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Alterations in Calcium Signaling Pathways in Breast Cancer

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Abstract

Breast cancer is the second most common cancer in women and the fifth cause contributing to death due to the cancer condition. It is essential to deeply understand the complex cellular mechanisms leading to this disease. There are multiple connections between calcium homeostasis alterations and breast cancer in the literature, but no consensus links the mechanism to the disease prognosis. Among the cells contributing to the breast cancer are the breast telocytes, which connect through gap junctions to other cells, including cancer cells and myoepithelial cells. Multiple proteins (i.e., voltage-gated calcium channels, transient receptor potential channels, STIM and Orai proteins, ether à go-go potassium channels, calcium-activated potassium channels, calcium-activated chloride channels, muscarinic acetylcholine receptors, etc.) coupled with calcium signaling pathways undergo functional and/or expression changes associated with breast cancer development and progression, and might represent promising pharmacological targets. Unraveling the mechanisms of altered calcium homeostasis in various breast cells due to the cancer condition might contribute to personalized therapeutic approaches.

Keywords: breast cancer, human breast stem cells, human breast epithelial cells, human breast myoepithelial cells, human breast adipocytes, human breast telocytes, calcium homeostasis alterations

1. Introduction

Breast cancer is the most common and the most frequently diagnosed cancer and is one of the most lethal malignant lesions in women worldwide, being the second leading cause of cancer death in women, after lung cancer [1].

1.1. Cell subtypes in human breast cancer and the alterations in calcium homeostasis

The mammary gland is an exocrine, compound tubuloalveolar gland [2]. Each mammary gland has 15–20 glandular lobes in its structure, each lobe being a separate gland with its excretory channel (galactophore channel) that opens at the level of the nipple via the galactophore pore [3]. The glandular lobe consists of glandular lobules delimited by fibrous connective tissue and fat. Lobules have a radiant arrangement, each opening through a lactiferous duct in the nipple, presenting a dilation called lactiferous sinus before opening. Lobules are composed of parenchyma and stroma, and made of loose connective tissue [4]. Each lobule consists of alveoli, lined by cuboidal epithelial cells, which secrete milk, and lactiferous ducts, lined by columnar epithelium, both epithelia surrounded by an outer layer of myoepithelial cells. The stromal cell population is composed of mesenchymal cells, including adipocytes, fibroblasts, and immune cells.

1.1.1. Human breast stem cells

The mammary gland structures are capable of self-renewal due to the presence of mammary stem cells (MSCs) and multipotent adult stem cells. Mammary stem cells lead to the growth of the mammary gland during puberty and are responsible for its further development during pregnancy. Besides their intrinsic self-renewal capacity, normal stem cells call for the adoption of other additional mechanisms to protect them from the microenvironmental pressure that may overdrive the stem cell pool [5]. MSCs are able to reconstitute a completely functional mammary gland upon orthotopic transplantation [6]. MSCs can differentiate into mature epithelial cells of either myoepithelial or luminal lineage through a series of lineage-restricted progenitor intermediate cells. Of major importance is also the long-term survival and expansion of MSCs [7] and the additional accumulation of genetic and/or epigenetic alterations in those cells that may increase the susceptibility of neoplastic transformation [8]. Van Keymeulen et al. demonstrated that the mammary gland contains different types of long-lived stem cells [9].

In breast cancer, both stem cells and progenitor cells are a potential candidate for tumorigenesis, and this could underpin the extraordinary phenotypic heterogeneity of malignant breast tumors. Also, different breast tumor subtypes might be linked to distinctive mammary stem cells and progenitor cells within the mammary epithelia as suggested by different studies [10]. Understanding how these cell subpopulations influence the normal epithelial differentiation hierarchy and the development of mammary tumors could have a significant impact on the proper taxonomy of these lesions.

Unlike the mouse counterparts, the detection of human breast stem cells may be difficult due to the lack of reliable surface makers. Also, the profile of MSCs is less predictable compared to mouse models [11].

To identify MSCs, several studies have employed multiple methods, including: 5-bromo-2-deoxy-uridine (BrdU) label-retention studies, nonadherent mammosphere cultures, cell-surface markers, such as Sca1 and CD49f, labeling MSCs with lipophilic fluorescent dye PKH26 during mammosphere growth and Hoechst dye efflux [12, 13]. These methods were

very helpful for the further detection and characterization of signal transduction pathways such as the Notch, Wnt, and Hedgehog pathways that may be crucial for the self-renewal and fate determination of MSCs.

Identification of breast cancer stem cells is strictly dependent on cell-surface markers. Several markers have been proposed such as hyaluronan receptor (CD44), signal transducer CD24 (CD24), CD133 (Prominin-1), integrins CD29 (β 1) and CD49f (α 6), aldehyde dehydrogenase-1 (ALDH1), as tumor-initiating cells in breast cancer progression with high metastatic potential and in high-grade tumors resistant to therapeutic treatments [14–17]. However, currently, there is no agreement regarding the phenotypic characterization of breast cancer stem cells. In addition to this discord, the great heterogeneity of breast tumors reflected by a myriad of histological subtypes with variable clinical presentations and diverse molecular signatures also contributes to this major shortcoming. The intrinsic molecular taxonomy describes five major subtypes of breast cancer (luminal-A, luminal-B, basal-like, HER2, and normal-like) which overlap with various clinicopathological classification systems and correlate with clinical behavior being vital for patient's management. In addition, different breast cancer stem cells phenotypes have been described contributing to the proper characterization and nomenclature of breast malignant lesions. Several immunohistochemical markers have been characterized showing that the prevalence of stem cell-like markers varies according to tumor histological subtype [18].

In the light of these facts, some studies have proposed ALDH1 as an independent prognostic marker in breast cancer. Ginestier et al. showed a prevalence of 30% for ALDH1 positivity in a cohort comprising 577 breast tumors from two independent tumor sets. They also showed that ALDH1 expression correlates with a high histological grade, human epidermal growth factor receptor type 2 (HER2) overexpression, and absence of estrogen receptor and progesterone receptor expression [19]. A similar study [20] highlighted the worst prognosis of breast cancer patients with ALDH1 expression. There is no agreement in this matter, as other studies fail to find these correlations [21] even in more aggressive breast tumor subtypes such as inflammatory breast cancer.

With all these conflicting results, the reliability of ALDH1 expression as a clinical prognostic factor is doubtful, thus increasing the need for a standard protocol and more rigorous evaluation criteria, as well as consideration of the dissimilarity between whole-tissue staining versus tissue microarray staining [22].

Alterations in calcium homeostasis frequently occur in some pathological conditions such as malignant proliferation and could have a key role to play in the near future of some targeted therapeutic approaches. Some recent studies have shown that exposure of breast cancer cells to chemotherapy (i.e., carboplatin) induces Ca^{2+} release and leads to an enrichment of breast cancer stem cells [23]. Lu et al. have documented that chemotherapy induces the expression of glutathione S-transferase omega 1 (GSTO1), a factor which is dependent on hypoxia-inducible factor 1 (HIF-1) and HIF-2. In turn, low level of GSTO1 revokes carboplatin-induced breast cancer stem cell enrichment, decreasing tumor initiation and metastatic potential and delaying tumor recurrence after chemotherapy. The authors also found that GSTO1 interacts with the ryanodine receptor (RYR1) and increases calcium release from the endoplasmic reticulum. In this manner, high levels of cytosolic calcium activate proline-rich tyrosine kinase 2 (PYK2)/tyrosine-protein kinase (SRC)/signal transducers and activators of transcription factors 3

(STAT3) signaling pathways, leading to an increased expression of pluripotency factors and breast cancer stem cell enrichment. Concurrent HIF inhibition blocks chemotherapy-induced GSTO1 expression and breast cancer enrichment [23]. The authors have concluded that these combining effects may improve clinical outcome in breast cancer patients.

Not just chemotherapeutic agents are responsible for the release of free intracellular calcium. Petrou et al. investigated the effect of several ion channel modulators such as amiodarone, dofetilide, furosemide, minoxidil, loxapine, and nicorandil in prostate and breast cancer cell lines, PC3 and MCF7, respectively and found that in all investigated cases, calcium levels were increased by modulator concentrations comparable to those used clinically [24]. However, the way these modulators act on breast cancer stem cells remains unknown.

Calcium-and integrin-binding protein (CIB1) depletion impairs cell survival and tumor growth in triple-negative breast cancer by inducing genetic programs that reduce proliferation and survival and mediate differentiation and cancer stem cell function and epithelial to mesenchymal transition [25]. The authors also observed an almost complete cell death in MDA-468 cells after extended CIB1 depletion, suggesting that CIB1-depleted cells do not become stem cells, but rather gain some stem-like features as they are dying.

1.1.2. Human breast epithelial cells

The lactiferous ducts spread into the breast through a series of branches decreasing in caliber from the nipple to the terminal ductal-lobular units (TDLU) that are surrounded in specialized, hormonally responsive stroma. Extralobular ducts are lined by columnar epithelium that is supported by myoepithelial cells and a basement membrane composed of elastic fibers. The epithelium of the luminal duct can give rise to ductules, and fully formed lobules can originate directly from these anatomic structures in the nipple or at deeper levels of the mammary ductal system [26]. The lobules that consist of groups of alveolar glands encompassed by specialized vascularized stroma are connected by intralobular ductules that combine to form a single TDLU that drains into the extralobular ductal system.

The normal microscopic appearance of the lobules is not constant because the structure and histological appearance of the lobule in the mature breast are subject to individual changes associated with the menstrual cycle, pregnancy, lactation, exogenous hormone administration, therapy, aging, and menopause. The inactive lobular glands are lined by a single layer of cuboidal epithelial cells supported by underlying, loosely connected myoepithelial cells. The intralobular stroma contains more capillaries and is less densely collagenized than the interlobular surrounding stroma. Immunoreactivity for hormone receptors (ER and PR) is also variably expressed in lobules in a checkerboard staining pattern: the intensity and frequency of staining varies considerably from patient to patient and in many individual slides from one area to another. ER PR are sporadically positive in normal luminal epithelial cells, but some areas are, however, completely negative for those receptors. ER, PR, and AR are almost always negative in myoepithelial cells.

The 3D cultures of human breast epithelial cells have been designed in order to mimic the normal and pathological tissue architecture [27].

The regulation of signaling pathways and homeostasis of free intracellular Ca^{2+} can entail many cellular and physiological consequences, which may lead to changes in Ca^{2+} levels during lactation [28]. The Ca^{2+} influx has a decisive part in determining the concentration of Ca^{2+} in breast epithelial cells. Breast glandular proliferation, differentiation, and lactation are regulated by several local and systemic hormones, of which estrogen is one of the most important hormones. The regulators of estrogen and its receptor are modulators of proliferation and differentiation of breast epithelial cells [29]. The effect of estrogen on epithelial breast cells is done mainly through genomic pathways, but nongenomic mechanisms are particularly dependent on Ca^{2+} signaling [30].

Some studies on the MCF-7 breast cell line concluded that breast epithelium proliferation is influenced by Ca^{2+} through activation of mitogen-activated protein kinase (MAPK) by 17β -estradiol [31]. It is known that several Ca^{2+} -related proteins can cause changes in cellular functions, leading to many breast lesions, including cancer and hypercalcemia-related malignancy, which have a poorer prognosis and have often a more aggressive nature been associated with metastasis [32]. The way calcium is involved in the differentiation of breast epithelial cells is closely dependent on vitamin D3. By modulating Ca^{2+} metabolism, vitamin D3 plays a crucial role in the regulation of cell proliferation and differentiation [33].

Various epidemiological studies [34, 35] suggest that vitamin D3 deficiency might increase cancer incidence, but no spontaneous tumors have been reported in mice models lacking $1,25(\text{OH})_2\text{D}_3$ or deficient in its receptor until recently [36]. The authors observed, for the first time, diverse types of spontaneous tumors in vitamin-D3-deficient mice for more than 1 year of age. The authors concluded that the tumors developed due to increased oxidative stress, cellular senescence, and senescence-associated secretory phenotype molecules, such as hepatocyte growth factor, mediated via its receptor c-Met. As such, vitamin D3 prevents tumorigenesis by inhibiting oxidative stress and inducing tumor cellular senescence in mice, and the study provides direct evidence supporting the role of vitamin D deficiency in increasing cancer incidence.

Calcium levels play an important role in mitochondria-induced apoptosis and epithelial breast cell necrosis [37], and reduction of Ca^{2+} content in the endoplasmic reticulum lumen is associated with resistance to apoptosis [38]. The release of calcium from the endoplasmic reticulum can be triggered by different molecules, even natural ones like resveratrol, a product commonly found in grapes. Resveratrol triggers the release of calcium from the endoplasmic reticulum, which in turn activates the calpain protease that ultimately leads to degradation of the plasma membrane by calcium-dependent ATPase isoform 1 [39].

Human breast epithelial cells with stem-like phenotype have been also demonstrated to be sensitive to the pathophysiological changes in calcium metabolism. To date, Wang et al. showed that how antioxidant medium is superior in terms of prolonged growth for normal breast epithelial cells that expressed stem cell phenotypes. The characteristics of these mammary stem cells include the deficiency in gap junctional intercellular communication, expression of Oct-4, and the ability to differentiate into basal epithelial cells and to form organoid showing mammary ductal and terminal end bud-like structures [40]. Their study concluded that using this new method of growing breast cancer epithelial cell with stem cell phenotype

in a medium with low calcium levels and antioxidants will be of real use in the future studies of mammary development, breast carcinogenesis, chemoprevention, and cancer therapy.

1.1.3. Human breast myoepithelial cells

Myoepithelial cells lie between the epithelial cell layer and the basal lamina, where they establish a network of slender processes covering the overlying epithelial cells. The branching cytoplasmic network of the myoepithelial cell processes can be seen especially in scanning electron micrographs [41]. Spindle-shaped ductal myoepithelial cells lie parallel to the long axis of the duct and form a continuous layer. Contraction of myoepithelial cells in lobules and around ducts contributes to the flow of milk during lactation. The location of the myoepithelial cells between luminal epithelial cells and the basal lamina is an ideal location for them favoring communication with both compartments.

The histologic appearance and immunoreactivity of myoepithelial cells are highly variable, especially in pathologic conditions, and depend on the degree to which the myoid or epithelial phenotype is accentuated in a particular situation. Myoepithelial cells usually display nuclear reactivity for p63, which is the most useful marker for detecting these cells in normal and lesional tissues. Epithelioid myoepithelial cells can have reduced p63 reactivity. Other useful myoepithelial markers are α -smooth muscle actin, calponin, CD10, CKS/6, myosin, p75, and S100 (**Figure 1**, left). The presence or absence of myoepithelial cells, at least as demonstrated by routinely used immunostains and is very valuable in discriminating against neoplastic in situ lesions (**Figure 1**, right) versus malignant, infiltrative ones.

While the luminal epithelial cell has received plenty of attention as the most functionally active milk-producing cells and as the most probable target cell for tumorigenesis, attention on myoepithelial cells has begun to grow with the acknowledgment that these cells play an active role in branching morphogenesis and tumor suppression.

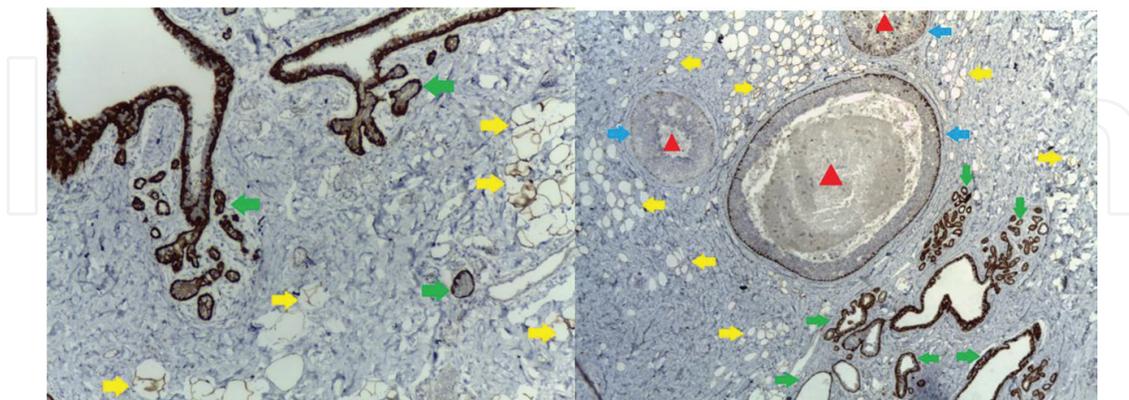


Figure 1. (Left) Immunostain for S100 highlighting the myoepithelial cells layer in a normal terminal ductal lobular unit (TDLU)—green arrow and some adjacent adipocytes—yellow arrow showing strong nuclear and cytoplasmic positivity (immunostain S100 with DAB chromogen, 100 \times magnification). (Right) Immunostain for S100 highlighting the intact myoepithelial cell layer (blue arrow) at the periphery of some foci of high-grade ductal carcinoma in situ (DCIS) with central comedo-type necrosis (red triangle). Also, note the adjacent normal TDLU (green arrow) and adipocytes (yellow arrow). Immunostain S100 with DAB chromogen, 40 \times objective.

The function of myoepithelial cells is strongly dependent on regulation of intracellular calcium. These cells contract in response to oxytocin secretion during lactation to generate the contractile force required for milk ejection. It is difficult to understand whether the alteration of calcium metabolism of myoepithelial cells plays a role, if any, in carcinogenesis. Even if it has been demonstrated that store-operated Ca^{2+} entry was mediated by a functional Orai3 in estrogen receptor-expressing (ER^+) breast cancer cells [42], the tumorigenesis impact of these findings on myoepithelial cell remains largely unknown, because it is known that most breast carcinomas originate in the epithelial cells and the spectrum of myoepithelial proliferative breast lesions is scarce.

However, disruption in calcium metabolism may alter the functionality of myoepithelial cells. On this issue, some studies have shown the important role of Orai1 store-operated calcium channels in lactation [43]. Davis et al., using genetically modified mouse models, observed that the store-operated Ca^{2+} channel Orai1 delivers over 50% of the calcium ions present in the secreted milk. They also demonstrated the role of Orai1 as a principal regulator of oxytocin-mediated alveolar unit contractility, milk ejection, general myoepithelial function, and survival.

S100 protein is expressed in myoepithelial cells. The S100 gene family is a Ca^{2+} -binding protein with low molecular weight [44]. The members of the S100 family have a myriad of cell functions such as cell proliferation, apoptosis, differentiation, cancer invasion, and metastasis. S100A2 is involved in breast tumorigenesis being downregulated in some cases, which led to the invasion of breast cancer cells [45]. The S100A4 expression is associated with tumor progression and metastatic potential [46]. In a similar manner, S100A7 is not only overexpressed in high-grade ductal breast carcinoma but also in in situ high-grade lesion (DCIS), and some studies suggested that the concomitant expression of S100A7, S100A8, and S100A9 in a class of breast cancers was associated with poor prognosis [47].

1.1.4. Human breast adipocytes

Apart from the epigenetic and genetic changes that occur within epithelial cells leading to breast proliferative lesions, it has shown that tumor initiation and progression also depend on the intricate intercellular dialog between tumor epithelial cells and the surrounding stromal cells [48]. Among the different cell types comprised in the breast stroma, the most abundant are those of adipose origin, mainly mature adipocytes, preadipocytes, and adipose-derived stromal/stem cells (ASCs). Besides the structural role that breast tissue has, it also has an important bioactive function [49, 50]. In pathologic condition, it is of great importance the interaction that is established between tumor cells and stromal adipocytes within the invasive front characteristic of breast cancers [51, 52]. Similar to breast myoepithelial cells, breast adipocytes also express s100 protein—**Figure 1** [53].

The role of calcium metabolism in such events is poorly understood, but some studies have found that some proteins such as calpains [54] and calpastatin [55] may be altered in adipose-derived stromal cells being responsible for their enhanced invasion potential.

Vitamin D3 triggers apoptosis in breast cancer cells and adipocytes by inducing an apoptotic signal by increasing the concentration of intracellular Ca^{2+} . This signal acts as an apoptotic

initiator that bluntly recruits calcium-dependent apoptotic effectors such as calpain and caspase 12, in both breast cancer cells and adipocytes. Some studies suggested that inducing apoptosis with vitamin D3, particularly in the tumor-surrounding adipose tissue involved in tumor progression, can contribute to the antitumoral effects of this hormone and may be of real therapeutic interest to include calcium-dependent apoptotic proteases as molecular targets for new therapeutic and preventive agents in breast cancer and obese patients [56].

1.1.5. Human breast telocytes

Recently, several papers were describing the presence of a new cellular type—the telocytes (TCs)—in the stroma of the mammary gland [57–59]. TCs are characterized by a small cellular body and extremely long telopodes with alternative regions of dilations called podoms and veil-like cytoplasmic extensions called podomeres [60–62]. Although there are numerous attempts to differentiate these cells from other cellular types such as fibroblasts, endothelial cells, mesenchymal stem cells, immune cells, a specific immunohistochemical marker was still not found [63]. The most specific markers, which are nowadays used for their identification, are CD34 and PDGFR alpha or beta [64, 65]. Genomic and proteomic approaches were also used to determine their uniqueness and have shown that telocytes are distinct from the other types listed above [66–70].

Mou et al. investigated the immunohistochemical characteristics and potential functions of TCs in reconstituted breast cancer tissue and found that they express c-kit/CD117, CD34, and vimentin. A very interesting observation is that TCs communicate with breast cancer cells as well as with other stromal cells [58, 71, 72]. Together with other stromal cells, TCs inhibited the breast cancer cell apoptosis and facilitated their proliferation and the formation of typical nest structure assembly in breast cancer, *in vitro* [58].

Rusu et al. described in an immunohistochemical study some CD34+/CD10±/c-kit-/vimentin-cells found in the inter- and intralobular stroma, which they considered to be TCs and suggested a stem cell-like features based on the expressed markers and changing phenotype [59]. Although a lot of studies are needed to talk about a certain function of TCs, a possible contribution to the mechanisms of carcinogenesis is not negligible, by the modification of the tumor microenvironment. As our team previously showed, TCs are not pacemakers but modulate the activity of the surrounding cells using calcium signaling [73]. Our results showed that uterine TCs express T-type calcium channels that might play a role in the generation of endogenous bioelectric signals responsible for the regulation of the surrounding cell behavior [74, 75].

2. Calcium signaling alterations in breast cancer

The most important proteins coupled with calcium signaling pathways and that have been described as key players in breast cancer cells are summarized in **Figure 2**.

2.1. Resting potential and calcium oscillations in breast cancer

The resting potential of human breast adenocarcinoma cells (estrogen receptor-positive MCF7 and triple-negative MDA-MB-231) is more positive (with approximately 27–30 mV) than normal

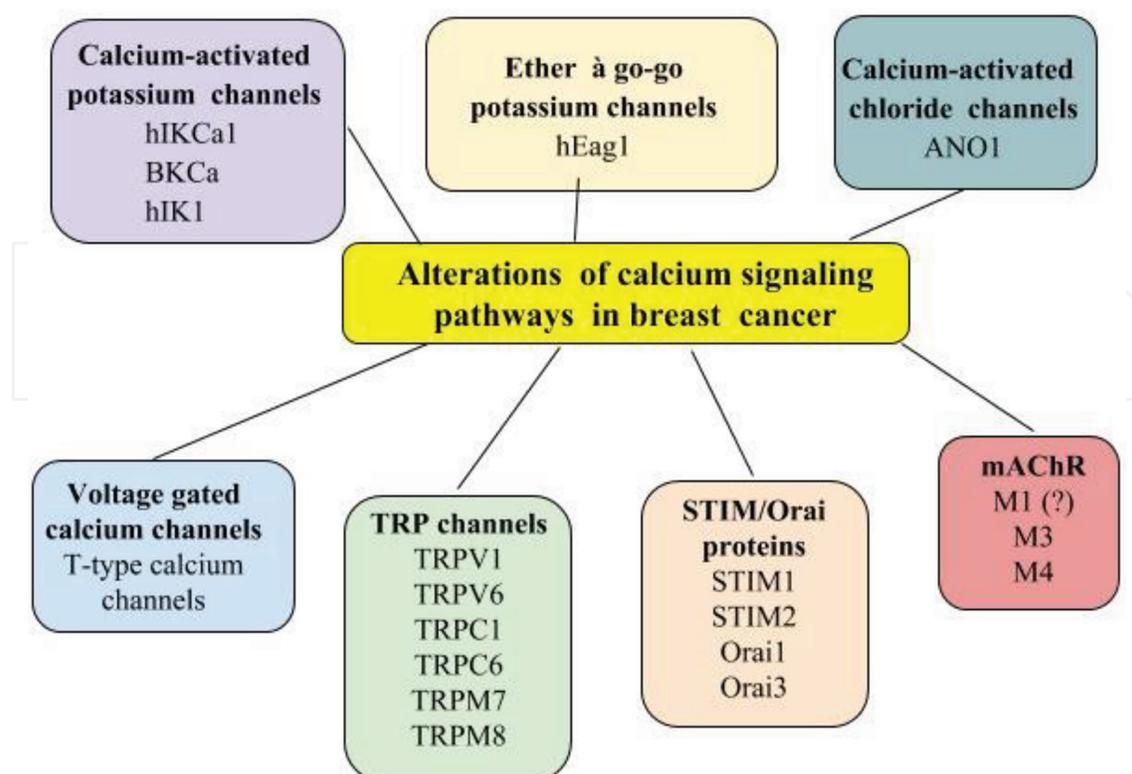


Figure 2. Proteins associated with calcium signaling pathways that have been described to undergo alterations in breast cancer cells.

human mammary epithelial cells [76]. Additionally, breast cancer cells react distinctly with respect to normal mammary epithelial cells in response to the changes in the extracellular ions (e.g., K^+ or Ca^{2+}) [76]. The differences in resting potential between breast cancer cells and normal cells may be useful in the development of anticancer-targeted therapies based on charged liposomes, which are considered among the promising liposome-based therapeutical approaches [77].

MDA-MB-231 breast cancer cells with high-metastatic potential also exhibit spontaneous Ca^{2+} oscillations in comparison with MCF7 breast cancer cells with a low-metastatic potential [78]. Interestingly, these spontaneous oscillations were absent in breast cancer cells with a low-metastatic potential, even in increased extracellular K^+ concentration conditions that determined the augmentation of their basal Ca^{2+} level [78]. This feature of presenting spontaneous calcium oscillations in metastatic cells might be further exploited in understanding the cellular mechanisms standing behind and in finding adequate therapies.

2.2. Voltage-gated calcium channels in breast cancer

Patch-clamp studies on human mammary epithelial cells (HMEC) indicated the absence of voltage-gated calcium currents [79].

The contribution of T-type calcium channels to breast cancer was investigated in several studies. To date, the expression of the $\alpha 1H$ subunit, but not the $\alpha 1C$ or $\alpha 1G$ subunits, of the voltage-gated calcium channels was demonstrated in MCF-7 breast cancer cells [80]. Among T-type calcium channels, Cav3.1, but not Cav3.2, was demonstrated to play an important

role in the inhibition of proliferation and apoptosis in MCF-7 human breast cancer cells [81]. Patch-clamp recordings demonstrated the presence of T-type voltage-gated calcium currents (ICaT) in MCF7 breast cancer cells [79].

A meta-analysis of public microarray datasets in clinical cancer tissue samples identified the upregulation of several VGCCs transcripts (e.g., *CACNA1C*, *CACNA1D*, *CACNA1B*, *CACNA1G*, and *CACNA1I*) in breast cancer and their involvement in the development and cancer progression [82]. Oppositely, another meta-analysis indicated the downregulation of the same VGCCs transcripts in breast cancer [83].

2.3. Transient receptor potential channels in breast cancer

Transient receptor potential (TRP) channels have been documented to play an important role in the development and progression of cancer. TRPC1, TRPC6, TRPM7, TRPM8, and TRPV6 channels were described to be upregulated in human breast ductal adenocarcinoma in comparison with the adjacent nontumoral tissue, and correlations with the proliferative parameters or the invasiveness cell capacity have been evidenced [84]. Moreover, several studies documented the role of TRP channels in different breast cancer cell lines. In detail, TRPM7 was demonstrated by silencing experiments to contribute to the migration and invasiveness of MDA-MB-435 breast cancer cells by signaling through the MAPK, but not through Akt, pathway [85]. TRPM8 was detected in BT-474 and MDA-MB-231, but not in MCF7, breast carcinoma cells, while menthol-evoked Ca^{2+} -oscillations were recorded in all three breast cancer cell lines [86]. TRPV6 had a high level of expression in breast adenocarcinoma tissue [87]. TRPV1 is functionally expressed in SUM149PT breast cancer cells, a model for a very aggressive form of breast cancer (i.e., triple-negative breast cancer) [88], and in MCF7 breast cancer cells [89, 90].

2.4. STIM and Orai proteins in breast cancer

In nonexcitable cells, such is the case of breast cells, both store-operated calcium entry (SOCE) and store-independent calcium pathways have been described, which involve the activation of stromal interaction molecules (STIM) and Orai proteins.

STIM/Orai proteins play an important role in the breast physiology. To date, in lactation, Orai1 is upregulated, while Stim1, but not Stim2, is downregulated [91]. Moreover, calcium-influx through Orai1 was described to be necessary for mammary epithelial cells for concentrating milk with Ca^{2+} and for milk expulsion through alveolar unit contraction [91].

In cancer metastasis, STIM/Orai proteins have been described to be involved by two major mechanisms: (i) upregulation or increased functional activation and (ii) molecular switching [92]. In estrogen-receptor-positive breast cancer cells (e.g., MCF7, BT474, ZR751, T47D, and HCC1500 cancerous cell lines) but not in estrogen-receptor-negative breast cancer cells (e.g., MCF10A and 184A1 normal breast epithelial cells, and MDA-MB231, BT20, and HCC1937 cancerous cell lines), Orai 3 mediates the STIM1/2 and Orai3 pathway and the Ca^{2+} -selective Ca^{2+} release-activated Ca^{2+} current (I(CRAC)), while Orai1 does not mediate the STIM1/Orai1 pathway [42]. Further on, estrogen-receptor- α knockdown downregulates Orai3 without any effect on Orai1, decreases Orai3-mediated SOCE, and diminishes the I(CRAC) current in estrogen-receptor-positive MCF7 cells [93].

The expression of Orai3 was identified to be higher in MCF-7 breast cancer cells versus normal MCF-10A mammary epithelial cells, while its silencing inhibits the MCF-7 cell proliferation, arrests the cell cycle in the G1 phase, downregulates cyclin-dependent kinases 4/2, cyclins E and D1, and determines the accumulation of p21(Waf1/Cip1) (a cyclin-dependent kinase inhibitor) and p53 (a tumor-suppressing protein) [94].

In STIM1 siRNA- or Orai1 siRNA-treated MDA-MB-231 human breast tumor cells, a reduction in the migration process was identified [95]. Additionally, overexpression of STIM1 and Orai1 in MCF-10A epithelial cells increased their invasiveness [95]. Experiments on immunodeficient NOD/SCID mice injected with MDA-MB-231 human breast tumor cells were stably transfected with STIM1 siRNA, or Orai1 siRNA, but not with control siRNA, which demonstrated the inhibition of metastasis [95].

Interestingly, a signaling pathway independent of endoplasmic reticulum calcium stores or STIM1 and STIM2 protein activation was identified in MCF-7 breast cancer cells (the secretory pathway calcium ATPase 2 (SPCA2)/Orai1 signaling) [96], where SPCA2 is located in the Golgi apparatus.

2.5. Ether à go-go (hEag1) K⁺ channels in breast cancer

Several pieces of evidence indicate the role played by Ether à go-go (hEag1) K⁺ channels in breast cancer cells invasiveness. To date, blocking or silencing hEag1 channels in MDA-MB-231 breast cancer cells induces membrane depolarization and subsequent diminishment of Ca²⁺ influx through Orai1, which affects cell migration and proliferation [97].

2.6. Calcium-activated potassium channels in breast cancer

Prolactin increases the current density of the human Ca²⁺-activated K⁺ channels (hIKCa1) in MCF-7 breast cancer cells, involved in the cell proliferation in a dose-dependent manner and activating the Janus kinase (JAK2)-coupled cytokine receptor pathway [98].

Large conductance Ca²⁺-activated K⁺ channels (BKCa) were described to be expressed in several breast cancer cell lines (e.g., UACC893, SK-BR-3, and MDA-MB-231) [99]. Intermediate-conductance, Ca²⁺-activated K⁺ channels (hIK1) are also functionally expressed in MCF7 breast cancer cells, and their current density and basal cytosolic Ca²⁺ concentration being augmented in cells synchronized at the end of the G1 or S phase with respect to the cells in the early G1 phase [100]. Caveolin-1 was demonstrated to colocalize with BKCa in MCF-7 breast cancer cells, and silencing caveolin-1 induces increased activation and upregulation of BKCa [101].

2.7. Calcium-activated chloride channels in breast cancer

Calcium-activated chloride channel anoctamin 1 (ANO1) was demonstrated to be highly expressed in breast cancer cell lines and primary tumors and was considered to be a predictive factor for the disease degree and poor prognosis [102]. ANO1 activation was demonstrated to be done via the EGF receptor and calmodulin-dependent protein kinase II signaling, and its expression was associated with tumor cell survival [102].

An extensive clinical study including 431 patients with invasive ductal breast carcinoma and 46 patients with fibroadenoma analyzed the expression of anoctamin 1 (Ano1, TMEM16A), one of the members in the Ano family [103]. The study identified a correlation between Ano1 overexpression and the good prognosis in patients with lower clinical stage (stage I or II) of the breast cancer or in patients with triple-negative breast cancers [103].

Integrating these results, it is still premature to evaluate if Ano1 is or is not a predictive factor in good/poor prognosis in breast cancer patients, and if it is a suitable pharmacological target. It would be very useful to have more insights into the cellular mechanisms related to Ano1 activation. In our opinion, a possible scenario would be that the opening of calcium-activated chloride channels induces chloride efflux, membrane depolarization followed by the calcium influx through VGCCs. The high level of expression of calcium-activated chloride channels in breast cancer cells might be correlated with the tendency of these cells to be more depolarized with respect to the normal surrounding cells. However, this scenario would explain only the clinical data showing the association between Ano1 overexpression and the poor prognosis in patients with breast cancer.

2.8. Muscarinic acetylcholine receptors in breast cancer

Muscarinic receptors have been described to be expressed in several non-neuronal cell types, including endothelial cells, smooth muscle cells, or bladder and gastrointestinal tract [104–107]. In multiple malignancies, including breast, prostate, lung, ovary, pancreas, prostate, skin, stomach, uterus and colon cancer, muscarinic receptors have been demonstrated to contribute to cell proliferation and cancer progression [108–110].

In particular, muscarinic receptors have also been described in normal murine mammary (NMuMG) cells, normal human-breast-derived MCF-10A cells, and homogenates of surgical samples derived from normal human mammary tissue by Western blot and radioligand binding assays [111–113]. The expression of muscarinic acetylcholine receptors, M_3 and M_4 , was detected in MCF-7 breast cancer cells [114]. Additionally, the expression of muscarinic acetylcholine receptors was evidenced in LM2, LM3, and LMM3 cell lines derived from spontaneous mammary adenocarcinomas developed in Balb/C mice [115–117]. These studies demonstrated the involvement of muscarinic receptors in the tumor cells proliferation, progression, and angiogenesis [115–117], by a mechanism involving the stimulation of the nitric oxide synthase activity [114].

Muscarinic receptors, M_1 , M_3 and M_5 , are coupled with $G_{q/11}$ proteins that activate phospholipase C (PLC) and determine the release of calcium from intracellular reservoirs. In MCF7, human breast cancer cells evidenced the activation of MAP by muscarinic receptors [118]. Considering that acetylcholine exerts neurocrine, paracrine, and autocrine regulation of cancer cell proliferation, migration, etc., we might consider muscarinic receptors among the master-players in breast cancer.

3. Calcium signaling pathways as pharmacological targets in breast cancer

3.1. Calcium oscillations as pharmacological targets in breast cancer

Tetrodotoxin (TTX), a blocker of voltage-gated sodium channels (VGSCs), was demonstrated to diminish the number of oscillating MDA-MB-231 breast cancer cells and to reduce the amplitude and the frequency of the Ca^{2+} oscillations (i.e., spontaneous calcium transients) in the same cells [78]. While TTX had no effect on the basal calcium level in nonoscillating MDA-MB-231 breast cells, when applied in high extracellular K^+ conditions augmented the intracellular calcium concentration in both oscillating and nonoscillating MDA-MB-231 cells [78].

Previous studies have demonstrated that the blockade of VGSCs inhibits the invasion of endocrine-resistant breast cancer cells [119]. It is very interesting the correlation between the blockade of VGSCs and the reduction of spontaneous calcium oscillations. Therefore, considering that only metastatic cells are characterized by spontaneous calcium transients in comparison with low-metastatic or normal cells, a possible therapeutic strategy would be to diminish calcium transients by applying targeted pharmacological agents against VGSCs. An interesting approach would be to encapsulate voltage-gated sodium channels antagonists in charged liposomes and to target only highly metastatic breast cancer cells.

3.2. Voltage-gated calcium channels as pharmacological targets in breast cancer

1 mM Mn^{2+} and 0.1 mM Ni^{2+} ions blocked the fast activation and inactivation of the T-type calcium currents in MCF7 breast cancer cells [79].

Low doses (10–20 μM) of verapamil, an antagonist of voltage-gated calcium channels, blocked the growth of triple-negative MDA-MB-231 breast cancer cells, while high doses (100 μM) reduced by 90% the triple-negative MDA-MB-231 and MCF7 breast cancer cells [76].

Cav3.2 channels were demonstrated to play an important role in the mechanisms involved in chemoresistance. To date, trastuzumab resistance was demonstrated to be correlated with high mRNA Cav3.2 levels in SKBR3 breast cancer cells [120]. Patients with estrogen receptor-positive breast cancer that had a poor clinical outcome presented a significant Cav3.2 upregulation [120]. Moreover, patients with HER2-positive breast cancer presented a positive correlation between the Cav3.2 expression and patient survival upon chemotherapy [120].

3.3. TRP channels as pharmacological targets in breast cancer

TRP channels have been demonstrated to be actively involved in the development and progression of breast cancer. Therefore, finding efficient strategies for blocking-/silencing-specific TRPs in breast cancer cells might represent a good strategy to diminish the breast cancer progression.

Among the active compounds that act on TRP channels, polyunsaturated fatty acids (PUFA) have been evidenced to act on TRPC3, to reduce MCF-7 breast cancer cell proliferation and migration and to inhibit the TRPC-mediated calcium entry [80]. These compounds are particularly interesting as they are common in the diet, and previous studies have already shown their positive effects in cardiovascular diseases, including atherosclerosis, arrhythmias, etc. [121, 122]. The increased tumor vascularization is among the typical alterations and some PUFA have been demonstrated to exert effects against angiogenesis, inflammation, and cancer [123]. In this context, the action of PUFA on TRP channels is explaining their effects against breast tumors, either by diminishing angiogenesis in tumors or by reducing the proliferation/migration of tumor cells.

The Rho-associated kinases, ROCK1 and ROCK2, are considered as important therapeutic targets in breast cancer, being already demonstrated their critical role in cancer cell migration and invasion [124]. Identifying efficient Rho-kinase inhibitors is of particular interest in several subtypes of cancer, including breast cancer. Some Rho-kinase inhibitors have been identified to inhibit TRP channels. To date, fasudil, a Rho-kinase inhibitor, upregulates the expression of TRPC1 and TRPV2 in breast cancer cell lines (e.g., ZR-75-1, MCF7, and MDA-MB-231) [125]. Additionally, another Rho-kinase inhibitor, Y-27632, was demonstrated to downregulate TRPM6 and to upregulate TRPC7 in the same breast cancer cells [125].

TRPV1 seems to be one of the most promising TRP channels as a pharmacological target in breast cancer. To date, capsaicin inhibits cancer growth in SUM149PT breast cancer cells, by triggering apoptotic/necrotic mechanisms, while capsazepine diminishes these effects [88]. MRS1477, a positive allosteric modulator of TRPV1, when co-applied with capsaicin, diminished the fraction of MCF7 breast cancer apoptotic cells but was ineffective against tumor growth in MCF7 tumor-bearing immunodeficient mice [89]. Another study demonstrated that doxorubicin and melatonin exert synergistic effects against apoptosis and mitochondrial oxidative stress in MCF-7 breast cancer cells by activating TRPV1 [126]. The chemotherapy agent, 5-fluorouracil, also exerts its apoptotic effect in MCF7 breast cancer cells via TRPV1 channels [127]. The anticancer effects of cisplatin mediated by TRPV1 have to be potentiated by co-application of selenium or alpha-lipoic acid on MCF-7 breast cancer cells [128, 129]. Interestingly, selenium was shown to diminish the electromagnetic radiation (900 MHz) effects in MDA-MB-231 breast cancer cell line mediated by TRPV1 activation [130].

Although, tamoxifen is commonly known as a selective estrogen receptor modulator and used in estrogen receptor-positive breast cancer cells, it was also shown to be effective in estrogen-receptors negative tumors. Recent data indicate that it exerts antiproliferative effects in MCF7 breast cancer cells by an estrogen receptor-independent pathway that involves TRPV6 channels [87].

N-(3-aminopropyl)-2-([(3-methylphenyl)methyl]oxy)(20)-N-(2-thienylmethyl)benzamide (AMTB), an inhibitor of TRPM8, was also described to diminish the proliferation and migration of MDA-MB-231 and SK-BR-3 breast cancer cells via a TRPM8-independent mechanism involving voltage-gated sodium channels [131].

3.4. STIM and Orai as pharmacological targets in breast cancer

Blocking Ca^{2+} influx with EGTA, Ni^{2+} , or SKF96365 in STIM1 siRNA-, or Orai1 siRNA-treated MDA-MB-231 human breast tumor cells decreased the number of invasive tumor cells [95].

BALB/c mice with 4 T1 tumor cells implanted in the mammary glands were injected with the store-operated channel blocker SKF96365 (10 mg/kg, daily, 20 days treatment), and the lung metastasis was diminished up to 20% [95]. Moreover, intraperitoneal administration of SKF96365 (10 mg/kg, daily, 4 weeks treatment) in NOD/SCID mice with MDA-MB-231 human breast tumor cells determined a significant reduction of lung metastasis after 1 week of treatment, and no metastasis recurrence was observed in 2 weeks after drug withdrawal [95].

3.5. hEAG1 channels as pharmacological targets in breast cancer

Astemizole, an antihistaminic drug, was shown to block hEAG1 channels in MDA-MB-231 breast cancer cells [97]. The authors have demonstrated that blocking hEag1 with astemizole or silencing induces the breast cancer depolarization and consequently reduces the calcium influx and the cell migration without any influence on the cell proliferation [97]. These data are clinically valuable as hEAG1 are overexpressed in invasive ductal carcinoma breast cancer or metastatic lymph nodes [97, 132] and their co-expression with HIF-1 α is correlated with tumor size, lymph node status, and tumor stage [132], and the possibility of pharmacologically blocking these channels might represent a promising therapy. Moreover, astemizole may be used to pharmacologically discriminate hEAG from the related hERG potassium channels in MCF-7 breast cancer cells [133].

Insulin-like growth factor-1 (IGF1) is known to ubiquitarily stimulate the growth of various cells in the human body, and also to strongly inhibit the programmed cell death [134]. IGF1 was shown to activate hEAG1 channels in breast cancer cells via an Akt-dependent signaling pathway [135]. Corroborating the antiapoptotic activity of IGF1 with its activating effect exerted on hEAG1, we can conclude that hEAG1 plays an important role in breast cancer proliferation and its blocking is of particular interest in finding an efficient anticancer therapy.

3.6. Calcium-activated potassium channels as pharmacological targets in breast cancer

The hIKCa1 blockers, TRAM-34, and clotrimazole, or siRNA-hIKCa1 inhibit the prolactin-induced proliferation of the MCF7 breast cancer cells [98].

Iberiotoxin inhibits large conductance of Ca²⁺-activated K⁺ channels (BKCa) in three types of breast cancer cell lines (e.g., UACC893, SK-BR-3, and MDA-MB-231), eliciting cellular depolarization, attenuating the anchorage-independent growth [99]. Oppositely, HER-2/neu-overexpressing SK-BR-3 cells were insensitive to iberiotoxin [99].

3.7. Calcium-activated chloride channels as pharmacological targets in breast cancer

CaCCinh-A01, an inhibitor of calcium-activated chloride channels ANO1, diminishes breast cancer cell viability and colony formation [102]. Recent clinical studies showed that ANO1 is upregulated in breast cancer in comparison with fibroadenoma [103]. Moreover, patients with progesterone receptor-positive or HER2-negative breast cancer, or breast cancer patients treated with tamoxifen, have an upregulation of ANO1, which can be considered as a predictive factor for longer overall survival [103].

3.8. Muscarinic acetylcholine receptors as pharmacological targets in breast cancer

IgG purified from the serum of breast cancer patients mimics the effect of carbachol by activating muscarinic acetylcholine receptors in MCF-7 breast cancer cells [136]. Moreover, these autoantibodies purified from the serum of breast cancer patients regulate the MCF7 breast cancer cell migration and the MMP-9 activity, and these effects are reduced by atropine, 4-DAMP (M_3 receptor antagonist), and tropicamide (M_4 receptor antagonist) [137].

Carbachol, an agonist of muscarinic acetylcholine receptors, acts on M_1 and M_3 receptors in the MCF7 breast tumor cells and potentiates tumor progression, by activating nitric oxide synthase via phospholipase C and protein kinase C signaling pathways [114]. Carbachol also elicits the mobilization of intracellular-free Ca^{2+} and induces the phosphorylation of MAPK/ERK in MCF-7 human breast cancer cells, while pretreatment with wortmannin or LY294002 (selective inhibitors of phosphoinositide 3-kinase), with genistein (nonselective inhibitor of tyrosine kinases) or with PP2 (specific Src tyrosine kinase inhibitor), diminished the carbachol-induced MAPK/ERK phosphorylation [118].

Moreover, carbachol upregulates the vascular endothelial growth factor-A in MCF7 tumor cells and determines angiogenesis, while atropine reverts its effects [136]. Carbachol treatment (20 hours) increased the tumor cell death and its administration in subthreshold concentrations in conjunction with paclitaxel potentiates cell death [138, 139], while atropine reverts these combined effects [138]. Interestingly, the combined treatment with carbachol (low doses) and paclitaxel induced the death of breast tumor MCF-7 cells, via the increased activity of nitric oxide synthase 1 and 3, and the reduced activity of arginase II, but the drug combination was ineffective against the nontumorigenic epithelial MCF-10A cell line, due to the absence of muscarinic acetylcholine receptors [140].

Although several preclinical studies indicated the pharmacological potential of M_3 antagonists in inhibiting tumor growth (e.g., melanoma, pancreatic, breast, ovarian, prostate, and brain cancers), no clinical trials have been done [109].

4. Conclusion

In conclusion, calcium signaling alterations occur in multiple cellular components, human breast stem cells, human breast epithelial cells, human breast myoepithelial cells, human breast adipocytes, human breast telocytes, etc., including those which contribute to the development and progression of breast cancer. Moreover, several molecular actors (e.g., voltage-gated calcium channels, TRP channels, STIM/Orai proteins, hEag1 K^+ channels, calcium-activated potassium channels, calcium-activated chloride channels, muscarinic acetylcholine receptors, etc.) are playing an important role in calcium-altered homeostasis associated with breast cancer that might be considered as potential pharmacological targets. Considering the interplay between the above-described calcium signaling pathways, the most efficient strategy against breast cancer would simultaneously target several molecular players.

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