Store-Operated Calcium Entry in Cancer Stem Cells

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Store-Operated Calcium Entry (SOCE), a major mechanism for Ca2+influx from the extracellular medium into excitable and non-excitable cells, is physiologically triggered by the activation of phospholipase C (PLC) and the production of IP3, which subsequently leads to the release of Ca2+from intracellular stores, mainly the ER, resulting in the activation of store-operated calcium channels in the plasma membrane and a rapid increase in cytosolic Ca2+concentration. SOCE is an extremely complex biological mechanism, with high dependency on the pattern of expression of its components-STIMs, Orai, and TRPC proteins- and its modulators in each cell type. Since the last decades of the 20th century, several studies, both in vivo and in vitro, have reported that an altered expression pattern of the proteins that mediate SOCE leads to unbalanced Ca2+homeostasis, which might contribute to tumor development, poor prognosis, and chemotherapeutic drug resistance.

store-operated calcium entry | Orai1 | cancer stem cells

1. Introduction

Normal stem cells are undifferentiated or partially differentiated cells that are characterized by their ability to self-renew, the process of bringing about indefinitely more cells of the same type, as well as to differentiate in more specialized mature cells. The term “stem cell” was coined by Ernst Haeckel in 1868 to describe the ancestor unicellular organism from which all multicellular organisms were supposed to evolve [1]. Normal stem cells can be found from the early embryos to the mature subject, where they can be present in different tissues, including the bone marrow, skin and hair follicles, muscle, brain, and epithelia, among others [2].

Cancer stem cells (CSC), also known as tumor-initiating cells, share features of both cancer and stem cells. These cells constitute a sub-population of tumor-resident malignant cells responsible for recurrence, metastasis formation, and chemoresistance. Experimental evidence indicates that CSC exhibit “stemness” properties, that is, the ability of cells to perpetuate their lineage, to bring about differentiated cells and to interact with their microenvironment to maintain a balance between quiescence, proliferation, and regeneration [3]. According to this, CSC exhibit low proliferative rates, self-renewing capacity, propensity to differentiate into proliferating tumor cells, resistance to apoptosis and senescence, as well as to chemo- and radio-therapy, evasion of immune attack, and are responsible for invasion and metastases [4][5].

2. Store-Operated Calcium Entry in Cancer Stem Cells and Cancer Hallmarks

Store-Operated Calcium Entry (SOCE), a major mechanism for Ca^{2+} influx from the extracellular medium into excitable and non-excitable cells, is physiologically triggered by the activation of phospholipase C (PLC) and the production of IP_3, which subsequently leads to the release of Ca^{2+} from intracellular stores, mainly the ER, resulting in the activation of store-operated calcium channels in the plasma membrane and a rapid increase in cytosolic Ca^{2+} concentration [6][7]. SOCE is an extremely complex biological mechanism, with high dependency on the pattern of expression of its components-STIMs, Orai, and TRPC proteins- and its modulators in each cell type. Since the last decades of the 20th century, several studies, both in vivo and in vitro, have reported that an altered expression pattern of the proteins that mediate SOCE leads to unbalanced Ca^{2+} homeostasis, which might contribute to tumor development, poor prognosis, and chemotherapeutic drug resistance [8].

The proteins of the STromal Interaction Molecule (STIM) family, STIM1 and STIM2, and their splice variants, possess a single transmembrane domain, with the N-region located either in the ER lumen or the extracellular medium, and a long cytosolic C-region [9][10]. Both, N- and C-terminal regions, present several key domains that enact STIM proteins' double function upon a diminishment of the luminal Ca^{2+} concentration in the intracellular stores: (1) as the Ca^{2+} sensors of intracellular organelles, mediated by EF-hand Ca^{2+}-binding domains in the N-terminus; and (2) as the transmitters of the filling state of intracellular Ca^{2+} stores to, and the activators of, Ca^{2+} channels in the plasma membrane. The latter is achieved by direct interaction between different domains within the STIM cytosolic C-region and the store-operated Ca^{2+} channels (STIM proteins structure is reviewed in [11][12][13]).

SOCE could be mediated by two types of channels with different biophysical properties: (1) the Ca^{2+} Release-Activated Ca^{2+} (CRAC) channels that exhibit high Ca^{2+} selectivity and an inwardly rectifying current, termed I_{CRAC}, which its exclusively conducted by members of the Orai family [14]; and (2) the Store-Operated Ca^{2+} (SOC) channels, responsible to mediate a non-selective cation current denominated I_{SOC}, formed by both, Orai1 and TRPC1, the first identified member of the canonical Transient Receptor Potential (TRPC) channel subfamily [15][16].

Orai1 was initially characterized as the main component of CRAC channel during a RNAi screening in 2006, when it was found that the Orai1 R91W mutation was responsible for abrogated CRAC channel function, critical for T-cell activation, in immunodeficient patients [17]. Orai1 and its paralogues, Orai2 and Orai3, present a unique structure among other Ca^{2+} channels, with four transmembrane domains spanning the PM and both, N- and C-terminus, facing the cytoplasm [18]. Originally, it was thought that Orai channels were formed by a homo-tetramer [19]; however, the crystal structure from *Drosophila melanogaster* Orai1 (dOrai1) presented a hexamer configuration, with the ion pore formed by the first transmembrane domain of the Orai subunits and located in the center of the complex surrounded by the remaining Orai plasma membrane domains [20]. The three members of the Orai family are capable to mediate store dependent Ca^{2+} influx, each of them with different biophysical properties that are extensively discussed here [21][22]. Some years ago, a shorter splicing variant for Orai1, Orai1β, lacking 64 aa in the N-terminus but able to generate functional Orai1 channels, was identified. Orai1β can be fully activated by STIM1 in a store-dependent manner but exhibits differential inactivation patterns as compared with the long variant,

Orai1α [16]. In addition, recent studies have shown that Orai proteins might have a role in non-capacitative Ca^{2+} influx forming heteromers, such as the arachidonate-regulated Ca^{2+} channels (ARC), where three Orai1 and two Orai3 subunits form a pentamer [23], or interacting with other proteins to mediate store-independent Ca^{2+} influx [24].

TRPC1 belongs to the TRP channel superfamily, whose members ubiquitously mediate ion fluxes across the whole animal kingdom in a cell type-dependent manner [25]. All TRPs possess a similar structure with six transmembrane domains and the pore located between the 5th and 6th transmembrane regions. TRPs exhibit N- and C-terminus of variable length, containing the TRP box and different functional domains, subfamily-dependent, which participate in the functions of TRP channels and their relationship with other molecules and proteins. A functional TRP channel is composed by four TRP subunits forming either a homo- or hetero-tetramer [26][27]. Prior to Orai1 characterization, TRPC1 was a suggested candidate as the channel responsible for SOCE as STIM1 is able to interact and activate TRPC1 channels [28][29]. The current hypothesis suggests that TRPC1, together with Orai1, is involved in the generation of I_{SOC} currents [16][30][31][32]. TRPC1 channels, permeable to Na^+, Ca^{2+}, and Cs^+ [33], are less selective for Ca^{2+} than Orai1 and allow a massive ion influx from the extracellular medium, required for the maintenance of SOCE and store replenishment [34].

Several stimuli might trigger intracellular Ca^{2+} stores depletion that will be sensed by STIM proteins. Minor reductions in luminal Ca^{2+} concentration will be detected by STIM2, which in turn, would momentarily trigger the opening of CRAC channels, allowing Ca^{2+} influx from the extracellular medium that will quickly be reintroduced into the stores by Ca^{2+}-ATPase pumps to revert to resting conditions [11]. More extensive discharge of intracellular Ca^{2+} stores would trigger the activation of STIM1, in addition to STIM2, which will fully generate the opening of CRAC channels, subsequently followed by a rapid and transient Ca^{2+} entry [14][35][36][37]. Ca^{2+} entry conducted by Orai1 will be severely inhibited after few milliseconds by Ca^{2+} itself [38][39] as well as after a longer period of time by the interaction of Orai1 N- and C-terminus with different proteins, such as SARAF [40][41][42] or by Orai1 serine phosphorylation at the N-terminus by kinases such as PKC or PKA [43][44]. Ca^{2+} influx through Orai1 leads to the recruitment of TRPC1 at the plasma membrane, which conducts further Ca^{2+} influx to reach the critical cytosolic Ca^{2+} concentration required for the physiological response evoked by the stimulus [34][45]. Next, the excess of intracellular Ca^{2+} is speedily removed, either by reintroducing the ion into the ER or by its extrusion to the extracellular medium via Ca^{2+}-ATPases [46][47]. When agonist stimulation ceases, replenishment of the Ca^{2+} stores leads to the incorporation of Ca^{2+} to STIM1/2 EF-hand domains, which return these proteins to their quiescent conformation, leading to the deactivation of SOCE [10][35].

The number of studies linking SOCE proteins with cancer stem cell properties is growing at an amazingly fast pace; however, the knowledge is still extremely limited. Regarding STIM proteins, it is known that STIM1 associates with the hypoxia-inducible factor-1 alpha (HIF-1α) modulating each other, in a reciprocal dependency, in hypoxic hepatocarcinoma cells (HCCs). HIF-1α up-regulates STIM1 transcription, which in turn, induces higher SOCE, activating the CaMKII and P300 pathways, which are required for the accumulation of HIF-1α in HCCs [48].

Even less is known about the role of TRPC1 in CSC, since some of the inhibitors used to block SOCE, act over both Orai1 and TRPC1 channels. For instance, treatment with SKF96365, a SOCE inhibitor, impairs CSC

proliferation in the glioblastoma stem-like cell line, TG1, triggering these cells to adopt a quiescent state by up-regulation of *CDKN1A* and *G0S2* and the down-regulation of *CCNB1* genes [49]. Similarly, SOCE impairment by SKF96365 in liver cancer stem cells (LCSCs) resulted in a drastic reduction in their ability to form spheroids, suppressing at the same time the expression of stemness-related genes. SOCE is activated in LCSC via the fibroblast growth factor 19 (FGF19), promoting the nuclear translocation of NFATc2 and self-renewal [50]. Even when the expression of Orai and STIM proteins was checked in both studies, TRPC1 was not considered and might be a possible candidate for future approaches.

3. Functional Role of Orai in Cancer Stem Cells and Cancer Hallmarks

As described above, native CRAC channels are hexameric structures comprised by the heteromeric association of Orai1, Orai2, and Orai3. Although all Orai family members can conform the channel, Orai2 and Orai3 also act as Ca^{2+} current modulators due to their lower Ca^{2+} conductivity and greater fast Ca^{2+}-dependent inactivation as compared to Orai1 [39][51]. Several studies have demonstrated that the three Orai proteins are overexpressed in tumor samples and different human cancer cell lines compared with their non-tumorigenic counterpart cell lines. Hence, Orai1 is overexpressed in oral/oropharyngeal squamous cell carcinoma cells (OSCC) [52][53], liver [54], and breast cancer cells [55][56], Orai2 expression is increased in gastric [57], breast [58], oral [53], and acute myeloid leukemia cancer cells [59], while Orai3 expression is enhanced in the luminal breast cancer subtype [56][60], as well as in lung [61], pancreatic [62], and prostate cancer cells [63] (for a more extensive one see [64][65][66][67][68]). Using pharmacological or gene silencing approaches, to inhibit protein function or to avoid protein expression, respectively, the mentioned studies showed that Orai proteins play a crucial role in both tumorigenesis and the development and maintenance of different cancer hallmarks, including resistance to apoptosis, proliferation, migration, invasion, and metastasis via SOCE. However, as mentioned above, Orai1 can also mediate cancer progression by regulating and driving different Ca^{2+} influx pathways that are independent of the filling state of intracellular Ca^{2+} stores [24]. These pathways include: (1) the arachidonic acid-regulated Ca^{2+} current mediated by a Orai1/3 channel [63][69][70], (2) the constitutive Ca^{2+} influx mediated by the physical interaction between Orai1 and secretory pathway Ca^{2+}-ATPase-2 [71][72][73], and (3) the Ca^{2+} influx mediated by the physical and functional interaction of Orai1 with the small conductance Ca^{2+}-activated K^+ channel 3 [74][75] or with the voltage-dependent Kv10.1 channel in the plasma membrane [76][77]. In the latter, a reciprocal positive feedback loop promotes the activation of both K^+ channels by Orai1-mediated Ca^{2+} entry, which in turn leads to plasma membrane hyperpolarization, thus maintaining the driving force for Ca^{2+} influx and Ca^{2+} entry through Orai1 channels [74][78][79].

The role of Orai family proteins has also been described in the induction of CSC phenotype in a variety of cancers, such as glioblastoma, lung, and OSCC cancer cells. This CSC phenotype includes self-renewal capacity, tumor spheres formation, drug resistance, increased migration ability, and enhanced expression of stemness-related transcription factors and CSC-related markers [52][80][81]. Lee et al. demonstrated that Orai1, the predominant Orai family member in OSCC, is overexpressed in OSCC-derived CSC and its function is required for the maintenance

of stemness and CSC phenotype through NFAT signaling pathway. Hence, Orai1 mediates the enhanced expression of stemness-related transcription factors, such as Nanog, Oct4 or Sox2, and promotes some CSC-related markers, including an increased ALDH1 activity and a higher CSC-related gene expression (Ezh2, Gli1, Hes1, Zeb2, FGF4, and IL4). The inhibition of Orai1 function in human tongue squamous carcinoma cell lines SCC4 and HOK-16B BapT by a pharmacological approach, using the Orai1 specific small molecular blocker compound 5D, impaired self-renewal capacity and reduced migration and invasion abilities in these cancer cells. Comparable results were also obtained by two different genetic approaches, using a specific siRNA to reduce Orai1 gene expression and inducing the overexpression of an Orai1 dominant negative mutant. Furthermore, Orai1 overexpression using viral vectors promoted CSC phenotype in non-tumorigenic immortalized oral epithelial cells HOK-16B [52]. Using related approaches, Singh et al. demonstrated that Orai1 and Orai2 overexpression is required for cell proliferation, migration, and colonization in SAS human tongue carcinoma cell line, processes that were found to be dependent on Akt/mTOR/NF-κB signaling pathway activation [53]. Analogous results were reported in glioblastoma stem cells derived from different human glioblastoma surgical samples. In these cells, the treatment with YM-58483, a CRAC current inhibitor, or with GSK-7975A, a more specific inhibitor of Orai1-mediated Ca^{2+} current, promoted a decrease in Sox2 expression, effect that was associated with reduced spheres formation and with the inhibition of their proliferation and self-renewal capacities [81]. Orai1 has been also related with chemoresistance, event that has been widely associated with CSC phenotype in cancer cells as previously mentioned. Hence, it has been demonstrated that ectopic overexpression of Orai1, using a plasmid vector, inhibited 5-fluorouracil-induced cell death in HepG2 hepatocarcinoma cells; meanwhile, Orai1 gene expression knockdown promoted the autophagic cell death induced by this pharmacological compound [54]. Similar findings were observed in cisplatin-resistant A2780 ovary carcinoma cells, in which Orai1 expression and SOCE are increased compared to therapy-sensitive parental cells. Pharmacological inhibition of Orai1 in cisplatin-resistant A2780 cells, using 2-aminoethoxydiphenyl borate (2-APB), promoted cisplatin-induced apoptotic cell death similarly to those observed in therapy-sensitive A2780 cells [82]. Conversely, an opposite effect has been reported in prostate cancer cells since the downregulation of Orai1 expression, caused by steroid-deprived conditions or by using specific siRNA against Orai1, and the impairment of Orai1 function by the overexpression of two Orai1 mutants, Orai1 R91W and Orai1 L273S, prevented the apoptotic cell death induced by different pharmacological compounds, including thapsigargin, TNFα, cisplatin, and oxaliplatin. Furthermore, the restoration of Orai1 expression in steroid-deprived cells by transfection with a Orai1 plasmid vector promoted the loss of chemoresistance in these cells [83].

Regarding the role of Orai3 in the CSC phenotype acquisition in cancer cells, it has been demonstrated that Orai3 overexpression is correlated with tumoral aggressiveness and chemoresistance acquisition in breast cancer cells [60][80]. Orai3 stable overexpressing T47D and MCF7 clones exhibited resistance to apoptotic cell death induced by thapsigargin, cisplatin, 5-fluorouracil, and paclitaxel compared with their parental cells transfected with the empty vector. This Orai3-dependent chemoresistance is acquired by ubiquitin ligase Nedd4-2-mediated p53 ubiquitination via the PI3K/Sgk-1 signaling pathway [60]. Previously, the same group demonstrated that Orai3 expression is also positively correlated with the oncogene c-myc expression in the ER-positive (luminal-like) breast cancer cell line MCF7 [84]. Daya et al. revealed that chemotherapy treatment increased Orai3 expression in primary human lung

adenocarcinoma cells derived from bronchial biopsy specimens. Similar findings were reported in lung adenocarcinoma cell lines A549 and NCI-H23 after treatment with cisplatin. Interestingly, cisplatin treatment increased SOCE without affecting the expression of other proteins involved in CRAC current activation, such as STIM1, STIM2, and Orai1, even a slight decrease in the expression of Orai1 was observed in A549 cells. Orai3 gene expression knockdown using a specific siRNA enhanced cisplatin-induced apoptotic cell death in both lung adenocarcinoma cell lines, while Orai3 overexpression drastically reduced cisplatin-induced cell death and enhanced stemness in non-small cell lung cancer cells, as demonstrated by the enhanced expression of the stemness-related transcription factors Nanog and Sox2 via PI3K/AKT pathway, which resulted to be dependent on the increase in Orai3 expression [80].

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