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Review

Altered calcium signaling in cancer cells☆

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ABSTRACT

It is the nature of the calcium signal, as determined by the coordinated activity of a suite of calcium channels, pumps, exchangers and binding proteins that ultimately guides a cell's fate. Deregulation of the calcium signal is often deleterious and has been linked to each of the 'cancer hallmarks'. Despite this, we do not yet have a full understanding of the remodeling of the calcium signal associated with cancer. Such an understanding could aid in guiding the development of therapies specifically targeting altered calcium signaling in cancer cells during tumorigenic progression. Findings from some of the studies that have assessed the remodeling of the calcium signal associated with tumorigenesis and/or processes important in invasion and metastasis are presented in this review. The potential of new methodologies is also discussed. This article is part of a Special Issue entitled: Membrane channels and transporters in cancers.

Contents

1. Introduction

Tightly controlled regulation of the calcium signal is essential for appropriate cellular functioning, as evidenced by the role of changes in cytosolic free Ca^{2+} in processes such as cell proliferation, gene transcription and cell death [1–5]. Typically cells at rest maintain an intracellular calcium concentration ($[Ca^{2+}]_i$) of approximately 100 nM, while extracellular calcium concentrations are much higher, generally within the range of 1–2 mM [3–5]. Specialized calcium pumps, channels and calcium binding proteins are used by cells to both maintain cellular homeostasis and carry out specific cellular functions, and have been referred to as the "molecular toolkit" for calcium signaling [1,2] (Fig. 1). Changes in cytosolic free Ca^{2+} can involve global increases that may be transient or sustained, or highly localized such as calcium sparks and puffs, or they may occur as waves or oscillations [1,5]. These changes can be "decoded" by the cell, which allows the ubiquitous calcium signal to specifically regulate cellular processes [1,2]. This complexity in calcium signaling means that the deregulation of the calcium signal can be a feature of certain pathological states, including cancer [5–7]. Much of the research assessing calcium signaling in cancer has focused on determining changes in the expression levels of proteins responsible

Abbreviations: ATP, adenosine triphosphate; EGF, epidermal growth factor; EMT, epithelial–mesenchymal transition; IP_3R2, inositol 1,4,5-triphosphate receptor, type 2; PMCA, plasma membrane Ca^{2+} ATPase; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; SOCE, store operated Ca^{2+} entry; STIM1, stromal interaction molecule 1; TRP, transient receptor potential

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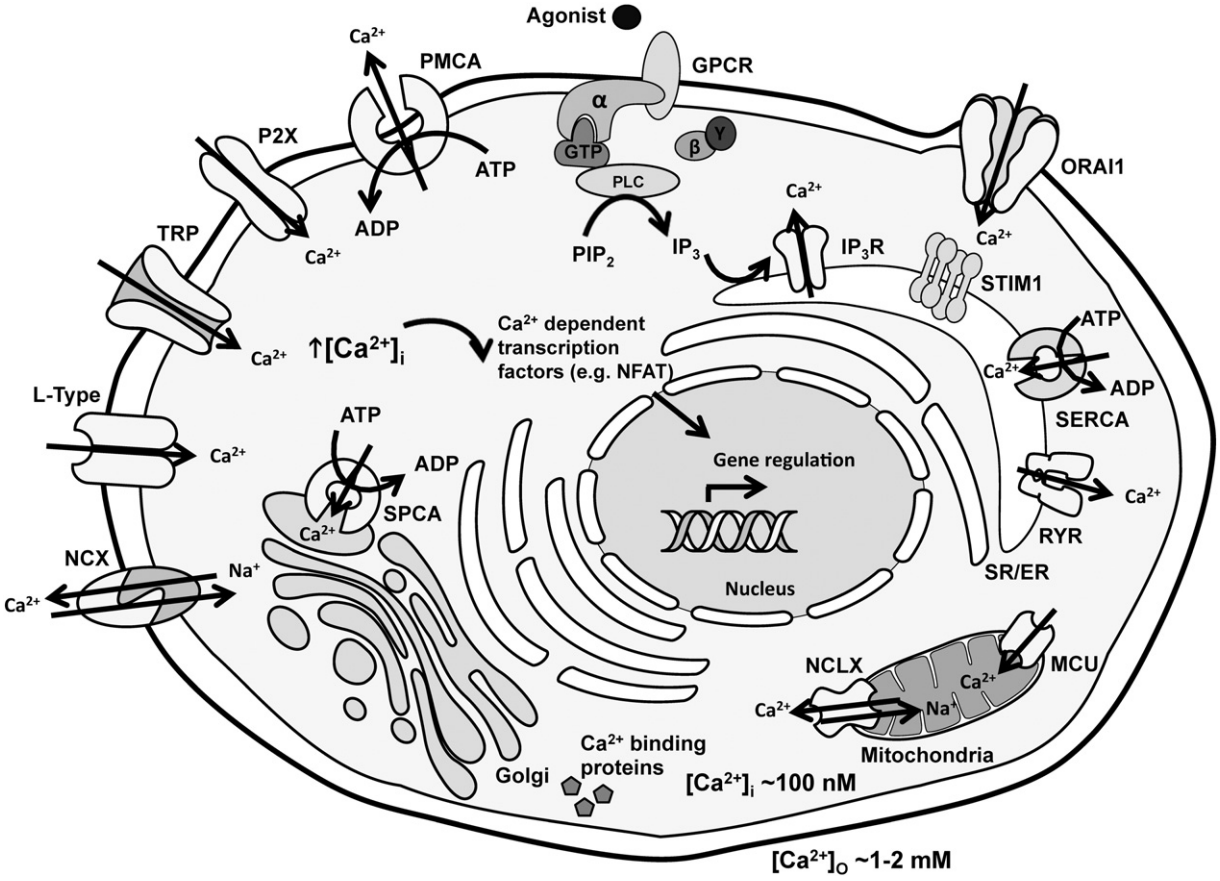

Fig. 1. Diagrammatic representation of major Ca^{2+} influx/efflux/release and resequestration pathways involved in the regulation of $[Ca^{2+}]_i$ homeostasis in mammalian cells and their associated proteins. Major Ca^{2+} influx pathways include those mediated by the transient receptor potential (TRP) family of Ca^{2+} permeable ion channels, voltage-gated Ca^{2+} channels (e.g. L-type), purinergic receptors (e.g. P2X), and the SOCE pathway components Orai1 and STIM1. Activation of plasma membrane localized G protein-coupled receptors (GPCRs) leads to generation of inositol triphosphate (IP_3) and subsequent stimulation of IP_3 receptors (IP_3Rs) located on the endoplasmic reticulum (ER), resulting in Ca^{2+} store release. ER localized ryanodine receptors (RYR) and mitochondrial Na^+/Ca^{2+} exchanger (NCLX) also regulate Ca^{2+} in organelles. The sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), secretory pathway Ca^{2+} ATPase (SPCA), and mitochondrial uniporter (MCU) all sequester cytosolic Ca^{2+} into intracellular organelles, while plasma membrane Ca^{2+}-ATPases (PMCA) actively extrude Ca^{2+} from the cytosol into the extracellular space, and together with the Na^+/Ca^{2+} exchanger (NCX) play a role in restoring resting $[Ca^{2+}]_i$. Ca^{2+} signaling also regulates various Ca^{2+} dependent transcription factors (e.g. NFAT) and Ca^{2+} binding proteins (e.g. calmodulin).
Adapted from references [8–10].

for regulating cytoplasmic free Ca^{2+} concentrations. Following the identification of aberrantly expressed calcium channels, pumps or exchangers, researchers often then rely on gene silencing approaches and/or chemical inhibitors/activators to evaluate their role in calcium signaling and cancer relevant processes (e.g. proliferation and migration). However, in the context of cancer, compared to some other disease states, there is a paucity of information regarding changes in the *nature* of the calcium signal that occurs in cancer cells compared to non-cancer derived cells. Elucidating such information would improve our understanding of the mechanisms underlying cancer progression, and may further help guide researchers to identify molecular targets not associated with changes in expression. This review will discuss the available evidence for the remodeling of the calcium signal in cancer, and briefly describe studies in other disease states to highlight potential approaches that could further improve our understanding of alterations in calcium signaling in cancer cells.

2. Remodeling of the calcium signal in disease

The development of Ca^{2+} sensitive indicators, such as the fluorescent dyes Fura-2 and Fluo-4, and genetically encoded Ca^{2+} indicators has been integral to our understanding and interpretation of intracellular calcium signaling by enabling quantitative analysis of Ca^{2+} in the cytoplasm and in subcellular organelles [11–16]. These tools have allowed a better understanding of how the nature of the calcium signal is remodeled in some diseases. A relatively well studied example of pathological remodeling of the calcium signal, reviewed in detail elsewhere [17–20], is that which occurs in smooth muscle cells as a consequence of vascular disease and injury, including pulmonary hypertension [21,22], atherosclerosis [23,24] and arterial restenosis following angioplasty [25,26].

Calcium signaling in smooth muscle cells regulates numerous cellular processes including proliferation, contraction and gene transcription [27–30]. During vascular injury (through mechanical stress and/or growth factors/cytokine exposure), vascular smooth muscle cells can undergo phenotypic switching from cells that are largely quiescent and contractile, to those exhibiting a more synthetic and proliferative phenotype [18,31,32]. This phenotypic switching [31], is associated with corresponding changes in the *nature* of the calcium signal, for example a transition from voltage-gated Ca^{2+} entry pathways typical of contractile cells to one resembling store-operated and receptor-operated Ca^{2+} entry (SOCE) in proliferating cells [20,33–35]. Kumar et al. demonstrated an example of such remodeling using an in vivo model of neointimal hyperplasia [25]. In this model, freshly isolated periadventitial cuff injured mouse carotid artery displayed increased $[Ca^{2+}]_i$ in response to reintroduction of Ca^{2+} following store depletion using the sarco/endoplasmic reticulum ATPase (SERCA) inhibitor thapsigargin, while K^+ induced depolarization failed to significantly increase $[Ca^{2+}]_i$ relative to uninjured arterial tissue [25]. These findings indicated a switch from a predominately voltage-gated calcium entry

pathway to one resembling SOCE following vascular injury. This change has been replicated in numerous in vitro models of vascular smooth muscle cell remodeling [33–37]. Changes in calcium signaling associated with neointimal hyperplasia are associated with increased expression of the transient receptor potential family member TRPC1 [25], a protein implicated in the SOCE pathway in some cells [38,39]. Subsequent papers, relevant to this model, also identified roles for Orai1 and stromal interaction molecule 1 (STIM1) [33,35,40,41], the canonical proteins involved in SOCE [38,42–46]. These included the ability of Orai1 or STIM1 silencing to reduce neointima formation following balloon-injury of rat carotid artery [40,47,48].

Vascular smooth muscle injury clearly represents an example of dynamic remodeling of calcium signaling in disease and how characterization of these calcium signaling changes can lead to a better understanding of disease mechanisms and/or help identify potential therapeutic targets. In addition to pathologies involving vascular smooth muscle cell remodeling, deregulated calcium homeostasis has also been linked to remodeling of airway smooth muscle cells in asthma [49–51], and various other conditions, including those of the brain [52] such as Alzheimer's disease [53–56]. These studies have included the use of advanced assessment of calcium signaling, such as in vivo multiphoton fluorescence lifetime imaging microscopy of Ca^{2+} levels in astrocytes in a mouse model of Alzheimer's disease versus wild type/non-transgenic control animals [56].

Numerous studies have demonstrated altered expression of various components of the "calcium signaling toolkit" [1,2] in cancer cell lines and in clinical samples (reviewed in [10,57–61]). However, there have arguably been less studies comparing how the nature of the calcium signal is altered in cancer and/or changes with tumor progression, especially when compared to other disease states (such as those described above). Although expression studies have identified specific Ca^{2+} channels and pumps as drug targets for various cancer types including ovarian [62], brain [63,64], prostate [65,66], breast [67,68], and esophageal [69], calcium signaling could be altered in cancer progression through other changes, such as altered calcium channel or pump localization or activity via disrupted post-translational modification. Such changes would be better identified through assessment of the calcium signal rather than protein or mRNA levels. Indeed Ingueneau et al. reported that oxidized low-density-lipoprotein induced Ca^{2+} influx, and consequent apoptosis in vascular smooth muscle cells requires translocation of TRPC1 from an intracellular compartment to caveolae/caveolin-1 containing regions of the plasma membrane [70]. The cellular localization and activity of other TRP family members are also altered in response to various activation pathways in other cell types [71]. Post-translational protein modifications may also mask alterations in cellular calcium signaling if interpreted in the absence of functional studies. For example, Sundivakkam et al. showed that phosphorylation of STIM1 inhibits SOCE in endothelial cells, and is potentially involved in regulating blood vessel permeability responses [72]. Some studies have compared calcium signaling in cancer derived and relevant non-cancer derived cells and/or other models such as those involving cancer cell differentiation. This review will provide a summary of such studies and their significance. We will also describe some of the experimental constraints and challenges in characterizing calcium signaling changes associated with cancer progression, before discussing some of the experimental approaches that may allow these challenges to be addressed.

2.1. Remodeling of the calcium signal in tumor derived cells versus normal cells

The nature of the calcium signal plays an important role in regulating cellular functions [1,3] including those defined as the "hallmarks of cancer" [59,73]. One way to identify and understand the possible remodeling of calcium homeostasis in some cancers is to compare the nature of the calcium signal in cells or cell lines derived from cancers with those derived from non-cancer tissue. Table 1 provides a summary of studies that have assessed differences in calcium homeostasis in tumor derived versus non-tumor derived cells. As discussed below, many of the changes between tumor and non-tumor derived cells are reflected in very specific changes in aspects of the calcium signal, such as the nature of Ca^{2+} influx or the rate of recovery of $[Ca^{2+}]_i$ after stimulation. Some of these changes are illustrated in Fig. 2.

Since the identification of the key molecular components of SOCE, namely STIM1 and Orai1, there has been a keen interest in determining the role of this calcium influx pathway in pathophysiology [88–91]. Indeed, altered expression of STIM1 and/or Orai1 has been reported in various cancer types including breast [67], cervical [92], and esophageal [81]. Prior to the identification of the STIM and Orai proteins, Baldi et al. conducted studies looking into the nature and remodeling of capacitative Ca^{2+} entry (a term used synonymously with SOCE) in the tumorigenic (luminal human epidermal growth factor receptor 2 positive) SKBR3 cell line, and HBL100, which they used to represent non-tumorigenic breast epithelial cells [78]. While both cell lines demonstrated classic SOCE in response to thapsigargin mediated store depletion in the absence of extracellular Ca^{2+}, the nature of store release and Ca^{2+} re-entry (influx) noticeably differed between the two cell lines. While the peak amplitude and initial rate of calcium influx was similar in both cell lines, Baldi et al. reported a more sustained and slower return to baseline $[Ca^{2+}]_i$ levels following SOCE in SKBR3 cells. Also, the amplitude of the initial peak representing store depletion appeared to be higher in HLB100 cells. The contribution of SOCE and the potential role of other Ca^{2+} entry pathways were also assessed using various surrogate divalent cations (Ba^{2+}, Mn^{2+} and Sr^{2+}), which demonstrate different permeabilities, and the lanthanide Gd^{3+}, which is known to block SOCE at low concentrations. From their studies, Baldi et al. characterized two main calcium influx pathways in both cell lines; however, their relative contributions to SOCE differed between the tumorigenic SKBR3 and non-tumorigenic HLB100 cell lines. These findings raised the question of whether differences in the nature of SOCE between both cell lines could be a function of tumorigenic remodeling, or merely due to other differences between these cell lines [78]. Interestingly, later studies by McAndrew et al. quantified the expression levels of Orai1, STIM1 and STIM2 in a panel of non-malignant and breast cancer cell lines [67]. Of the six breast cancer cell lines assessed, SKBR3 cells expressed the lowest levels of Orai1 mRNA (relative to the non-tumorigenic cell line 184A1), and an altered STIM1/STIM2 ratio compared to other tumorigenic cell lines. Unfortunately, while mRNA expression levels of the STIM and Orai family members were comprehensively characterized, a comparison of calcium signaling dynamics in all cell lines was not performed. Such a study may provide insight into the functional consequences of altered Orai1, STIM1 and STIM2 expression not only between non-tumorigenic and tumorigenic cell lines, but also between breast cancer cell lines representing different molecular subtypes.

Investigation into the contribution of SOCE, this time in glial cells, revealed a remodeling of this Ca^{2+} entry pathway in the form of a two-fold increase in the amplitude of SOCE in cultured human primary malignant glioblastoma multiforme cells relative to a non-malignant human primary astrocyte control [63]. Further investigation revealed an increase in Orai1 mRNA levels in only two of the three glioblastoma multiforme cell lines assessed, despite SOCE being increased in all three malignant cell lines. This further supports the importance of comparing functional calcium signaling in cancer and non-cancer control cells in addition to assessment of gene expression. Enhanced SOCE indicated by significantly increased $[Ca^{2+}]_i$ peak amplitude following store depletion, and corresponding to increased Orai1 protein expression, was also shown in four metastatic melanoma cell lines relative to a control melanocyte cell line [82]. Zhu et al. recently identified a remodeling of the SOCE pathway in an esophageal squamous cell carcinoma derived cell line KYSE-150, relative to a non-tumorigenic esophageal epithelial cell line HET-1A [81]. In addition to assessing SOCE specifically, the authors also evaluated differences in global calcium signaling using live cell

Table 1
Examples of differences in Ca^{2+} homeostasis from studies comparing tumor versus non-tumor models.

Model studied[a]	Observed change in Ca^{2+} signaling in tumor model(s) relative to control(s)[b]	Potential consequence(s) of altered Ca^{2+} signaling	References
Human J82, RT24, T24 and 5637 bladder urothelial carcinoma cell lines vs. normal bladder urothelial cells from healthy human subjects	Absence of carbachol stimulated $[Ca^{2+}]_i$ increases	Possible alterations in cell adhesion	[74]
Human 5367 bladder urothelial carcinoma cell line vs. normal human urothelial primary cell lines from healthy controls	↓ mechanically stimulated Ca^{2+} wave propagation	Altered intercellular communication via gap junctions	[75]
Human breast lobular infiltrating carcinoma derived endothelial cells vs. adult human dermal microvascular endothelial cells	↑ 4αPDD (a selective TRPV4 agonist) and arachidonic acid mediated $[Ca^{2+}]_i$ influx	Role in tumor angiogenesis and tumor derived endothelial cell migration	[76,77]
Human SKBR3 breast cancer cell line vs. non-tumorigenic HBL100 mammary epithelial cell line	Remodeled SOCE and store depletion kinetics	Different contribution of calcium influx pathways and hence altered cellular responses to stimuli	[78]
Murine RAW 264.7 monocytic cell line treated with human MDA-MB-231 metastatic breast cancer cell line conditioned media vs. non-tumorigenic MCF-10A breast cell line conditioned media	Induction of sustained $[Ca^{2+}]_i$ oscillations following treatment of RAW 264.7 cells with MDA-MB-231 conditioned media	Potential role in osteoclast formation and bone metastases	[79]
Rat colonocytes from DMH procarcinogen treated animals vs. colonocytes from vehicle treated animals	↓ basal $[Ca^{2+}]_i$ in colonocytes from DMH treated rats	Reduced apoptosis	[80]
Human KYSE-150 esophageal squamous cell carcinoma cell line vs. non-malignant HET-1A esophageal epithelial cell line	↑ SOCE and spontaneous $Ca^{2+}{}_i$ oscillations	May promote cell proliferation, migration and invasion	[81]
Human U251 glioblastoma cell line, GBM1 and GBM8 primary glioblastoma multiforme cell lines vs. non-malignant human primary astrocytes	↑ SOCE	Promotion of cell invasion	[63]
Human SK-Mel-2, SK-Mel-24, C8161 and UACC257 metastatic melanoma cell lines vs. control HEMA-LP melanocyte cell line	↑ SOCE	Promotion of cell proliferation and migration	[82]
Rat pancreatic acinar carcinoma cells vs. normal acinar cells	↑ rate of $[Ca^{2+}]_i$ recovery following stimulation by carbamylcholine and peptidergic agonist cholecystokinin octapeptide	Altered cellular responses to some stimuli and possible reduced apoptosis sensitivity via enhanced Ca^{2+} efflux	[83]
Human endothelial progenitor cells from renal cellular carcinoma patients vs. control endothelial progenitor cells	↑ SOCE	Enhanced proliferation and tubulogenesis	[84]
Human peripheral blood lymphocytes from CLL patients vs. normal peripheral blood lymphocytes from healthy controls	↑ ATP stimulated $[Ca^{2+}]_i$ in some CLL patient samples	Altered response to ATP effect	[85]
Human peripheral blood leukocytes from CML patients vs. normal peripheral blood leukocytes from healthy controls	↓ IP_3, ATP and ionomycin stimulated $[Ca^{2+}]_i$	Altered oxidative stress response	[86]
Human CEM and Jurkat malignant T cell lines vs. normal human peripheral blood T cells	↑ $[Ca^{2+}]_i$ in response to L-type Ca^{2+} channel activation by BAYK8644 and ionomycin stimulation	Altered sensitivity to apoptotic inducing agents and/or differential regulation of Ca^{2+} sensitive pathways	[87]

Abbreviations: 4αPDD, 4α-phorbal 12,13-didecanoate; ATP, adenosine triphosphate; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; DMH, 1,2-dimethylhydrazine dihydrochloride; IP_3, inositol 1,4,5-trisphosphate; SOCE, store operated calcium entry; TRPV4, transient receptor potential cation channel, subfamily V, member 4.
[a] Includes human and/or animal models of both solid and hematological cancers as well as those using conditioned media treatments.
[b] Arrows indicate either an increase (↑) or decrease (↓) in the nature of the Ca^{2+} signal in tumor model(s) relative to the non-tumor control(s).

imaging in the absence of a specific stimulus. Esophageal squamous cell carcinoma cells displayed a significantly higher degree of spontaneous intracellular Ca^{2+} oscillations compared to normal cells, 76% versus 26%, respectively [81]. These oscillations could be inhibited by pharmacologically mediated SOCE blockade with SKF96365 and Orai1 silencing [81]. This is significant because of the importance of the nature of

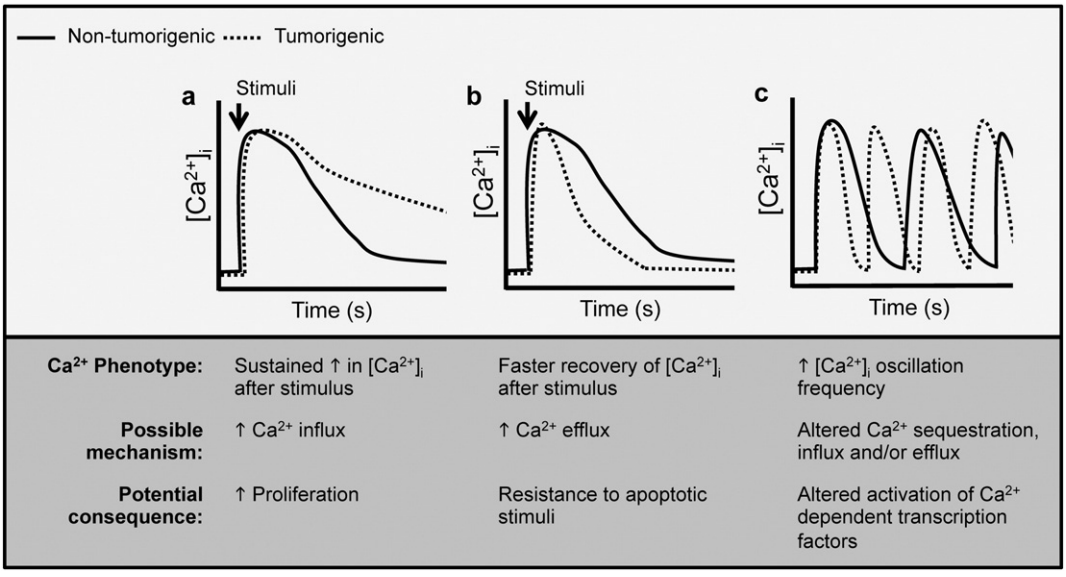

Fig. 2. Examples of hypothetical remodeling of the Ca^{2+} signal in tumorigenic versus non-tumorigenic cells. Each stylized Ca^{2+} trace depicts a remodeling of various aspects of intracellular Ca^{2+} signaling pathways, including (a) Ca^{2+} influx pathways, (b) Ca^{2+} efflux pathways, and (c) intracellular Ca^{2+} oscillatory behavior.

calcium oscillations in the regulation of the transcription factors that regulate genes important in cell proliferation and/or migration [93–96].

Altered expression of other Ca^{2+} entry pathways, in particular certain members of the transient receptor potential (TRP) family of Ca^{2+} permeable ion channels has also been identified in various cancer types including breast [68,97,98], prostate [61,99], ovarian [62], esophageal [69], and brain [64] (reviewed in [61,100]). Studies comparing cancer and non-cancer derived cells have also shown changes in calcium signaling mediated by some members of the TRP channel family. Early studies by Fiorio Pla et al. investigating mechanisms of endothelial cell migration, a process important in angiogenesis in tumors, showed that the second messenger arachidonic acid induced a significantly higher Ca^{2+} response in breast tumor derived endothelial cells relative to normal dermal endothelial cells [76]. This difference appears to be mediated by the TRP channel family member TRPV4, since arachidonic acid and the TRPV4 agonist 4αPDD stimulated Ca^{2+} entry in tumor derived endothelial cells are attenuated by TRPV4 silencing [77]. Another TRP family member showing altered activity in certain cancer types is the cold stimulus activated TRPM8, which mediated significantly greater current density (pA/pF) (determined via whole cell patch clamp) in an androgen sensitive primary prostate cancer cell line relative to normal primary prostate epithelial cells in response to icilin stimulation [66].

It is now well recognized that tumor cells do not exist in isolation but are rather part of a larger microenvironment comprising multiple cell types, including immune, endothelial and other supporting stromal cells, which together act to maintain and promote tumor growth and progression (reviewed in [101–104]). There is increasing evidence to suggest that the recruitment of endothelial progenitor cells from the bone marrow plays an important role in tumoral neoangiogenesis (reviewed in [105,106]), which is essential for sustaining tumor growth and facilitating metastatic spread [107]. Remodeling of calcium signaling was recently identified in endothelial progenitor cells derived from patients with renal cell carcinoma relative to healthy controls [84]. Lodola et al. showed that the amplitude of SOCE was significantly higher in endothelial progenitor cells derived from renal cell carcinoma patients compared to those derived from healthy controls. This finding was supported by a corresponding increase in the transcript and protein levels of STIM1, Orai1 and TRPC1 in endothelial progenitor cells derived from renal cell carcinoma patients. Through a series of experiments, the authors provided evidence to support the hypothesis that the increase in SOCE in endothelial progenitor cells derived from renal cell carcinoma patients increased proliferation and tubulogenesis, processes important in endothelial progenitor cell mediated neoangiogenesis [84].

Another example of the importance of calcium signaling in cells of the tumor microenvironment was shown by Tiedemann et al. when they demonstrated significantly increased intracellular Ca^{2+} oscillatory behavior in osteoclast (bone resorbing) precursor cells following stimulation with conditioned media from tumorigenic breast cancer cells, but not non-tumorigenic breast epithelial cells [79]. This increase in conditioned media stimulated Ca^{2+} oscillations was associated with a significant increase in osteoclast number and size, an effect that was effectively inhibited by treatment with the Ca^{2+} chelator BAPTA (1,2-bis(*O*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid). This further supported a role for Ca^{2+} in the process of breast cancer cell induced osteoclastogenesis [79]. This finding is significant as increased osteoclast activity mediated by metastatic breast cancer cells in the bone microenvironment can lead to bone destruction, increased fracture risk and pain in cancer patients [79,108].

The studies described above provide evidence for the value of comparing calcium signaling in tumor-derived cells with those derived from non-diseased tissue. However, in some cases experimental limitations make it difficult to determine how well these changes in calcium signaling actually reflect the changes associated with tumor development. In Section 4 we will describe some of the methodological advancements that may address these limitations.

3. Differentiated cell lines as a model to study remodeling of calcium signaling in cancer cells

In order to overcome some of the difficulties associated with matching cancer cells to suitable normal control cells, a number of researchers have turned to tumorigenic cell lines in which a differentiated phenotype can be induced as a surrogate for 'normal' or at least less tumorigenic cells. The use of specific cell culture methods and/or stimulation with selected growth factors/pharmacological agents to induce a different cellular phenotype is not unique to cancer cell line models. Researchers investigating methods of culturing primary vascular smooth muscle cells observed that under certain culture conditions, smooth muscle cells can transition between a differentiated contractile and typically quiescent phenotype to a dedifferentiated and synthetic phenotype, characterized by a loss of contractile response, altered morphology and increased proliferative and migratory potential [109–114]. The phenotypic switching of vascular smooth muscle cells in culture is associated with changes in the nature of the calcium signal [33–35]. These changes include an increase in Ca^{2+} influx via SOCE. Any in vitro model cannot be expected to fully recapitulate conditions and remodeling events that occur in vivo. However, similar calcium signaling remodeling has been reported using an in vivo model of vascular injury [25]. Such agreement between in vivo and in vitro models of smooth muscle injury suggests that the differentiation or dedifferentiation of cell lines may be a useful approach to study the remodeling of calcium signaling in other disease states, including cancer. As described below, alterations in calcium signaling with differentiation have been reported in a variety of cancer cell lines including those of the breast, colon and lung.

In a similar way to groups culturing vascular smooth muscle cells to induce a 'switch' from a contractile to a synthetic phenotype [33,35,41], Bidaux et al. developed a model of prostate epithelial cell dedifferentiation to aid in characterizing expression and activity of the TRP family member, TRPM8, during prostate cancer progression [66]. The Na^{+} and Ca^{2+} permeable TRPM8 channel has been identified as a potential diagnostic/prognostic marker in prostate cancer [99,115–118]. Prolonged culture of primary prostate epithelial cells led to a less differentiated phenotype corresponding with a loss of androgen receptor expression, which was associated with decreased TRPM8 activity in response to stimulation with the TRPM8 activator menthol. Confocal imaging of TRPM8 localization demonstrated a loss of TRPM8 from the plasma membrane as a consequence of dedifferentiation [66]. Other changes in Ca^{2+} influx pathways as a consequence of differentiation are reflected by the effects of the differentiating agent 9-*cis* retinoic acid on N- and S-type neuroblastoma cells derived from the SH-SY5Y neuroblastoma cell line [119]. Differentiation of the more malignant N-type cells is associated with the downregulation of SOCE, leading the authors to propose the utility of therapeutically targeting SOCE as a means for promoting neuroblastoma cell differentiation [119]. In the A549 lung cancer cell line, differentiation via all-*trans*-retinoic acid is associated with enhanced Ca^{2+} influx following trypsin-mediated Ca^{2+} store depletion [120], demonstrating that the downregulation of Ca^{2+} influx is not a ubiquitous feature of the differentiation of cancer cell lines.

Pharmacologically induced differentiation of cancer cells using agents such as those acting on histone deacetylase, including short chain fatty acids and their derivatives, and the protein kinase C activator phorbal 12-myristate 13-acetate (PMA) is another method used to study altered calcium signaling and homeostasis in cancer cells [121–125]. Recently, Varga et al. showed that short chain fatty acid and/or PMA induced differentiation of the MCF-7 breast cancer cell line correlates with an increase in plasma membrane Ca^{2+} ATPase isoform 4b (PMCA4b) protein and mRNA expression [121]. PMCA4 was also detected in normal breast tissue sections following immunohistochemical staining [121]. These findings are in agreement with an earlier study, which showed decreased PMCA4 mRNA expression in a panel of breast cancer cell lines relative to non-tumorigenic breast epithelial cell

lines [126]. In order to further investigate the effect of differentiation on the nature of the calcium signal, Varga et al. developed an MCF-7 cell line stably expressing the genetically encoded Ca^{2+} indicator GCaMP2 [121]. Valerate and/or the combination of valerate and PMA induced differentiation resulted in a remodeling of the calcium signal characterized by reduced peak $[Ca^{2+}]_i$ following adenosine triphosphate (ATP) mediated store depletion, as well as a much faster recovery of $[Ca^{2+}]_i$ to baseline, relative to undifferentiated control cells. Peak $[Ca^{2+}]_i$ was also greatly reduced in differentiated cells following calcium ionophore treatment. The authors proposed a role for PMCA4b in maintaining Ca^{2+} homeostasis in normal mammary epithelial cell physiology, a function that may be lost as cells progress to a tumorigenic phenotype [121].

Breast cancer cells are not the only cancer cell type demonstrating a differentiation induced remodeling of the calcium signal corresponding to altered PMCA expression and function. Indeed, Ribiczey et al. showed increased PMCA4b expression following short chain fatty acid induced differentiation of a gastric and colon carcinoma cell line, corresponding to a two to three fold increase in PMCA transport activity in isolated microsomal membrane vesicles [123]. Pharmacologically induced differentiation also resulted in increased PMCA2, PMCA3 and PMCA4 isoform expression in a human neuroblastoma cell line, which is associated with a faster recovery rate of $[Ca^{2+}]_i$ to baseline after depolarization [127].

In addition to plasma membrane localized Ca^{2+} efflux pathways, evidence of remodeling of intraorganellar Ca^{2+} sequestration pathways and homeostasis, specifically those involving the sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCAs), has also been demonstrated following differentiation of cancer cells. SERCAs play an important role in the regulation of endoplasmic reticulum Ca^{2+} homeostasis and altered expression of the SERCA isoforms, particularly SERCA3, has been identified in various cancer subtypes [124,128–130]. Arbabian et al. demonstrated an increase in SERCA3 protein expression upon short chain fatty acid induced differentiation in a panel of lung adenocarcinoma cell lines [122]. Using A549 lung carcinoma derived cell lines stably expressing the genetically encoded calcium indicator GCaMP2, the authors reported significantly decreased Ca^{2+} store release induced by the SERCA inhibitor thapsigargin in phenylbutyrate differentiated cells relative to untreated control, whereas subsequent SOCE was essentially unaltered [122]. This finding was partially attributed to increased levels of the lower Ca^{2+} affinity SERCA3 isoform relative to that of the higher Ca^{2+} affinity SERCA2 isoform in differentiated cells [122]. Increased SERCA3 expression and altered Ca^{2+} homeostasis were also shown in the gastric carcinoma cell line, KATO-III, following butyrate induced differentiation [124]. Differentiation of KATO-III cells was associated with increased basal cytosolic Ca^{2+} relative to non-differentiated cells, and decreased thapsigargin induced endoplasmic reticulum Ca^{2+} store release [124], as was reported by Arbabian et al. in differentiated A549 cells [122].

The findings from the various models of differentiation described above provide further evidence of a remodeling of calcium signaling during the process of tumorigenesis. They also provide a rationale for future in vitro investigations using matched normal and tumorigenic primary and/or immortalized cell lines, as well as carefully designed in vivo experiments, to further study remodeling of the calcium signal in the context of cancer.

4. Modulation of the calcium signal during processes associated with invasion and metastasis

The transformation from benign to malignant disease is a leading cause of cancer related death [131,132], and therapies targeted towards inhibiting processes important in the invasion–metastasis cascade are a major focus of current cancer research [131–136]. Therefore understanding the remodeling of the calcium signal in invasion and metastasis could aid in the identification of novel therapeutic targets. Recently, researchers have used various models to replicate processes important in invasion and metastasis in order to help define changes in calcium signaling that may be important in tumor progression.

In order for cancer cells to successfully metastasize they must first acquire the ability to invade and migrate into their surrounding microenvironment and local vasculature before disseminating to a distant site [137–140]. A functional role for Ca^{2+} in the directional migration of lung fibroblasts was elegantly demonstrated using real time confocal Ca^{2+} imaging, where highly organized Ca^{2+} signals known as "calcium flickers", which are tightly regulated in both space and time were visualized in response to a chemotactic agent [141]. The stretch activated Ca^{2+} permeable TRP family member, TRPM7, together with inositol 1,4,5-triphosphate receptor, type 2 (IP_3R2) stimulated Ca^{2+} release was implicated in mediating these calcium flickers. Subsequent studies have shown a role for TRPM7 in the migration of both nasopharyngeal [142] and pancreatic carcinoma cell lines [143]. For a detailed review of the role of calcium in cancer metastasis refer to [59].

The SOCE pathway appears to play an important role in migration and invasion in various cancer cell types [63,81,82,144–146]. Indeed, evidence of a role for Orai1 and/or STIM1 in cell migration and invasion has been shown both in vitro and in vivo [81,82,92,144]. Recently, Umemura et al. showed pharmacological inhibition of SOCE and/or gene silencing of either STIM1 or Orai1 significantly decreased melanoma cell line migration in vitro, and the formation of lung metastasis in vivo [82]. Some studies have also provided important evidence that some cancer cell lines are more sensitive to silencing of SOCE components than their appropriate non-malignant cell line controls. Indeed, Motiani et al. showed that the silencing of STIM1 and Orai1 significantly reduces serum stimulated invasion of glioblastoma cells relative to normal human primary astrocytes [63].

Vascularization of tumoral tissues via induction of angiogenesis represents one of the original cancer hallmarks [73] and intracellular calcium signaling plays an important role in regulating this process [147–149]. Using live cell imaging Fiorio Pla et al. showed that migrating breast tumor endothelial cells, located at the wounded edge of a scratched cell monolayer, responded with a significantly higher Ca^{2+} influx with TRPV4 activation compared to non-migrating endothelial cells located away from the wound edge [77]. These studies suggest that migrating endothelial cells, which are important in tumor angiogenesis, undergo a remodeling of their calcium signaling and this is associated with increased responsiveness to TRPV4 activators. This remodeling may be due to dynamic changes in TRPV4 localization, since arachidonic acid (an effector in the angiogenic growth factor signaling pathway) caused redistribution of TRPV4 to the plasma membrane in endothelial cells, a function related to remodeling of the actin cytoskeleton. TRPV4 gene silencing abolished the migratory response of breast tumor endothelial cells to arachidonic acid [77], providing further evidence of a role for TRPV4 mediated Ca^{2+} influx in arachidonic acid stimulated breast tumor endothelial cell migration.

The ability of polarized cancer epithelial cells to transition to a mesenchymal and migratory state via epithelial–mesenchymal transition (EMT), a process thought to play a role in cancer metastasis [150–152], has recently been linked to calcium signaling [153]. Davis et al. demonstrated that mechanical wounding of an MDA-MB-468 breast cancer epithelial cell monolayer, a process shown to induce expression of the EMT marker vimentin in other tumorigenic and non-tumorigenic breast epithelial cell lines [154,155], resulted in the propagation of a calcium wave from the site of wounding [153]. In addition, treatment of MDA-MB-468 breast cancer cells with exogenous epidermal growth factor (EGF) (another inducer of EMT in this model) also resulted in increases in cytosolic free Ca^{2+}. Although the buffering of intracellular Ca^{2+} inhibited EMT induction by EGF, the nature of the calcium signal was critical to the ability of agents to induce EMT, and the Ca^{2+} permeable ion channel TRPM7 was identified as a key regulator of EGF-induced EMT in MDA-MB-468 breast cancer cells [153]. Further remodeling of the calcium signal was also demonstrated in this model of EMT, whereby cells induced to undergo EMT exhibited decreased Ca^{2+} influx

induced by the purinergic receptor agonist ATP and the protease activated receptor 2 activator trypsin, as well as reduced SOCE [156]. In addition, cells in the more invasive mesenchymal state displayed significantly faster recovery of $[Ca^{2+}]_i$ after ATP stimulation [157]. Hence, EMT at least in this breast cancer cell line model is associated with a remodeling of the calcium signal [156]. A different remodeling of Ca^{2+} signaling as a consequence of EMT has been reported by Hu et al., who used TGFβ to induce EMT in MCF-7 breast cancer cells, where EMT was associated with an increase in SOCE [158]. Further studies in these and other models of changes implicated in metastasis are required to fully understand how Ca^{2+} signaling may be altered in cells undergoing changes associated with the acquisition of a more invasive phenotype.

5. Novel methods for studying changes in the nature of the calcium signal in cancer

While valuable in contributing to our understanding of the remodeling of the nature of the calcium signal in tumorigenesis, findings from studies such as those presented above can suffer limitations by virtue of the low throughput nature of the methods used. Methods often restrict the number of tumorigenic and normal (or non-tumorigenic) cell lines that can be compared. This may be particularly important in research using breast cancer cell lines, which are commonly classified according to molecular subtype [159–162] and represent an example where the use of multiple controls, both tumorigenic and non-tumorigenic, is often desirable in order to represent the heterogeneity seen in breast cancer [163–165]. This limitation can be partially addressed through use of high throughput devices, such as the fluorometric imaging plate reader (FLIPR®, Molecular Devices), which could enable the simultaneous analysis of multiple cell lines in microplates of 96, 384 or 1536 wells [166]. Although such instrumentation has been used to assess Ca^{2+} signals in breast cancer cells [67,68,153,156,157], their potential in fully characterizing many tumorigenic and non-tumorigenic cell lines has not been fully utilized.

Development of the Ca^{2+} sensitive fluorescent dyes (e.g. Fura-2 and Fluo-4) has been integral to our current understanding of intracellular calcium signaling in both physiology and pathophysiology. However, as discussed in Section 2.1, there is an increasing interest in the remodeling of the calcium signal as a consequence of the interaction between tumor cells and cells of their microenvironment. The assessment of calcium signaling in vivo using advanced imaging techniques such as multi-photon microscopy is increasing, and enables investigation of calcium signaling in the context of the cellular microenvironment [56,167–169]. While the development of the acetoxymethyl ester forms of the fluorescent dyes (e.g. Fura-2/AM), has provided a relatively non-invasive means of measuring intracellular calcium signaling in living cells [170], their ability to become sequestered into Ca^{2+} containing intracellular organelles and/or extruded by multidrug transporters, including MDR1 (also known as P-glycoprotein) [171–173] (up-regulated in many cancer cells [174]), can limit their utility in long term studies of calcium signaling in vivo. The range of genetically encoded Ca^{2+} indicators now available (reviewed in [175–177]), which can be stably transfected into living cells, allows the measurement of calcium events over extended periods [176]. In vivo Ca^{2+} imaging has provided enormous insight into the role of calcium signaling in living systems (reviewed in [178]). While this method is predominately currently used to study calcium signaling in neuronal networks [179–181], the potential exists for these tools to provide insight into the remodeling of the calcium signal during tumorigenic processes such as invasion and metastasis in vivo.

6. Conclusion

While expressional changes in proteins responsible for regulating intracellular calcium signaling, such as channels, pumps and exchangers, have been characterized in various cancer cell lines and tissues, specific alterations in the *nature* of the calcium signal as a cause and/or consequence of tumorigenesis are less well described. Remodeling of the calcium signal is a feature of various disease states, and has been well characterized in models of vascular injury and disease. The important role of calcium signaling in proliferation, cell death, and invasion and metastasis, represents multiple opportunities for targeting altered calcium signaling during the course of tumorigenesis. Models enabling comparison of the calcium signal between tumorigenic and non-tumorigenic phenotypes combined with the use of high throughput and/or advanced Ca^{2+} imaging methodologies may further aid in the translation of altered calcium signaling assessment into the development of targeted cancer therapeutics.

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