, 0.2% TritonX-100, 1% BSA) for one hour. Primary antibodies were added at 1:500 in
IMF buffer and incubated overnight at 4°C. Slides were prepared for secondary antibody by three, consecutive IMF buffer for 5, 10 and 15 minutes. Alexa Fluor® 488 (goat anti-rabbit) and Alexa Fluor® 568 (goat anti-mouse) were used as secondary immunofluorescence staining at 1:1000 dilutions in IMF wash buffer for 1 hour. The embedded cells were then washed three times with 1xPBS and DAPI stained. Cells were washed, briefly in sterile water then mounted in Dako fluorescent mounting media and allowed to dry overnight. Images were taken shortly after on a Zeiss Axio Scope fluorescence microscope (Carl Zeiss, Germany).
Figure 4-1: Expression of CALTRANS components in nontransformed (MCF10A), tumorigenic (MCF7) and migratory (MDA-MB 231) cell lines. A) Calcium pump expression and B) channel expression elevation in MCF7 cells. C) Elevation of Ca\textsuperscript{2+} buffers, GPCRs and GPCR-coupled factors in background of consistent expression of STIM1 and 2 a store-operated calcium regulator.
Figure 4-2: Hypothesized calcium dysregulation CALTRANS mechanisms in MCF7 and MDA-MB 231 cell lines. A) Based on mRNA data collected we expect that stores are calcium rich in MCF7 cells which are highly tumorigenic, due to enrichment of intracellular and plasma membrane calcium pumps and channels. While B) we hypothesize MDA-MB 231 cells are calcium-starved and responding to extracellular signals to obtain calcium in their highly calcium-purged stores, due to low abundance of intracellular calcium pumps coupled with high IP3R and phospholipase expression. In response to highly expressed GPCRs.
Figure 4-3: Overexpression of (A) SPCA2C and SPCA2 in MDA-MB 231 cells confers increased cell proliferation (B) in MDA-MB 231 cells. Overexpression of a CMV promoter coupled (C) to an SPCA2 C-terminal truncation (SPCA2C), an ATP deficient mutant (D379N) or in its full form (SPCA2) confers graded tumorigenicity in MDA-MB 231 cells.
Figure 4-4: A) Overexpression of SPCA2 in MDA-MB 231 cells changes the signature of store-dependent calcium influx when compared to SPCA2C (C-terminal truncation), SPCA2 D379N (catalytically “dead” mutant of SPCA2), and our control cells. B) Overexpression of SPCA2 increases total intracellular calcium. C) Store-independent calcium signature changes upon overexpression of SPCA2.
Figure 4-5: (A) Cannabidiol (CBD) is a known ligand which releases intracellular calcium from the stores. However, (B) we see that in addition to the intracellular store release, there is a coupled calcium influx which is magnified by CBD treatment.
Figure 4-6: (A) CBD reduces cell density in the breast cancer cell line MCF7. B) Expression of TRPV1 and SPCA2 in MCF7 cells with either vehicle-only (VO) or CBD treatment (10μM). Cellular growth rate over 3 days upon treatment with CBD, thapsigargin (Thap) or vehicle-only (VO).
Figure 4-7: Untreated (A) and CBD treated (B), MCF7 cells labeled for e-cadherin (red) or CB2 receptor (CNR2)(green). Average colony diameter in untreated (C) and CBD treated (D) MCF7 cells.
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