Roles of store-operated Ca\(^{2+}\) channels in regulating cell cycling and migration of human cardiac c-kit\(^{+}\) progenitor cells

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Che H, Li G, Sun HY, Xiao GS, Wang Y, Li GR. Roles of store-operated Ca\(^{2+}\) channels in regulating cell cycling and migration of human cardiac c-kit\(^{+}\) progenitor cells. Am J Physiol Heart Circ Physiol 309: H1772–H1781, 2015. First published October 9, 2015; doi:10.1152/ajpheart.00260.2015.—Cardiac c-kit\(^{+}\) progenitor cells are important for maintaining cardiac homeostasis and can potentially contribute to myocardial repair. However, cellular physiology of human cardiac c-kit\(^{+}\) progenitor cells is not well understood. The present study investigates the functional store-operated Ca\(^{2+}\) entry (SOCE) channels and the potential role in regulating cell cycling and migration using confocal microscopy, RT-PCR, Western blot, immunoprecipitation, cell proliferation, and migration assays. We found that SOCE channels mediated Ca\(^{2+}\) influx, and TRPC1, STIM1, and Orai1 were involved in the formation of SOCE channels in human cardiac c-kit\(^{+}\) progenitor cells. Silencing TRPC1, STIM1, or Orai1 with the corresponding siRNA significantly reduced the Ca\(^{2+}\) signaling through SOCE channels, decreased cell proliferation and migration, and reduced expression of cyclin D1, cyclin E, and/or p-Akt. Our results demonstrate the novel information that Ca\(^{2+}\) signaling through SOCE channels regulates cell cycling and migration via activating cyclin D1, cyclin E, and/or p-Akt in human cardiac c-kit\(^{+}\) cells.

NEW & NOTEWORTHY

The present study demonstrates that Ca\(^{2+}\) signaling through SOCE channels regulates cell cycling and migration via activating cyclin D1, cyclin E, and/or p-Akt in human cardiac c-kit\(^{+}\) cells. Understanding the regulation of these cellular functions by SOCE channels is beneficial for developing new approaches to improve myocardial repair/regeneration.

CARDIAC C-KIT\(^{+}\) PROGENITOR CELLS are located in the myocardial interstitium and assembled with other cells to maintain the cardiac homeostasis (15, 39). It has been recognized that c-kit\(^{+}\) progenitor cells communicate with surrounding cardiomyocytes and fibroblasts, asymmetrically differentiate into committed cells (5), and therefore regulate cardiomyogenesis during heart development and provide various cardiac cell progenies in the developed heart (17). The stimulated expansion of in situ c-kit\(^{+}\) progenitor cell expansion or injection of in vitro expanded c-kit\(^{+}\) progenitor cells to infarcted myocardium has been found to enhance cardiac repair and improve heart function and survival after myocardial infarction (15, 53, 56).

However, cellular physiology and biology of cardiac c-kit\(^{+}\) progenitor cells are not well understood. It is well known that intracellular Ca\(^{2+}\) (Ca\(^{2+}\)i) is an important second messenger that plays an essential role in regulating many cellular activities, e.g., muscle contraction, gland secretion, cell growth, differentiation, migration, etc. (9). In general, Ca\(^{2+}\)i signals are mediated by different pathways, including membrane ion channels, e.g., L-type Ca\(^{2+}\) channels, transient receptor potential (TRP) channels, membrane Ca\(^{2+}\) pump, Na\(^{+}\)-Ca\(^{2+}\) exchanger, intracellular Ca\(^{2+}\) store ER/SR (endoplasmic reticulum/sarcoplasmic reticulum), store-operated Ca\(^{2+}\) entry (SOCE) channels, etc. (18). SOCE channels mediate Ca\(^{2+}\) signaling in nonexcitable cells (14). It is activated by Ca\(^{2+}\) store depletion of ER, and the opening of SOCE channels allows the replenishment of the Ca\(^{2+}\) store (6). SOCE channels consist of STIM1 (stromal interaction molecule 1) located on ER, Orai1 (Ca\(^{2+}\) release-activated Ca\(^{2+}\) modulator 1), and TRPC (TRP canonical) channels on plasma membrane. STIM1 can sense the Ca\(^{2+}\) concentration in the ER and interact with Orai1 when Ca\(^{2+}\) concentration is decreased (19). The participation of TRPC channels in SOCE is cell type dependent. TRPC4 and TRPC6 were found to contribute to the formation of SOCE in human skeletal muscles (4), TRPC5 was found to contribute to the formation of SOCE in smooth muscle cells isolated from rabbit pial arterioles (54), and TRPC1 and TRPC6 were reported to contribute to SOCE in rat kidney fibroblasts (2). However, no TRPC7 involvement in SOCE channels was reported (43).

SOCE channels have been reported to participate in cell growth of adult hippocampal neural progenitor cells (32) and regulate migration of endothelial progenitor cells (28). Roles of SOCE channels in regulating cellular function are unknown in cardiac c-kit\(^{+}\) progenitor cells. The present study was designed to investigate whether/how SOCE channels regulate the important cell cycling progression and migration in human cardiac c-kit\(^{+}\) progenitor cells. Our results demonstrated that TRPC1, STIM1, and Orai1 are involved in the formation of SOCE channels and participate in the regulation of cell cycling and migration by activating cyclin D1, cyclin E, and/or p-Akt in human cardiac c-kit\(^{+}\) cells.

MATERIALS AND METHODS

Cell culture. Human cardiac c-kit\(^{+}\) cells were isolated from atrial specimens obtained from coronary artery bypass surgery. The tissue collection was approved by the Ethics Committee of the University of Hong Kong based on the patients’ consent; cell isolation was performed following a modified procedure, as described previously (5, 60). Briefly, the tissue was cut into 1-mm\(^3\) pieces in oxygenated cardioplegic solution, washed with PBS, and digested with 0.2% trypsin (Invitrogen, Hong Kong, China) and 0.1% collagenase II (Worthington Biocheam Lakewood, NJ) at 37°C for 3 × 5 min, and the softened tissues were washed with IMDM containing 10% FBS. These tissue chunks were plated in six-well plates in IMDM contain-
ing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 5 µg/ml bFGF, and 5 ng/ml hEGF at 37°C in 5% CO2. The tissue fragments were discarded after fibroblast-like cell colonies formed. Cells were resuspended in PBS at room temperature after aggregates were removed with a 40-µM strainer (BD Falcon; www.bdbiosciences.com) and then incubated with rabbit c-kit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in MACs buffer for 20 min at 4°C. C-kit+ cells were sorted with magnetic immunomagnetic beads (Miltenyi, Bergisch Gladbach, Germany) coated with anti-rabbit IgG at 4°C. Our recent study demonstrated that >98.0% of purified c-kit+ cells were mononuclear cells expressing c-kit protein (60), and >99% cells expressed the stem cell markers CD29 and CD105; the adult somatic cell marker CD8A was present in a very limited population of cells (<1%), and hematopoietic stem cell markers CD34 and CD45 were mostly absent (60), which was consistent with previous reports by other research groups (5, 22).

**Solutions and reagents.** Tyrode’s solution contains (in mM) 140 NaCl, 5 KCl, 1.0 MgCl2, 1.8 CaCl2, 10 HEPES, and 10 glucose, with pH adjusted to 7.3 using NaOH. All chemicals and reagents were purchased from Sigma-Aldrich Chemicals unless otherwise specified.

**Reverse transcript polymerase chain reaction.** The reverse transcript polymerase chain reaction (RT-PCR) was performed with a procedure described previously (51). Briefly, total RNA was isolated using the TRIzol method (Invitrogen) from human cardiac c-kit+ progenitor cells and then treated with DNase I (Invitrogen). Reverse transcription (RT) was performed with an RT system (Promega, Madison, WI) protocol in a 20-µl reaction mixture. RNA (1 µg) was used in the reaction, and a combination of oligo(dT) and random hexamer primers was used for the initiation of cDNA synthesis. After RT, the reaction mixture (cDNA) was used for polymerase chain reaction (PCR), with the forward and reverse PCR oligonucleotide primers listed in Table 1. PCR was performed by a Promega PCR system with Taq polymerase and accompanying buffers. The cDNA in 2-µl aliquots was amplified by a DNA thermal cycler (Mycycler; Bio-Rad, Hercules, CA) in a 25-µl reaction mixture containing 1.0× thermophilic DNA polymerase reaction buffer, 1.25 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.6 mM of each forward and reverse primer, and 1.0 U of Taq polymerase under the following conditions: the mixture was annealed at 50 – 60°C (1 min), extended at 72°C (2 min), and denatured at 95°C (45 s) for 30 cycles. This was followed by a final extension at 72°C (10 min) to ensure complete product extension. The PCR products were electrophoresed through a 1.5% agarose gel, and the amplified cDNA bands were visualized by ethidium bromide staining. The bands were imaged by Chemi-Genius Bio Imaging System (Syngene, Cambridge, UK).

**Western blotting analysis and coimmunoprecipitation.** The membrane proteins were determined with Western immunoblotting analysis, as described previously (51, 59). Briefly, cells were lysed with modified RIPA buffer (50 mM Tris-Cl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS) for 30 min at 4°C, and cell lysates were then centrifuged at 12,000 g for 15 min at 4°C. After the supernatant was transferred to a fresh ice-cold tube, protein concentration was determined with Bio-Rad protein assay. Equal concentrations of proteins were mixed with SDS sample buffer and denatured at 95°C for 5 min. Samples were resolved with 8% SDS-page gel. Gels were then transferred onto nitrocellulose membrane paper, and membranes were blocked with 5% nonfat dried milk in TTBS (0.1% Tween-20) for 1 h. After blocking, the blots were incubated overnight at 4°C in the primary antibodies (1:1,000 –2,000) anti-cyclin D, anti-cyclin E, anti-pERK1/2, anti-ERK1/2, anti-pAkt, and anti-Akt (Santa Cruz Biotechnology). After washing with TTBS, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies (1:5,000) (Santa Cruz Biotechnology) at room temperature for 1 h. Membranes were washed again with TTBS and then processed to develop X-ray film using an enhanced chemiluminescence detection system (GE Healthcare, Bio-Science, Uppsala, Sweden). The relative band intensities were measured by the image analysis software Gel-Pro Analyzer.

**Coimmunoprecipitation.** The interaction was used to determine protein-protein interaction, as described previously (59). Briefly, equal amounts of proteins were immunoprecipitated with 2 µg of antibody at 4°C overnight. Then, 20 µl of protein A/G agarose beads (Santa Cruz Biotechnology) was added and incubate for 2–4 h at 4°C. Immunoprecipitated proteins bound to the pelletted protein A/G beads were washed thoroughly in PBS, denatured in Laemmli sample buffer, electrophoresed, and blotted. Proteins were detected by using anti-TRPC1, anti-STM1, or anti-Orai1 primary antibody. As a negative control, protein samples were mock immunoprecipitated with preimmuno-IgG and treated in the same way as described above.

**Ca2+ signaling measurement.** Cytosolic Ca2+ (Ca2+i) activity was measured with a confocal microscopy scanning technique, as described previously (11, 51). Briefly, the cultured human cardiac c-kit+ progenitor cells were loaded with 5 µM fluo-3 AM (Biotium, Hayward, CA) serum-free IMDM for 30 min at 37°C and incubated in Tyrode’s solution for 1 h. Fluor 3 was excited by a 488-nm argon-ion laser, and emission was detected at 525 nm. Ca2+ i activity was monitored every 10 s using confocal microscopy (Olympus FV300, Tokyo, Japan) at room temperature (23–24°C).

**Table 1. Human gene-specific primers for RT-PCR**

<table>
<thead>
<tr>
<th>Gene (Accession No.)</th>
<th>Primer sequences (5’-3’)</th>
<th>Fragment Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPC1 (NM_003304)</td>
<td>CTGGATAGGGCTTGGAAAGAA</td>
<td>451</td>
</tr>
<tr>
<td>Reverse</td>
<td>AAGACAGGACGGATTTACAG</td>
<td></td>
</tr>
<tr>
<td>TRPC3 (NM_003305)</td>
<td>ATGACTGGTGCAGCGGGAGAG</td>
<td>430</td>
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<tr>
<td>Reverse</td>
<td>CTTCTGGCTTCCAGAAAGAT</td>
<td></td>
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<tr>
<td>TRPC4 (NM_016179)</td>
<td>TGGTAGATTATTTACGTTG</td>
<td>345</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTTAAATGTTCCAGAGGCA</td>
<td></td>
</tr>
<tr>
<td>TRPC5 (NM_012471)</td>
<td>CTCTTGTGCTACTGGGAAATCTGAT</td>
<td>477</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCCCTACCAAGACTGTTGAT</td>
<td></td>
</tr>
<tr>
<td>TRPC6 (NM_004621)</td>
<td>TTTACTGTTTTGCTGATGCC</td>
<td>500</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGAAGGGGTGCCCATTTATGCC</td>
<td></td>
</tr>
<tr>
<td>TRPC7 (NM_020389)</td>
<td>GAGGACGACCTTCACTTCTT</td>
<td>389</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCCCACTCAAAACACTTT</td>
<td></td>
</tr>
<tr>
<td>STIM1 (NM_003156)</td>
<td>GAGGAGTTTTGCGAGATTTG</td>
<td>499</td>
</tr>
<tr>
<td>Reverse</td>
<td>TGGAGTTATTAGGGGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Orai1 (NM_032790)</td>
<td>GATTTACCCAGGATGTAGA</td>
<td>307</td>
</tr>
<tr>
<td>Reverse</td>
<td>GACCAGGTGAGATGTTGC</td>
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<tr>
<td>Orai2 (NM_032832)</td>
<td>CATGATTACCGGGACTG</td>
<td>460</td>
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<td>Reverse</td>
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<tr>
<td>Orai3 (NM_032790)</td>
<td>TTCGGAATGGTGCTCCTG</td>
<td>364</td>
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<tr>
<td>Reverse</td>
<td>TTCCTAGGTCTGCTGTTAG</td>
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<tr>
<td>GAPDH (J02642)</td>
<td>AAGGAGGACACCCACGTCCTC</td>
<td>258</td>
</tr>
<tr>
<td>Reverse</td>
<td>GAGGAGGAGGATTTCATGTTG</td>
<td></td>
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</table>

**TRPC:** transient receptor potential canonical; **STIM1:** stromal interaction molecule 1; **Orai1:** calcium-release-activated calcium modulator.
from Santa Cruz Biotechnology. These siRNAs are target-specific 19- to 25-nt siRNA designed to knock down gene expression with the previously described procedure (50). Human cardiac c-kit+ cells at 60–70% confluence were transfected with siRNA molecules at 10–40 nM using Lipofectamine 2000 reagent (Invitrogen). The siencer negative control siRNA (no. AM4611; Ambion, Austin, TX), which had no known target in human genomes, was applied as negative control. Sixty to 72 hours after transfection, cells were used for determining Ca2+ activity, related gene and protein expression, and cell proliferation and migration assays.

Cell proliferation assays. Methyl thiazolyl tetrazolium (MTT) assay was applied to assess the effect of siRNAs of SOCE components on cell proliferation (50) in human cardiac c-kit+ progenitor cells. Briefly, the cells were plated into 96-well plates at a density of 4,000 cells/well in 200 µl of normal culture medium for 24 h. The culture medium was then replaced with medium containing ion channel blockers or specific siRNAs. After 72 h of incubation, 20 µl of MTT stock solution (5 mg/ml) was added to each well, and the plates were incubated at 37°C for an additional 4 h. The medium was removed, and formazan crystals were dissolved in 150 µl of DMSO. The optical density values of the samples were read using a µQuant microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT). Results were normalized using vehicle control.

[3H]thymidine incorporation assay was introduced to assess proliferating cell rate (50). Human cardiac c-kit+ progenitor cells were plated into 96-well plates at a density of 4 × 103 cells/well in 200 µl of complete culture medium. After a 24-h subculture, the culture medium was replaced with a medium containing ion channel blockers or specific siRNAs and incubated for 60–70 h. [3H]thymidine (1 µM; GE Healthcare) was then added into each well. After incubation for another 12 h, the cells were harvested and transferred to a UniFilter-96 GF/C microplate via cell harvester (Perkin-Elmer, Waltham, MA). The microplate was washed with water and air-dried at 37°C overnight. Liquid scintillation cocktail (20 µl/well) was then added to each well. The labeled DNA in each well was measured as counts per minute by a TopCount microplate scintillation and a luminescence counter (Perkin-Elmer).

Wound-healing assay. The wound-healing technique was employed to determine cell migration, as described previously (10). Briefly, human cardiac c-kit+ cells were cultured to a confluent monolayer and scraped with a linear strip across the well with a standard 200-µl pipette tip. The floating cells were immediately removed with PBS, and a fresh 1% FBS medium was added to prevent cell proliferation. Wound closure of various treatments was detected for 8–12 h, and then five representative images of the scratched area of each treatment were photographed under a phase contrast microscope (Olympus, Tokyo, Japan). The number of migrated cells was counted to evaluate the migration rate of various conditions. Experiments were performed in triplicate.

Transwell assay. Transwell assay with a modified Boyden chamber with 8-µm pore polycarbonate membranes (Corning, Corning, NY) was performed to determine cell migration, following the previously described procedure (10). The chambers were precoated with 0.1 ml of serum-free medium for ≥1 h. After the precoated medium was removed, 5,000 viable human cardiac c-kit+ cells were plated into the upper chamber in 200 µl of medium containing 1% FBS and the lower chamber containing 600 µl of 1% FBS medium. The plates were incubated at 37°C in 5% CO2 for 8 h. Then the chambers were washed with PBS for three times, fixed with formaldehyde for 15 min at room temperature, and stained with crystal violet for 15 min. After being washed with PBS to thoroughly remove the dye, nonmigrated cells on the upper surface of the membrane were scraped off by cotton swabs. The migrated cells on the lower surface of the membrane were counted in five to eight representative fields under a microscope.

Statistical analysis. Group data are expressed as means ± SE. Paired and/or unpaired Student’s t-tests were used as appropriate to evaluate the statistical significance of differences between two group means, and ANOVA was used for multiple groups. Values of P < 0.05 were considered to be statistically significant.

RESULTS

Store-operated Ca2+ entry in human cardiac c-kit+ progenitor cells. It is well recognized that activation of SOCE channels is dependent on the depletion of ER Ca2+ store (7). Figure 1 illustrates the Ca2+ activity mediated by SOCE channels with Ca2+ store depletion by thapsigargin (a SERCA pump inhibitor) in human cardiac c-kit+ progenitor cells. A transient increase of Ca2+; i was induced by depleting the ER Ca2+ store with thapsigargin (1 µM) in the absence of bath Ca2+ (0 Ca2+), and second Ca2+; i transient was followed upon bath Ca2+ (2 mM Ca2+) being restored through SOCE channels (Fig. 1A). Previous studies demonstrated that the trivalent ion La3+ blocked the Ca2+ influx through SOCE channels in different cell systems (for review, see Ref. 24); this was tested in human cardiac c-kit+ progenitor cells. The Ca2+ influx through SOCE channels was also inhibited (Fig. 1B) or prevented (Fig. 1C) by 100 µM La3+ in human cardiac c-kit+ progenitor cells.

In Jurkat human T cells and rat basophilic leukemia cells, the IP3Rs antagonist 2-aminoethoxydiphenyl borate (2-APB) elicited both stimulatory and inhibitory effects on Ca2+ influx through SOCE channels (38). To determine whether it is the case in human cardiac c-kit+ progenitor cells, a low concentration of 5 µM and a high concentration of 50 µM 2-APB were tested. The SOCE-mediated Ca2+ transient was slightly increased by 2-APB at 5 µM (Fig. 1D), highly decreased (Fig. 1E), and prevented (Fig. 1F) by 2-APB at 50 µM. These pharmacological properties of SOCE channels in human cardiac c-kit+ progenitor cells are similar to those observed in other cell systems (35, 38, 48).

Molecular components of SOCE channels in human cardiac c-kit+ progenitor cells. It is generally believed that SOCE channels are composed of three molecular components, including TRPC channels, STIM1, and Orai1. All TRPC channels (except for TRPC7) can function as SOCE channels in heterogeneously expressed in HEK 293 cells (57); however, only TRPC1 channels are found to be abundantly expressed in several native mammalian cells, and contribute to the SOCE channels (26, 37, 59).

To demonstrate the molecular components of SOCE channels, we first detected gene expression of TRPC channels, STIM1, and Orai1 in human cardiac c-kit+ progenitor cells with specific primers (Table 1) and then determined the specific protein expression and the protein-protein interaction. The images (Fig. 2A) of RT-PCR showed that gene expressions for TRPC1, STIM1, and Orai1 were abundant, whereas the expression for TRPC3, TRPC4, Orai2, and Orai3 was relatively weak. Therefore, we postulated that TRPC1, STIM1, and Orai1 might contribute to SOCE channels in human cardiac c-kit+ progenitor cells. Western blots (Fig. 2B) showed significant protein expression of TRPC1, STIM1, and Orai1.

The possible interaction among TRPC1, STIM1, and/or Orai1 was determined by communoprecipitation. Anti-TRPC1, anti-STIM1, or anti-Orai1 antibody was used to pull down each corresponding protein. Communoprecipitation revealed the protein bands at the expected molecular weight (Fig. 2C). The protein-protein interaction was further confirmed by

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immunostaining in human cardiac c-kit+ progenitor cells with anti-TRPC1, anti-Orai1, and anti-STIM1 antibodies (Fig. 2D). These results suggest that TRPC1, STIM1, and Orai1, as in human atrial myocytes (59), form the complex of SOCE channels in human cardiac c-kit+ progenitor cells.

Silence of TRPC1, STIM1, or Orai1 and function of SOCE channels. To determine whether TRPC1, STIM1, and Orai1 contribute to the formation of the ternary complex of SOCE channels, specific siRNA molecules were employed to silence TRPC1, STIM1, or Orai1 in human cardiac c-kit+ progenitor cells. Figure 3 displays the gene and protein levels of TRPC1, STIM1, and Orai1 in human cardiac c-kit+ progenitor cells transfected with control siRNA and was significantly reduced in cells transfected with TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA (Fig. 4A). The relative mean values of peak Ca2+ influx through SOCE channels are illustrated in Fig. 4B. The peak of Ca2+ influx through SOCE channels was decreased in cells transfected with TRPC1 siRNA, STIM1 siRNA, and Orai1 siRNA to 47, 30, and 57%, respectively, compared with cells transfected with control siRNA (n = 15, P < 0.01). These results confirm that SOCE channels are composed by TRPC1, STIM1, and Orai1 in human cardiac c-kit+ progenitor cells.

SOCE channels and proliferation and migration in human cardiac c-kit+ progenitor cells. Recent studies have demonstrated that SOCE channels participate in regulating cell proliferation and/or migration in cultured rat aortic vascular smooth muscle cells (8) and mouse and rat airway smooth muscle cells (46, 61). To study whether this is the case in human cardiac c-kit+ progenitor cells, we determined cell proliferation and/or migration in cells transfected with control siRNA, TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA. The transfection of 40 nM TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA for 72 h decreased cell proliferation significantly (n = 6, P < 0.01 vs. control siRNA). Flow cytometry analysis revealed that silencing TRPC1, STIM1, or Orai1 arrested the cells at the G0/G1 phase, and the mean percent values of cells at the G0/G1 phase, and the mean percent values of cells at the G0/G1 phase.
phase were significantly increased (Fig. 5C), whereas the S phase was reduced in cells transfected with TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA (n = 6, P < 0.05 or P < 0.01 vs. control siRNA). These data indicate that SOCE channels regulate cell cycle progression in human cardiac c-kit+ progenitor cells.

Cell migration was determined with the wound-healing and the transwell methods (10) in cells transfected with 40 nM control siRNA, TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA for 72 h. The confluent cells were scraped off with a 200-μl pipette tip to make a wide acellular area. After 8 h, cells that migrated into this area were counted (Fig. 6A). The mean value of the migrated cells was decreased significantly in cells transfected with TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA (n = 6, P < 0.01 vs. control siRNA; Fig. 6B). Transwell assay was used to exclude the potential contamination of cell migration by proliferation. Figure 6C shows the migrated cells on the lower surface of the transwell membrane in cells transfected with 40 nM corresponding siRNA. The migrated cell number is clearly reduced in cells transfected with TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA (n = 6, F < 0.01 vs. control siRNA; Fig. 6D).

These results indicate that in addition to the regulation of proliferation, SOCE channels participate in modulating migration of human cardiac c-kit+ progenitor cells.

Signal pathway involvement of SOCE channels in regulating cell cycling and migration. To determine the potential signal pathway involvement of SOCE channels in regulating cell progression and migration, the cell cycle-related kinases cyclin D1 and cyclin E, the mitogen-activated protein kinases ERK1/2, and the cell survival kinase Akt were determined in human cardiac c-kit+ progenitor cells transfected with control siRNA, TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA. Cyclin D1 (Fig. 7A) and cyclin E (Fig. 7B) were significantly reduced in cells transfected with TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA (n = 3, P < 0.05 or P < 0.01 vs. control siRNA). However, the phosphorylated ERK1/2 was not affected by silencing TRPC1, STIM1, or Orai1 (Fig. 7C). Interestingly, phosphorylated Akt (Fig. 7D) was decreased in cells transfected with TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA (n = 3, P < 0.01 vs. control siRNA). These results suggest that cyclin D1, cyclin E, and/or p-Akt are involved in the regulation of cell proliferation and/or migration by SOCE channels, and silencing any component of the SOCE complex would impair cell growth or migration in human cardiac c-kit+ progenitor cells.
DISCUSSION

The present study demonstrated that activation of SOCE channels in human cardiac c-kit⁺ progenitor cells, as in other cell types (35, 38, 48), occurs on the depletion of intracellular Ca²⁺ store by thapsigargin. The nonselective cation channel blocker La³⁺ remarkably blocked or prevented the Ca²⁺ influx mediated by SOCE channels. The IP₃ receptor antagonist 2-APB increased the Ca²⁺ influx through SOCE channels at a low concentration (5 μM), whereas it remarkably inhibited the Ca²⁺ influx at a high concentration (50 μM), which is consistent with the observation in Jurkat human T cells and basophilic leukemia cells (38).

It is generally recognized that SOCE channels are a complex composed of STIM1, Orai1, and TRPC. STIM1 functions as an endoplasmic reticulum Ca²⁺ sensor, and Orai1 is the pore subunit located on the plasma membrane (47). However, there is no definite conclusion for the contribution of type(s) of TRPC channels to SOCE channels. The experiment results from the heterogeneous expression in HEK-293 cells showed that all TRPC channels (except for TRPC7) can function as part of the SOCE channels (57). In EA.hy926 cell line derived from human umbilical vein endothelial cells, TRPC3 was found to be involved in the formation of SOCE channels (3). TRPC4 interacts with TRPC1 to form the SOCE channels in human myoblast (4). Nonetheless, most results support the notion that TRPC1 is the major player in the formation of SOCE channels (13). TRPC1 contributes to the SOCE channels in pulmonary artery smooth muscle cells (36), human atrial myocytes (59), human endometrial stromal cells (27), HEK-293 cells (44), human platelet (25), and secretory epithelial cells (23).

The results from the present study support the notion that TRPC1 contributes to the formation of SOCE channels with STIM1 and Orai1 in human cardiac c-kit⁺ progenitor cells, which is confirmed by communoprecipitation and immunostaining for the protein-protein interaction between TRPC1, STIM1, and Orai1. The Ca²⁺ influx through SOCE channels was also reduced when TRPC1 was silenced with TRPC1 siRNA. Ca²⁺ signaling through SOCE channels generates cellular Ca²⁺ elevations and activates downstream molecular signaling by coupling to the related enzymes and transcription factors that regulate many functions, including gene expression, exocytosis, and cell proliferation and migration (12, 24, 52). Silencing SOCE components STIM1 and/or Orai1 signif-
Significantly attenuates cell proliferation in mouse embryonic and adult mouse neural stem/progenitor cells (1), mouse airway smooth muscle cells (46), rat vascular smooth muscle cells (20), and human retinal pigment epithelial cells (55). In addition, silencing TRPC1 inhibited the proliferation of canine kidney cells (33). Inhibition and/or silencing TRPC1 resulted in the inhibition of proliferation by arresting cells at the G0/G1 phase in adult mouse neural progenitor cells (32). Consistent with these reports, we demonstrated that silencing any component of SOCE channels, TRPC1, STIM1, or Orai1 decreased Ca\(^{2+}\)/H\(_{11001}\) signaling through the channel, thereby suppressing proliferation in human cardiac c-kit\(^{+}\) progenitor cells by arresting the cells at G0/G1 via reducing cyclin D1 and cyclin E.

Migration of cardiac c-kit\(^{+}\) progenitor cells is an essential cellular function for the developing heart (17) and cardiac repair, in which cardiac stem/progenitor cells are required to migrate first to the infarcted myocardial zone and then proliferate (29, 45). However, the mechanisms underlying cardiac c-kit\(^{+}\) progenitor cell migration are not fully understood. It has been proposed that the dynamics of the cytoskeleton and multiple signal transduction pathways, including intracellular Ca\(^{2+}\) signaling, are involved in the process of cell migration (49). Schmid et al. (41) demonstrated that increase in SOCE channels by serum- and glucocorticoid-inducible kinase isoform SGK3 enhanced dendritic cell migration via activating downstream target of PI3K. In the present study, silencing TRPC1, STIM1, or Orai1 significantly decreased the migration in human cardiac c-kit\(^{+}\) progenitor cells, which is consistent with the results observed in human glioblastoma (34), HEK-293 cells (40), and vascular smooth muscle cells (31).

It is well known that Akt kinase plays important roles in regulating cell survival, growth, proliferation, migration, and differentiation (30). In the present study, we demonstrated that phosphorylated Akt was decreased in human cardiac c-kit\(^{+}\) progenitor cells transfected with TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA, suggesting that the Ca\(^{2+}\) signaling through SOCE channels may increase the phosphorylation of Akt kinase, accordingly affecting cell proliferation and migration of human cardiac c-kit\(^{+}\) progenitor cells. Our observation is supported by recent reports that Ca\(^{2+}\) entry through TRPC1 channels regulates myoblast migration and differentiation via Akt activation (58). Overexpression of functional TRPC1 significantly protected against neurotoxin-induced loss of SOCE channels in mouse dopaminergic neurons by regulating Ca\(^{2+}\) homeostasis via activating Akt (42). However, no alteration in phosphory-

![Fig. 4. Ca\(^{2+}\) influx through SOCE channels in human cardiac c-kit\(^{+}\) progenitor cells after silencing TRPC1, STIM1, or Orai1. A: averaged data (means ± SE) of normalized Ca\(^{2+}\)i levels recorded with Ca\(^{2+}\) store depletion and followed by restoring bath Ca\(^{2+}\) in cells transfected with control siRNA, TRPC1 siRNA, STIM1 siRNA, and Orai1 siRNA (n = 15 for each treatment). B: mean %values of Ca\(^{2+}\) peak influx through SOCE channels in cells transfected with control siRNA, TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA (n = 15 for each treatment). **P < 0.01 vs. control siRNA.](http://ajpheart.physiology.org/)

![Fig. 5. Silencing TRPC, STIM1, or Orai1 channels and cell proliferation. A: cell proliferation determined with MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] in cells transfected with control siRNA, TRPC1 siRNA, STIM1 siRNA, and Orai1 siRNA (n = 6). B: mean %values of [\(^{3}\)H]thymidine incorporation in cells transfected with control siRNA, TRPC1 siRNA, STIM1 siRNA, and Orai1 siRNA (n = 6). C: mean %values of cell cycling population at different phases in cells transfected with corresponding siRNAs (n = 4). *P < 0.05; **P < 0.01 vs. control siRNA.](http://ajpheart.physiology.org/)
lated ERK1/2 was detected in cells after silencing TRPC1, STIM1, or Orai1. This suggests that ERK1/2 may not be a downstream target of SOCE channels in human cardiac c-kit⁺ progenitor cells.

In addition, it has been reported that the SOCE component STIM1 is involved in both early neural differentiation and survival of early differentiated cells in mouse embryonic stem cells [21], and the Orai1 and STIM1 complex controls human cardiac c-kit⁺ progenitor cell migration [40 nM].

Fig. 6. Silencing TRPC1, STIM1, or Orai1 and cell migration in human cardiac c-kit⁺ progenitor cells. A: images of wound-healing assay in cells transfected with corresponding siRNAs (40 nM). B: mean values of the migrated cell number (under microscopic field; bar, 150 μm) in cells transfected with control siRNA, TRPC1 siRNA, STIM1 siRNA, and Orai1 siRNA (n = 6). C: images of migrated cells on the lower chamber membrane in cells transfected with corresponding siRNAs (40 nM). D: mean values of migrated cell number (under each microscopic field; bar, 150 μm) in human cardiac c-kit⁺ progenitor cells transfected with control siRNA, TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA (n = 6). **P < 0.01 vs. control siRNA.

Fig. 7. Intracellular signals involvement of SOCE channels in regulating proliferation and migration. A: protein level of cyclin D1 in cells transfected with control siRNA, TRPC1 siRNA, STIM1 siRNA, or Orai1 siRNA (40 nM). B: protein level of cyclin E in cells transfected with corresponding siRNAs. C: relative level of phosphorylated (p)-ERK1/2 in cells transfected with corresponding siRNAs (n = 3 each treatment). *P < 0.05; **P < 0.01 vs. control siRNA.
dendritic cell maturation (16). The present study did not investigate whether SOCE channels regulate the differentiation of human cardiac c-kit+ progenitor cells into cardiomyocytes, endothelial cells, and/or vascular smooth muscle cells. This remains to be studied in the future. However, this study conclusively shows that SOCE channels regulate cell proliferation and migration in human cardiac c-kit+ cells.

Collectively, the present study provides the novel information that SOCE channels are involved in the functional Ca2+ influx in human cardiac c-kit+ progenitor cells, and the molecular components of SOCE channels are TRPC1, STIM1, and Orai1. Ca2+ signaling through SOCE channels is involved in regulating cell cycling progression and migration via cell cycle kinase cyclin D1 and cyclin E and/or phosphorylation of Akt in human cardiac c-kit+ progenitor cells. Understanding the regulation of human cardiac c-kit+ progenitor cell proliferation and migration by SOCE channels would be beneficial for developing new approaches to improve myocardial repair/regeneration.

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DISCLOSURES
The authors declare that no competing interests exist.

AUTHOR CONTRIBUTIONS
H.C., G.-S.X., Y.W., and G.-R.L. conception and design of research; H.C., G.-S.X., and G.-R.L. performed experiments; H.C., G.-R.L., and H.-Y.S. analyzed data; H.C. and G.-R.L. approved final version of manuscript; G.-S.X. and G.-R.L. revised manuscript.

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