The Calcium-Signaling Toolkit in Cancer: Remodeling and Targeting

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Processes that are important in cancer progression, such as sustained cell growth, invasion to other organs, and resistance to cell death inducers, have a clear overlap with pathways regulated by Ca^{2+} signaling. It is therefore not surprising that proteins important in Ca^{2+} signaling, sometimes referred to as the “ Ca^{2+} signaling toolkit,” can contribute to cancer cell proliferation and invasiveness, and the ability of agents to induce cancer cell death. Ca^{2+} signaling is also critical in other aspects of cancer progression, including events in the tumor microenvironment and processes involved in the acquisition of resistance to anti-cancer therapies. This review will consider the role of Ca^{2+} signaling in tumor progression and highlight areas in which a better understanding of the interplay between the Ca^{2+} -signaling toolkit and tumorigenesis is still required.

The calcium ion (Ca^{2+}) is a crucial regulator of a plethora of diverse cellular processes. These Ca^{2+} signal-sensitive processes control events as distinct as muscle contraction to gene transcription, and hormone release to neurotransmission (Carafoli 2002). As coined by Bootman et al. (2001), a “toolkit” of different Ca^{2+} channels, pumps, and exchangers regulate cellular Ca^{2+} levels in compartments (Fig. 1), enabling precise control of these different processes. Although beyond the scope of this review to provide a complete overview of Ca^{2+} signaling, readers are encouraged to consult comprehensive reviews (Bootman et al. 2001; Carafoli 2002; Monteith et al. 2017). Briefly, a variety of cell membrane hormone/growth factor receptors can

induce increases in cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{CYT}}$) by generating inositol 1,4,5 trisphosphate (IP_3) and releasing Ca^{2+} from internal endoplasmic reticulum Ca^{2+} stores via the opening of IP_3 -activated Ca^{2+} channels (Fig. 1A). Refilling these stores after depletion occurs by a specific Ca^{2+} influx pathway referred to as store-operated Ca^{2+} entry (SOCE). SOCE involves Ca^{2+} influx via ORAI1 Ca^{2+} channels after activation by the endoplasmic reticulum Ca^{2+} store sensor stromal interaction molecule (STIM) 1 (Fig. 1A). Other Ca^{2+} influx pathways include those activated by changes in membrane potential (e.g., L-type and T-type voltage-gated Ca^{2+} channels) and external stimuli such as temperature (e.g., transient receptor potential [TRP] channels)

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Figure 1. An overview of Ca²⁺ signaling pathways and toolkit members. (A) Activation of plasmalemmal receptors including G-protein-coupled receptors (GPCRs) can initiate the release of internal Ca²⁺ stores from the endoplasmic reticulum (ER) through the formation of secondary messenger IP₃. The reduction in ER Ca²⁺ levels is sensed by STIM1, which subsequently activates ORAI1 channels to permit Ca²⁺ influx and ER refilling via the ER Ca²⁺ pump sarco/endoplasmic Ca²⁺ ATPases (SERCA) (Trebak and Putney 2017). Collectively, this process constitutes store-operated Ca²⁺ entry (SOCE). (B) In addition to internally activated pathways, Ca²⁺ influx can be initiated by external stimuli acting on a plethora of Ca²⁺ toolkit members (Berridge et al. 2003). Examples include voltage-gated calcium channels, of which L-type and T-type channels are subtypes, stretch-activated PIEZO channels, and the transient receptor potential (TRP) superfamily of ion channels, sensitive to a range of stimuli, including warm (TRPV4) and cool (TRPM8) temperatures (Berridge et al. 2003; Pedersen et al. 2005; Martinac and Poole 2018). (C) Cytosolic Ca²⁺ levels are tightly controlled through the orchestrated work of pumps and exchangers. Ca²⁺ toolkit members SERCA and secretory pathway Ca²⁺ ATPases (SPCAs) sequester Ca²⁺ within internal stores of the ER and Golgi, respectively, whereas plasma membrane bound toolkit members plasma membrane Ca²⁺-ATPases (PMCAs) and Na⁺/Ca²⁺ exchanger expel Ca²⁺ through active or Na⁺ gradient-dependent means. Mitochondrial Ca²⁺ influx and efflux occur via the mitochondrial calcium Ca²⁺ uniporter (MCU) and the mitochondrial Na⁺/Ca²⁺ exchanger (NCLX), respectively (Berridge et al. 2003; Rizzuto et al. 2012).

and stretch (e.g., PIEZO channels) (Fig. 1B; Berridge et al. 2003; Pedersen et al. 2005). Other members of the Ca²⁺ tool kit include Ca²⁺ pumps that actively expel Ca²⁺ across the plasma membrane (plasma membrane Ca²⁺-ATPases [PMCAs] and Na⁺/Ca²⁺ exchangers [NCX]) (Fig. 1C). The toolkit also includes members that sequester Ca²⁺ into the endoplasmic reticulum (sarco/endoplasmic Ca²⁺ ATPases [SERCAs]) or the Golgi (secretory pathway Ca²⁺ ATPases [SPCAs]), or can regulate Ca²⁺ levels in intracellular organelles such as those recently identified in the mitochondria (Fig. 1C).

Distinct cellular outcomes as a result of Ca²⁺ signaling are achieved by differential regulation of the magnitude of [Ca²⁺]_{CYT} increases, as well as the frequency or localization of Ca²⁺ increases (Berridge 1997; Kar and Parekh 2015). As will be evident throughout this review, there are cases in which the expression of members of this toolkit

are altered in cancer, and, in some cases, this altered expression is manifested predominantly in a specific cancer subtype.

Studies in both cancer and noncancer cells have linked Ca²⁺ signaling with key events relevant to tumor progression such as proliferation, migration, and apoptosis. Although cardiovascular and neurological diseases were the initial focus of Ca²⁺ signaling in disease, the connections between Ca²⁺ signaling and processes important in the hallmarks of cancer (Hanahan and Weinberg 2000, 2011) have resulted in Ca²⁺ signaling being studied in cancer. Although studies initially examined in vitro cancer cell processes, development of the field has identified roles for Ca²⁺ toolkit members in the growth and metastasis of cancer cells in vivo. Even the acquisition of resistance to some cancer therapies is linked to specific Ca²⁺-permeable ion channels. As cancer research turns toward the

tumor microenvironment as an important mediator in cancer progression, recent studies in Ca^{2+} signaling have begun to define a role for Ca^{2+} in the tumor microenvironment (Weaver et al. 1996; Kenny et al. 2007; Bissell and Hines 2011; Boudreau et al. 2012; Mao et al. 2013). Collectively, this work has led to many researchers proposing that components of the Ca^{2+} signaling toolkit may represent new therapeutic targets in cancer therapy (Monteith et al. 2007; Dubois et al. 2013). Although it is not possible to provide a comprehensive evaluation of the entire field in this short review, we have aimed to provide an overview of what we consider to be some of the key areas and issues. The topics we discuss include examples and mechanisms of changes in the expression of Ca^{2+} signal regulators in some cancers, and the importance of the Ca^{2+} signal in cells of the primary tumor and the tumor microenvironment. We also seek to provide a snapshot of issues related to the targeting of members of the Ca^{2+} signaling toolkit in cancer therapy.

REMODELING OF THE CALCIUM-SIGNALING TOOLKIT IN CANCER CELLS

Studies have identified particular Ca^{2+} -permeable ion channels or Ca^{2+} pumps that are expressed at higher levels in cancers compared with normal surrounding tissue. TRPM8, a Ca^{2+} -permeable ion channel, which is now mostly studied for its role in the detection of lower temperatures in sensory neurons, was actually first reported in a study identifying proteins overexpressed in prostate cancers (Tsavaler et al. 2001). Elevated TRPM8 expression occurs in many malignancies, including those of the breast, lung, and colon (Tsavaler et al. 2001), although TRPM8 appears to be lost in some cancer cell lines (Yapa et al. 2018). The remodeling of expression of specific members of the Ca^{2+} signaling toolkit in cancer has been extensively reviewed (Monteith et al. 2007, 2012; Lee et al. 2011; Lastraioli et al. 2015; Shapovalov et al. 2016). Similarly to TRPM8, there are Ca^{2+} channels and pumps that are overexpressed in more than one malignancy, such as TRPV6, which is overexpressed in several cancers including prostate and breast (Zhuang et al. 2002; Fixemer et al.

2003; Bolanz et al. 2008). Indeed, a TRPV6 inhibitor, SOR-C13, underwent a phase I clinical trial in patients with advanced solid cancers, including colorectal, prostate, and breast (Fu et al. 2017), and has U.S. Food and Drug Administration approval as an orphan drug for pancreatic and ovarian cancers. In other cases, alterations may be more predominant in a particular cancer as exemplified by the Ca^{2+} -permeable ion channel TRPC6, in which overexpression occurs in breast and prostate cancers, but with lower expression in colorectal cancers (Guilbert et al. 2008; Yue et al. 2009; Sozucan et al. 2015). Examples also exist in which the overexpression of a particular Ca^{2+} channel or pump is specific to a cancer subtype. The expression of the aforementioned highly Ca^{2+} -permeable TRPV6 is correlated with estrogen receptor levels in breast cancer. Patients with estrogen receptor-positive breast cancers can be treated with hormone therapies, including estrogen receptor blockers or aromatase inhibitors. Estrogen receptor-negative breast cancers are intrinsically resistant to these hormone therapies, limiting the therapeutic options available for these patients. Considering this, the correlation of increased TRPV6 expression in estrogen receptor-negative breast cancers, associated with an increase in TRPV6 gene copy number, is a significant finding (Peters et al. 2012). Although TRPV6 expression is associated with breast cancer hormone receptor expression, some members of the Ca^{2+} signaling toolkit are linked to specific molecular subtypes, where cancers are classified by their expression of groups of genes. For TRPV4, expression is elevated in breast cancers of the basal molecular subtype compared with subtypes known as luminal A, luminal B, and HER2 breast cancers (Peters et al. 2017). In the case of MDA-MB-468 basal breast cancer cells, the degree of TRPV4 overexpression is sufficient for the cell line to be highly sensitive to cell death initiated by the TRPV4 activator GSK1016790A (Peters et al. 2017).

Functional consequences can arise from remodeling of the Ca^{2+} signaling toolkit in cancer, potentially impacting on the nature of Ca^{2+} signaling in cancers. This is evident with the Ca^{2+} channel ORAI3. In contrast to the canonical

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SOCE channel, ORAI1, few studies have identified endogenous roles for ORAI3 in SOCE. In estrogen receptor–positive breast cancer cells, but not in estrogen receptor–negative breast cancer cells; however, ORAI3 expression levels are increased (Motiani et al. 2010). Assessment of clinical samples shows higher levels of ORAI3 in luminal breast cancer subtypes compared with the basal molecular subtype, which overlap with estrogen receptor–positive and estrogen receptor–negative breast cancer subtypes, respectively (Azimi et al. 2019). Unlike in most other cell types, and in contrast to the estrogen receptor–negative breast cancer cell lines tested, the overexpression of ORAI3 in estrogen receptor–positive breast cancer cells is associated with ORAI3 also contributing along with ORAI1 to SOCE (Motiani et al. 2010). This suggests that the remodeling of the Ca^{2+} signaling toolkit in cancer can involve a switch, not just in toolkit component expression, but also the nature of their contribution to Ca^{2+} transport or homeostasis. Another example of this switch is evident in prostate cancer cells. Prostate cancer cells also have elevated expressions of ORAI3 (Dubois et al. 2014), and in this context high levels of ORAI3 protein appear to promote formation of a heteromeric ORAI1/3 channel, which is more responsive to pro-proliferative signals such as arachidonic acid and a cellular phenotype with less SOCE (Dubois et al. 2014). This reduction in SOCE bestows a reduced sensitivity to some apoptotic stimuli that normally induce cell death through increases in Ca^{2+} influx (Dubois et al. 2014). Another aspect of remodeling of Ca^{2+} signaling in cancer could involve changes in the relationship between key players in Ca^{2+} homeostasis and Ca^{2+} -dependent processes, such as the interface between mitochondria and the endoplasmic reticulum, as has been recently reviewed (Kerkhofs et al. 2017).

Changes in Ca^{2+} signaling can also be consequential to gene mutations. Genetic mutations are a cancer hallmark and occur in proto-oncogenes or tumor suppressor genes acting as drivers for cellular transformation. Indeed, as reviewed elsewhere, there are important links between many oncogenes, tumor suppressors, and Ca^{2+} signaling (Marchi et al. 2014; Bittre-

mieux et al. 2016). The RAS family of genes are proto-oncogenes that are frequently mutated in human cancers (Prior et al. 2012). RAS proteins are GTPases with a crucial role in proliferation pathways (Prior et al. 2012). Mutations in the K-RAS isoform (specifically G13D) drive changes in members of the Ca^{2+} signaling toolkit, with effects on Ca^{2+} release from the endoplasmic reticulum and on SOCE. K-RAS mutations alter the suite of endoplasmic reticulum Ca^{2+} regulators expressed in colorectal cancer cells, including the expression of Ca^{2+} release channel inositol 1,4,5-trisphosphate receptor (IP_3R) isoforms. Accompanying these expression changes is a suppression of IP_3 -dependent Ca^{2+} release (Pierro et al. 2014). A colorectal cancer cell line where the oncogenic K-RAS G13D mutation was deleted had increased IP_3 -mediated Ca^{2+} release. Moreover, silencing of K-RAS phenocopied this effect in the isogenic colorectal cancer cell line with the K-RAS G13D mutation (HCT116) (Pierro et al. 2014). Many growth factors drive proliferation through IP_3 -dependent Ca^{2+} release; hence, suppression of IP_3 -mediated Ca^{2+} release in K-RAS-mutated cancer may seem counterintuitive. However, mutated K-RAS remodeling of IP_3 -dependent Ca^{2+} release bestows reduced sensitivity to apoptotic stimuli via alteration of mitochondrial Ca^{2+} levels (Pierro et al. 2014). K-RAS mutations in colorectal cancer also alter SOCE and the relative expression of the SOCE regulators STIM1 and STIM2 (Pierro et al. 2018). The ability of the phosphorylation state of K-RAS to regulate Bcl-xl-mediated regulation of IP_3 receptors adds another dimension to the intersection between K-RAS, cell survival, and Ca^{2+} signaling (Sung et al. 2013). PTEN is another gene that is frequently mutated in cancer (Milella et al. 2015). Loss of PTEN function drives changes in Ca^{2+} signaling via IP_3Rs by increasing FBXL2-dependent degradation of $\text{IP}_3\text{R3}$ (Kuchay et al. 2017). The tumor suppressor BRCA1-associated protein 1 (BAP1) is also a regulator of $\text{IP}_3\text{R3}$ stability and its loss reduces $\text{IP}_3\text{R3}$ levels and Ca^{2+} release, which may be important in bestowing apoptosis insensitivity to a variety of genomic stress inducers (Bononi et al. 2017). Although the above examples illus-

trate that genes commonly mutated in cancer can affect Ca^{2+} signaling, there are clear future opportunities to define and assess gene mutations in members of the Ca^{2+} signaling toolkit and their consequences. Indeed, the recent identification of rare ORAI1 mutations in some cancers, and the association of some of these mutations with enhanced ORAI1 constitutive channel activity (Frischauf et al. 2017), highlights potential significance and the need for further studies.

With disease progression, there can also be a remodeling of Ca^{2+} channels, pumps, or signaling. Epithelial-mesenchymal transition (EMT) is a cancer progression mechanism whereby epithelial cancer cells adopt a phenotype that has less epithelial markers but is rich in mesenchymal markers, and become less proliferative with an up-regulation of pathways associated with metastasis and therapeutic resistance (Ye and Weinberg 2015; Zhang and Weinberg 2018). EMT modulators have been proposed as potential therapeutic agents (Davis et al. 2014b). In both hypoxia and epidermal growth-factor-induced EMTs in MDA-MB-468 breast cancer cells, there is reduced sensitivity to the $[\text{Ca}^{2+}]_{\text{CYT}}$ -increasing effects of ATP (Davis et al. 2011; Azimi et al. 2016). Indeed, changes in Ca^{2+} signaling and/or expression of members of the Ca^{2+} signaling toolkit occur as a consequence of EMT (Davis et al. 2011, 2012, 2013; Mahdi et al. 2015; Qi et al. 2016; Wu et al. 2017; Gershkovitz et al. 2018b). Hence, a variety of factors may contribute to altered Ca^{2+} signaling and/or expression of specific Ca^{2+} channels and pumps in cancer cells, including increased gene copy number, altered transcription pathways, and microenvironmental factors such as growth factors and hypoxia.

CANCER CELLS AND THE CALCIUM SIGNAL

The intersection between events within the cancer cell that are important in tumor progression and Ca^{2+} signaling have been extensively reviewed (Roderick and Cook 2008; Prevarskaya et al. 2011; Azimi et al. 2014; Stewart et al. 2015; Monteith et al. 2017). Examples include the roles of specific Ca^{2+} signals during cell cycle

progression (Roderick and Cook 2008), the importance of Ca^{2+} signals during cancer cell migration and release of matrix metalloproteinase (MMP) during cancer cell invasion (Prevarskaya et al. 2011), and the roles of Ca^{2+} signals in cell death pathways (Danese et al. 2017; Ivanova et al. 2017). The expansive involvement of Ca^{2+} toolkit members in the progression of a single type of malignancy, breast cancer, is summarized in Figure 2. Given the scope of these phenomena and their discussions in other recent reviews, here we will highlight some selected specific examples that clearly show the significance of Ca^{2+} signals in cancer cell processes important in tumor progression. Studies using in vivo models, in particular those involving silencing of a specific Ca^{2+} channel or pump have been prioritized for discussion, and where possible, more recent examples from different cancer types and members of the Ca^{2+} toolkit are presented.

Proliferation

Ca^{2+} signaling has an important role in the proliferation of different cancer cell lines. In cervical cancer, STIM1 expression is elevated compared with adjacent nonneoplastic epithelia, is positively correlated with tumor size, and is higher in patients with lymph node metastasis (Chen et al. 2011). STIM1 silencing significantly reduces cell cycle progression of the cervical cancer cell line SiHa with arrest in the S and G₂/M phases and an increase in p21 protein levels (Chen et al. 2011). STIM1 silencing also reduces tumor weight in cervical cancer in vivo models. Conversely, the ability of STIM1 overexpression to increase tumor weight places STIM1 as a driver of cervical cancer cell growth in vivo (Chen et al. 2011). However, this effect may not be solely caused by the aforementioned cell cycle effects, because STIM1 promotes the secretion of the proangiogenic factor VEGF-A and stimulates cervical cancer angiogenesis (Chen et al. 2011). Using a small cohort of glioma brain cancer samples, TRPC6 expression was shown to be elevated at the protein and messenger RNA (mRNA) levels compared with normal control brain tissue controls, with

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Figure 2. Examples of the diversity and multitude of Ca²⁺ toolkit members implicated in four aspects of breast cancer progression: proliferation, metastasis, cell death and resistance, and the tumor microenvironment. Note that not all targets have been assessed in vivo or using pharmacological agents. TRPA1 (Takahashi et al. 2018), TRPC1 (El Hiani et al. 2009; Faouzi et al. 2016; Schaar et al. 2016), TRPC3 (Zhang et al. 2012), TRPC5 (Ma et al. 2012, 2014; Zhu et al. 2015), TRPC6 (Aydar et al. 2009), TRPM2 (Hopkins et al. 2015; Koh et al. 2015; Gershkovitz et al. 2018a,b,c), TRPM7 (Guilbert et al. 2009; Kim 2012; Davis et al. 2014a), TRPV1 (Weber et al. 2016), TRPV4 (Fiorio Pla et al. 2012; Lee et al. 2016; Peters et al. 2017), TRPV6 (Bolz et al. 2008; Peters et al. 2012), ORAI1 (Yang et al. 2009; Feng et al. 2010; Badaoui et al. 2018; Liu et al. 2018b), ORAI3 (Faouzi et al. 2013; Motiani et al. 2013; Hasna et al. 2018), STIM1 (Yang et al. 2009, 2018), MCU (Curry et al. 2013; Tosatto et al. 2016), IP₃R3 (Szatkowski et al. 2010; Mound et al. 2017), Ca_v3.1 (Ohkubo and Yamazaki 2012), PMCA2 (VanHouten et al. 2010; Curry et al. 2016; Peters et al. 2016), PMCA4 (Curry et al. 2012), PIEZO1 (Li et al. 2015), and PIEZO2 (Pardo-Pastor et al. 2018).

TRPC6 protein expression greater in higher grade gliomas (Ding et al. 2010). TRPC6 shRNA-mediated silencing in glioma cell lines attenuates platelet-derived growth factor-mediated increases in [Ca²⁺]_{CYT}, inhibits colony formation in vitro, and produces cell cycle arrest at the G₂/M phase of the cell cycle (Ding et al. 2010). An association between TRPC6 and glioma growth also occurs in both subcutaneous and intracranial xenograft models where suppression of TRPC6 expression, using a dominant-negative construct, attenuated growth in vitro (Ding et al. 2010).

In prostate cancer, mRNA levels of the human analogue of a voltage-gated Ca²⁺ channel

subunit gene (Tedeschi et al. 2016), CACNA2D2, are elevated and its silencing reduces the proliferation and percentage of LNCaP cells in the G₂/M and S phases (Warnier et al. 2015). Moreover, exogenous overexpression of CACNA2D2 significantly increases the size of LNCaP tumors in vivo (Warnier et al. 2015). Of significance in Ca²⁺ signaling research, and particularly important in the context of cancer, silencing or inhibiting a Ca²⁺-permeable ion channel expressed in a cancer cell line does not necessarily produce antiproliferative effects (Monteith et al. 2007); our own, and other groups, have observed many cases in which the silencing of a specific Ca²⁺ channel has no effect on cancer cell prolifer-

eration. There are even cases in which a Ca^{2+} -permeable ion channel component may actually have a tumor-suppressive role. Indeed, the voltage-gated Ca^{2+} channel subunit encoded by the *CACNA2D3* gene is such an example. Overexpression of *CACNA2D3* reduces the proliferation of glioma cell lines in vitro and in vivo (Jin et al. 2017), the channel has reduced expression in clinical samples of gliomas compared with normal controls, and low expression of this channel is correlated with poorer patient prognosis than high expression (Jin et al. 2017). Diverse tumor-suppressing/-promoting roles of specific Ca^{2+} channels maybe because of subtle differences in the remodeling of the Ca^{2+} signal. In this context, in gastric cancer, higher *CACNA1G* expression is associated with better overall survival, but the expression of two other T-type Ca^{2+} channels (*CACNA1H* and *CACNA1I*) is associated with poorer prognosis (Forzano et al. 2017).

Metastasis

Although inherently more difficult to study in vivo compared with tumor growth, numerous studies have defined roles for Ca^{2+} signaling toolkit members in metastatic progression; the ultimate cause of mortality in most cancers. Many studies have linked the molecular components of SOCE with processes important in cancer cell metastasis, such as migration and invasion (Chen et al. 2011; Hammadi et al. 2012; Yang et al. 2013; Kim et al. 2014; Umemura et al. 2014; Xia et al. 2016; Diez-Bello et al. 2017; Gueguinou et al. 2017). ORAI1 and STIM1 were first linked with metastasis in breast cancer in which ORAI1 and STIM1 silencing in MDA-MB-231 breast cancer cells reduced invasiveness in vitro and metastasis in vivo (Yang et al. 2009). Mechanistically, in vitro studies implicate a change in focal adhesion protein turnover as a consequence of suppression of SOCE by ORAI1 and STIM1 silencing, resulting in the suppression of the motility and invasiveness capacity of cancer cells (Yang et al. 2009). L-type voltage-gated Ca^{2+} channels were recently associated with collective migration of squamous cell carcinoma cells (Grasset et al. 2018). At high con-

centrations, diltiazem and verapamil, clinically used L-type Ca^{2+} channel blockers, significantly reduce the invasive properties of patient-derived spheroid cultures, as well as invasion of squamous cell carcinoma patient-derived xenografts in an in vivo mouse model (Grasset et al. 2018). Although gene silencing experiments are still required to confirm that the L-type voltage-gated channel responsible for these effects is the proposed $\text{Ca}_v1.1$ subtype, this study is an exemplar for the use of more diverse in vivo models to supplement the commonly used cell line xenograft and tail injection metastasis models.

The importance of Ca^{2+} signaling in metastasis is not confined to mediators of Ca^{2+} influx. Despite the relatively recent molecular identification of the mitochondrial Ca^{2+} uniporter (MCU), studies have already identified its important role in breast cancer and hepatocellular carcinoma metastasis. The MCU, which is a gateway for Ca^{2+} uptake into the mitochondria, is an obvious controller of cellular metabolism, and in breast cancer cells it is a regulator of mitochondrial relative oxygen species and hypoxia-inducible factor 1 α (Tosatto et al. 2016). These effects and others may explain the ability of MCU silencing to reduce the migration of MDA-MB-231 breast cancer cells in vitro as well as their metastatic progression in vivo as assessed by lymph node and lung metastasis size (Tosatto et al. 2016). In addition to high-MCU expression being associated with poor survival in hepatocellular carcinoma patients, silencing of MCU reduces the migration and invasiveness of SMMC7721 hepatocellular carcinoma cells in vitro and lung metastasis formation in vivo (Ren et al. 2017). Data from the same study assessing the consequences of MCU overexpression, suggest that elevated MCU expression in some hepatocellular carcinomas promotes migratory and invasive pathways via elevated ROS production, subsequent promotion of JNK phosphorylation, and activation of MMP-2, focal adhesion turnover and lamellipodia formation (Ren et al. 2017). Melanoma cells with a mutation in the proto-oncogene *BRAF* appear to have a specific association with the plasma membrane Ca^{2+} pump alternative splice variant PMCA4b. The expression of PMCA4b is

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induced in BRAF-mutated melanoma cell lines after treatment with the clinically used mutant BRAF inhibitor vemurafenib (Hegedüs et al. 2017). Induced overexpression of PMCA4b in BRAF-mutated A375 melanoma cells significantly reduces their migration in vitro and the formation of lung metastasis in a tail-vein injection model (Hegedüs et al. 2017). As well as links to cancer cell migration and invasion, Ca^{2+} signaling is an important regulator of the ability of some cancer cells to transition to a more invasive phenotype through EMT (Davis et al. 2014a). Targeting specific Ca^{2+} -permeable ion channels or modifying cellular Ca^{2+} signals are approaches that can be used to regulate EMT in various cancer cell lines (Davis et al. 2014a; Liu et al. 2014; Zhang et al. 2017; Zhu et al. 2017).

Cancer Cell Death

Given the intrinsic link between increases in $[\text{Ca}^{2+}]_{\text{CYT}}$ and/or mitochondrial Ca^{2+} levels and cell death (Rizzuto et al. 2003; Giacomello et al. 2007; Zhivotovsky and Orrenius 2011; Dubois et al. 2013; La Rovere et al. 2016; Bultynck and Parys 2018; Parys and Bultynck 2018), it is not surprising that members of the Ca^{2+} signaling toolkit have been investigated as potential ways to promote the death of cancer cells. The most direct evidence for the ability of the Ca^{2+} signal to induce cancer cell death and its potential clinical application is seen with Ca^{2+} electroporation. This method involves the application of high-voltage pulses after injection of a Ca^{2+} solution to a tumor region, which produces an overload of intracellular Ca^{2+} and cancer cell death (Frandsen et al. 2012). The process is effective in clinical trials (Falk et al. 2018) and offers potentially less damage to normal surrounding tissue than coadministration with agents such as bleomycin, because many cancer types are more sensitive to Ca^{2+} electroporation, perhaps because of compromised Ca^{2+} efflux pathways (Frandsen et al. 2017). An analogous phenomenon is seen with pharmacological Ca^{2+} channel activators producing death in cancer cells that overexpress their Ca^{2+} channel target (Zhang and Barritt 2004; Akbulut et al. 2015; Peters et al. 2017). The selective TRPV4 activator

GSK1016790A induces cell death in MDA-MB-468 breast cancer cells overexpressing this Ca^{2+} -permeable ion channel. This death is via two mechanisms, oncosis and apoptosis (Peters et al. 2017), with oncosis associated with close to maximal activation of TRPV4-mediated ion influx, and apoptosis associated with lower concentrations of GSK1016790A (Peters et al. 2017). The association between excessive $[\text{Ca}^{2+}]_{\text{CYT}}$ accumulation and cell death is also seen following PMCA1 silencing in MDA-MB-231 breast cancer cells, where compromised Ca^{2+} efflux results in greater sustained levels of global $[\text{Ca}^{2+}]_{\text{CYT}}$ and increased sensitivity to necrosis induced by the Ca^{2+} ionophore ionomycin (Curry et al. 2012).

Although the aforementioned studies provide clear examples of the ability of excessive global $[\text{Ca}^{2+}]_{\text{CYT}}$ to induce cancer cell death and how this might be exploited, there are examples of more nuanced contributions of members of the Ca^{2+} signaling toolkit to cancer cell death. One example is seen in the diverse effects of PMCA isoforms (Ca^{2+} efflux pumps) in breast cancer cell death. MDA-MB-231 breast cancer cells express PMCA1, PMCA2, and PMCA4, but only PMCA1 has an appreciable ability to regulate acute changes in global $[\text{Ca}^{2+}]_{\text{CYT}}$ (Curry et al. 2012, 2016). However, silencing of PMCA4 or PMCA2 promotes the sensitivity of this breast cancer cell line to the proapoptotic effects of the Bcl-2 inhibitor navitoclax (Curry et al. 2012, 2016). Under some circumstances, cancer cells may be uniquely sensitive to targeting specific aspects of the Ca^{2+} signaling toolkit, as evidenced by the ability of disruption of endoplasmic reticulum to mitochondrial Ca^{2+} transfer to induce death in cancer cell lines, rather than autophagy as seen in nonmalignant cells (Cárdenas et al. 2010, 2016). Silencing of IP₃R1 and IP₃R3 or MCU disrupts endoplasmic reticulum to mitochondrial Ca^{2+} transfer and induces cell death in transformed but not normal human fibroblasts (Cárdenas et al. 2016). Indeed, pharmacological inhibition and silencing of IP₃Rs promotes autophagic cell death in MCF-7 breast cancer cells (Singh et al. 2017). There are also examples in which current agents used to treat cancer or those undergoing clinical

trials act, at least in part, through Ca^{2+} signaling to induce cancer cell death. This has been extensively reviewed elsewhere (Bonora et al. 2015; Bong and Monteith 2018; Kerkhofs et al. 2018). One specific example of how Ca^{2+} signaling and therapy intersect is seen in studies assessing the tumor suppressor p53 (Giorgi et al. 2015). Levels of Ca^{2+} in the endoplasmic reticulum are higher in cells with p53, as p53 is an activator of the pump that transports Ca^{2+} into this store (SERCA). Loss of p53 therefore reduces the amount of Ca^{2+} that is available to be transferred from the endoplasmic reticulum to the mitochondria, which is a requirement for apoptosis induced by some chemotherapeutic agents (Giorgi et al. 2015).

THE TUMOR MICROENVIRONMENT AND THE CALCIUM SIGNAL

Although the initial studies of Ca^{2+} signaling in cancer focused quite reasonably on the cancer cells themselves, cancer research has shifted focus toward the contribution of the tumor microenvironment to tumor progression. Depending on the cell type or context, cells in the tumor microenvironment can be contributors to tumor progression or they may help contain or suppress tumor growth.

The tumor microenvironment often includes infiltrating immune cells that can assist the removal of tumor cells, or alternatively, contribute to tumor progression via the release of agents such as growth factors and cytokines (Hanahan and Weinberg 2011). Although the role of Ca^{2+} signaling in immune-cancer cell interactions is still an understudied area, there is clear evidence for the role of SOCE in antitumor immunity in mice. Stim1 and Stim2 knockdown produces a phenotype of reduced activity of CD8^+ T cells (cytotoxic lymphocytes), which are critical for suppressing the establishment and eventual growth of melanoma and colon cell carcinomas in mouse syngeneic allograft models (Weidinger et al. 2013). Although this suggests that agents that inhibit SOCE may always be detrimental in the context of tumor cell immunity, recent work suggests the area is complex, and that there may be an optimal level of

SOCE suppression whereby the activity of cytotoxic T lymphocyte and natural killer cells is actually increased with partial suppression of Orai1 (Zhou et al. 2018). Although studies with the SOCE pharmacological inhibitor BTP2 (Weidinger et al. 2013) did not show any such immune cell activation, studies with other SOCE inhibitors (such as Synta66 [Azimi et al. 2017, 2018]) now seem warranted to fully define this phenomena. The role of SOCE, and other forms of Ca^{2+} influx, in immune cell-mediated tumor-promoting inflammatory signaling and other pathways also requires attention. This is elegantly exemplified by the recent study of T-cell acute lymphoblastic leukemia (T-ALL). Mice lacking SOCE have reduced cancer-induced inflammation in organs with leukemia invasion in a NOTCH1-dependent model of T-ALL. Consequently, there is reduced necroinflammatory responses in leukemia-infiltrated organs and improved survival in mice with leukemic cells that lack SOCE (Saint Fleur-Lominy et al. 2018).

One of the most important requirements of the tumor microenvironment is the establishment and maintenance of a tumor vasculature to supply oxygen and nutrients for tumor growth. Disruption of the tumor vasculature is the mechanism underlying clinically used angiogenesis inhibitors. A Ca^{2+} -permeable ion channel linked to angiogenesis and the tumor vasculature is TRPV4. Although, whether inhibition or activation of TRPV4 is the best strategy for reducing angiogenesis, or whether either may be appropriate, is a matter of debate. TRPV4 expression is elevated in endothelial cells derived from breast cancers (Fiorio Pla et al. 2012). Pharmacological inhibition and knockdown of TRPV4 reduces arachidonic acid-induced promotion of migration in endothelial cells from breast cancers, implicating an important role for TRPV4 in mediating the effects of some proangiogenic stimuli (Fiorio Pla et al. 2012). Hence, inhibition of TRPV4 could be analogous to antiangiogenesis drugs used in cancer therapy. Conversely, direct pharmacological activation of TRPV4 partly restores normal vasculature in tumors in vivo, and through this mechanism, TRPV4 activation may help improve therapy effectiveness by improving the de-

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livery of cytotoxic therapy to the tumor mass as has been shown in vivo (Adapala et al. 2016). Clearly there will be other Ca^{2+} channels that play critical roles in angiogenesis. Indeed, ORAI1 is linked to the ability of hypoxia to promote colon cancer cells to activate angiogenesis. Silencing of ORAI1 in HCT-116 and SW480 colon cancer cells reduces the ability of conditioned media derived from these cells under hypoxic conditions to promote tube formation of a human endothelial cell line (HMEC-1), a tool to assess angiogenesis pathways (Liu et al. 2018a). ORAI1-mediated SOCE drives colon cancer cell promotion of angiogenesis by hypoxia via a NOTCH1-dependent mechanism that involves the Ca^{2+} -dependent transcription factor NFATc3 (Liu et al. 2018a). Another study highlighting the importance of studying other ion channels in angiogenesis is the work defining an important role for the stretch-activated Ca^{2+} channel Piezo2 in angiogenesis in a variety of models (Yang et al. 2016).

Fibroblasts and adipocytes are other components of the tumor microenvironment that can contribute to tumor progression (Bussard et al. 2016). Despite their clear importance in many cancer types, the contribution of these cells and many other stromal components to tumorigenesis has been the focus of very few studies. Ca^{2+} is likely to contribute to processes in carcinogenesis in these cell types. Highlighting why this is an area of potentially rich study for Ca^{2+} signaling researchers, a Ca^{2+} -dependent signaling pathway is implicated in cancer-associated fibroblast (CAF)-induced drug resistance in the ovarian cancer tumor microenvironment (Leung et al. 2018). Which Ca^{2+} pathway was not identified however, hence this is still an area for further study. Similarly, new work has defined a role for Ca^{2+} signaling in CAFs in a colon cancer cell spheroid model (Stadler et al. 2017), but again the exact member(s) of the Ca^{2+} signaling toolkit involved remains unclear. One member of the Ca^{2+} signaling toolkit that is linked to CAFs in prostate cancer is TRPA1. Prostate cancer-derived CAFs express and have functional TRPA1 channels. Moreover, studies with the TRPA1 inhibitor HC-030031 (at a relatively high concentration of 50 μM) in a CAF

and prostate cancer cell (LNCaP) coculture suggest a role for TRPA1 in regulating apoptotic sensitivity to resveratrol (Vancauwenbergh et al. 2017).

Adipocytes in serous ovarian cancer can activate salt-inducible kinase 2 (SIK2) in ovarian cancer cells to alter their metabolism and promote proliferation and survival (Miranda et al. 2016). Coculture experiments and intracellular Ca^{2+} chelation using BAPTA-AM show the importance of the Ca^{2+} signal in adipocyte-mediated activation of SIK2, but again the exact Ca^{2+} channels or pumps involved have not been defined (Miranda et al. 2016). So, although specific members of the Ca^{2+} signaling toolkit are linked to the tumor microenvironment in the context of immune cell function and angiogenesis, more work is required to fully define other aspects of the tumor microenvironment and their relationship with Ca^{2+} signaling.

TARGETING THE CALCIUM-SIGNALING TOOLKIT

Given the involvement of the Ca^{2+} signaling toolkit in key cancer processes, Ca^{2+} channels and pumps are proposed as drug targets for cancer therapeutics. In the discussion above, we highlighted examples in which silencing and/or pharmacological modulation of Ca^{2+} toolkit components inhibits proliferation or metastasis in vivo, or promotes cancer cell death. Figure 3 depicts a range of potential strategies for targeting the Ca^{2+} signal in cancer. Either pharmacological inhibition or pharmacological activation may be appropriate approaches, depending on whether the resultant alteration to $[\text{Ca}^{2+}]_{\text{CYT}}$ is prodeath or prosurvival, and in some cases this dichotomous approach may be possible for the same target. TRPV4 is such a target. As discussed above, not only has TRPV4 inhibition or activation both been proposed as ways to target the tumor vasculature (Fiorio Pla et al. 2012; Adapala et al. 2016), but TRPV4 overexpression in some cancer cells could also be targeted through inhibition or activation. Inhibition of TRPV4 expression reduces metastasis of breast cancer cells that have TRPV4 overexpression in vivo (Lee et al. 2016). There may be some cases

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Figure 3. Selected examples of the myriad of strategies to target the Ca^{2+} toolkit in cancer. Pharmacological modulation of overexpressed channels can achieve a reduction in tumor progression through (A) inhibition of Ca^{2+} channels to suppress proliferative or survival pathways, as exemplified by studies antagonizing overexpressed Ca^{2+} -permeable channels TRPV6 and TRPA1 using selective inhibitors SOR-C13 and AM-0902, respectively (Takahashi et al. 2018; Xue et al. 2018), or (B) activation of overexpressed Ca^{2+} -permeable channels, including TRPV4 and TRPC4/5 via GSK101670A and Englerin A, respectively, to induce cell death via multiple pathways (Akbulut et al. 2015; Peters et al. 2017). (C) Prodrug analogues of classic Ca^{2+} modulators use tumor activation to selectively target malignant cells. Prodrug mipsagargin cannot translocate the plasma membrane unless cleaved by prostate-specific membrane antigen (PSMA) enzyme into the membrane-permeable cytotoxic SERCA inhibitor thapsigargin. Expression of PSMA is low in normal tissues but up-regulated in some tumor microenvironments (Mahalingam et al. 2016). (D) Modification of Ca^{2+} channel interactions, as achieved by Bcl-2/IP $_3$ R Disruptor-2 (BIRD-2). This agent has cytotoxic capacity in cells reliant on these interactions for survival. High levels of Bcl-2 expression can induce pro-survival signaling following B-cell receptor (BCR) activation by suppressing intracellular Ca^{2+} release through IP $_3$ Rs. Abrogation of the Bcl-2 and IP $_3$ R interaction by BIRD-2 can induce proapoptotic Ca^{2+} signaling in some lymphoma and leukemia cells (Bittremieux et al. 2018). (E) Targeting Ca^{2+} signaling in the tumor microenvironment can enhance chemotherapeutic potential. Cancers have aberrant vasculature formation. Activation of TRPV4 via a selective activator GSK1016790A normalizes vascular maturation by actions on endothelial cells and enhances delivery of the chemotherapeutic cisplatin, improving the efficacy of this agent (Fiorio Pla et al. 2012; Adapala et al. 2016). Note that these examples are often in different cancer types.

in which TRPV4 expression is so high that its pharmacological activation may promote cell death and suppress tumor growth (Peters et al. 2017). Although the ideal target for the channel-activation approach would be one that does not contribute in any way to tumor progression (to

avoid any risk of promotion of metastatic or proliferative pathways), appropriate dosing, patient selection, and combination therapies may allow for such an approach. In summary, there are diverse ways to target members of the Ca^{2+} toolkit in cancer, and consideration will need to

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be made as to whether the target is in the cancer cell or cells of the tumor microenvironment, and/or whether an inhibitor or activator of the target is most appropriate.

How a member of the Ca^{2+} signaling toolkit is prioritized as a target for future therapy will depend on a variety of factors. This could include the risk of toxicity to normal cells, which may be predicted based on studies of pharmacological inhibitors, or when such agents are not available the phenotype of knockout mice. The degree of overexpression of the member of the Ca^{2+} signaling toolkit may also be a consideration. Pronounced overexpression may bestow increased sensitivity of cancer cells to an agent and allow a therapeutic window for future therapies that do not affect noncancer cells. Targets whose expression levels are related to prognosis and/or are relevant to cancer types in which therapeutic options are limited and clinical outcomes are still poor could also be prioritized. One key aspect as this field moves forward will be considerations related to combination therapies and what combinations based on mechanistic insight should be the focus of initial assessments. Given that an increasing number of studies have identified that agents currently used to treat cancer modify Ca^{2+} signaling, understanding the interactions on Ca^{2+} signaling will be important. Although this area is still relatively underexplored, examples include the ability of doxorubicin to induce changes in $[\text{Ca}^{2+}]_{\text{CYT}}$ in MDA-MB-231 breast cancer cells and changes in the levels of the Ca^{2+} channels TRPC1 and TRPC3 (Abdoul-Azize et al. 2018).

In addition to the basic concept of a direct pharmacological inhibitor or activator of a member of the Ca^{2+} signaling toolkit for cancer therapy, there may be other ways to exploit the Ca^{2+} signal in cancer therapy. Mipsagargin, a prodrug of the SERCA inhibitor thapsigargin, is converted to its active form by the prostate-specific membrane antigen enzyme, which is elevated in the tumor microenvironment of malignancies including nonprostate cancers (Mahalingam et al. 2016). Cytotoxic effects of SERCA inhibition are targeted to cancer sites because expression of the prostate-specific membrane antigen enzyme is low in normal tis-

sue (Mahalingam et al. 2016). Mipsagargin has undergone phase I clinical trials in patients with refractory and advanced solid tumors (Mahalingam et al. 2016) and it adds another tool to how the Ca^{2+} signaling toolkit may be targeted in cancer. Another example of how future therapies may go beyond direct pharmacological activation or inhibition of Ca^{2+} signals is seen in the effects of the peptide Bcl-2/IP₃R Disruptor-2 (BIRD-2). By disrupting Bcl-2 interactions with IP₃Rs, BIRD-2 can convert constitutive IP₃R Ca^{2+} release in some lymphoma and leukemia cells to a level that promotes their death (Zhong et al. 2011; Bittremieux et al. 2018). Another dimension in the targeting of cancer cells through the Ca^{2+} signaling toolkit is via mechanisms to prevent or reverse drug resistance to other therapies. The clearest example of this potential approach is seen in the role of TRPC5 in breast cancer multidrug resistance. TRPC5 is critical for the induction of multidrug resistance ATPase 1 (MDR-ATPase 1) expression in breast cancer cells (Ma et al. 2012). MDR-ATPase 1 is an important drug-resistance mechanism in many cancers because of its ability to efflux a diverse array of anticancer agents from the cancer cell cytoplasm. The ability of TRPC5 silencing to reduce MDR-ATPase 1 induction and reverse doxorubicin resistance in vitro and in vivo models (Ma et al. 2012), suggests that further study of other members of the Ca^{2+} signaling toolkit may reveal additional strategies for overcoming cancer drug resistance.

CONCLUSION

Some cancer cells appear to recruit and remodel their Ca^{2+} signaling toolkit to proliferate, invade, and avoid cell death. This remodeling, although not always a driver for oncogenesis, could potentially be targeted to attenuate these hallmarks of cancer. The role and importance of the Ca^{2+} signal in tumor progression goes beyond the cancer cell itself and may involve the regulation of the tumor microenvironment. This diversity of contributions by the Ca^{2+} signaling toolkit to tumor progression has resulted in specific Ca^{2+} channels and pumps being proposed as drug targets for individual cancer types and even sub-

types. Indeed, clinical trials in different malignancies have begun on agents that act through the Ca^{2+} signal. The future should see an expansion of such trials to agents that may act more effectively in combination or those that may reverse resistance to current cancer therapies.

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