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Orai1 and STIM1 are critical for cell migration and proliferation of clear cell renal cell carcinoma

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ABSTRACT

The intracellular Ca^{2+} regulation has been implicated in tumorigenesis and tumor progression. Notably, store-operated Ca^{2+} entry (SOCE) is a major Ca^{2+} entry mechanism in non-excitable cells, being involved in cell proliferation and migration in several types of cancer. However, the expression and biological role of SOCE have not been investigated in clear cell renal cell carcinoma (ccRCC). Here, we demonstrate that Orai1 and STIM1, not Orai3, are crucial components of SOCE in the progression of ccRCC. The expression levels of Orai1 in tumor tissues were significantly higher than those in the adjacent normal parenchymal tissues. In addition, native SOCE was blunted by inhibiting SOCE or by silencing Orai1 and STIM1. Pharmacological blockade or knockdown of Orai1 or STIM1 also significantly inhibited RCC cell migration and proliferative capability. Taken together, Orai1 is highly expressed in ccRCC tissues illuminating that Orai1-mediated SOCE may play an important role in ccRCC development. Indeed, Orai1 and STIM1 constitute a native SOCE pathway in ccRCC by promoting cell proliferation and migration.

1. Introduction

The ubiquitous second messenger Ca^{2+} regulates various cellular processes such as proliferation and migration [1–5]. The Ca^{2+}-mediated signaling pathways have been implicated either directly or indirectly in tumorigenesis and tumor progression [1,2,4]. Store-operated Ca^{2+} entry (SOCE) is a major Ca^{2+} entry mechanism in non-excitable cells [5] including epithelial cells which are the most common origin of cancer [2]. The canonical components of SOCE are Orai and STIM proteins [5]. Orai proteins (Orai1-3) are pore-forming subunits in the plasma membrane, and STIM1 is a Ca^{2+} sensor in the endoplasmic reticulum. STIM1 is oligomerized and translocated to the plasma membrane during Ca^{2+} store depletion, thereby triggering Ca^{2+} entry via Orai1 [5]. Orai1 and STIM1 are involved in a variety of physiological and pathological processes including tumor progression [2,5].

Altered expression and function of SOCE such as Orai1, 3 and STIM1 are characterizing features of several types of cancer. Orai1 exaggerates cell proliferation, migration, invasion and evasion of apoptosis in breast cancer [6], glioblastoma [7], prostate cancer [8] and hepatocellular carcinoma (HCC) [9] in a STIM1-dependent manner. Orai1 also upregulates tumorigenesis of mammary tumors [10,11] independently of STIM1. Similarly, overexpression of STIM1 accelerates tumor growth, angiogenesis and migration in a cervical cancer [12] and tuberous sclerosis complex (TSC)-related tumor [13], independently of Orai1 expression. Another pore-forming subunit of SOCE, Orai3 constitutes a native SOCE pathway in the estrogen receptor α (ERα)-positive breast cancer [14–16] and non-small cell lung carcinoma (NSCLC) cells [17]. Orai3 expression and Orai3-mediated SOCE are positively correlated with tumor progression as well as Orai1 and STIM1.

Renal cell carcinoma (RCC) is the most common type of kidney cancer, accounting for 2–3% of all malignancy in adults [18,20] with increasing incidence [19,20]. The most common subtype of RCC is clear cell renal cell carcinoma (ccRCC) [20]. Though surgical procedures remain the gold standard for the treatment of localized RCC, 20–40% of patients with initially localized RCC develop

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metastasis after curative surgery [18,20,21]. Also, one third of patients with RCC already have metastasis at the time of diagnosis. Unlike localized RCC, metastatic RCC has a poor prognosis because it is resistant to radiotherapy and chemotherapy [20] and there is no specific therapeutic target for RCC up to date.

Although intracellular Ca^{2+} signaling has a crucial role in cancer hallmarks, little is known about the pathological function of Ca^{2+} dysregulation and expression of Ca^{2+}-permeable channels involved in ccRCC biology. Therefore, we examined the expression of specific components of SOCE (Orai1, Orai3 and STIM1) which are implicated in tumor progression. We also studied the biological role of Orai1 and STIM1 in ccRCC cell migration and proliferation.

2. Materials and methods

2.1. Patients and tissue samples

We collected tissue samples from patients with ccRCC who had undergone radical surgery at the Yonsei University Wonju Severance Christian Hospital. Formalin fixed paraffin embedded (FFPE) and frozen fresh tissue were collected for immunohistochemistry (IHC) and immunoblotting assay, respectively. We reviewed pathologic reports and clinical records. The collection of surgical specimens was approved by the institutional review board of Yonsei University Wonju College of Medicine (YWMR-12-0-014).

2.2. Cell culture, siRNA and drugs

The stable cell lines of ccRCC, ACHN and Caki1, were cultured under high glucose DMEM medium supplemented with 10% FBS and 1% penicillin at 37 °C in a humidified atmosphere. Oligonucleotides for non-targeting control and human Orai1 and STIM1 siRNA was previously described [14]. For knockdown by siRNA, sense and antisense oligonucleotides (20 nM final concentrations) were introduced to ccRCC cells with DharmaFect, (Thermo Scientific, Lafayette, CO, USA) following the manufacturer's instructions. 2-Aminoethyl diphenylborinate (2-APB, Sigma–Aldrich, St. Louis, MO, USA) and SKF96365 (Tocris, Ellisville, MO, USA) were used as pharmacological inhibitors of SOCE.

2.3. Immunohistochemistry

FFPE samples were used for IHC. The 4 μm sections of paraffin blocks were cut and attached onto coated slides using the automatic immunostaining machine (Ventana Benchmark XT, Roche Diagnostics, Basel, Switzerland). The sections were deparaffinized in xylene, rehydrated in graded alcohols, and subjected to pretreatment with CC1 (Roche Diagnostics). The sections were washed with reaction buffer followed by incubation with primary antibodies against Orai1, Orai3 and STIM1 (Abcam, Cambridge, MA, US), in dilution of 1:100, 1:50, and 1:100, respectively, for 60 min at room

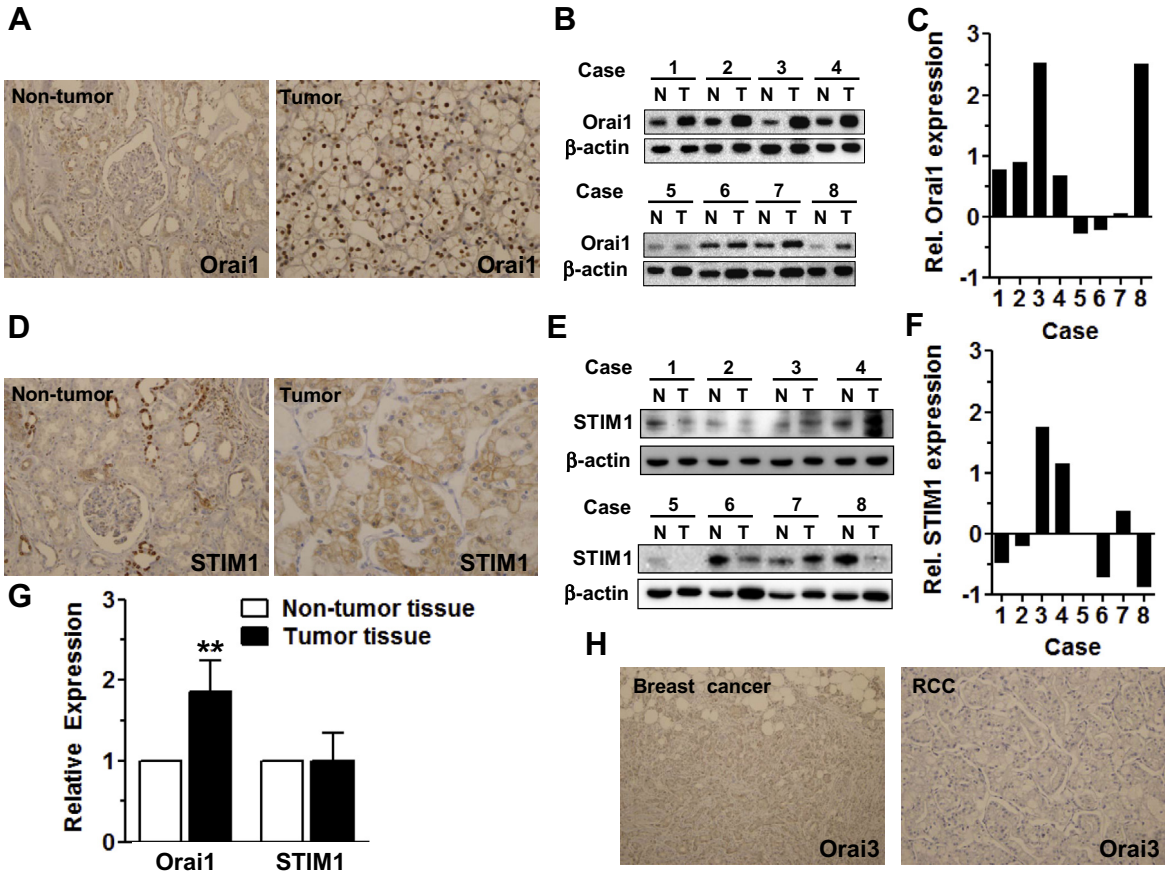

Fig. 1. Orai1, but not STIM1 and Orai3, is overexpressed in ccRCC. (A) IHC of Orai1 in normal renal parenchyma (Non-tumor) and adjacent tumor tissues (Tumor) from ccRCC patients. (B) Expression pattern of Orai1 in ccRCC. Individual ccRCC with the pair tissues of tumor (T) and adjacent non-tumor (NT) were analyzed by Western blotting. Case, individual patient case number. (C) Quantitative analysis of results in panel 1B. Orai1 expression level in normal tissue was used as control and those in tumor tissues were expressed as relative of control. Positive and negative values reflect overexpression or downexpression, respectively. (D) IHC of STIM1 in normal (Non-tumor) and adjacent tumor tissues (Tumor) from ccRCC patients. (E) Representative immunoblotting of STIM1 in individual pair tissues of ccRCC. (F) Quantitation of the results in panel 1E. Relative STIM1 expression levels were analyzed the same way as the panel 1C. (G) Summary of relative expression level of Orai1 and STIM1 from pair tissues of 18 ccRCC patients. A double asterisk denotes $p < 0.01$ versus adjacent non-tumor tissues. (H) IHC of Orai3 in breast cancer (left panel) and ccRCC (right panel) tissues. All photos were taken with ×400 magnification.

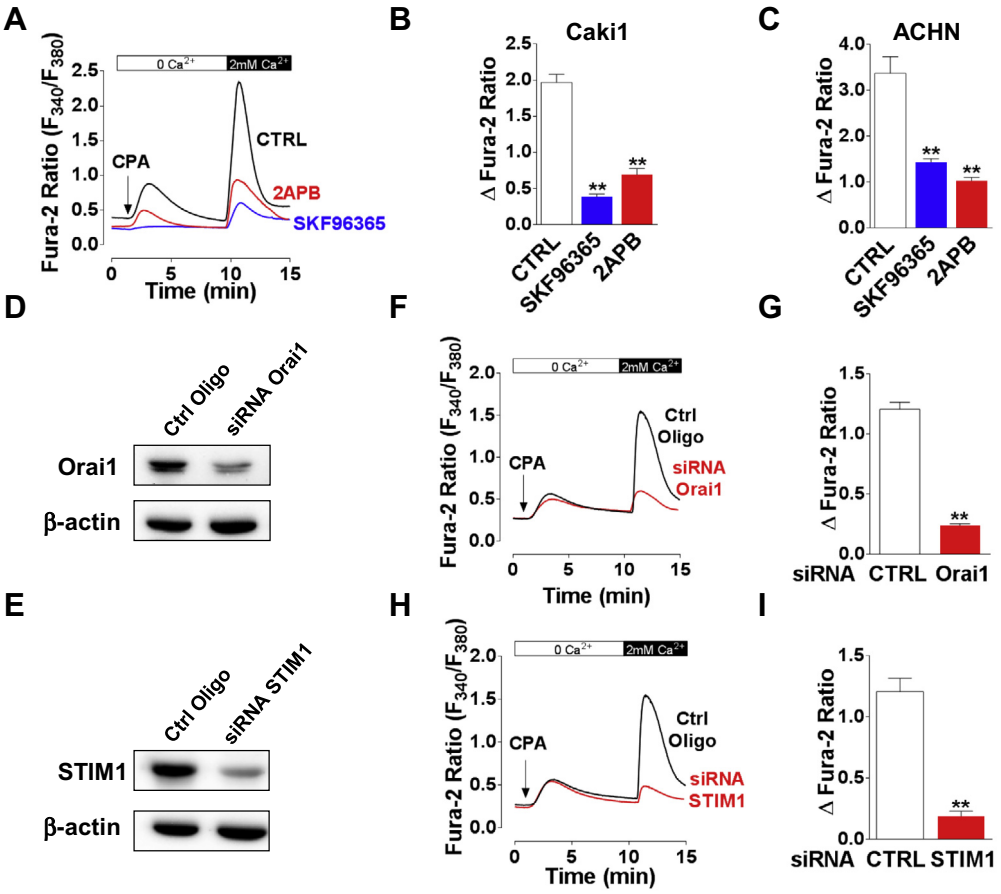

Fig. 2. Orai1 and STIM1 constitute a native SOCE in ccRCC. To record SOCE, cells were stimulated by 10 μM CPA in the a Ca^{2+}-free physiological salt solution to passively deplete the intracellular Ca^{2+} stores followed by application of 2 mM Ca^{2+} in bath. (A) Representative traces illustrating time course of SOCE with or without either SKF96365 (50 μM) or 2-APB (50 μM) in Caki1 cells. (B and C) Summary of effect of SKF96365 and 2-APB on SOCE in Caki1 and ACHN RCC cell lines, respectively. ΔFura-2 ratio denotes change of $[Ca^{2+}]_i$ induced by SOCE. Double asterisk denotes $p < 0.01$ versus vehicle (CTRL). (D and E) Successful knockdown of Orai1 and STIM1 (against Orai1 and STIM1, 2D and 2E, respectively) were evident by western blot analysis. (F and H) Representative traces showing SOCE recorded in cells transfected with control oligo and siRNA against Orai1 or STIM1, respectively. (G and I) Summary of results in panel 2F and 2H, respectively. A double asterisk denotes $p < 0.01$ versus control oligo (CTRL). Data points (B, C, G and I) are mean ± SEM. Knockdown effect in ACHN cells is similar to that of Caki1 cell (data not shown). All experiments were performed at least 4–5 times with similar results.

temperature. The antibodies were detected with the Ultra View Universal DAB kit (Roche Diagnostics) and counterstained with hematoxylin (Roche Diagnostics). A positive and negative control stains were also performed according to the manufacturer's recommendation.

2.4. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurement was previously described [22]. A normal physiological salt solution used for bath solution that contained (in mM) 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.4). Fura-2 signals were obtained by alternating excitation at 340 or 380 nm, and detecting emission at 510 nm. Data were analyzed using MetaFluor (Sutter Instruments) software.

2.5. Western blots

For immunoblotting, fresh ccRCC tissues were lysed using PRO-PREP lysis buffer (iNtRon Biotechnology, Daejeon, Korea). Equal amounts of protein lysates were separated by SDS–PAGE, and transferred to the immobilon-P membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk and incubated with primary antibodies, and incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies. Bands in the immunoblotting were detected and quantified using Biospectrum Imaging System (UVP, Upland, CA, USA) and Image J software (NIH, USA), respectively.

2.6. In vitro scratch assay

Scratch assay (wound-healing assay) was performed as previously described with minor modifications [23]. Caki1 and ACHN cells were plated at 1×10^7 and 1×10^6 per well in a 6-well plate, respectively, and grown to confluence. Cells were scratched with a 200 μl pipette tip across the center of the wells. In order to distinguish cell migration from proliferation, all wound-healing assay were performed in the presence of antitumor drug mitomycin C (Sigma–Aldrich, final concentration of 0.1 μg/ml in Caki1 and 0.5 μg/ml in ACHN cells) [24].

2.7. Colony formation and cell viability assays

In order to carry out the colony formation assay, ccRCC cells were first transfected with Orai1, STIM1 and control siRNA. One day after transfection, cells were trypsinized and re-plated into new 6-well plates (100 cells/well) and allowed to grow for ten days until they formed colonies. Cells were also cultured in the same maneuver in the presence of SKF96365 and 2-APB, SOCE

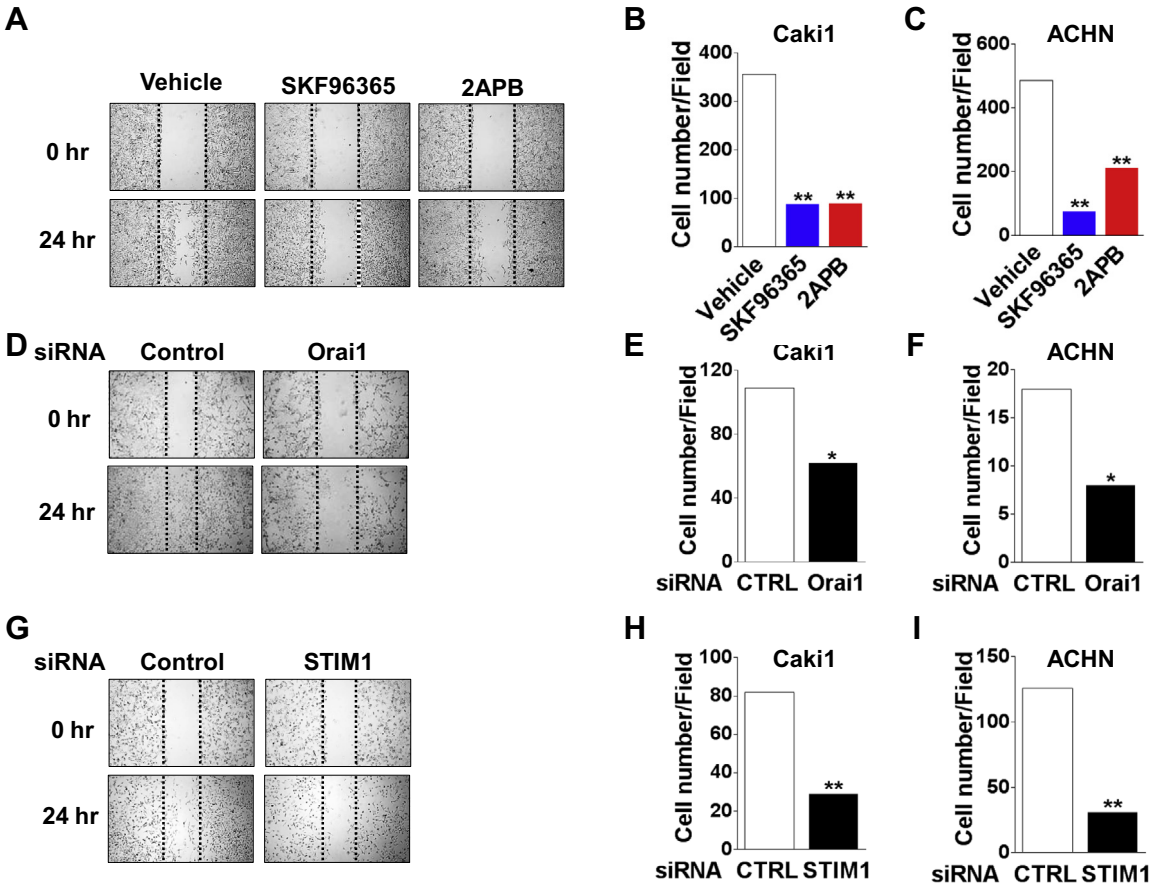

Fig. 3. Orai1 and STIM1 are crucial for ccRCC cell migration. RCC cell migration was examined using wound-healing assay which was performed in the presence of mitomycin C. (A) Representative photos of wound-healing assays showing that either SKF96365 (50 μM) or 2-APB (50 μM) inhibited the migration of Caki1 cells. (B and C) Summary of effect of SKF96365 and 2-APB on migration of Caki1 and ACHN cells, respectively. Double asterisk denotes $p < 0.01$ versus vehicle. (D) Wound-healing assays showing Orai1 silencing effect on Caki1 cell migration. (E and F) Summary of knockdown effect of Orai1 on migration of Caki1 and ACHN cells, respectively. An asterisk denotes $p < 0.05$ versus control oligo. (G) Effects of silencing of STIM1 on Caki1 cell migration. (H and I) Summary of STIM1 knockdown effect on migration of Caki1 and ACHN cells, respectively. Double asterisk denotes $p < 0.01$ versus control oligo. Data points (B, C, E, F, H and I) are mean ± SEM. All experiments were performed 3–4 times with similar results.

blockers. Medium was changed every three days, and colonies were visualized with 1% methylene blue.

For cell viability assay, cells were transfected with Orai1 and STIM1 siRNA alongside control siRNA. Two days after transfection, cells were trypsinized and re-plated into new 6-well plates (400 cells/well). After 9 days, cells were counted using a hemocytometer.

2.8. Data analysis

Data analysis was performed using the GraphPad Prism Software (version 5.0, GraphPad Software, San Diego, CA, USA). Statistical comparisons between two groups of data were made using a two-tailed unpaired Student's *t*-test. Multiple comparisons were determined using one-way ANOVA followed by Tukey's multiple comparison tests. *p* values less than 0.05 and 0.01 were considered significant for single and multiple comparisons, respectively. Data were presented as mean ± SEM.

3. Results

3.1. Orai1 is highly expressed in clear cell renal cell carcinoma (ccRCC)

Expression of Orai1 and STIM1, the canonical components of SOCE, was altered in many types of cancer. To explore whether Orai1 and STIM1 are involved in ccRCC development, the expression of Orai1 and STIM1 was analyzed from pair tissues of ccRCC (tumor and adjacent normal renal parenchyma) using IHC and immunoblotting. In IHC, Orai1 was expressed in both tumor and normal renal parenchyma (Fig. 1A). The immunoreactivity of Orai1 was much higher in tumor tissues with nuclear staining pattern, compared with that in the adjacent normal parenchymal tissues including podocytes of glomeruli, tubular epithelium and endothelial cells of blood vessels (Fig. 1A). Next, the expression pattern of Orai1 in individual pair tissues was examined by immunoblotting. In comparison to the protein level of Orai1 in non-tumor tissues, the expression level of Orai1 in ccRCC tissues was markedly increased (Fig. 1B and C).

As well as Orai1, STIM1 was expressed in tumor and adjacent normal tissues (Fig. 1D). However, the intensity and pattern of STIM1 immunoreactivity between tumor and adjacent non-tumor tissues showed no differences in IHC (Fig. 1D). We next examined the STIM1 protein level in individual pair tissues. Compared with that of non-tumor tissue, the expression level of STIM1 was not changed in tumor tissue (Fig. 1E and F). We further compared the relative expression levels of Orai1 and STIM1 in 18 pair tissues using immunoblotting. As summarized in Fig. 1G, the level of Orai1 expression in tumor tissues was significantly higher than that of normal tissues whereas STIM1 expression was not different between normal and tumor tissues.

In addition, Orai3 is a part of a native SOCE pathway in ERα-positive breast cancer cells and NSCLC [14–17]. Those studies on

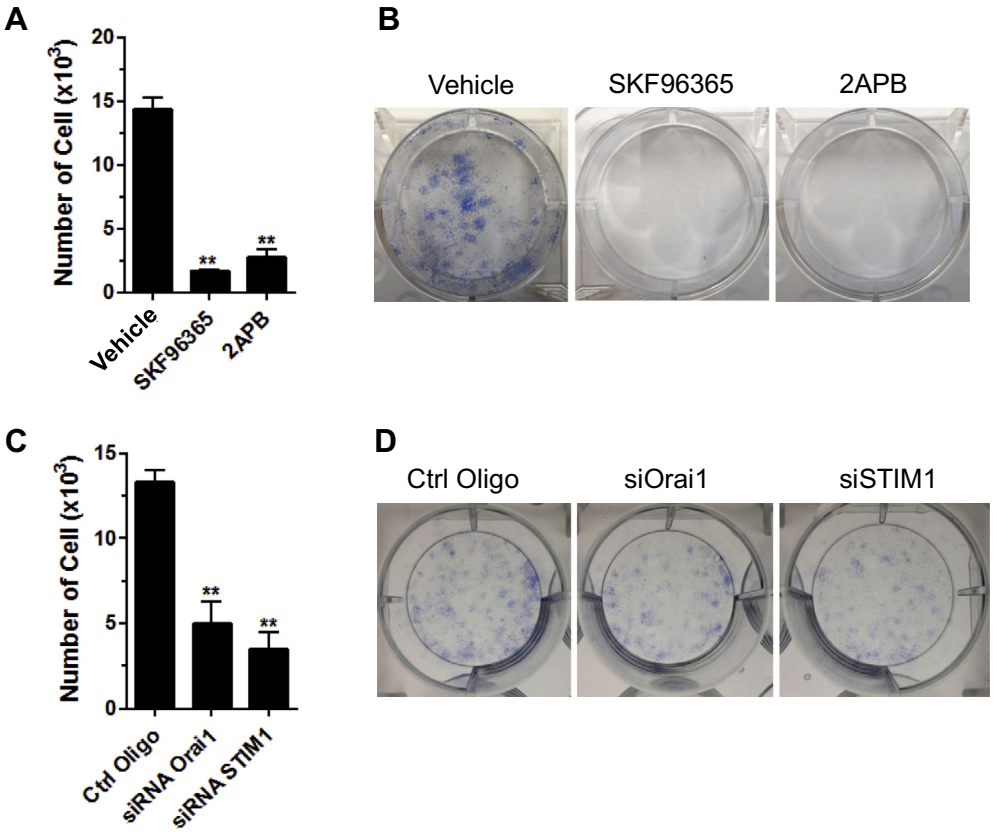

Fig. 4. Orai1 and STIM1 are involved in ccRCC cell proliferation. (A) Effect of SKF96365 (5 μM) or 2-APB (30 μM) on Caki1 cell viability. Double asterisk denotes $p < 0.01$, versus vehicle. (B) Proliferative capability of Caki1 cells was assessed by colony forming assay in the presence of SKF96365 or 2-APB. (C) Effect of silencing Orai1 and STIM1 on Caki1 cell viability. Double asterisk denotes $p < 0.01$, versus control oligo (Ctrl oligo). (D) Colony forming assay showing the knockdown effect of Orai1 or STIM1 on proliferative capability of Caki1 cells. Data points (A and C) are mean ± SEM. All experiments were performed at least 3–4 times with consistent results.

Orai3 prompted us to investigate the Orai3 expression in ccRCC. Orai3 was expressed in breast cancer tissues (Fig. 1H) as reported previously, but the immunoreactivity of Orai3 was negligible in ccRCC tissues (Fig. 1H). Taken together, these data elucidate that Orai1 overexpression may contribute to the development of ccRCC.

3.2. Orai1 and STIM1 are responsible for native SOCE in ccRCC

We next characterized properties of SOCE in RCC cell lines, Caki1 and ACHN, using Fura-2 imaging. All three Orai homologs exhibit different pharmacological sensitivity [25]. Hence, we examined pharmacological sensitivity of SOCE using its blockers, SKF96365 and 2-APB (Fig. 2A–C). Native SOCE mediated by Orai1 has been inhibited by both 2-APB and SKF96365, whereas Orai3 has been activated by 2-APB or inhibited by SKF96365 [25]. CPA-induced SOCE activation was blocked by both SKF96365 and 2-APB in both Caki1 and ACHN cells (Fig. 2A–C). These pharmacological profiles indicate that SOCE is functional in ccRCC. To determine the molecular identity of SOCE, knockdown experiment was performed using small interfering RNA (siRNA) against Orai1 and STIM1. Immunoblotting analysis confirmed successful knockdown of endogenous Orai1 or STIM1 in ccRCC cells (Fig. 2D and E). SOCE was abrogated by silencing Orai1 or STIM1 (Fig. 2F–I), supporting that Orai1 and STIM1 are pivotal molecular identities of SOCE in ccRCC.

3.3. Orai1 and STIM1 are critical for ccRCC cell migration

SOCE has been considered as a major Ca^{2+} influx pathway involved in cancer cell migration and metastasis [6,10,12]. Thus, we investigated whether SOCE is critical for ccRCC cell migration via wound-healing assay. All wound-healing assays were performed after treating with mitomycin C to exclude the effect of cell proliferation [24]. Migration ability of Caki1 and ACHN cells was blunted by SOCE inhibitors, SKF96365 and 2-APB (Fig. 3A–C). We also found that knockdown of endogenous Orai1 or STIM1 inhibited migration of Caki1 and ACHN (Fig. 3D–I), signifying that Orai1 and STIM1 contribute to ccRCC migration.

3.4. Orai1 and STIM1 regulate proliferative capability of ccRCC cells

The role of SOCE mediated by Orai1 and/or STIM1 in regulating cancer cell proliferation remains still controversial. Cell growth rate of Caki1 was markedly reduced by blockade of SOCE by SKF96365 or 2-APB (Fig. 4A). Reduction of proliferation capability was further confirmed by colony formation assay. As shown in Fig. 4B, colonigenicity of Caki1 was also significantly decreased by SOCE blockers. Knockdown of Orai1 or STIM1 also reduced cell growth rate and proliferation of Caki1 cell confirmed by cell viability and colony forming assays (Fig. 4C and D). All in all, these results indicate that Orai1 and STIM1-dependent pathway plays a crucial role in ccRCC cell proliferation.

4. Discussion

Deregulation of Ca^{2+} signaling pathways has been implicated in tumorigenesis and tumor progression [1–4]. However, Ca^{2+} signaling in regulating ccRCC progression remains elusive. Moreover, the level of expression and activity of SOCE components in ccRCC have not been investigated. Deregulation of SOCE involved in tumor progression is possibly attributed to the altered expression and

activity of molecular components of SOCE. Orai1 and Orai3 are highly expressed in ERα-negative and ERα-positive breast cancer cells, respectively, [14,15] while STIM1 is overexpressed in cervical cancer and HCC cells [9,12]. As several observations argue that essential components of SOCE and their activation mechanism depend on the types of cancer cells, we examined ccRCC. In ccRCC, we first revealed that the expression levels of Orai1, but not STIM1 and Orai3, were altered. Orai1 is expressed in both normal and tumor tissues, but its expression in tumor tissues was much higher than that of adjacent normal tissues suggesting that the increased Orai1 expression may lead to ccRCC development. These results also raise the possibility that Orai1 expression could be a potential prognostic marker for ccRCC. Whether the level of Orai1 expression is related with clinicopathology and survival of ccRCC patients awaits future investigation.

Orai1, along with STIM1, was shown to be the predominant components of native SOCE in many cancer cells, including breast, glioblastoma, and cervical cancer [6,7,12]. Orai1 also mediates STIM1- and store-independent Ca^{2+} influx via the secretory pathway Ca^{2+}-ATPase (SPCA2) in mammary tumors [10]. On the other hand, Orai3 constitutes a native SOCE in ERα-positive breast cancer and NSCLC that regulates their progression [14–17]. In ccRCC cells, SOCE was markedly inhibited by silencing Orai1 and STIM1 or by applying 2-APB which is known as Orai3 activator [25]. Our results suggest that Orai1 and STIM1 not Orai3, are predominant molecular components of native SOCE in ccRCC cells.

Multiple studies have reported that Orai1 and STIM1 are crucial for cancer cell migration and proliferation [2–4]. Orai1/STIM1 pathway stimulated migration and invasion in various cancer cells [6,7,9,12,13]. Consistent with these observations, we found that Orai1/STIM1-mediated SOCE was critical for ccRCC cell migration. Notably, Orai1 and STIM1 upregulated ccRCC cell motility independently of cell proliferation (Fig. 3). Orai1 along with STIM1 stimulated migration in most cancer cells [6,15]. Contrast to migration, the role of Orai1/STIM1 pathway in regulating cancer cell proliferation remains controversial. Orai1 stimulated cell proliferation of mammary and breast tumors independently of STIM1 [10,11]. In HEK293 cell, a non-tumor cell line, Orai1 and not STIM1, but STIM2 regulate cell proliferation [26]. Moreover, Orai1 overexpression inhibited cell proliferation via attenuation of SOCE-mediated signaling pathway in A549 lung cancer cells [27]. Our results clearly show that Orai1 and STIM1 upregulate ccRCC cell proliferation. Strikingly, the inhibitory effect of SKF96365 on proliferation was much higher than that of silencing Orai1 or STIM1. Indeed, proliferation of ACHN cell has been stimulated by TRPC6 whose activity is also blocked by SKF96365 [28]. Thus, it is conceivable that TRPC6 may also regulate proliferation of RCC as well as Orai1/STIM1 pathway. Our study expands the list of Ca^{2+}-permeable channels that are potentially involved RCC development. The list will continue to grow.

In conclusion, our study demonstrates that Orai1 is highly expressed in ccRCC tissues suggesting that Orai1 is involved in RCC development. Moreover, Orai1/STIM1 pathway constitutes a native SOCE functioning as a major regulator of migration and proliferation in ccRCC. Our study opens the possibility that selective Orai1 overexpression and Orai1/STIM1-dependent signaling could be potential prognostic markers and attractive targets for therapeutic intervention in ccRCC.

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