Calcium signalling pathways in prostate cancer initiation and progression

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Abstract

Cancer cells proliferate, differentiate, and migrate by repurposing physiological signalling mechanisms. In particular, altered calcium signaling is emerging as one of the most profound and widespread adaptations in cancer cells. Alterations in calcium signals drive the onset and development of several malignancies, including prostate cancer (PCa). *In vitro*, *in vivo* and bioinformatic studies of human PCa patient- and xenograft-derived gene expression data have identified significant changes in the expression and function of various components of the calcium signalling toolkit. Indeed, discrete alterations in calcium signaling have been implicated in hormone-sensitive, castration-resistant, and aggressive variant forms of PCa. Hence, modulation of calcium-dependent signalling is a plausible therapeutic strategy for both early and late stages of PCa. Based on this evidence, clinical trials have been undertaken to establish the feasibility of targeting calcium signalling. In this review, we summarize both the etiology of PCa and the evidence for altered calcium signalling as a critical component of the molecular re-programming of prostate cells. We highlight links between pre-clinical and clinical results relevant to PCa progression. A model is proposed in which specific calcium signalling alterations, commonly involving crosstalk between calcium and other cellular signaling pathways, underpin the temporal progression of prostatic malignancies.

Introduction

Prostate cancer (PCa) is one of the most common malignancies and a leading cause of cancer-related death in men, with ~400,000 deaths per year worldwide in 2020[1]. In the early stages, PCa cells grow within the prostate gland. Thereafter, spreading to surrounding tissues and distant metastatic sites during the advanced forms of the disease. Early intervention with chemotherapy and surgery can be effective, and the prognosis is generally favourable[2]. However, as the cancer starts disseminating, these approaches lose their applicability and effectiveness, and the prognosis significantly worsens[3]. Since malignant prostate cells heavily rely on androgen signalling for their growth and survival, androgen deprivation therapy (ADT) has become the treatment of choice for advanced PCa[4]. Although ADT is initially effective, castration-resistant PCa (CRPC) eventually emerges, usually 2-3 years post-treatment[4,5]; CRPC is characterized by the activation of the intracellular androgen signalling pathway, despite androgen deprivation[6].

CRPC can further evolve towards more rapidly progressing anaplastic forms of PCa known as aggressive variant prostate cancer (AVPC), which show a marked metastatic behavior[7]. AVPC often expresses neuroendocrine markers, defining the PCa subtype known as neuroendocrine prostate cancer (NEPC)[7]. While CRPC still depends on the intracellular androgen receptor (AR) signalling axis, AVPC activates alternative pathways for survival and growth; thus none of the androgen-based therapies is effective for its treatment, making AVPC invariably fatal, with an average survival of less than one year[8].

In the last decades, studies have unveiled the role of calcium signalling in many cellular processes, including the cell cycle, migration, and apoptosis[9]. When dysregulated, these processes can confer a malignant phenotype, driving cancer onset and progression[10]. Not surprisingly, calcium signalling mediators, such as the transient receptor potential (TRP) channels or the voltage-gated calcium channels (VGCC), are becoming an attractive therapeutic target for many malignancies, including lung, colon, breast, prostate cancer and other types of tumours[11]. In recent years, many clinical trials have been designed to evaluate the safety and activity of calcium signalling-targeting drugs[11]. Although none of these drugs is currently used in clinical practice to treat solid cancers, many are showing promising results and could become crucial elements for developing single-agent or combined therapies for PCa and other malignancies. Here, we present an overview of the role of calcium as a driver of PCa onset and progression, along with a discussion of the most current therapies targeting the calcium signalling machinery to treat this malignancy.

The clinical evolution of prostate cancer

The malignant transformation of prostate cells results from a complex interaction between epigenetic and genetic alterations triggered by signalling and remodelling processes within the nascent tumour microenvironment (TME). These aberrations lead to the onset and development of prostate cancer, driving the early development, the metastatic spread, the acquisition of drug resistance and, eventually, the emergence of the aggressive neuroendocrine phenotype. Identifying the events involved in each step of prostate cancer progression and understanding their specific role is crucial to comprehend this malignancy.

Genetic and epigenetic aberration in PCa

Among the earliest events in PCa development is the dysregulation of pathways affecting DNA repair, cell cycle progression, and apoptosis; often mediated by epigenetic changes. For example, hypermethylation in the promoter of the *GSTP1* gene can be observed during pre-malignant conditions, with a frequency of about 70%, which further increases to about 90% with the onset of PCa[12]. Similarly, tumour suppressor genes, such as *NKX3.1* and *PTEN,* are often downregulated during pre-malignant conditions[13–18]. These epigenetic changes promote the onset of genetic aberrations, including the TMPRSS2-ERG (T2E) fusion/PTEN loss (found in about 50 and 40% of primary PCa, respectively)[19–22] and *SPOP/CHD1* mutations (5-15% of PCa)[23], that results in the activation of the mitogenic PI3K/AKT and AR signalling axes, promoting cancer cells' proliferation[21,24] (FIG. 1a).

When the cancer starts disseminating, the activation of epithelial-mesenchymal transition (EMT), migration and invasion programs become prominent. In PCa, *T2E* fusion and the overexpression of the TRPchannels enhance the expression of several matrix metallopeptidases (MMPs) and other EMT markers that mediate the degradation of the extracellular matrix (ECM) and promote the evasion of PCa cell from its primary site[25–28]. An extensive genetic reprogramming also occurs, orchestrated by the H2K27 methyltransferase Enhancer of Zeste homologue 2 (EZH2), which seems critical in promoting PCa cell dedifferentiation, invasiveness, metastasis and in the acquisition of a castration-resistant phenotype[29–31]. PCa cells can acquire resistance to ADT by several escape mechanisms that allow the activation of the AR signalling axis through alternative routes: **(i)** AR gene amplification/mutation; **(ii)** AR ligand-binding domain deletions; **(iii)** overexpression of the AR co-activators SRC1/TIF2; **(iv)** non-canonical activation of the AR signalling pathway through the glucocorticosteroid receptor[32] (FIG. 1b). Since CRPC still relies on AR signalling pathway activation, studies are ongoing to develop new AR-targeting strategies that could improve patients'

prognosis[33–35]. However, such AR-targeting strategies are ineffective against the AR-independent forms of PCa, such as NEPC (FIG. 1b)[8].

The activation of the EZH2/CREB/TSP1 axis[36] and the expression of the long non-coding RNA (lncRNA) *MIAT*[37] may drive the neuroendocrine trans-differentiation (NED) in NEPC by mediating the expression of neural tissue specification genes, such as *n-MYC*[38,39] and t-type calcium channels (TTCC)[40–42], and inhibiting the neural repressors *REST* and *FOXA1*[36]. NEPC cells exploit AR-independent mechanisms for their growth, which include the activation of RET, WNT, and STAT3 pathways[7] and the overexpression of cell cycle-related proteins Aurora Kinase A (AURKA), PEG10, the MYST/Esa1-associated factor 6 (MEAF6), and cyclin D[43]. Additionally, lncRNA *LINC0026*1 and the transcription factor ONECUT2 enhance cell proliferation and cell cycle progression by activating the CBX2 and TGF-β axis[36,44]. Both *LINC00261* and ONECUT2 interact with SMAD3 promoting its expression and recruitment onto *FOXA2* promoter. The resulting overexpression of *FOXA2* increases the metastatic potential of NEPC by enhancing cell migration and invasion[36,37]. Lastly, although the exact mechanisms remain elusive, the concomitant loss of TP53, RB1 and PTEN seems determinant in NED[23,36,45]. Interestingly, a recent study highlighted a link between TP53 loss and the overexpression of the TRPM4 channel, which, as we will discuss further on, participates in PCa progression by enhancing the proliferation rate and the migratory ability of PCa cells[46].

The role of microenvironment in PCa

Crosstalk between tumour cells and their microenvironment was shown to be a crucial aspect in the development of PCa[47]. Chronic inflammation caused by microbial infections, physical trauma, or lifestyle, creates a microenvironment rich in reactive oxygen species and cytokines [48]. In response to these stimuli, PCa cells promote the recruitment of myeloid-derived suppressor cells/tumour-associated macrophages (MDSC/TAM) that release additional cytokines, chemokines and reactive oxygen species in a positive feedback loop[49,50]. These molecules activate AR signalling through the JAK/STAT pathway, leading to enhanced PCa proliferation and survival, favouring DNA breaks and genomic translocation of AR-related genes such as *T2E*[48,49,51].

Moreover, inflammation is amplified by severe hypoxia within the tumour tissues, which are characterized by a very low level of oxygen (0.3–1.2%) with respect to the physiological level in normal tissue cells (3.4–3.9%)[52–54]. Hypoxia plays a crucial role in tumorigenic processes, leading to a plethora of adaptative events and treatment resistance acquisition principally mediated by hypoxia-inducible factor 1 (HIF-1)-related pathways[55–60]. Inflammation and hypoxia promote the

morphological transition of peritumoral stromal fibroblasts into cancer-associated fibroblasts (CAF), forming the so-called reactive stroma (RS)[61,62].

CAFs and RS participate in ECM remodelling by affecting the expression of EMT markers and releasing a broad range of cytokines, and angiogenetic factors[60,63]. These latter factors promote angiogenesis through the VEGF/VEGFR axis, providing the vascularization needed for tumour growth and dissemination[63].

Moreover, under hypoxic stimuli and androgen deprivation, CAFs that express myofibroblast markers are activated by HIF-1 combined with autocrine TGF-β signalling[64,65]. Myofibroblasts are the major source of CXCL13, the chemokine involved in the recruitment of the B lymphocytes in intra-tumoral regions, amplifying the inflammation and promoting CRPC progression in murine models[66]. Furthermore, hypoxia shapes the tumour microenvironment by means of exosomes secreted by cancer cells. These vesicles are laden with growth factors, cytokines, proteinases and lipids that contribute to stemness, invasiveness and EMT in naïve PCa cells[67–71].

Modification of the future metastatic sites' microenvironment begins when cancer cells are still confined within the prostate gland in a remodelling process promoted by soluble factors, such as cytokines, and vesicles released into the bloodstream by the cancer cells[63] This remodeling process promotes the formation of premetastatic niche, which favors the homing of cancer cells to their metastatic sites. Thus, cytokines are essential for homing PCa to metastatic sites as they create the premetastatic niche, favouring the endothelial attachment of circulating cancer cells and promoting the remodeling of the microenvironment. Additionally, evidence suggests that an acid microenvironment stimulates the secretion of MMP9 and VEGF from PC3 cells, resulting in increased invasiveness and promoting bone metastasis[72]. Moreover, an acid TME seems to impair the anticancer effect of ascorbic acid in the PCa cell lines DU145 and PC3[73]. Microenvironment signalling is also critical for the NED of PCa[74,75]. Indeed, cancer cells induce axonogenesis through the secretion of neurotrophins and axon guidance molecules such as S4F, mimicking the processes observable during embryonic development. Additionally, granulocyte colony stimulating factor seems to potentiate PCa growth and metastasis by promoting autonomic innervation. Notably, the expression of neurotrophins and the chemokines CCL2 and CXCL12 by PCa cells could induce the differentiation of neural progenitors within the tumour microenvironment[76,77]. In PCa and other malignancies, the CCL2-CCR2 axis plays a critical role in perineural invasion (PNI), in which cancer cells invade distant sites along nerves[77]. Targeting the molecular players of PNI, such as CCL2 and CCR2, may inhibit the communication between cancer cells and nerve microenvironment, reducing

the metastatic potential of PCa[77]. Understanding the crosstalk among the tumor cells and all the contributing elements of the microenvironment will help to identify new therapeutic strategies targeting these interactions[78].

Introduction to calcium signaling

Calcium is a universal messenger employed by all cell types to regulate their activities in response to numerous extrinsic and intrinsic stimuli[79]. The diversity of calcium signals that underlies the physiology of different cell types derives from a broad toolkit of calcium channels, transporters, and effectors. The repertoire of calcium signalling toolkit components expressed by a particular cell type suits that cell's physiology[80]. For example, a non-excitable epithelial cell that functions with relatively slow calcium signals would express a different selection of calcium channels and transporters compared to a striated muscle cell that requires rapid calcium signals to function[79]. Whilst specific cell types express calcium signalling toolkit components that suit their physiological roles, it is important to note that calcium signalling is highly plastic. Cells alter the expression of calcium signalling toolkit components in response to environmental and intrinsic cues. It is this plasticity that underpins the ability of cellular calcium signalling to deviate from physiological functions to driving various pathological outcomes[81], including cancer[82]. A complexity in understanding how altered calcium signals impact on the development of cancer is that both decreases and increases in calcium signalling, as well as de novo expression/repression of calcium toolkit components, have been implicated in oncogenesis[83]. A substantial body of evidence shows that cancer cells dampen calcium signalling, for example, to avoid cell death[84–86], but at the same time cancer cells can be addicted to calcium signalling to support their metabolism and survival[87,88]. It has been known for some time that calcium signals with different kinetics and spatial effects can occur simultaneously within the same cell[89,90]. For example, there can be temporally and spatially discrete calcium signals that affect cytosolic versus nuclear processes[91]. Understanding how calcium affects PCa development therefore requires a careful dissection of changes in the location, kinetics, and downstream outcomes of calcium signals during the development of a malignancy[92].

Physiology of calcium signalling

The basal cytosolic calcium concentration in unstimulated cells is maintained at ~100 nM (approximately 15,000-fold less than the calcium concentration in the extracellular milieu). Stimulation of cells, which can arise in numerous ways, increases the cytosolic calcium concentration and thus activates specific effector pathways to generate a cellular response[80].

Cellular calcium signals encode information in their frequency, kinetics, amplitude and/or spatial extent. The characteristics of cytosolic calcium signals depend on the cell type and the nature and intensity of stimulation. Physiological stimuli, such as hormones and growth factors, give rise to controlled, reversible cytosolic calcium signals that are generally less than 1 μM[79]. Pathological stimuli can lead to aberrant calcium signals that may spiral out of control by overwhelming homeostatic mechanisms and even provoke cell death[93,94]. Cellular calcium signalling does not only involve the cytosol. Several organelles, including mitochondria and the endoplasmic reticulum (ER), serve as sources of calcium in the generation of signals[95]. Moreover, organelles can sequester calcium following a cytosolic calcium increase and thereby alter their function[79].

ER and mitochondria are intimately linked and participate in the generation and sensing of calcium signals[96]. Due to their proximity within cells, cytosolic calcium signals that are caused by the activation of channels within the ER membrane are sequestered by adjacent mitochondria[97] (FIG. 2). The sequestration of calcium by mitochondria stimulates respiration and biosynthetic processes, but it can also promote cell death[98]. Many cancer cells possess mechanisms that reduce the frequency and amplitude of cytosolic calcium signals and attenuate mitochondrial calcium sequestration, thus acquiring a survival advantage by decreasing their susceptibility to cell death[84,85].

The calcium signalling toolkit

Whilst all cell types have the same basal calcium concentration, the mechanisms by which calcium homeostasis and calcium signalling are mediated can be strikingly different[79].

Non-electrically excitable cells, akin to non-malignant prostate tissues, typically activate physiological calcium signalling via G protein-coupled receptors or tyrosine kinase receptors[81]. The activation of these receptors stimulates phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), yielding diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Following its production, IP_3 diffuses away from the cell membrane and binds to inositol 1,4,5-trisphosphate receptors (IP_3R); calcium channels that are primarily located on the ER[99]. Calcium signals within non-excitable cells are typically observed as a series of oscillatory elevations in the cytosolic calcium level that are rapidly arise from the basal calcium level and reach peak concentration of ~1 micromolar. Such calcium oscillations are sensed by calcium-binding proteins such as calmodulin (CAM), which then activate downstream signalling or effector processes[100–103].

While the calcium signalling toolkit is too broad in scope to describe here fully, some of its principal components are briefly mentioned below and schematized in FIG. 2. The key sources of calcium used in the generation of signals involve the influx of calcium from the extracellular space and the release of calcium from organelles[79]. The channels that mediate calcium influx include transient receptor potential (TRP) channels, VGCCs, and ORAI[100]. The TRP superfamily consists of seven subfamilies (TRPC, TRPV, TRPM, TRPA, TRPP and TRPML)[104].

TRPs can be activated through multiple mechanisms, including metabolites such as DAG, ADP-ribose, NAD, growth factors, depletion of ER calcium, mechanical stretching, noxious and environmental stimuli[104]. Consistent with their sensitivity to a wide range of stimuli, TRPs are involved in many physiological processes, mainly related to sensory physiology and sensing environmental changes[105]. The growing interest in the TRP superfamily of ion channels and their involvement in cancer biology is shedding new light on the importance of these genes in PCa[106].

There are ten members of the VGCC superfamily, organized into three subfamilies (Ca_v1, Ca_v2, and Ca_v3). Each VGCC is activated by membrane depolarization but can also be modulated by cellular metabolites and accessory proteins such as CAM[107]. VGCCs initiate contraction in muscle cells, and participate in synaptic transmission, hormone secretion, regulation of enzyme activity, and gene expression in a wide range of cell types[107].

Intracellular calcium stores have critical roles in cellular calcium signalling but possess a finite capacity for releasing calcium and activating cellular processes. Ultimately, the calcium needed for sustained signalling and replenishment of intracellular stores derives from the extracellular milieu[108,109]. In non-excitable cells, and some excitable cells, a process known as store-operated calcium entry (SOCE) coordinates the influx of calcium with the release of calcium from the ER. Two widely-expressed proteins mediate SOCE: ORAI (three isoforms; Orai1, 2 and 3) and stromal interaction molecule (STIM; two homologues; STIM1 and 2)[108]. ORAI is a calcium channel expressed on the cell membrane, whilst STIM is a transmembrane protein located on the ER. Reduction of the calcium concentration within the lumen of the ER causes the redistribution and oligomerisation of STIM1 to the ER membrane in close apposition with the plasma membrane.

These events cause STIM to change conformation and physically interact with ORAI, activating calcium influx across the cell membrane into the cytosol. The activation of IP_3Rs and RyRs on the ER leads to the depletion of ER calcium. SOCE is, therefore, a mechanism for replenishing calcium stores following the activation of intracellular calcium stores. In addition, the influx of calcium mediated

by SOCE has been shown to activate cellular processes distinct from those sensitive to calcium release[110].

The various calcium fluxes into the cytosol are counteracted by ATPase pumps and exchangers that transport calcium across the cell membrane or sequester it into organelles. Three primary pumps/exchangers mediate these processes: plasma membrane Ca^{2+}-ATPases (PMCAs 1-4), sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs 1-3), and Na^{+}/Ca^{2+} exchangers (NCXs 1-3)[81]. SERCAs and PMCAs transport calcium up its concentration gradient from the cytosol into the ER and the extracellular space, respectively, and are fueled by ATP hydrolysis. NCXs transport calcium from the cytosol across the plasma membrane, fueled by the concomitant movement of Na^{+} ions down their concentration gradient. The activity of these pumps/exchangers is essential for several reasons: it maintains the basal calcium concentration, allows the replenishment of intracellular stores, and prevents the cytotoxic effects of sustained cytosolic calcium elevations[81].

Calcium signalling in cancer-related pathways

Cancer hallmark pathways are cellular processes that, when dysregulated, can drive carcinogenesis. These processes include cycle progression, cellular migration, and apoptosis[111]. In the last decades, many studies highlighted the role of calcium signalling in each of the cancer hallmark pathways.

The cell cycle initiates upon external stimuli that trigger the transition from a resting state (G0) to a proliferative state (early G1 phase). These stimuli activate c-AMP responsive element-binding protein (CREB), the nuclear factor of activated T-cell (NFAT), and AP1 transcription factors involving FOS and JUN family members, eventually triggering cell cycle progression[112]. It is known that, by binding with CAM and calcineurin, and activating CAMKs, Ca^{2+} can promote the transcription of CREB and the nuclear translocation of NFAT[112–114]. Additionally, calcium influx can promote the activity of the cyclin/CDK complexes through calcium/CAM-activated kinases, indicating the importance of calcium in cell cycle activation and progression[114,115].

Cellular migration requires a combination of cyclic events that include the formation of lamellipodia, their adhesion with the ECM (focal adhesion), cellular contraction mediated by actin and myosin and, lastly, the disassembly of focal adhesion complexes (FAC)[116]. Calcium participates in all these steps by affecting the cytoskeleton dynamics via interaction with actin regulators such as protein kinase C (PKC), calcium/CAM-dependent kinases, and myosin[116]. Moreover, the calcium/CAM-dependent kinase CAMKII regulates focal adhesion kinase (FAK) activity, which is crucial for the disassembly of the FAC[116,117]. Increased cellular migration can result in the acquisition of a metastatic phenotype when coupled with the EMT[54]. As we will discuss further on, dysregulated

calcium signalling can induce EMT by promoting the expression of the MMPs, N-cadherin and other markers important for mediating the proteolytic degradation of the ECM and cellular adhesion[54].

Apoptosis is a type of programmed cell death that involves the release of cytochrome *c* (cyt *c*) from the mitochondrial intermembrane space to trigger the formation of the caspase-activation platform (apoptosome)[118]. In addition to the Bax/Bak-dependent cyt *c* release, calcium and oxidative stress can also promote cyt *c* loss from mitochondria through a process known as mitochondrial permeability transition (MPT)[119,120]. While the identity of the protein responsible for MPT is still debated, it is widely accepted that substantial mitochondrial calcium sequestration, mainly resulting from sustained IP_3R-mediated cytosolic calcium signalling, promotes MPT[114]. Intriguingly, several oncogenes/tumour suppressors have been linked with the regulation of calcium uptake by mitochondria and prevention of MPT[85,119,121]. For example, the oncogene AKT and the antiapoptotic proteins of the BCL2 family can inhibit the activity of IP_3Rs, decreasing cytosolic calcium signals and exerting an antiapoptotic function[85,121,122]. Additionally, by phosphorylating the regulatory subunit (MICU1) of the mitochondrial calcium uniporter (MCU), AKT impairs its function leading to an increase in the basal mitochondrial Ca^{2+} concentration and promoting cancer progression[123].

Conversely, tumour suppressors such as PTEN and TP53 promote mitochondrial overload by facilitating the activity of IP_3Rs and SERCA, respectively, thereby triggering apoptosis[114,124,125].

IP_3Rs bind to many accessory proteins that can thereby modulate cellular calcium signalling and are also implicated in cancer[84]. Although there is an overlap in the expression of some components of the calcium signalling toolkit in different tissues, each cell type expresses a unique calcium signalling proteome, which is plastic and can be remodeled, depending on environmental cues[81]. It is worth noting that the same calcium signalling mediator (i.e., channel, transporter, effector etc) expressed in a different cellular context may acquire an alternative function[126]. Thus, characterizing the expression pattern and the biological function of each calcium signalling mediator in different types of cancer, and at different stages of cancer progression, can provide crucial information to understand cancer pathogenesis and to identify new therapeutic strategies.

Epigenetic regulation of calcium signalling

The epigenetic regulation of gene expression results from interconnected and coordinated elements acting at transcriptional and post-transcriptional levels, including DNA methylation, histone modifications, lncRNA, and microRNA (miRNA) regulation[127]. Epigenomes are dysregulated in many malignancies[128], with the cancer landscape generally characterised by a global DNA hypomethylation and specific hypermethylation in CpG-rich regions[129]. In solid cancers, calcium

signalling-related genes show altered methylation levels, with hypermethylation reported for *CACNA1A*, *CACNA1B*, *CACNA1H* and *ORAI2*[130,131], associated with diminished gene expression and, in some cases, a worse prognosis[131]. Few studies have evaluated the epigenetic reprogramming of calcium signalling-related genes in PCa[15]. A hypomethylation of *CACNA1D* gene was reported in T2E-positive PCa compared with T2E-negative ones; as expected reduced DNA methylation correlated with higher *CACNA1D* mRNA levels[132,133].

Conversely, the promoter region of *S100P* gene (a calcium binding protein that mediates cytoskeletal dynamics, protein phosphorylation and transcriptional control) was often hypermethylated in PCa, with reduced mRNA expression[134,135]. Additionally, the epigenetic regulation of other calcium-related genes, including *EGFR*, *ITPKA*, *BST1* and *PTGER1*, seems to be involved in the development of docetaxel-refractory metastatic CRPC[136]. In a panel of cancer cells, including the PCa cell lines PC3, LNCaP and 22Rv1, miR-25 seems to exert a post-transcriptional regulation of the mitochondrial uniporter MCU, which mediates the mitochondrial calcium uptake. When upregulated, miR-25 inhibits MCU resulting in an imbalance of the mitochondrial calcium homeostasis, and leading to increased apoptotic resistance[137].

Moreover, the chromatin remodelling factor EZH2 can lead to the epigenetic silencing of calcium-related and tumour suppressor genes involved in PCa progression[138–141]. In CRPC, EZH2 up-regulation inactivates the AR-repressed tumour suppressor gene *CCN3*, promoting the acquisition of the androgen-independent phenotype[142]. Moreover, the overexpression of EZH2 in prostate stem cells, NEPC cells, and NEPC mouse models suggested its involvement also in the NED[143]. Although the mechanisms by which these processes are affected and the link to calcium signalling are not fully elucidated[144].

In undifferentiated human mesenchymal stem cells (hMSCs), EZH2 transcriptionally represses the *PIP5K1C* gene to maintain intracellular calcium at a low level while neuronal differentiation is induced. When differentiation processes start, a transient increase in intracellular calcium levels is detected. Among the various mechanisms involved[145,146], the dissociation of EZH2 from the *PIP5K1C* promoter triggers the increase of PIP_2 formation and the activation of IP_3-mediated calcium signalling that support hMSCs neuronal differentiation[144].

EZH2 could affect cell fitness through the downregulation of miR-708, which modulates the phosphorylation of AKT/FOXO1 through the post-transcriptional inhibition of sestrin 3 (*SESN3*)[143], triggering cell proliferation, survival and NED. MiR-708 also modulates the expression of the ER protein neuronatin, an inhibitor of the SERCA pump. Through this mechanism, miR-708 reduces the

activation of ERK and FAK, suppressing cell migration and metastases[147] and induces apoptosis through the ER-stress pathway[148].

EZH2 also regulates cell fate by modulating mitochondrial calcium uptake. In head and neck cancer, the inhibition of EZH2 mediated by DZNep (3-deazaneplanocin A) triggers mitochondrial-mediated apoptosis by affecting the activity of calcium uniporter regulator MICU1[149]. Notably, DZNeP has been employed successfully in pre-clinical models of PCa. The authors reported inhibition of Polycomb repressive complex 2 (PRC2; composed of EED, EZH2, SUZ12) in prostate cells treated with a nontoxic dose of DZNeP but not in non-tumour cells. The treatment caused G0/G1 arrest in the LNCaP and apoptosis in the DU145 cells. In addition, SNAIL and TGFBR2 were inhibited by DZNeP treatment in DU145, affecting cell invasion processes. Thus, this epigenetic drug reduces stemness markers and affects EMT through increased expression of E-cadherin (CDH1), which is usually downregulated by EZH2/SNAIL cooperation[150]. These evidence highlight a critical role for epigenetic modifications in PCa progression and of particular interest is the intricate network through which EZH2 orchestrates calcium signalling, which has just started to be unveiled, especially in PCa.

Calcium signalling in PCa progression

PCa progression is marked by alterations in cellular calcium influx, efflux, and storage[92] (FIG. 3). At each stage of PCa, different alterations of calcium-dependent signalling play a key role.

Calcium signalling in PCa proliferation and survival

Changes in the expression of calcium toolkit genes can be determinant in early cancer development, diminishing cell death and apoptosis and enhancing cell proliferation[114,115,124]. For example, in PCa cells, the expression of ORAI1/STIM1 is required for pro-apoptotic stimuli to cause cell death[151]. SOCE mediated by ORAI1/STIM1 was observed to be the principal source of calcium influx that was involved in triggering apoptosis. By affecting the SOCE activity, downregulation of ORAI1 protects the cells from diverse apoptosis-inducing pathways, and is associated with apoptosis resistance in androgen-independent PCa cells[152,153].

Slightly conflicting evidence emerged when evaluating the role of ORAI3[154,155]. Dubois et al. reported overexpression of ORAI3 in PCa tissues from 15 patients compared with normal-matched tissues. The expression of ORAI3 progressively decreased when comparing LNCaP, DU145, and PC3. When silencing ORAI1, ORAI2, ORAI3, or STIM1, the authors observed that only STIM1 and ORAI1 affected SOCE in LNCaP, DU145, and PC3 cells, suggesting that ORAI2 and ORAI3 did not participate in SOCE.

Moreover, the silencing of any ORAI family member, but not STIM1, resulted in increased NFAT-mediated proliferation in LNCaP, suggesting a SOCE-independent effect.

The overexpression of ORAI3 in PC3 cells, but not in LNCaP, resulted in a significant reduction of thapsigargin-induced SOCE and a consequent apoptotic resistance. Similar results were obtained in xenograft models, where the overexpression of ORAI3 led to increased tumour size due to the enhanced proliferation rate and apoptotic resistance[154].

Interestingly, ORAI3 overexpression promotes the formation of ORAI1-ORAI3 hetero-multimeric calcium-selective channels that are activated by arachidonic acid, and mediate calcium influx independently of SOCE. The ORAI1/ORAI3 ratio can affect the formation of ORAI1 homo-multimers, which are essential in supporting susceptibility to calcium-dependent apoptosis. Based on these results, the authors concluded that ORAI1-ORAI3 channel predominance confers apoptosis resistance by inhibiting SOCE, and enhances proliferation in PCa cells via an NFAT-dependent pathway [154,156].

On the other hand, Holzmann et al. reported lower levels of ORAI3 in PCa than in normal tissues[155]. According to the authors, the ORAI1/ORAI3 ratio progressively increased when comparing primary cultures of human prostate epithelial cells (hPEC), LNCaP, DU145, and PC3.

Concerning the involvement of ORAI3 in SOCE, the authors observed that siRNA-mediated silencing of ORAI3 caused a significant increase of thapsigargin- and IP_3-induced SOCE in LNCaP but did not affect DHT-induced SOCE in hPEC[155]. Despite some differences in the results, which may depend on the patient heterogeneity and different experimental conditions, both groups showed that, under certain circumstances, an imbalance in the ORAI1/ORAI3 ratio could inhibit the activation of SOCE, resulting in an ontogenetic shift. However, additional studies are needed to better characterize the expression profile and the clinical relevance of ORAI3 in PCa patients.

Other calcium signalling mediators also participate in cellular proliferation and tumour growth in PCa. For instance, a study on LNCaP cells revealed that the enhanced cell growth promoted by EGF (epidermal growth factor) correlates with SERCA2b expression, leading to increased organellar calcium storage without any variation in cytosolic concentration. The authors propose that the increase of SERCA2b protein expression regulates ER luminal calcium concentration thereby promoting cell proliferation[157].

Modulation of TRP superfamily expression is linked to PCa development and can enhance cellular proliferation through different mechanisms[106]. In PCa, TRPM7-dependent increase in cytosolic calcium concentration leads to the activation of calcium/CAM-dependent kinases, which, in turn,

mediates the activation of ERK[158,159]. Phosphorylated ERK modulates the expression and activity of cyclin D1, Cdk4/6 and other cell cycle-related proteins, leading to increased proliferation[160,161].

Similar mechanisms may drive the cell cycle progression promoted by TRPC6. In DU-145 and PC-3 cells, stimulation with Hepatocyte Growth Factor (HGF) resulted in a TRPC6-mediated calcium entry and enhanced proliferation. The inhibition of TRPC6 abolished the HGF-induced proliferation and caused a G2/M cell cycle arrest. Moreover, the overexpression of TRPC6 resulted in an HGF-independent cellular proliferation. These data suggest that TRPC6 could enhance the proliferation rate by affecting the G2/M transition[162]. Interestingly, a study of oesophageal cancer cell lines revealed that the blockade of TRPC6 inhibited both the calcium entry and the activation of the Cdk2. The authors hypothesized that TRPC6 could promote the G2/M progression through the activation of Cdk2, possibly mediated by CaM or the calcium/CaM dependent phosphatase calcineurin, known for their role in the cell cycle progression[83].

Similarly, the expression of TRPM4, increases in PCa compared with matched non-cancerous tissues, and is associated with PCa progression[163,164]. TRPM4 promotes the inactivating phosphorylation of glycogen synthase kinase, GSK-3β; a kinase involved in the proteolytic degradation of several targets[165]. This TRPM4-mediated inactivation of GSK-3β stabilizes the transcription factor β-catenin, promoting the expression of c-Myc and cyclin D1, thereby enhancing cellular proliferation[166,167].

As summarized in **FIG. 2** and **Table 1**, other TRPs, including TRPM2[168], TRPM8[169,170], and TRPV6[171] are dysregulated in early-stage PCa and contribute to cancer growth and progression, albeit through mechanisms that are not yet fully elucidated.

Table 1*: TRP superfamily components involved in regulation of calcium signalling during PCa progression*

Channel	Regulation	PCa Stage	Ion flux	Mechanisms of action	Phenotypic effect	Ref.
TPRM2	↑	HG PCa	↑Ca^{2+}[i]	TPRM2 expression negatively influences with expression of apoptosis and autophagy-related genes and promotes cellular proliferation	Autophagy and proapoptotic stimuli inhibition and increased proliferation	(168,172,173)
TRPM4	↑	HG PCa	↑Ca^{2+}[i]	TRPM4 mediates the activation of β-catenin (via GSK-3β inhibitions) and AKT phosphorylation	Cell cycle related genes activation	(166,174)
				TRPM4 mediates the inhibition of GSK-3β stabilizing Snail1 and enhancing the expression of EMT markers	Acquisition of metastatic phenotype	
TRPM7	↑	LG PCa	↑Ca^{2+}/Mg^{2}[i]	TRPM7 promotes the activation of the MAPK/ERK pathway enhancing the expression of cell cycle genes	Enhanced proliferation rate	(159,175–177)
				Activation of EMT-related transcription factors via PI3K/Akt pathway	Acquisition of metastatic phenotype	
TRPM8	↑	LG PCa	↑Ca^{2+}[i]	In hypoxic conditions, TRPM8-induced calcium entry results in the inactivating de-phosphorylation of RACK1 and HIF-1α activation	Expression of growth-related genes	(178–182)
	↓	HG PCa	↓Ca^{2+}[i]	TRPM8 degradation leads to the activation of FAK	Suggest tumour-suppressive role in advanced PCa	
TRPV2	↑	HG PCa	↑Ca^{2+}[i]	TRPV2 enhances the expression of EMT markers	Enhanced cellular migration and adhesion	(183,184)
TRPV6	↑	LG PCa HG PCa	↑Ca^{2+}[i]	TRPV6 stimulates NFAT-dependent genes transcriptions	Cell proliferation/ apoptosis resistance	(171,185)
				Activation of EMT markers	Acquisition of metastatic phenotype	
TRPC6	↑	LG PCa	↑Ca^{2+}[i]	TRPC6 affects the G2/M transition	Enhanced proliferation rate	(83,162)

↓: Downregulation; ↑: Upregulation; HG: high grade; LG: low grade; [i]: intracellular concentration

Calcium signalling in metastatic castration-resistant PCa

Metastatic spreading is a multistep process leading to the dissemination of primary cancer cells to distant organs[186]. Emerging evidence shows that calcium-dependent processes are essential in metastatic steps, including cell deformation, invasion, migration and adhesion[80]. In PCa cellular models, STIM1 promotes the EMT and cellular migration and invasion through the activation of PI3K/AKT, resulting in the acquisition of a metastatic phenotype[187]. The overexpression of TRPM4 in PCa has been linked to increased migration in DU145 and PC3 cells[163]. This result has been confirmed in a TRPM4 knockout cell line DU145 that, compared with wild-type DU145 cells, exhibited reduced migratory ability and reduced adhesion rate[188].

As discussed above, TRPM4 promotes the inhibitory phosphorylation of GSK-3β through the modulation of intracellular calcium levels[165]. GSK-3β mediates the proteolytic degradation of Snail1, a transcription factor critical for the expression of EMT markers[165]. Thus, the TRPM4-mediated inhibition of GSK-3β stabilizes Snail1, thereby inducing the expression of EMT markers N-cadherin, vimentin, and MMP9[174].

Other TRP channels are also involved in the metastatic transformation of PCa. Among these, TRMP7, which is upregulated in metastatic PCa tissues[175], and promotes the EMT by stimulating the expression of MMPs in PC3 and DU145[175], possibly through the activation of PI3K/AKT as shown in other cell types[189–191]. Intriguingly, the TRPM7-induced EMT in PCa could depend on the TRPM7-mediated Mg^{2+} influx rather than on calcium dynamics[176], as also suggested by studies showing a link between TRPM7, Mg^{2+} homeostasis, and the PI3K/AKT pathway[192,193].

Of particular interest is the role of TRPM8, which seems to exert a protective effect in advanced, androgen-insensitive forms of PCa. TRPM8 expression changes during the various stages of PCa progression, with a high expression characterizing the initial, androgen-sensitive stages, followed by a marked downregulation in the more advance and aggressive forms of PCa[194,195]. In androgen-sensitive LNCaP cells, TRPM8 seems to promote cellular proliferation and survival[169,196,197]. However, in androgen-insensitive DU145 and PC3 cells, TRPM8 exerts an anti-proliferative and pro-apoptotic effect[179,180,198]. Moreover, a study by Grolez et al. using a prostate orthotopic xenograft mouse model highlighted that the overexpression of TRPM8 inhibited tumour growth and metastases[181]. In this study, the authors reported that the observed growth inhibition was mediated by a cell cycle arrest in the G_0/G_1 phase, accompanied by a downregulation of Cdk4/6. Additionally, TRPM8 reduced the Cdc42 and Rac1 activity and inhibited the phosphorylation of ERK and FAK, which are essential for cell adhesion and migration[181]. Overall, most lines of evidence indicate a protective role for TRPM8 in androgen-insensitive PCa stages[179–181,198]. TRPV2 and TRPV6 are other TRPs channels upregulated in advanced PCa that promote the acquisition of a metastatic behaviour by enhancing the expression of EMT markers through mechanisms that still need to be elucidated[183–185].

Taken together, these research works suggest that alterations in the expression or activity of calcium-signalling mediators drive the acquisition of a metastatic phenotype by inducing the expression of various EMT-related proteins such as MMPs, N-cadherin, and cathepsin B.

Interestingly, AKT could stabilize the EMT-inducing transcription factors (SNAIL, TWIST and ZEB), either by their direct phosphorylation or by promoting the degradation of GSK-3β[165]. At the same

time, the activity of STIM, TRPM4, and TRPM7 has been linked with the activation of the PI3K/AKT pathway and increased migratory ability[166,175,187,192,193]. Thus, we could speculate that some calcium signalling mediators may participate in the metastatic process by inducing the activation of AKT, which, in turn, stabilizes the EMT-related transcription factors leading to the increased expression of the EMT-markers.

TTCCs in neuroendocrine PCa trans-differentiation

The acquisition of androgen-independent phenotypes and the appearance of differentiated neuroendocrine cells are critical steps in the progression of PCa[8]. Dysregulation of AR signalling in PCa cells evokes an overexpression of TTCCs and increased cytosolic calcium, resulting in significant morphological and biochemical changes[42,199]. In LNCaP cells, the differentiation of neurite-like processes and the expression of tubulin IIIβ and neurotensin neuroendocrine markers were detected after treatment with bicalutamide or hormone-depleted media[42]. The resulting increased expression of TTCCs correlates with the morphological differentiation observed in cells undergoing NED and the reversion of NED phenotypes after the blockade of functional channels. Comparable results have been obtained after stimulation of LNCaP cells with sodium butyrate (NaBu). NaBu-induced NED has been associated with increased mRNA and protein expression of Cav3.2 and TRPM8[200,201]. Additionally, the current density of Cav3.2 was significantly increased during the NED in LNCaP cells, where Cav3.2 mediates the calcium-dependent secretion of mitogenic factors upregulated during neuroendocrine-like differentiation[40,41,202]. This evidence indicates the involvement of Cav3.2 in NED during PCa progression.

Calcium signalling and the PCa microenvironment

Cancer stroma is a complex environment that includes noncellular extracellular matrix (ECM), fibroblasts, epithelial, endothelial, and immune cells[203]. The stroma provides nutrients, oxygen, and signalling molecules supporting tumour growth[204]. Generally, the growing tumour triggers an unphysiological pressure against the surrounding stroma leading to pathological cellular responses mediated by mechanosensitive ion channels[205]. In different cellular cancer models, including PC3 cells, the pressure-sensitive calcium channel Cav3.3 promotes cellular proliferation in response to the growing extracellular pressures, involving the PKC-β/IKK/IkB/NF-κB pathway[206] (FIG. 4). Hypoxia is another common characteristic of PCa and is associated with aggressiveness and resistance to treatments[207]. Organotypic PCa culture revealed expression of EMT-related transcription factors Snail, Zeb1 and SK3, which are triggered by hypoxia and enhanced SOCE-mediated calcium influx, increasing PCa cell migration and aggressiveness[208,209]. Moreover, SOCE activity increase as a result

of the CaV1.3 overexpression that occurs during ADT[210]. Previously work found CaV1.3a1 isoform overexpressed at both mRNA and protein levels, especially among CRPC, and mediates Ca2+ influx under androgen stimulation[211]. Moreover, under hypoxic conditions, cells knockdown for CAV3.1 and treated with ADT showed a lower HIF-1α expression in ADT-sensitive cells but increased in CRPC, with a significant reduction of cell survival. These works suggest that CaV1.3 promotes the upregulation of SOCE and modulates HIF signalling contributing to treatment resistance and CRPC progression[199,211] (FIG. 4).

Additionally, TRPM8 overexpression promotes cell growth under hypoxic conditions in LNCaP cells and LNCaP-derived xenograft models[178]. The hypoxic cellular growth is mediated by HIF-1, tightly regulated by the ubiquitin-mediated degradation of its α subunit (HIF-1α). Mechanistically, TRPM8-induced calcium entry results in the inactivating de-phosphorylation of RACK1. Since RACK1 mediates the proteolytic degradation of HIF-1α, its inactivation promotes the stabilization of this latter, allowing the expression of the growth-related downstream genes[178] (FIG. 4). A similar mechanism was reported by Yang et al. for TRPM7 in DU145 and PC3 cells, in which knocking down the expression of TRPM7 resulted in increased RACK1-mediated inhibition of HIF-1α and a consequent reduction of cell growth under hypoxic conditions[177]. Moreover, evidence suggest the involvement of reactive oxygen species (ROS) in enhancing intracellular Ca^{2+} in PCa cells[212,213]. H_2O_2 exacerbates its function through TRPM2 channel causing an actin cytoskeleton remodeling, which results in enhanced cell migration[212]. Despite TRPM2 mediates the influx of both, Ca^{2+} and Zn^{2+}, Zn^{2+} concentration has the predominant role in regulating the ROS-related response in cancer cells[212]. Moreover, an increased ORAI1/ORAI3 ratio makes prostate cancer cells especially prone to H2O2-induced SOCE inactivation, and sensible to ROS-induced cell death[213].

Calcium signalling also participates in bone homing during the metastatic process[92]. The high calcium concentration in the bone microenvironment activates the calcium-sensing receptor (CaSR), which is frequently overexpressed in PCa cells derived from skeletal metastasis (e.g., PC-3 cells)[214]. Activation of the CaSR evokes cytosolic calcium signals and increases cellular proliferation and attachment to the bone ECM[215]. While the exact mechanisms through which CaSR exerts its effects on metastasis are still debated, its activation correlates with the stabilization of proteins involved in the cell cycle progression. Additionally, calcium-mediated activation of AKT has been observed during the PCa bone homing, although the putative link with the CaSR needs to be elucidated[214].

In conclusion, the microenvironment provides cancer-promoting signals that are translated through calcium-mediated pathways, setting up the cascade of events that lead to the onset of a more severe cancer phenotype.

Clinical significance of calcium signalling disruption in PCa

With calcium signalling participating in most of the cancer hallmark processes, it is not surprising that much research has focused on targeting components of the calcium signalling toolkit for potential novel therapies. However, no calcium signalling-targeting drug is currently used to treat solid tumours[9], partially due to the universality of cellular calcium signalling and the consequent challenge of targeting molecules expressed by cancer cells without affecting critical physiological processes elsewhere.

Mipsagargin derives from the SERCA inhibitor thapsigargin conjugated with a prostate-specific membrane antigen (PSMA)-recognized peptide carrier, which limits its toxicity to PSMA-expressing cells and their microenvironment with limited adverse effects[216]. By inhibiting the SERCA pump, mipsagargin causes prolonged depletion of ER calcium storage, persistent activation of SOCE and chronic increased cytosolic calcium concentration, leading to the induction of MPT and apoptosis[217,218]. Numerous studies have identified that thapsigargin treatment leads to acute cell death. However, increased expression of Bcl-2 promotes the survival of cancer cells by ameliorating the toxic effects of chronic calcium signalling[219]. In human prostatic adenocarcinoma DU145 cells, increased Bcl-2 expression significantly increased chemoresistance to thapsigargin[220].

In phase I and II clinical trials involving patients with advanced solid tumours, mipsagargin was well tolerated, with limited severe adverse effects (SAE)[221,222]. Moreover, even if no objective responses were observed, mipsagargin prolonged disease stabilization in hepatocellular carcinoma patients[221,222]. These observations were encouraging, and several trials are ongoing to assess the antitumor potential of mipsagargin[223–225], with the PSMA-mediated activation suggesting the potential of this drug also for the treatment of PCa.

The observation that blocking TTCCs reduces cell proliferation by inducing a G1/S cell cycle arrest suggests that TTCC blockers could sensitize cancer cells to classical chemotherapeutic drugs[226,227]. A sequential therapy based on the TTCC blocker mibefradil and the chemotherapeutic drug temozolomide (TMZ) has been proposed to treat glioblastoma multiforme[228]. In phase I clinical trial enrolling 27 high-grade gliomas (HGGs) patients, this combination was well tolerated, with only three Grade 3 Adverse Events (AE) reported[228]. Interestingly, a significant reduction in standardized uptake value (SUV) signal was reported in 2 out of 10 patients who underwent PET imaging,

suggesting the potential anticancer activity of this combination[228]. However, this first trial had some limitations. Firstly, the reasons behind the reduction in SUV peak and its clinical significance remained elusive. Additionally, since the trial included TMZ, establishing the actual contribution of mibefradil in the observed response was not feasible. Nonetheless, these results pave the way for further studies investigating the role of mibefradil as an anticancer agent in cancer expressing TTCCs, including NEPC.

TRPV2 and TRPV6 represent other targets with potential clinical implications for PCa[27]. The search for specific TRPV6 inhibitors culminated with the development of SOR-C13, a soricidin-based high-affinity antagonist of TRPV6[229]. SOR-C13 recently underwent a phase I clinical trial involving 23 patients affected by solid tumours of epithelial origin[230]. During this trial, 16 patients experienced AE possibly related to SOR-C13 administration. No SAE was observed, confirming that SOR-C13 was well tolerated. Additionally, a promising anticancer activity was observed, with disease stabilization reported in 12 out of 22 patients. Interestingly, a tumour diameter reduction (up to 27%) was reported in two patients affected by pancreatic ductal adenocarcinoma[230]. A phase Ib trial is currently ongoing on a second cohort of patients to determine the maximum tolerated dose and further evaluate the anticancer potential of SOR-C13[231].

With respect to TRPV2, tranilast is the most widely studied inhibitor. Tranilast induces cell cycle arrest and apoptosis, and reduces the release of TGF-β1 from bone-derived stromal cells, suggesting that it could suppress metastatic phenotypes[232]. In the first-in-human pilot study in 21 advanced CRPC patients, the administration of tranilast was safe and well-tolerated, with AEs occurring only in two patients[233]. Interestingly, cancer progression was inhibited in five CRPC patients with bone metastases. Moreover, tranilast improved the overall survival of CRPC patients when compared to the standard docetaxel-based regimen, with a reported overall survival of 74.5% and 61.5% at 12 and 24 months, respectively. However, different experimental settings complicated an accurate comparison between the two treatment regimens, and additional data are warranted to establish the clinical efficacy of tranilast. In this respect, a phase I/II trial on patients affected by oesophageal cancer is ongoing to evaluate the safety and activity of tranilast in a combination therapy regimen[234].

SOCE is another mechanism of Ca^{2+} entry involved in PCa development. Targeting SOCE by inhibiting STIM1 or ORAI1 could reduce cancer cell proliferation and metastatic potential. Carboxyamidotriazole (CAI) is an inhibitor of calcium entry active on SOCE, VGCE, and RMCE[235–237] that significantly inhibits cell proliferation and invasiveness in LNCaP, DU145, and PC3 cells[238]. In a Phase I clinical trial, CAI was administered to 49 patients with refractory solid tumours. Among the

evaluable patients, 49% showed disease stabilization[239]. Two other trials on patients with refractory solid tumours, evaluating the clinical benefits of combining CAI with the cytotoxic agent paclitaxel (PAX), reported encouraging results[240,241]. In these trials, the combination of CAI and PAX did not result in cumulative/additive toxicity, and grade 3 toxicity was rare, suggesting the tolerability of this regimen[240]. When evaluated in 27 patients with relapsed refractory solid tumours, this regimen led to a response rate of 5/11 (45%) in relapsed epithelial ovarian cancer and 1/4 (25%) squamous cell cervical carcinoma patients, suggesting the potential benefits of this regimen, especially in treating patients with gynaecological malignancies[241]. However, in the only trial enrolling patients with androgen-independent PCa and soft tissue metastases, CAI did not show any clinical activity, with all the 14 evaluable patients showing progressive disease after two months[242]. Other SOCE inhibitors exist, some of which have shown promising results in pre-clinical models, but trials are needed to evaluate the actual clinical benefit of these molecules for the treatment of PCa and other malignancies[243].

Concerning the chromatin remodelers, different drugs are available for targeting EZH2[244]. Tazemetostat, an inhibitor of the EZH2 methyltransferase activity, is the most studied: its safety and efficacy were evaluated in 126 patients affected by haematological malignancies and 105 patients affected by solid cancers in four different phase I/II clinical trials[245–247]. These trials showed that tazemetostat was well tolerated, with patients experiencing mainly low-grade AE. SAEs were reported only by two patients with epithelioid sarcoma[246]. Moreover, tazemetostat induced an objective response rate between 38% and 69% in haematological patients[245,247,248] and 5% and 15% in patients affected by advanced solid cancers[245,246]. Phase I and I/II clinical trials are currently ongoing to evaluate the safety and activity of tazemetostat in a combination therapy regimen for metastatic CRPC[249,250].

Other inhibitors of EZH2 methyltransferase activity exist, including CPI-1205[251–253], GSK126[254,255], PF-06821497[256] and the dual inhibitor of EZH1 and EZH2 DS-3201b[257–261], as summarized in Table 2. Notably, since EZH2 can activate AR[262], clinical trials are ongoing to assess whether combining EZH2 and AR inhibitors can improve their anticancer effect[250,253]. The next few years will be crucial to determine the clinical relevance of calcium signalling-targeting strategies for treating PCa. However, these results suggest that targeting dysregulated or remodelled calcium signalling machinery may lead to the development of novel and effective agents for cancer treatment.

Table 2: calcium-signalling targeting drugs under development for cancer therapeutic purposes.

Drug	Target	Type	Mechanisms	Key References	Clinical Trials	Trials Phases	Trials Conditions
Mipsigargin	SERCA-pump	PSMA-activated inhibitor of the SERCA pump	Triggers apoptosis through the inhibition of the SERCA pump	(263–265)	(216,221–225)	I/II	Reccurrent/progressive glioblastoma; clear renal cell carcinoma; prostate cancer
Mibefradil	T-type VGCC	Inhibitor of VGCCs	Promotes a G1/S cell cycle arrest by blocking the TTCC-mediated Ca^{2+} current	(266)	(228)	I	Recurrent glioblastoma multiforme; reccurrent glioma
SOR-C13	TRPV6	Sorcidin-based inhibitor of TRPV6	Inhibits the calcium uptake via TRPV6, reducing cell proliferation	(229)	(230,231)	I	Advanced TRPV6-expressing cancers; advanced refractory solid cancers
Tranilast	TRPV2	Inhibitor of TRPV2 and other targets	Suppresses the metastatic phenotype by inhibiting the TGF- β1 release from bone-derived stromal cells	(232)	(233)	I	Metastatic castration resistant prostate cancer; esophageal cancer
Tazemetostat CPI-1205 GSK126 PF-06821497 DS-3201b	EZH2	EZH2 inhibitors	Inhibit the EZH2 methyltransferase activity	(244)	(246–253,256–261)	I/II	Wide range of solid and haematological cancers, including prostate cancer

Conclusions

Altered calcium signalling plays a pivotal role in a plethora of cellular events promoting PCa development, drug resistance and metastatic dissemination. Evaluations using different PCa models and patient databases (Box 1) have contributed to identifying the calcium signalling-related genes, pathways, and downstream effectors involved in these oncogenic processes. Research on patient-derived xenograft models has corroborated these observations and highlighted the clinical significance of calcium signalling alterations, allowing the identification of novel putative therapeutic targets. These emerging lines of evidence suggest a preliminary map of the complex interactions between calcium signaling and clinical prostate cancer progression (FIG. 5). Whilst a complete understanding of this phenomenon is still lacking, it is evident that some calcium-relevant genes (e.g. EZH2) promote oncogenic progression at all stages of malignant transformation. Other genes (e.g. ORAIs) play a stage-specific role and may work as oncogenes or tumour suppressors, depending on cancer cell context and on the interaction between the tumour and its microenvironment. Due to the crucial role of calcium in most physiological processes, targeting cellular calcium signalling machinery is proving a difficult task. Indeed, to avoid unacceptable adverse effects, ideal therapeutic targets should be expressed only by cancer cells, or their

681 expression should be associated with an entirely discrete gain/loss of function. In this context, data
682 on the expression/function of the calcium signalling mediators in PCa patients is needed and would
683 represent a precious resource for developing specific drugs with limited side effects. Nevertheless,
684 many drugs that target calcium signalling have been developed, some of which have undergone
685 phase I/II clinical trials showing a good safety profile. Currently, most of these drugs have been
686 evaluated in a limited number of heterogeneous patients affected by different malignancies, and
687 only a few studies exist specifically enrolling PCa patients. Currently, none of these studies has led
688 to the identification of a drug with significant clinical activity. Undeniably, calcium signalling
689 machinery represents a fascinating target for cancer therapy. However, the pharmacological
690 opportunities offered by calcium signalling and its clinical benefits need further elucidation. In the
691 future, intensive investigations in this field are likely to produce specific drugs that could act as a
692 single agent or in combination with current therapies for the treatment of PCa and other
693 malignancies.

- **Cell lines**[267]

  RWPE1: normal human prostate cell line used as control for PCa studies.

  LNCaP: prostatic adenocarcinoma cell line isolated from lymph node metastasis; hormone-sensitive, positive for AR and PSA and negative for neuroendocrine markers; frequently used to test hormone sensitivity.

  DU145: moderately differentiated prostatic adenocarcinoma cell line isolated from brain metastasis; hormone-independent, negative for PSA, AR and neuroendocrine markers, with moderate metastatic potential; frequently used to test therapies for PCa.

  PC-3: isolated from bone metastasis of a grade IV prostatic adenocarcinoma; hormone insensitive, negative for AR, PSA and neuroendocrine markers, with high metastatic potential; used as a model of aggressive PCa in cancer research[268,269].

  NCI-H660: small cell prostatic carcinoma cell line isolated from lymph node metastasis; negative for AR and PSA, positive for neuroendocrine markers; used as a model of NEPC.

  LASCPC-01: NEPC cell line; negative for AR, positive for neuroendocrine markers; frequently used to test treatments for NEPC.

- **Xenograft models**[270,271]

  Adenocarcinoma: LTL-310, 311, 313A, 313B, 313C, 313D, 313H, 331, 412, 418, 467, 484.

  CRPC: LTL-310FR, LTL-331BR, 313HR, LTL-573R.

  NEPC: LTL-331R, LTL-352, LTL-370, LTL545, LTL-610

- **Genomic data in prostate cancer patients**

  cBioPortal: database containing 16 PCa datasets including adenocarcinoma, CRPC and NEPC[272].

  The Genomic Data Commons: database providing information through the prostatic adenocarcinoma dataset (PRAD) of "The Cancer Genome Atlas" (TCGA) containing samples from 500 prostatic adenocarcinoma patients[273].

  GEO: database reporting gene expression information with 79 PCa datasets available[274].

Box 1: major cell lines, xenograft models, and patients databases available for the study of PCa

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Key points

- Calcium is a ubiquitous ion playing crucial roles in many cellular pathways.
- Aberrations in calcium signalling can result in pathogenic phenotypes, including cancer.
- The onset and progression of prostate cancer are characterized by a deregulation of several calcium signalling mediators.
- Targeting calcium signalling mediators is a promising strategy for developing novel drugs for treating prostate cancer and other malignancies.