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An Updated Review of Oral Cancer Stem Cells and Their Stemness Regulation

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Abstract

Cancer stem cells (CSCs; also known as tumor-initiating cells) are a small population of cancer cells that retain characteristics similar to those of normal stem cells. CSCs are known to be responsible for metastasis, drug resistance, and cancer recurrence. Thus, controlling CSCs may provide an effective therapeutic intervention that inhibits tumor growth and aggressiveness. Despite the importance of targeting CSCs in cancer therapy, the detailed nature of oral CSCs remains underexplored. This article reviews the current understanding of oral CSCs, with emphasis on recent advances in novel signaling pathways involved in their stemness regulation.

Keywords

oral cancer; cancer stem cell; stemness pathway

I. INTRODUCTION

Oral/oropharyngeal squamous cell carcinoma (OSCC), a common malignant tumor of the head and neck, is currently the sixth most common cancer worldwide.¹ The projected 5-yr survival for OSCC is ~ 50%, and this statistic has remained the same during the past decade. As such, there is an urgent demand for new directions in therapeutics. Risk factors for OSCC development in the elderly are typically associated with a life-long history of cigarette smoking and heavy alcohol consumption.^{2,3} But recently, the incidence of oral cancer among young adults has been alarmingly elevated,^{4,5} and a sizeable proportion of cases has been associated with factors other than tobacco and alcohol use.⁶ Indeed, 34% of the cases in young adults did not show any tobacco/alcohol-related habits⁷; but rather, changes in sexual behaviors was one of the contributing factors for this increasing incidence of oral cancer in young adults. These data indicate that oral cancer is an emerging public health concern.⁸ Clinically, well-defined lesions, such as leukoplakia that is histologically classified as dysplastic or nondysplastic leukoplakia, often precede OSCC. Dysplastic leukoplakia is defined as an oral premalignant lesion and is associated with a likely progression to cancer; however, dysplastic leukoplakia is not an accurate predictor of cancer risk.^{9,10} Early-stage tumors can usually be managed through surgery and radiotherapy, but successful treatment is

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inversely proportional to extent of disease at the time of treatment. A combination of chemotherapy and radiation therapy, although effective in treating 97% of early-stage tumors, was only 33% effective in treating advanced tumors.¹¹

Recent studies have unveiled and validated the pathophysiologic role of cancer stem cells (CSCs; also called tumor-initiating cells) in long-term sustenance of cancers.^{12,13} CSCs are small subpopulations of tumor cells that share many molecular similarities to embryonic and normal adult stem cells. CSCs have been isolated from various primary tumors and established cancer cell lines, including OSCC.^{14–20} They play a crucial role in tumorigenicity, metastasis, and recurrence and are thus considered to be the root of the cancer. Therefore, advancing our understanding in the molecular properties and signaling pathways unique to oral CSCs is crucial for developing novel targeted and effective therapies for OSCC. This review focuses on several aspects of oral CSCs and their stemness signaling and also provides current advances in these fields.

II. ISOLATION OF ORAL CSCs

The existence of a CSC population was first reported in leukemic cells.²¹ The leukemic CSC population was positively stained with CD34 and negatively for CD38 (CD34⁺/CD38) and became capable of developing leukemia when inoculated onto immunocompromised mice. Since then, CSC populations have been extensively examined and successfully identified in various solid tumors, such as brain, breast, prostate, colon, pancreas, and head and neck.^{22–25} The presence of subpopulations of oral CSCs was originally suggested by the observation that only a subpopulation of OSCC cells can form an expanding tumor mass.²⁶ Subsequently, Chiou and coworkers demonstrated that a subpopulation of OSCC cells derived from cultured OSCC cell lines possessed properties of both stem cells and aggressive malignant tumors, including self-renewal, tumorigenic potential, migratory ability, and radioresistance.¹⁴ Numerous research groups have reported successful isolation of oral CSC populations using various markers.^{14–20,27,28} In general, CSCs in OSCC can be isolated by either cell-surface markers or their unique functional properties.^{29–31} Nevertheless, no single marker and CSC property are capable of specifically isolating oral CSC populations from OSCC cells, suggesting the heterogeneity of CSC populations.^{32,33} Therefore, identification of additional oral CSC markers and their unique cellular characteristics is warranted.

Many studies have reported differential expression of CD44 on CSCs versus non-CSCs in various solid tumors,^{34,35} and such CD44⁺ CSC populations were successfully isolated from head and neck cancer by flow cytometry sorting using the CD44 antibody.^{25,27,28} CD44, a multifunctional transmembrane glycoprotein, binds to hyaluronan and behaves as a vital surface molecule that can interact with various intrinsic and extrinsic signals to regulate a variety of gene expressions. CD44⁺ cells can be fractionated from heterogeneous single-cell-prepared cancer cells using CD44 specific antibody labeling followed by flow cytometry sorting.³⁶ These CD44⁺ cells display discriminative traits of stem cells, including self-renewal capacity, high tumorigenic potential, and metastasis and drug resistance.^{34,35} When CD44 is experimentally suppressed, CD44⁺ CSCs diminish their stemness traits, indicating that CD44 expression is imperative for preservation of the CSC phenotype.³⁵

The activity of aldehyde dehydrogenase 1 (ALDH1) has been used as a CSC marker for different types of cancer, including OSCC.^{37–39} ALDH1 is a cytosolic isoenzyme responsible for oxidizing intracellular aldehydes, thus contributing to the oxidation of retinol to retinoic acid in early stem cell differentiation.⁴⁰ A subpopulation of cancer cells with enriched CSC activity shows high ALDH1 activity (ALDH1^{HIGH} or ALDH1⁺) compared to the non-CSC population, suggesting that ALDH1⁺ cells may be a source of CSCs.^{15,41,42} Moreover, ALDH1^{HIGH} cancer cells display enhanced CSC properties compared to ALDH1^{low} cells.^{43–45} Targeting ALDH1 significantly suppresses multiple CSC properties in human cancer cells.⁴⁶ However, it remains controversial as to whether CD44 or ALDH1 alone can be defined as a single molecule to identify CSCs. Thus, the combination of ALDH1 and CD44 has been commonly used as a marker for isolating CSCs in head and neck cancer cells.²⁵ Indeed, ALDH1^{HIGH}/CD44⁺ cancer cells display elevated CSC traits compared to those of ALDH1^{low}/CD44[–] cancer cells,^{47,48} indicating that the combination of CSC markers may improve specificity of CSC isolation.

CSCs can be enriched in nonadherent tumor spheres cultured in ultra-low attachment plates to support the undifferentiated growth of self-renewing stem cells.⁴⁹ The sphere medium is in a serum-free condition supplemented with adequate mitogens, such as basic fibroblast growth factor and epidermal growth factor (EGF).^{50–52} Abundance and growth kinetics of tumor spheres are indicative of self-renewal capacity in a given culture of heterogeneous cancer cells, indicating CSC content. Thus, the tumor sphere-forming assay is a common technique used to isolate CSCs from heterogeneous cancer cell populations by means of the unique functional trait of CSCs. Tumor sphere-forming cells identified in multiple primary tumors and cultured cancer cell lines displayed enhanced CSC characteristics compared to those of corresponding adherent monolayer cells that were considered to be non-CSCs.⁵³ Tumor sphere-forming cells possess high tumorigenicity, metastatic potential, and drug resistance and show the robust expression of stemness factors, suggesting their central role in pathogenesis and progression of cancer.^{54–59} Similarly, tumor spheres derived from OSCC cells display enhanced stem-like properties and express higher levels of pluripotent transcription factors including Nanog, Oct4, KLF4, Lin28, and Sox2, compared with their corresponding adherent monolayer cells.^{14,52,60,61} Oral tumor sphere-forming cells also express robust expression of CSC specific markers, such as CD44 and ALDH1.^{51,52,62} They are highly tumorigenic when inoculated into nude mice and maintain their self-renewal capacity for multiple generations.⁶²

III. PROPERTIES AND STEMNESS PATHWAYS OF ORAL CSCs

CSCs share common properties with normal stem cells and have multiple unique properties that maintain tumor growth and aggressiveness. A key feature of CSCs is their self-renewal capacity, which appears to be a driving force for initiating and maintaining tumorigenicity.⁶³ Self-renewal of CSCs can be maintained by several endogenous signaling pathways, such as Notch, Hedgehog, Wnt, B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi1), Pten, Bmp, and TGF- β ,^{64–70} which are frequently activated in human cancers.^{65,71,72} Among these pathways, the roles of Notch and Bmi1 signaling in oral cancer stemness have been extensively documented. Activation of the Notch1 signaling pathway is critical for the maintenance of CSCs and requires binding of its ligands Jagged 1 (JAG1),

JAG 2, and δ -like, followed by proteolytic release of the Notch intracellular domain (NICD) and activation of NICD downstream target genes.⁷³ Previously, we reported that prolonged exposure of OSCC cells to the proinflammatory cytokine TNF α enhances self-renewal capacity and tumorigenicity, which is associated with activation of the Notch pathway.⁵⁰ In TNF α -induced oral cancer stemness, Hes1 is the target of activated Notch1, and its knockdown suppresses self-renewal capacity of TNF α -treated OSCC cells. Hes1 is commonly expressed in most undifferentiated cell types in a developing mouse embryo and has a critical role in maintenance of progenitor cell fate. Hes1-deficient mice displayed premature differentiation, progenitor cell depletion, and consequent lethality.⁷⁴ Collectively, these findings suggest that the Notch1–Hes1 axis is a novel axis for regulating self-renewal of oral CSCs.

Bmi1, a member of the polycomb group transcription repressor, is implicated in oral cancer.^{75,76} Recent studies showed a critical role of Bmi1 in maintaining the self-renewal capacity of oral CSCs.^{77,78} Furthermore, using genetic lineage tracing, the *in vivo* role of Bmi1 in regulation of oral CSC stemness was clearly demonstrated, including its self-renewal and tumorigenic potential.⁷⁷

A unique property of CSCs is their metastatic potential.⁶³ Epithelial–mesenchymal transition (EMT) is known to confer migratory potential in cancer cells, and this process has crucial roles in cancer metastasis. EMT is a process by which epithelial cells lose their characteristics to gain the mesenchymal phenotype, thus leading to cell migration and invasion.^{79,80} During EMT, epithelium-specific protein expressions (e.g., cytokeratins and E-cadherin) are diminished, whereas expressions of mesenchymal-specific proteins (fibronectin, vimentin, and N-Cad) are elevated. Master transcription factors for EMT including SNAIL, TWIST, and LEF-1 have been identified, and their overexpression promoted EMT.^{81,82} Fractionated CSCs overexpress EMT transcription factors and demonstrate great *in vivo* metastatic potential compared to that in unfractionated cancer cells, suggesting that CSCs are the major source of the metastatic cancer cell population.⁸³ In addition, other reports also revealed the crucial roles of the zinc-finger E-box-binding homeobox (Zeb) in maintenance of CSC properties and EMT.⁸⁴ Zeb1 and Zeb2 are significantly increased in head and neck CSCs compared to those in non-CSCs.⁸⁵ Knockdown of Zeb1 and Zeb2 in head and neck cancer cells decreased their CSC properties such as migration, self-renewal capacity, and expression of stemness markers. Moreover, their suppression inhibited *in vivo* tumor growth and rate of metastasis to distant sites.⁸⁵ Conversely, co-overexpression of Zeb1 and Zeb2 enhanced the migration ability of head and neck cancer cells.⁸⁵

The CSC population can be enriched following chemoradiotherapy, suggesting that therapy results in chemoradioresistance and/or selectively enriches the resistant cell population. Various molecular determinants for CSC chemoradioresistance have been reported. Among these, the roles of adenosine triphosphate (ATP)-binding cassette (ABC) transporters are well documented to be key players in therapy resistance.⁸⁶ ABC transporters are membrane transporters that can pump various small molecules, for instance anticancer drugs, out of cells at the cost of ATP hydrolysis, thereby resulting in low intracellular drug concentrations. Overexpression of ABC transporters is a common occurrence observed in multidrug

resistance in cancer.⁸⁷ Normal cells and CSCs express high levels of ABC transporters, and overexpression of ABC transporters in cancer cells increased their chemoradioresistance.⁸⁸ Suppression of ABC transporters increases anticancer drug sensitivity in cancer.⁸⁹ These reports collectively indicate that ABC transporters are indeed key molecular determinants of CSC chemoradioresistance. Small populations of CSCs possessing high efflux capacity due to increased ABC transporters can be isolated by treatment of cells with Hoechst 33342 dye and then designated as side population (SP). Numerous studies have demonstrated successful isolation of CSCs using this technique, and SP cells harbor a greater capacity for the CSC phenotype than do non-SP cells.^{90,91} The presence of SP cells in oral cancer has been reported, and oral SP cells, when compared with non-SP cells, possess not only increased anticancer drug resistance but also the stem cell phenotype.^{91–93} Therefore, there is general consensus that CSCs are intrinsically resistant to chemoradiotherapy and contribute to tumor relapse.¹³

IV. ROLE OF HISTONE DEMETHYLASES IN THE REGULATION OF ORAL CANCER STEMNESS

Emerging evidence has indicated that oral CSCs could be epigenetically regulated by histone demethylases or microRNAs.^{51,94–96} A group of histone demethylases epigenetically modulated gene transcription by removing histone methylation marks.⁹⁷ As such, histone demethylases have a crucial role in governing gene transcription by altering chromatin accessibility and transcriptional machineries. Compelling evidence indicates that histone demethylases are implicated in various cellular processes, including carcinogenesis, cell fate choices, and cell differentiation.^{98–100} Recently, a growing body of evidence has indicated an important role of histone demethylases, including LSD1, JARID1, KMD3, KDM4, KDM5, KDM6A, KDM6B, and Jumonji domain-containing protein 6 (JMJD6), in the CSC phenotype in multiple cancer types.^{51,101–109}

JMJD6 is identified as a novel molecular regulator of oral CSCs.⁵¹ JMJD6 is a histone arginine demethylase that preferentially removes methyl groups from dimethylated arginine 2 of histone 3 (H3R2me2) and arginine 3 of histone 4 (H4R3me2),¹¹⁰ thereby enabling dynamic regulation of transcription. JMJD6 also regulates gene expression by modulating RNA splicing,¹¹¹ suggesting that JMJD6 is a multifaceted regulator of gene expression. Elevated JMJD6 expression has been reported in various human cancers, including breast cancer,¹¹² lung cancer,¹¹³ and colon cancer.¹¹⁴ A high expression of JMJD6 protein is also strongly linked to poor prognosis and aggressive behavior in human cancers. The level of JMJD6 in nonmalignant oral epithelial cell lines is much lower than that in OSCC cell lines *in vitro*.⁵¹ JMJD6 is highly expressed in OSCC compared to in normal tissues *in vivo*, indicating that JMJD6 plays an important part during oral carcinogenesis.

We have found that JMJD6 is enriched in OSCC CSC populations (i.e., tumor spheres and the ALDH1^{HIGH} cell population) compared to that in OSCC non-CSC populations (adherent monolayer cells and the ALDH1^{LOW} cell population).⁵¹ Silencing JMJD6 led to loss of self-renewal capacity, migration ability, and chemoresistance in OSCC cells. It was also reported that knockdown of JMJD6 in invasive breast cancer cell lines decreased cell migration, but

its overexpression promoted cellular motility.¹¹² JMJD6 interacts with splicing factor U2AF65 and modulates alternate splicing of the vascular EGF (VEGF) receptor.¹¹¹ Alternate splicing of the VEGF receptor by U2AF65 promoted endothelial cell migration, and silencing of JMJD6 in endothelial cells led to decreased migration.¹¹⁵ Thus, the effects of JMJD6 on EMT warrants investigation. Conversely, JMJD6 overexpression promotes not only such CSC properties but also the number of CSCs, indicating that JMJD6 is an important regulator of the CSC phenotype and genesis in OSCC. JMJD6 induces robust expression of pluripotent transcription factors and CSC-related genes, such as *Oct4*, *Lin28A*, *Lin28B*, *FGF4*, *Zeb1*, *Zeb2*, *Gli1*, and *Gli3* in OSCC.¹¹² Studies have shown the important role of these genes in acquisition and maintenance of CSC phenotype. For instance, Oct4 is detected in CSCs of various cancers including melanoma, prostate cancer, lung cancer, and oral cancer.^{14,116,117} Oct4 promotes self-renewal and the tumorigenic potential of melanoma cells.¹¹⁸ Lin28A is up-regulated in the CSC-enriched OSCC population, and overexpression of Lin28A in oral cancer cells increased their proliferation, colony formation, and migration.¹⁷ These CSC-related genes are up-regulated by JMJD6 overexpression and down-regulated by JMJD6 knockdown in OSCC cells. These results collectively suggest that JMJD6 enhances oral cancer stemness by modulating various CSC factors.

A recent study has shown a critical role for inflammatory cytokines in the tumor microenvironment and regulation of cancer stemness.¹¹⁹ Inflammatory cytokine TNF α enhances the CSC phenotype of OSCC.⁵⁰ Multiple cytokines (e.g., TNF α , IL4, etc.) are commonly up-regulated in OSCC CSCs compared to the non-CSC population (our unpublished data). IL4 is known to promote tumor formation by inhibiting apoptosis and enhancing proliferation.^{120,121} The autocrine production of IL4 by cancer cells confers resistance to chemotherapy-induced cell death¹²² and promotes tumor growth and metastasis.¹²⁰ Moreover, the production of IL4 in colon CSCs is responsible for their chemoresistance, which is a key CSC characteristic.^{123,124} Our study reveals that up-regulation of IL4 is an important event during JMJD6-induced self-renewal in OSCC CSCs. IL4 treatment rescues CSC properties suppressed by JMJD6 knockdown, and JMJD6 up-regulates IL4 by binding to the IL4 promoter region. Because CSCs interact with stromal cells in the tumor microenvironment, involvement of cytokines in this interaction is probable and also a crucial event for cancer progression and propagation. Collectively, these studies conclude that JMJD6 is a novel epigenetic regulator of CSC properties in OSCC by up-regulating IL4, suggesting that the JMJD6-IL4 axis could be an important therapeutic target in OSCC CSCs. Because chemical inhibitors readily inhibit histone demethylases, targeting JMJD6 may reveal a plausible therapeutic modality against oral CSCs.^{125,126}

V. ROLE OF CALCIUM CHANNELS IN REGULATING ORAL CANCER STEMNESS

Recent studies suggested the importance of calcium signaling in regulating oral cancer stemness properties.^{52,127–129} Ca²⁺ is a universal second messenger that regulates many physiological processes, and its homeostasis is disrupted during carcinogenesis, thereby leading to deregulated cell proliferation, migration, and suppression of apoptosis.^{130–133} In most nonexcitable cells, Ca²⁺ influx is tightly regulated by the store-operated Ca²⁺ entry

(SOCE) pathway and mediated through store-operated Ca^{2+} release-activated Ca^{2+} (CRAC) channels.¹³⁴ Orai1 is an essential pore subunit of CRAC channels.^{135–137} After stimulation, cells release Ca^{2+} from the endoplasmic reticulum (ER), followed by extracellular Ca^{2+} influx through SOCE. SOCE not only refills the depleted ER Ca^{2+} stores but also provides a direct Ca^{2+} signal to activate downstream responses, including the nuclear factor of activated T-cells (NFAT) signaling pathway.^{138,139} Orai1 has been extensively studied in immunology because NFAT is the transcription factor that is necessary for activation, differentiation, and effector functions of T-cells.¹⁴⁰ Emerging evidence indicates the crucial role of Orai1 in carcinogenesis.^{128,141–150}

Elevated expression of Orai1 is observed in various human cancers, including head and neck cancer,^{52,128,145,147,150} and strongly linked to poor overall and recurrence-free survival in human cancers.^{145,150} Orai1 is required for tumor growth.^{128,142–146,151} Our recent study revealed that Orai1 is increased in a stepwise manner during oral carcinogenesis.⁵² Orai1 is highly expressed in OSCC compared to precancerous and normal tissues *in vivo*. Precancerous oral epithelial cells express higher Orai1 protein than normal oral epithelial cells. Inhibition of Orai1 function by expressing the dominant negative Orai1 E106Q mutant¹³⁷ in OSCC cells abolishes SOCE and subsequently inhibits tumorigenic potential *in vitro* and *in vivo*. Conversely, ectopic Orai1 expression successfully converts nontumorigenic oral epithelial cells into tumorigenic cells. These findings indicate that Orai1 is required for tumorigenicity of OSCC.

Orai1 is highly expressed in CSC-enriched cell populations, such as tumor spheres and the ALDH1^{HIGH} population of OSCC.⁵² Furthermore, Orai1 can endow nontumorigenic immortalized oral epithelial cells with self-renewal and concomitantly increases pluripotent transcription and CSC-related factors, including Nanog, Oct4, Sox2, KLF4, Bmi1, Zeb1, and Zeb2. Ectopic Orai1 expression increases the ALDH1⁺ CSC population in nontumorigenic oral epithelial cells and promotes metastatic potential of OSCC. The latter finding is consistent with other reports showing the importance of Orai1 in the migration ability of invasive breast cancer cells.¹¹² In multiple OSCC cell lines, inhibition of Orai1 led to suppression of CSC properties. Thus, it is hypothesized that Orai1 promotes malignant progression of OSCC by enriching the CSC phenotype. However, the underlying mechanism by which Orai1 regulates oral cancer stemness remains largely unknown.

The main downstream target of Orai1-mediated Ca^{2+} is NFAT, which is dephosphorylated by a protein phosphatase complex of calmodulin and calcineurin.^{152,153} This process results in translocation of cytoplasmic NFAT to the nucleus. Once in the nucleus, NFAT binds to target promoter elements and induces gene transcription. Four members of the NFAT family (i.e., NFATc1–c4) that are regulated by Ca^{2+} signaling have been identified.¹⁵⁴ They share a highly conserved DNA-binding domain but depend on interacting partners including other transcription factors and coactivators for their target gene specificity. It is known that NFAT has an important role in tumorigenesis by regulating various target genes involved in cancer development.^{155,156} For instance, NFATc1 induced the malignant cell growth phenotype in pancreatic cancer cells by up-regulating Myc¹⁵⁷ and promoted metastasis of mammalian cancer cells via MMP-2 up-regulation.^{158–160} NFATc2 was overexpressed in multiple cancer types,^{159,160} and its depletion suppressed the migration/invasion of cancer cells.¹⁵⁹ In

addition, the crucial role of NFAT in maintenance of CSCs was reported in human cancers, including melanoma and colonic, pancreatic, and lung.^{161–164} In our unpublished study, we found that NFATc3, among four NFAT isoforms, is the dominant isoform in human oral epithelial cells and greatly increases in OSCC compared to normal oral epithelial cells. Interestingly, Orai1-mediated SOCE activates NFATc3.⁵² Silencing NFATc3 in cells with ectopic Orai1 overexpression in OSCC cells led to suppression of the CSC phenotype. An NFAT chemical inhibitor also successfully inhibited cancer stemness in cells. These results indicate that NFATc3 is required for the Orai1-induced CSC phenotype, suggesting the functional role of the Orai1/NFATc3 axis in regulation of oral CSC.

Ca²⁺ oscillation (spatiotemporal regulation calcium signaling) is more essential than global changes in cytosolic Ca²⁺ concentration in the context of tumor invasion, growth, and cancer stemness.^{127–129} Ca²⁺ oscillation is the end result of Orai1-mediated SOCE, and Orai1 is enriched in oral CSCs. Because suppression of Orai1 channel function resulted in complete shutdown of Ca²⁺ oscillation in OSCC cells, Orai1-mediated Ca²⁺ oscillation could be a potential selective target for treatment of oral CSCs.

VI. CONCLUSIONS

The pathophysiologic role of CSCs in sustaining and maintaining cancers is increasingly evident in numerous studies. These small subpopulations of tumor cells are responsible for tumorigenicity, metastasis, and recurrence and are thus considered to be the root of the cancer. Our understanding of the molecular signaling pathways specific to CSCs, such as histone modifications and calcium regulations, may provide unique opportunities to develop new strategies in targeting CSCs for cancer treatment.

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ABBREVIATIONS:

ABC	adenosine triphosphate (ATP)-binding cassette
ALDH1	aldehyde dehydrogenase 1
CSC	cancer stem cell
EMT	epithelial–mesenchymal transition
JMJD6	Jumonji domain–containing protein 6
NFAT	nuclear factor of activated T cells
OSCC	oral/oropharyngeal squamous cell carcinoma
SOCE	store-operated Ca ²⁺ entry
SP	side population

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