Regulation of cell proliferation by intermediate-conductance Ca\(^{2+}\)-activated potassium and volume-sensitive chloride channels in mouse mesenchymal stem cells

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1Department of Medicine, and Research Centre of Heart, Brain, Hormone and Healthy Aging, Li Ka Shing Faculty of Medicine, The University of Hong Kong, and 2Department of Physiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR, China

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Tao R, Lau CP, Tse HF, Li GR. Regulation of cell proliferation by intermediate-conductance Ca\(^{2+}\)-activated potassium and volume-sensitive chloride channels in mouse mesenchymal stem cells. *Am J Physiol Cell Physiol* 295: C1409–C1416, 2008. —Bone marrow stem cells (MSCs) are a promising cell source for regenerative medicine; however, their cellular physiology is not fully understood. The present study aimed at exploring the potential roles of the two dominant functional ion channels, intermediate-conductance Ca\(^{2+}\)-activated potassium (IK\(\text{Ca}\)) and volume-sensitive chloride (Cl\(\text{vol}\)) channels, in regulating proliferation of mouse MSCs. We found that inhibition of IK\(\text{Ca}\) with clotrimazole and Cl\(\text{vol}\) with 5-nitro-1-(3-phenylpropylamino) benzoic acid (NPPB) reduced cell proliferation in a concentration-dependent manner. Knockdown of K\(\text{Ca}3.1\) or Cl\(\text{cn}3\) with specific short interference (si)RNAs significantly reduced IK\(\text{Ca}\) or Cl\(\text{vol}\) density and channel protein and produced a remarkable suppression of cell proliferation (by 24.4 ± 9.6% and 29.5 ± 7.2%, respectively, P < 0.05 vs. controls). Flow cytometry analysis showed that mouse MSCs retained at G\(_0\)/G\(_1\) phase (control: 51.65 ± 3.43%) by inhibiting IK\(\text{Ca}\) or Cl\(\text{vol}\) using clotrimazole (2 \(\mu\)M: 64.45 ± 2.20%, P < 0.05) or NPPB (200 \(\mu\)M: 82.89 ± 2.49%, P < 0.05) or the specific siRNAs, while distribution of cells in S phase was decreased. Western blot analysis revealed a reduced expression of the cell cycle regulatory proteins cyclin D1 and cyclin E. Collectively, our results have demonstrated that IK\(\text{Ca}\) and Cl\(\text{vol}\) channels regulate cell cycle progression and proliferation of mouse MSCs by modulating cyclin D1 and cyclin E expression.

Intermediate conductance Ca\(^{2+}\)-activated K\(^{+}\) current; volume-sensitive Cl\(^{-}\) current; cell cycle progression; short interference RNA

Mesenchymal stem cells (MSCs) from bone marrow are pluripotent adult stem cells that can differentiate into a broad spectrum of cells, including osteoblasts, chondrocytes, adipocytes, neurons, skeletal muscle cells, and cardiomyocytes (25, 31, 43). Multidisciplinary researchers have paid great attention to MSCs, which have been considered as a source of cells for cell-based therapy in regenerative medicine, especially in repair of damaged myocardium (24, 27).

Clinical trials showed that implantation of human MSCs into infarcted myocardium tissues improved cardiac function (39). Although the therapeutic effects are encouraging, potential proarhythmic effect of transplanted MSCs was observed in both in vitro and in vivo studies (5). Therefore, the electro-physiological properties of MSCs from different species (i.e., human, rabbit, rat, and mouse) and tissues (i.e., bone marrow, umbilical cord blood, and fat tissue) have recently been studied (1, 8, 9, 13, 22, 23, 30, 37). Results from these studies demonstrated that expression of multiple ion channels was heterogeneous and species dependent (9, 22, 23, 37) or cell cycle dependent (8). This information is important for the application of bone marrow MSCs-based therapy for repairing myocardium; however, the roles of these ion channels in the regulation of stem cell behaviors (e.g., cell proliferation) are not well understood. Our previous study demonstrated that three functional ion channels, intermediate-conductance Ca\(^{2+}\)-activated K\(^{+}\) channel (IK\(\text{Ca}\) or K\(\text{Ca}3.1\)), volume-sensitive chloride channel (Cl\(\text{vol}\) or Cl\(\text{cn}3\)), and inward rectifier K\(^{+}\) channel (Kir2.1), were present in mouse MSCs (37). IK\(\text{Ca}\) and Cl\(\text{vol}\) were recorded in most mouse MSCs (95% and 88%, respectively), while Kir2.1 channel expressed only in a small population of cells (16%). The present study was designed to explore the potential roles of these two dominant ion channels (IK\(\text{Ca}\) and Cl\(\text{vol}\)) in regulation of proliferation and cell cycle progression in mouse MSCs.

Materials and Methods

**Cell culture.** Mouse bone marrow MSCs (passage 5) from C57Bl/6 mice were kindly provided by Dr. Darwin J. Prockop, Center for Gene Therapy, Tulane University (http://www.som.tulane.edu/gene_therapy). Cells tested positive for Sca-1 and negative for CD34, CD45, and CD11B-C surface markers and can be differentiated into adipocytes and osteocytes. MSCs were cultured and passaged as described previously (37). Cells used in this study were from the early passages 6 to 8 to limit the possible variations in functional ion channel currents, gene expression, and protein levels induced by cell aging and senescence in later passages, since it has been reported that cell senescence occurs in later passages of in vitro MSC culture (2, 4).

**Drugs and reagents.** The chloride channel blocker 5-nitro-1-(3-phenylpropylamino) benzoic acid (NPPB) was purchased from Tocris (Bristol, UK). Rabbit polyclonal anti-K\(\text{Ca}3.1\), goat polyclonal anti-C\(\text{cn}3\)-3, mouse monoclonal anti-cyclin D1 and anti-cyclin E, goat anti-rabbit, donkey anti-goat IgG-horseradish peroxidase (HRP), and goat polyclonal anti-GAPDH-HRP antibodies were products of Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). Other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell proliferation assays.** 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was applied to assess the effects of ion channel blockers on cell proliferation (19). Mouse MSCs were

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plated into 96-well plates at a density of $5 \times 10^4$ cells per well in 200 µl complete culture medium. After 8 h recovery, the culture medium containing ion channel blockers was used. Following 72 h incubation, 20 µl PBS-buffered MTT (5 mg/ml) solution was added to each well and the plates were incubated at 37°C for an additional 4 h. The medium was removed, and 100 µl/well DMSO was added to each well to dissolve the purple formazan crystals. The plates were read (wavelengths: test, 570 nm; reference, 630 nm) using a µQuant microplate spectrophotometer (Bio-Tek Instruments). Results were standardized using control group values.

[1^H]thymidine incorporation assay was introduced to assess proliferating cell rate (19). Mouse MSCs were plated into 96-well plates at a density of $5 \times 10^4$ cells per well in 200 µl complete culture medium. After 8 h recovery, the culture medium was replaced with a medium containing ion channel blockers or specific short interference RNAs (siRNAs) and incubated for 48 h, and 1 µCi (0.037 MBq) [1^H]thymidine (GE Healthcare, Hong Kong) was then added into each well. Following an additional 24 h of incubation, cells were harvested and transferred to a nitrocellulose-coated 96-well plate via suction. Nitrocellulose membrane was washed with water flow, and plate was air dried at 50°C overnight. Liquid scintillant (20 µl/well) was then added to each well. Counts per minute (cpm) for each well was read by a TopCount microplate scintillation and luminescence counter (PerkinElmer, Waltham, MA).

RNA interference. siRNA molecules targeting different exons of KCa3.1 (i.e., KCNN4 for IKCa) and Clcn3 (for ICa.vol) genes were synthesized by Ambion (Austin, TX). Specific siRNA sequences for target genes are shown in Table 1. A FAM-labeled sequence that had no known target in the mouse genome was used as a negative control (i.e., control siRNA). The siRNA molecules (final concentrations at 50 nM as recommended by the supplier) were transfected into mouse MSCs at 50–60% confluence using Lipofectamine 2000 (Invitrogen). Transfection efficiency was monitored by control siRNA by counting transfected mouse MSCs at 50–60% confluence using Lipofectamine 2000 (Invitrogen). Transfection efficiency was monitored by control siRNA by counting transfection efficiency was monitored by control siRNA by counting transfection efficiency was monitored by control siRNA by counting.

Electrophysiology. Membrane ionic currents were recorded using an EPC-10 amplifier and Pulse software (HEKA, Lambrecht, Germany). Briefly, borosilicate glass electrodes (1.2-mm outer diameter) were pulled with a Brown-Flaming puller (model P-97; Sutter Instrument, Novato, CA) and had tip resistances of 2–3 MΩ. Membrane ionic currents were recorded using the whole cell patch-clamp technique as described previously (37) and an EPC-10 amplifier and Pulse software (HEKA, Lambrecht, Germany). Briefly, borosilicate glass electrodes (1.2-mm outer diameter) were pulled with a Brown-Flaming puller (model P-97; Sutter Instrument, Novato, CA) and had tip resistances of 2–3 MΩ when filled with pipette solution. Tip potentials were compensated before the pipette touched the cell. After a gigaohm seal was obtained by negative suction, the cell membrane was ruptured by gentle suction to establish whole cell configuration. Membrane currents were low-pass filtered at 5 kHz and stored on the hard disk of an IBM-compatible computer.

For IKCa recording, cells were perfused with Tyrode solution containing (in mM) 136 NaCl, 5.4 KCl, 1.0 MgCl2, 1.8 CaCl2, 0.33 NaH2PO4, 10 glucose, and 10 HEPES (pH 7.3). The pipette solution contained (in mM) 20 KCl, 110 K-aspartate, 1.0 MgCl2, 10 HEPES, 0.1 GTP, 5.0 Na2-phosphocreatine, 5.0 Mg-ATP, and 800 nM free Ca2+ (pH 7.2). For ICa currents recording, cells were initially perfused in 1.0T solution containing (mM) 110 NaCl, 5.0 KCl, 1.8 CaCl2, 10 glucose, 10 HEPES and 60 mannitol (pH 7.3). When whole-cell configuration was obtained, cells were perfused with 0.8T solution (omitted mannitol from 1.0T solution). The pipette solution used for ICa recording contained (mM) 110 CsCl, 20 Cs-aspartate, 5 EGTA, 1.0 MgCl2, 10 HEPES, 0.1 GTP, 5.0 Na2-phosphocreatine, 5.0 Mg-ATP. The experiments were conducted at room temperature (22–23°C).

Reverse transcriptase polymerase chain reaction. Total RNA of mouse MSCs was extracted, and RT-PCR was conducted according to the established protocol as described previously (37). Semiquantitative PCR was run for 30 cycles for KCa3.1 and Clcn3, and 28 cycles for GAPDH as a reference control. The PCR products were resolved through 1.5% agarose gel electrophoresis, and the amplified cDNA bands were visualized by ethidium bromide staining and imaged using Chemi-Genius Bio Imaging System (Syngene, Cambridge, UK).

Western blot analysis. Cells lysates were extracted via a modified RIPA buffer, and protein concentrations were determined by Bio-Rad protein assay. Western blot analysis of protein levels was conducted as previously described (37). To quantitatively analyze ion channel protein levels, membranes were stripped with stripping buffer (62.5 mM Tris, 2% SDS, and 100 mM 2-mercaptoethanol; pH adjusted to 6.8) at 55°C for 30 min and were then probed with HRP-conjugated goat polyclonal anti-GAPDH antibody (1:1,000). To develop X-ray films, membranes were prepared using an enhanced chemiluminescence detection system (GE Healthcare). The expression of GAPDH levels was used as an internal control to standardize the relative levels of KCa3.1 and Clcn3 protein. The relative band intensities of Western blot were measured by quantitative scanning densitometer and image analysis software (Bio-1D version 97.04).

Flow cytometry and cell cycle analysis. Cell cycle distribution of mouse MSCs was determined by flow cytometry (FC500, Beckman Coulter). Briefly, cells were lifted using 0.25% trypsin, washed with PBS, and fixed with ice-cold ethanol (70%). Ethanol was removed by centrifugation, and cell pellets were washed with PBS. Cells were incubated in a propidium iodide/PBS staining buffer (20 µg/ml propidium iodide, 100 µg/ml RNaseA, and 0.1% Triton X-100) at

<table>
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<th>siRNA Sequence (5’-3’)</th>
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</tr>
<tr>
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<td>60763</td>
<td>A</td>
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<td>10</td>
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KCNN4/KCa3.1, accession no. NM_008433; Clcn3, accession no. NM_007711. siRNA, short interference RNA.
control). In addition, \(^{3}H\)-thymidine incorporation assay effectively blocked functional IK_{Ca} and Cl_{vol} inhibition.

Our previous study showed that clotrimazole and NPPB (Fig. 1A) decreased the proliferation by 25.5 \% (\(P < 0.01\) vs. vehicle control). NPPB (200 \(\mu M\)) reduced the proliferation by 25.5 \% (\(P < 0.01\) vs. vehicle control). In addition, \(^{3}H\)-thymidine incorporation assay showed that both clotrimazole and NPPB decreased DNA incorporation rate in a concentration-dependent manner (Fig. 1B). Clotrimazole (2 \(\mu M\)) inhibited the DNA synthesis rate by 22.0 \% \pm 4.8\% (\(P < 0.01\) vs. vehicle), and NPPB (200 \(\mu M\)) decreased the DNA synthesis rate by 34.3 \% \pm 7.8\% (\(P < 0.01\) vs. vehicle control). These results suggest that the inhibition of IK_{Ca} or Cl_{vol} channels is required for cell proliferation in mouse MSCs.

**RESULTS**

**Effects of IK_{Ca} and Cl_{vol} blockades on mouse MSC proliferation.** Our previous study showed that clotrimazole and NPPB effectively blocked functional IK_{Ca} and Cl_{vol} in mouse MSCs, respectively (37). These two pharmacological compounds were therefore applied in the present study to investigate cell proliferation. Cell proliferation assessed by MTT assay demonstrated that inhibition of the IK_{Ca} by clotrimazole and of the Cl_{vol} by NPPB resulted in a concentration-dependent reduction of proliferation in mouse MSCs after a 72-h incubation (Fig. 1A). Clotrimazole (2 \(\mu M\)) inhibited the proliferation by 20.5 \% \pm 2.7\% (\(P < 0.01\) vs. vehicle control), NPPB (200 \(\mu M\)) reduced the proliferation by 25.5 \% \pm 2.5\% (\(P < 0.01\) vs. vehicle control). In addition, \(^{3}H\)-thymidine incorporation assay showed that both clotrimazole and NPPB decreased DNA incorporation rate in a concentration-dependent manner (Fig. 1B). Clotrimazole (2 \(\mu M\)) inhibited the DNA synthesis rate by 22.0 \% \pm 4.8\% (\(P < 0.01\) vs. vehicle), and NPPB (200 \(\mu M\)) decreased the DNA synthesis rate by 34.3 \% \pm 7.8\% (\(P < 0.01\) vs. vehicle control). These results suggest that the inhibition of IK_{Ca} or Cl_{vol} decreases proliferation of mouse MSCs.

**Effects on ion channel currents, ion channel mRNA, and protein expression.** To exclude the possibility that inhibition of cell proliferation by ion channel blockers is related to a nonselective inhibition of cell proliferation and to ascertain whether activity of IK_{Ca} and Cl_{vol} channels is required for cell proliferation in mouse MSCs, we used the specific siRNA technique to knock down the KCa3.1 (for IK_{Ca}) gene or Clcn3 (for Cl_{vol}) gene. Three specific siRNA molecules targeting different exons of mouse KCa3.1 or Clcn3 mRNA are shown in Table 1. We found that the transfection efficiency of siRNA is very high (>90\% cells showing FAM fluorescence in control siRNA group, data not shown).
Figure 2A displays the membrane currents (IKCa) recorded with the protocol shown in the inset using a K+ pipette solution containing 800 nM free Ca2+ in a mouse MSC transfected with 50 nM control siRNA (left) and a cell transfected with 50 nM KCa3.1 siRNA C (right). The current showed a property of inward rectification at potentials positive to +10 mV in cells transfected with control siRNA, typical of IKCa. The current also showed an inward rectification in cells transfected with KCa3.1 siRNA; however, the current amplitude was much smaller than that of control siRNA cells. Current-voltage (I-V) relation curves of IKCa recorded with a ramp voltage protocol from -100 to +60 mV showed an inward rectification in cells transfected with control siRNA and KCa3.1 siRNA (A, B, or C). IKCa density was significantly smaller in cells transfected with KCa3.1 siRNAs than that in cells transfected with control siRNA (Fig. 2B). Figure 2C shows the mean values of IKCa density at +30 mV. IKCa density was significantly suppressed by KCa3.1 siRNA (A, B, or C) transfection (n = 7 for each group, P < 0.01 vs. control siRNA).

Figure 2D shows the membrane current (ICl.vol) recorded with the protocol voltage shown in the inset using a K+ free pipette solution under conditions of 0.8T external solution and symmetrical Cl− in a cell transfected with control siRNA (left) and a cell transfected with Clcn3 siRNA C (right). ICl.vol amplitude was significantly larger in control siRNA cells than that in Clcn3 siRNA cells. The mean value of ICl.vol density was smaller in cells transfected with three types of Clcn3 siRNAs than that in cells transfected with control siRNA at all test potentials (Fig. 2E). Figure 2F displays the mean ICl.vol density at +30 mV in cells transfected with control siRNA or three types of Clcn3 siRNAs (n = 5 for each group, P < 0.01 vs. control siRNA).

The effects of siRNAs on ion channel mRNA and protein expression were determined by RT-PCR and Western blot analysis, respectively. The mRNA levels of KCa3.1 and Clcn3 were remarkably reduced by the corresponding siRNAs (Fig. 3, A and D). Similarly, the protein levels of KCa3.1 and Clcn3 were also decreased by the corresponding siRNAs (Fig. 3, B and E). The protein levels of KCa3.1 were reduced to 28.21 ± 5.59%, 23.99 ± 1.16%, and 12.14 ± 1.41% by the KCa3.1 siRNA molecules A, B, and C, respectively (P < 0.01 vs. control siRNA), and the protein levels of Clcn3 were reduced to 34.60 ± 5.80%, 14.88 ± 2.81%, and 21.69 ± 2.68% by the Clcn3 siRNA molecules A, B, and C, respectively (P < 0.01 vs. control siRNA).

Effect of KCa3.1 and Clcn3 knockdown on mouse MSC proliferation. Figure 4 illustrates the effects of knockdown of KCa3.1 or Clcn3 on proliferation in mouse MSCs. All three KCa3.1 siRNAs or Clcn3 siRNAs (50 nM each) remarkably slowed cell growth; the control siRNA had no significant effect on cell growth in mouse MSCs compared with control group with the transfection reagent Lipofectamine 2000 (Fig. 4, A and B). The [3H]thymidine incorporation assay revealed that DNA synthesis rates were significantly slowed by KCa3.1 siRNAs or Clcn3 siRNAs (Fig. 4C). The KCa3.1 siRNA molecules A, B, and C reduced the DNA synthesis rate by 7.55 ± 5.45%, 15.87 ± 4.35%, and 29.60 ± 7.67% (P < 0.05 or P < 0.01 vs. control siRNA), respectively. The Clcn3 siRNA molecules A, B, and C decreased the DNA synthesis rate by 16.00 ± 3.06%, 31.56 ± 6.63%, and 21.29 ± 4.73% (P < 0.05 or P < 0.01 vs. control siRNA), respectively. These data indicate that activity of IKCa and Clcn3 channels is essential in the regulation of cell proliferation.

Effects of ion channel blockers and siRNAs on cell cycle progression. To determine how IKCa and ICl.vol participate in regulating cell proliferation, we used flow cytometry to analyze cell cycle progression with the IKCa inhibitor clotrimazole or the ICl.vol blocker NPPB (Fig. 5A). Figure 5B shows that clotrimazole and NPPB reduced the percentage of cells in S phase (43.04 ± 5.61% for vehicle control; 30.60 ± 4.21% for 2 μM clotrimazole; 12.01 ± 2.03% for 200 μM NPPB, n = 3, P < 0.05 vs. vehicle control) and increased the percentage of cells in G0/G1 phase (51.65 ± 3.34% for vehicle control; 64.45 ± 2.20% for 2 μM clotrimazole; 82.89 ± 2.49% for 200 μM NPPB, n = 3, P < 0.05 vs. vehicle control). The cells in G2/M phase were not affected (5.31 ± 0.57% for control, 5.59%, 23.99 ± 1.16%, and 12.14 ± 1.41% by the KCa3.1 siRNA molecules A, B, and C, respectively (P < 0.01 vs. control siRNA), and the protein levels of Clcn3 were reduced to 34.60 ± 5.80%, 14.88 ± 2.81%, and 21.69 ± 2.68% by the Clcn3 siRNA molecules A, B, and C, respectively (P < 0.01 vs. control siRNA).

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modulation was examined in mouse MSCs. Clotrimazole and NPPB reduced both cyclin D1 and cyclin E protein levels in a concentration-dependent manner (Fig. 6, A and B). Similarly, KCa3.1 siRNAs or Clcn3 siRNAs also significantly decreased cyclin D1 and cyclin E proteins (Fig. 6, C and D). These results indicate that IKCa and ICl.vol channels participate in the regulation of cell cycle progression by modulating expression of cyclin D1 and cyclin E proteins in mouse MSCs.

**DISCUSSION**

In the present study, we demonstrated that inhibition of IKCa (with clotrimazole) or blockade of ICl.vol (with NPPB) produced a significant reduction of proliferation in mouse MSCs. Similarly, knockdown of KCa3.1 or Clcn3 gene with specific siRNA to decrease IKCa or ICl.vol mRNA and KCa3.1 or Clcn3 protein caused a suppression (20–30%) of mouse MSC prolif-

The effect of knockdown of IKCa and ICl.vol channels on cell cycle progression was determined by transfecting KCa3.1 and Clcn3 siRNAs into mouse MSCs. Control siRNA had no significant effect on cell cycle progression, compared with Lipofectamine 2000 (Fig. 5, A and B). KCa3.1 siRNAs or Clcn3 siRNAs reduced the cell number in S phase and increased the cell number in G0/G1 phase. The highly efficient KCa3.1 siRNA molecule C or Clcn3 siRNA molecule B caused an accumulation of 68.59 ± 3.72% or 77.30 ± 3.12% cells (control siRNA: 53.47 ± 2.79%, \( P < 0.05 \)) at G0/G1 phase, an effect comparable with the IKCa inhibitor clotrimazole (2 μM; 64.45 ± 2.20%) or the ICl.vol blocker NPPB (200 μM; 82.89 ± 2.49%), respectively. These results suggest that IKCa and ICl.vol channels regulate cell cycle progression at the G0/G1 transition or the G1/S boundary.

**Effects of ion channel blockers and siRNAs on cell cycle regulatory protein expression.** It is generally believed that the cell cycle regulators cyclin D1 and cyclin E play an important role in early G1 and late G1 progression. Therefore, whether the G0/G1 accumulation induced by ion channel blockers or the corresponding siRNAs is related to cyclin D1 and/or cyclin E
The cell cycle progression was accumulated at G0/G1 phase by ion channel blockers or siRNAs of IKCa and Cl.vol channels participate in the regulation of mouse MSC proliferation. Therefore, expression levels of IKCa channel or changes in permeability. In addition to this physiological function, it is generally believed that Clcn3 is the candidate for molecular identity of Cl.vol in a variety of mammalian tissues (10, 21, 46). However, some reports have demonstrated that Clcn3 is primarily expressed in intracellular organelles, serves as a H+/Cl− exchanger, and contributes to organelle acidification and secretion in hepatocytes (12) and osteoclasts (26). The activation of Clcn3 is not only volume dependent but also Ca2+/calmodulin dependent (32). Our recent study showed that I_{Cl.vol} was activated by hyposmotic challenge and likely encoded by Clcn3 gene in mouse MSCs (37). The present study further demonstrated that knockdown of the Clcn3 gene with specific siRNAs remarkably reduced I_{Cl.vol} (Figs. 3 and 4), supporting the notion that Clcn3 encodes I_{Cl.vol} in mouse MSCs.

I_{Cl.vol} is responsible for regulation of membrane potential in excitable cells and transmembrane Cl− transport in epithelial cells (14, 20) and also plays a role in regulatory volume decrease (20). In addition, I_{Cl.vol} has been found to regulate cell proliferation in smooth muscle cells (7, 44); the antisense oligonucleotide specific to Clcn3 reduces proliferation in rat aortic smooth muscle cells (41). In the present study, we demonstrated that blockade of I_{Cl.vol} with NPPB or downregulation of Clcn3 with the specific siRNAs significantly reduced cell proliferation (Figs. 1–3) and accumulated cells at G0/G1 phase (Fig. 5); this finding is consistent with recent observations in cancer cells and fibroblasts (6, 34, 45). Our results demonstrated that this effect is related to the downregulation of the cell cycle regulatory proteins cyclin D1 and cyclin E (Fig. 6).

It is generally recognized that as a crucial cellular function, cell proliferation is very strictly controlled by several indepen-

**Fig. 6. Effects of channel blockers and siRNAs on expressions of cyclin D1 and cyclin E.** A: Western blot images of cyclin D1 and cyclin E with clomotizolole and NPPB. B: mean values of protein levels of cyclin D1 and cyclin E in mouse MSCs treated with clomotizolole or NPPB for 72 h (n = 3; *p < 0.05, **p < 0.01 vs. vehicle control, relative to GAPDH levels). C: Western blot images of cyclin D1 and cyclin E levels in mouse MSCs treated with lipofectamine, control siRNA, KCa3.1 siRNAs, or Clcn3 siRNAs. D: mean values of cyclin D1 and cyclin E levels in mouse MSCs treated with lipofectamine, control siRNA, KCa3.1 siRNAs, or Clcn3 siRNAs. **p < 0.01 vs. control siRNA group (n = 3), relative to GAPDH levels.
dent mechanisms (28). The present study showed that blockade or knockdown of IK_{Ca} or I_{Cl,vol} caused a 20–30% reduction of cell proliferation in mouse MSCs, suggesting that ion channels participate in regulation of cell proliferation but are not a determinant of cell proliferation. Although the detailed mechanisms underlying regulation of cell proliferation by ion channels remain to be clarified, ion channels are believed to regulate cell proliferation by modulating cell membrane potential and/or cell volume. Cell proliferation eventually requires an increase of cell volume. Alterations of cell volume require the presence that IK_{Ca} and I_{Cl,vol} regulate cell cycle and proliferation by modulating expression of cyclin D1 and cyclin E proteins in mouse MSCs.

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