Endothelial progenitor cells functionally express inward rectifier potassium channels

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1Department of Physiology, 2Program in Neuroscience, Seoul National University College of Medicine, Seoul, Korea; 3National Research Laboratory for Cardiovascular Stem Cell, Seoul National University College of Medicine, Seoul, Korea; 4Division of Pediatrics, Seoul National University College of Medicine, Seoul, Korea; 5Division of Pediatric Neurosurgery, Seoul National University College of Medicine, Seoul, Korea; and 6Department of Brain and Cognitive Sciences, Seoul National University, Seoul, Korea

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Jang SS, Park J, Hur SW, Hong YH, Hur J, Chae JH, Kim SK, Kim J, Kim HS, Kim SJ. Endothelial progenitor cells functionally express inward rectifier potassium channels. Am J Physiol Cell Physiol 301: C150–C161, 2011. First published March 16, 2011; doi:10.1152/ajpcell.00002.2010.—Since the first isolation of endothelial progenitor cells (EPCs) from human peripheral blood in 1997, many researchers have conducted studies to understand the characteristics and therapeutic effects of EPCs in vascular disease models. Nevertheless, the electrophysiological properties of EPCs have yet to be clearly elucidated. The inward rectifier potassium channel (Kir) performs a major role in controlling the membrane potential and cellular events. Here, via the whole cell patch-clamp technique, we found inwardly rectifying currents in EPCs and that these currents were inhibited by Ba2+ (100 μM) and Cs+ (1 mM), known as Kir blockers, in a dose-dependent manner (Ba2+, 91.2 ± 1.4% at −140 mV and Cs+, 76.1 ± 6.9% at −140 mV, respectively). Next, using DiBAC3, a fluorescence indicator of membrane potential, we verified that Ba2+ induced an increase of fluorescence in EPCs (10 μM, 123 ± 2.8%), implying the depolarization of EPCs. At the mRNA and protein levels, we confirmed the existence of several Kir subtypes, including Kir2.x, 3.x, 4.x, and 6.x. In a functional experiment, we observed that, in the presence of Ba2+, the number of tubes on Matrigel formed by EPCs was dose-dependently reduced (10 μM, 62.3 ± 6.5%). In addition, the proliferation of EPCs was increased in a dose-dependent fashion (10 μM, 157.9 ± 17.4%), and specific inhibition of Kir2.1 by small interfering RNA also increased the proliferation of EPCs (116.2 ± 2.5%). Our results demonstrate that EPCs express several types of Kir which may modulate the endothelial function and proliferation of EPCs.

Ion channels are accountable for a variety of physiological events, and their dysfunction leads to several diseases by the disruption of balance of cells, tissues, and organs. In recent years, with further developments in stem cell research, several ion channels have been identified and considered as novel modulators that control the differentiation and modulation of stem cells (4, 13, 31, 42). The inward rectifier K+ channel (Kir), as one of the K+ channels, is important for the maintenance of the resting membrane potential (RMP). Kir is composed of seven families. Importantly, Kir2.x has an interesting property in which inward rectifying K+ conductance increases when the membrane potential is held below the equilibrium potential for K+, by which RMP of cells is stably maintained. Also, some Kir subtypes, such as G protein-activated inward rectifier K+ channel (Kir3.x) and ATP-sensitive K+ (KATP) channel (Kir6.x), triggered by extracellular ligands and intracellular ATP, have been established to modulate a variety of cellular events and signaling cascades. Recently, many reports have suggested that voltage-dependent Kir expressed in neural stem cells determines electrical properties, which results in the increase of cell proliferation (49), KATP is associated with myelination and proliferation of oligodendrocyte precursor cells (40), and the hyperpolarization of membrane potential induced through overexpression of Kir2.1 leads to the differentiation of human cardiomyocyte progenitor cells (46). How-
ever, despite many results about Kir in various stem cells, the expression and function of Kir on the membrane of EPCs are not yet understood. In the present study, we focused primarily on Kir and established its expression and physiological role in EPCs using a variety of experimental tools.

MATERIALS AND METHODS

Isolation and culture of EPCs. This study was approved by the Institutional Review Board of Seoul National University Hospital. Human umbilical cord blood-derived late EPCs were isolated and cultured as described previously (50). In brief, human umbilical cord blood (50 ml) obtained with informed consent from donors was fractionated via centrifugation of the Ficoll-Plaque (GE Healthcare) gradient. According to the manufacturer’s instructions, isolated mononuclear cells were resuspended using an EGM-2-MV system (catalog no. CC-3162; Clonetics) consisting of endothelial basal medium, 5% fetal bovine serum, hEGF, VEGF, hFGF-B, IGF-I, ascorbic acid, and heparin. Mononuclear cells (5 × 10^6 per well) were seeded on 1.5% fibronectin-coated (Sigma, St. Louis, MO) six-well plates and incubated in a 5% CO_2 incubator at 37°C. Under daily observation, the first media change was conducted within 6 days after plating. Thereafter, media were changed every 3 days. Late EPCs from mononuclear cells appeared within 2 wk after plating as colonies consisting of cells with different morphology from early EPCs.

Confirmation of EPC molecular phenotype. To confirm the EPC phenotype, adherent cells were incubated with Dil-labeled ac-LDL (Molecular Probes) for 1 h, fixed, and then incubated for 1 h with FITC-labeled VE-cadherin antibody (BenderMed Systems). Cells were visualized using an inverted fluorescent microscope (Olympus IX71), and adherent cells staining positive for both FITC-VE-cadherin and Dil-ac-LDL were identified as EPCs. Fluorescence was excited at 488 nm and emission was recorded at 530 nm and 620 nm, respectively. To evaluate the surface marker phenotype of the cultured EPC population, the cells were detached with trypsin-EDTA and filtered at 4 kHz. Patch pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) using a vertical pipette puller (Narishige PC-10). These pipettes had a resistance of 5–7 MΩ filled with a solution containing (in mM) 140 KCl, 5 NaCl, 0.5 MgCl_2, 3 CaCl_2, 3 Mg-ATP, 10 EGTA, 10 HEPES, and 5 glucose. The pH was adjusted to 7.4 with KOH. The external solution containing (in mM) 145 NaCl, 5 KCl, 1 MgCl_2, 1 CaCl_2, 10 HEPES, and 5 glucose was used as a normal bath solution. The pH was adjusted to 7.4 with NaOH. Each pipette was generated for high-resistance seals without fire polishing. For whole cell recording, a coverslip attached with EPCs was transferred into a chamber. After the GFS seal was made by attachment to the cell membrane and negative suction, the pipette capacitive currents were compensated. To establish the whole cell configuration, the cell membrane was ruptured by weak negative suction. We did not compensate for cell capacitance and series resistance in each cell. EPCs were held at ~70 mV. A series of voltage steps from ~140 to +60 mV for 500 ms per pulse at an interpulse interval of 5 s were used to record the voltage-dependent currents. To minimize and isolate the inward potassium currents, the potassium concentration in the extracellular solution was increased to 140 mM by iso-osmotic substitution of NaCl for KCl. To minimize other currents, we inhibited the voltage-gated potassium channel, KvATP channel, and Ca^{2+}-dependent Cl\(^{-}\) channel using tetraethylammonium (TEA, 1 mM), glibenclamide (10 μM), and niflumic acid (100 μM), respectively. Under these conditions, the holding potential of EPCs was changed to 0 mV. All recordings were conducted at room temperature. The acquired whole cell current was analyzed with the Pulse-Fit program (HEKA).

Measurement of changes in the membrane potential. The membrane potential of EPCs was assessed using the fluorescent indicator DiBAC_3 (Molecular Probes). EPCs on coverslips were transferred to a chamber adapted to a confocal laser-scanning fluorescence microscope (Olympus) and incubated for 20 min in external solution with 0.3 μM DiBAC_3 at room temperature. The fluorescence signal intensity was evaluated at 10-s intervals. Using 5, 60, and 140 mM KCl solutions with 0.3 μM DiBAC_3, we optimized the recording conditions in our experiment. To confirm the change in membrane potential in EPCs by blockage of Kir, we added 10 μM Ba^{2+} to block Kir and 140 mM KCl as experimental controls. Fluorescence was excited at 488 nm and emission was recorded at 530 nm.

Reverse transcription-polymerase chain reaction. Total mRNA was extracted from EPCs using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. The first-strand cDNA was synthesized using the reverse-transcription system (Promega) and amplified by NOVA-Taq DNA polymerase (Genemed) in a 25-μl reaction mixture. Each cDNA was subjected to polymerase chain reaction (PCR) amplification using gene-specific primers and was sequenced by chain termination using an ABI PRIZM3100 genetic analyzer (Applied Biosystems). The primer sequences and PCR conditions for each of the Kir subtypes and the GADPH housekeeping gene are listed in Table 1. Control reactions consisted of the above

<table>
<thead>
<tr>
<th>Gene</th>
<th>Access no.</th>
<th>Forward Primer Sequence (5’-3’)</th>
<th>Reverse Primer Sequence (5’-3’)</th>
<th>Length, bp</th>
<th>T(_{anneal}), °C</th>
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<tr>
<td>Kir1.1</td>
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<td>CACAGACAGGGAGTGGTGG</td>
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<tr>
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<td>TTGGGGTTGGGTTGGTGGTGG</td>
<td>298</td>
<td>58</td>
</tr>
<tr>
<td>Kir2.4</td>
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</tr>
<tr>
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<td>CGTATGGGAGGAGGAGGAGGAGG</td>
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<td>54</td>
</tr>
<tr>
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<td>Kir4.1</td>
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<tr>
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Kir, inward rectifier potassium channel; T\(_{anneal}\), annealing temperature.
mentioned PCR amplification mix with primers but no cDNA template. PCR was conducted using a PCR system 3700 (Applied Biosystems). PCR cycles were programmed as follows: 94°C for 10 min, 35 cycles consisting of 30 s denaturation at 94°C, 30 s at the annealing temperature indicated in Table 1, and 45 s extension at 72°C, with a final extension at 72°C for 5 min. The mRNA expression of GAPDH was used utilized as an internal control. The size of the PCR product was confirmed by running 10 µl of product on 1.0% agarose gel electrophoresis and visualized via ethidium bromide staining. Commercially available human heart mRNA (Ambion) and water were used as positive and negative controls, respectively.

Western blot analysis. To extract total protein from EPCs, EPCs were lysed with a buffer [25 mM Tris, pH 7.4, 10 mM NaCl, 1% Triton X-100, 1% cholic acid, and phosphatase inhibitor cocktail (PIC)]. Lysates were clarified via centrifugation at 7,300 g for 10 min at 4°C. Protein concentrations were then determined via a Bradford assay. The lysates (40 µg) were solubilized in a loading buffer containing 250 mM Tri-Cl, pH 6.8, 357.7 mM β-mercaptoethanol, 10% SDS, 0.5% bromphenol blue, 50% glycerol, and PIC, boiled for 10 min, and separated via 10% SDS-polyacrylamide gel electrophoresis. The proteins were then transferred electrophoretically onto a polyvinylidene difluoride membrane. The membrane was blocked with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 2.5% skim milk for 1 h. After being blocked, the transferred membranes were incubated with rabbit polyclonal anti-Kir2.1 (Santa Cruz Biotechnology), goat polyclonal anti-Kir2.4 (Santa Cruz Biotechnology), rabbit polyclonal anti-Kir3.1 (Santa Cruz Biotechnology), goat polyclonal anti-Kir6.2 (Santa Cruz Biotechnology), and GAPDH (Santa Cruz Biotechnology) in PBS containing 0.05% Tween 20. After three washings with PBS, the membrane was reacted with the appropriate horseradish-peroxidase (HRP)-conjugated rabbit IgG as a secondary antibody (Santa Cruz Biotechnology) and HRP-conjugated goat IgG as a secondary antibody (Santa Cruz Biotechnology). The membranes were then immunoblotted. The bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Immunofluorescence. EPCs were seeded onto a slide and fixed with 4% paraformaldehyde at pH 7.4. After being rinsed three times with PBS, EPCs were permeabilized with 0.1% Triton in PBS buffer for 2 min, blocked with 5% bovine serum albumin in physiological saline solution with 0.1% Triton for 10 min at room temperature, and exposed to rabbit polyclonal anti-Kir2.1 antibody (1:50 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. After three washes with PBS containing 0.1% Triton X-100, EPCs were incubated with a secondary antibody conjugated to Alexa Fluor 488 goat anti-rabbit IgG (1:200 dilution; Molecular Probes) for 50 min in the dark at room temperature and treated with DAPI (Sigma) to stain the nuclei in the EPCs. After several additional washes, coverslips were mounted with Gel/Mount (Biomega). Immunofluorescent images were captured with a confocal fluorescence microscope (Zeiss LSM410). The excitation and emission wavelengths were 488 nm and 525 nm, respectively.

In vitro tube formation on Matrigel plate. Matrigel (Becton-Dickinson Labware) basement membrane matrix was added to a chamber slide. After 1 h of incubation at room temperature, 1 × 10^5 EPCs/ml were added to the chamber slide with 100 µl of EGM-2-MV media containing 0, 1, and 10 µM BaCl2. After 16–24 h of incubation, we randomly chose 10 representative fields from an unbiased examiner in each plate, and the averages of the total number and area of complete tubes formed by EPCs per unit area were compared by Image-Pro Plus.

Assessment of EPC number and cell cycle analysis. The number of EPCs was determined in EGM-2-MV including 0, 1, and 10 µM BaCl2. EPCs were detached on culture dishes using 0.25% trypsin-EDTA and counted with a hemocytometer (Reichert) under an in-
verted microscope. To determine the percentages of G0/G1, S, and G2/M phase cells, detached EPCs were washed in PBS and fixed with 70–100% ethanol. Ethanol was removed with a centrifuge, and the cell pellets were washed with PBS. EPCs were incubated with PBS including propidium iodide (Sigma) and analyzed using FACS. Data were acquired with Cell Quest software.

RNA interference. The small interfering RNAs (siRNAs) were transfected into EPCs at 50–60% confluence using siRNA transfection reagent (Santa Cruz Biotechnology) for 6 h, and then transfection media were exchanged for EGM-2-MV. The efficiency of transfection was confirmed by a comparison of the expression of target protein in each group treated with negative universal control siRNA and target siRNA (Santa Cruz Biotechnology) 24 h after treatment. EPCs were detached on culture dishes using 0.25% trypsin-EDTA and counted with a hemocytometer (Reichert) under an inverted microscope, 2–3 days after transfection.

Data analysis. Data are expressed as means ± SE. Statistical analyses were conducted using paired Student’s t-test (ANOVA, independent). The differences between groups were considered significant at $P < 0.05$.

[Fig. 2. Whole cell currents and inwardly rectifying K+ currents in EPCs. A: morphology of late EPCs seeded on a coverslip for whole cell patch-clamp recording. Scale bar, 30 μm. B: established voltage protocol used to measure whole cell currents. EPCs were changed from a holding potential of −70 mV to pulse potentials between −140 mV and +60 mV for 500 ms. C: representative trace of currents that were recorded by the voltage protocol. D: current-voltage (I-V) relationship plotted from average currents recorded using step pulses from −140 to +60 mV during 400–500 ms, which shows two types of currents including inwardly rectifying currents and outwardly rectifying currents. E: representative trace of inward rectifying currents measured in each condition containing 5, 60, and 140 mM KCl with tetraethylammonium (TEA; 1 mM), glibenclamide (10 μM), and niflumic acid (100 μM) to inhibit the voltage-gated potassium channels, ATP-sensitive potassium channels, and Ca2+-dependent Cl− channels, respectively. F: I-V relationship plotted from average currents recorded using step pulses from −140 to +60 mV during 400–500 ms (5, 60, and 140 mM KCl) ($n$ = 17, 7, and 17, respectively). The capacitive current traces in C and E are truncated.]
RESULTS

Characterization of human umbilical cord blood-derived EPCs. Late EPCs appeared in cultured human umbilical cord blood mononuclear cells after 2–3 wk. The colonies evidenced characteristic homogeneity and “cobblestone” morphology on the third-fifth passages, as previously reported (Fig. 1A) (14, 50). To further characterize cell outgrowth, in accordance with previous reports (15), we conducted several experiments. EPCs were identified as double-positive for DiI-Ac-LDL uptake (Fig. 1B) and VE-cadherin (Fig. 1C) under a fluorescence microscope. Additionally, we confirmed the expression of endothelial-specific markers: CD34, KDR/VEGFR-2, VEGF-3, ICAM-1, Tie-2, CD44, Ib-1, and Ib-2 via flow cytometry (Fig. 1D). These results demonstrated that the late EPCs for recording electrophysiological properties are well isolated and identified.

Whole cell currents and the existence of inwardly rectifying K⁺ currents in EPCs. In the electrophysiology experiments, we used the third and fourth passage EPCs that appeared at 28 ± 3 days. The whole cell configuration was made to record currents through membrane channels in EPCs (Fig. 2A). The membrane capacitance was 29.8 ± 1.8 pF (n = 27). The RMP was −49.6 ± 6.8 mV (n = 8), which was measured via the previously recorded calibration procedure (36). Whole cell membrane currents of EPCs were recorded with 500-ms voltage steps between −140 mV and +60 mV from a holding potential of −70 mV. The majority of EPCs that we recorded showed electrophysiological homogeneity, which supports our previous notion that the population of late EPCs is homogeneous in vitro (14). Figure 2D demonstrates that inwardly rectifying currents were induced at hyperpolarized membrane potentials. To prove whether these inwardly rectifying currents are dependent on the extracellular potassium concentration, we established experimental conditions in which inward rectifying currents could be isolated by using TEA (1 mM), glibenclamide (10 μM), and niflumic acid (100 μM) to inhibit voltage-gated K⁺ channels, KATP channels, and Ca²⁺-dependent Cl⁻ channels, respectively. As the concentration of potassium was increased to 5, 60, and 140 mM KCl, the amplitudes of inward rectifying currents were elevated (5 mM, −83.1 ± 9.5 pA; 60 mM, −226.6 ± 17.1 pA; 140 mM, −500.0 ± 30.4 pA at −140 mV) (Fig. 2F). These data suggested that inwardly rectifying K⁺ currents exist in EPCs and the properties of these inwardly rectifying currents are similar to those of Kir.

The relationship of inwardly rectifying K⁺ currents and Kir in EPCs. To confirm whether Kir is associated with inwardly rectifying currents recorded in EPCs, we conducted whole cell patch clamping in an experimental condition which was established to isolate and maximize inwardly rectifying K⁺ currents. Under the established condition, we attempted to confirm whether these currents are inhibited by Ba²⁺ and Cs⁺, both of which are known as Kir blockers. The application of extracellular Ba²⁺ (1, 10, and 100 μM) inhibited inwardly rectifying currents in a dose-dependent fashion (1 μM, 26.2 ± 4.1%; 10 μM, 88.5 ± 4%; 100 μM, 91.2 ± 1.4% at −140 mV, respectively) and the pattern of Ba²⁺ blockage was similar to that of Cs⁺ blockage reported in other cells (Fig. 3, A and B) (7, 51). In addition to Ba²⁺, the application of extracellular Cs⁺ (10, 100, and 1,000 μM) also inhibited inwardly rectifying currents in a dose-dependent fashion (10 μM, 53 ± 3.7%; 100 μM, 57.6 ± 9.0%; 1,000 μM, 76.1 ± 6.9% at −140 mV, respectively), and the pattern of Cs⁺ blockage was also similar.
to that of Cs\(^+\) blockage reported in other cells (Fig. 4, A and B) (7, 51). Our data showed that inward rectifying K\(^+\) currents are dose-dependently inhibited by Kir blockers.

**Change of the membrane potential by Kir in EPCs.** Next, to elucidate how Kir influences the electrophysiological properties of EPCs, we assessed the changes of membrane potential caused by the blockage of Kir in EPCs. Since it was difficult to measure membrane potential of EPCs under current-clamp mode, we used the voltage-sensitive dye DiBAC\(_3\) to estimate the membrane potential. We hypothesized that the application of Ba\(^2+\) may alter the membrane potential of EPCs. Many papers have reported that the blockage of Kir depolarizes the membrane potential and induces a change in calcium concentration (10). Before conducting the experiment, we optimized the experimental conditions by changing the membrane potential. As shown in Fig. 5B, we observed that the fluorescence of EPCs was linearly elevated under conditions of increasing potassium concentration of external solution with DiBAC\(_3\) (5 mM, 100%; 60 mM, 117 \pm 1.7%; 140 mM, 139.2 \pm 3.7%), which implies the depolarization of EPCs. After the recording conditions were optimally established, we confirmed that Ba\(^2+\) induced an increase of fluorescence in EPCs (10 \(\mu\)M Ba\(^2+\), 123.8 \pm 2.8%; 140 mM KCl, 159.3 \pm 4.8%), demonstrating that Kir blockage depolarizes membrane potential of EPCs. At the end of each experiment, we treated 140 mM KCl for saturation of fluorescence and 5 mM KCl for the decrease of fluorescence level to the baseline (data not shown). Taken together, the results of this experiment suggested that Kir contributes to the resting membrane potential of EPCs.

**The mRNA and protein expression of Kir subtypes in EPCs.** After observing the fact that Kir functionally exists and controls the membrane potential in EPCs, we tried to prove whether EPCs express several Kir subtypes at the mRNA and protein levels. First, we conducted RT-PCR analysis with specific primers (Table 1). Using RT-PCR, the selected 10 representative Kir subtypes were tested, as follows: Kir1.1, Kir2.1, Kir2.2, Kir2.3, Kir3.1, Kir3.4, Kir4.1, Kir6.1, and Kir6.2; GAPDH was used as a PCR control. We also evaluated the expression of human ether-a-go-go-related gene 1 (hERG1), known as Kv11.1, which can pass inwardly rectifying currents. We used commercial mRNA from the human heart as a positive control to determine the optimal annealing temperature for each primer and water without cDNA template as a negative control. The PCR results demonstrated that mRNA expression of Kir2.1, Kir2.2, Kir2.4, Kir3.1, Kir3.4, Kir4.1, Kir6.1, and Kir6.2 was positive (Fig. 6A), whereas the mRNA expression of Kir1.1 and Kir2.3 was not detected. To confirm expression at the protein level, we selected four representative subtypes (Kir2.1, Kir2.4, Kir3.1, and Kir6.2) among Kir2.x, Kir3.x, and Kir6.x. Each subtype is known to be activated by membrane hyperpolarization, triggered by the activation of G protein-coupled receptors, and modulated by intracellular ATP, respectively. Selected subtypes were detected by Western blotting in EPC lysates (Fig. 6B). Moreover, to elucidate the distribution of Kir protein in EPCs, we chose a Kir2.1 antibody for immunostaining on EPCs. Figure 6C shows that Kir is widely localized in EPCs. As shown in Fig. 6, we demonstrated that various subtypes of Kir are expressed in EPCs.

**Endothelial function of Kir in EPCs.** To investigate the functional roles of Kir in EPCs, we evaluated the capacity of tube formation of EPCs on Matrigel under different Ba\(^2+\)
concentrations. Many reports have established the capacity of tube formation on Matrigel as an experiment that can demonstrate the endothelial function of EPCs. After seeding EPCs on Matrigel and incubating for 16–24 h, we counted the number of tubes and calculated the area of each tube, based on the following standards to count a complete tube:

1) intact and complete hedron and
2) hedron that consists of more than four cells. We observed that the application of Ba\(^{2+}\)/H11001 resulted in a numerical reduction of complete tubes formed by EPCs on Matrigel (1 \(\mu\)M, 18.1 ± 11.4%; 10 \(\mu\)M, 157.9 ± 17.4%, \(P < 0.01; n = 15\)), respectively) (Fig. 7B). Additionally, we observed that the percentage of EPCs in the S phase related to DNA synthesis among G0/G1, S, and G2/M phase was increased under Ba\(^{2+}\) (10 \(\mu\)M, 26.5 ± 1.5%, \(P < 0.05; n = 3\)) (Fig. 8C). To reduce concerns for Ba\(^{2+}\) specificity as Kir blocker and further elucidate the role of Kir2.1 and Kir2.4 in EPC proliferation, we treated siRNA transfection reagent with siRNA Kir2.1 and Kir2.4 on EPCs. After 18–24 h, the effect of siRNA was determined by Western blotting. Figure 8D shows the decrease of Kir2.1 and Kir2.4 expression in EPCs, implying that the knockdown was successfully conducted. Our siRNA data proposed that the number of EPCs was elevated in the group treated with Kir2.1 siRNA (116.2 ± 2.5%, \(P < 0.01; n = 5\)) but was not changed in the group treated with Kir2.4 siRNA (100.4 ± 2.0%; \(n = 5\)). The change of proliferative capacity in EPCs under Ba\(^{2+}\) blockage conditions and the knockdown system suggested that Kir is an essential channel for the regulation of the proliferative activity and replicative capacity of EPCs. Also, the fact that specific inhibition of Kir2.1 through siRNA induced the increase in the number of EPCs concentration.

Proliferative capacity through Kir and the relation of proliferation and Kir2.1 in EPCs. In addition to the endothelial functions of Kir, we attempted to determine how Kir regulates the cellular proliferation of EPCs. To assess the proliferative effect of Kir in EPCs, we seeded EPCs on six-well culture plates with EGM-2-MV containing 0, 1, and 10 \(\mu\)M Ba\(^{2+}\) followed by incubation at 37°C. After 3–5 days, we manually calculated the number of EPCs on a hemocytometer and analyzed the cell cycle progression of EPCs via FACS analysis. Our data demonstrated that the number of EPCs was dose-dependently elevated in EGM-2-MV containing Ba\(^{2+}\) (1 \(\mu\)M, 137.4 ± 11.4%; 10 \(\mu\)M, 157.9 ± 17.4%, \(P < 0.01; n = 15\)), respectively) (Fig. 8B). Additionally, we observed that the percentage of EPCs in the S phase related to DNA synthesis among G0/G1, S, and G2/M phase was increased under Ba\(^{2+}\) (10 \(\mu\)M, 26.5 ± 1.5%, \(P < 0.05; n = 3\)) (Fig. 8C). To reduce concerns for Ba\(^{2+}\) specificity as Kir blocker and further elucidate the role of Kir2.1 and Kir2.4 in EPC proliferation, we treated siRNA transfection reagent with siRNA Kir2.1 and Kir2.4 on EPCs. After 18–24 h, the effect of siRNA was determined by Western blotting. Figure 8D shows the decrease of Kir2.1 and Kir2.4 expression in EPCs, implying that the knockdown was successfully conducted. Our siRNA data proposed that the number of EPCs was elevated in the group treated with Kir2.1 siRNA (116.2 ± 2.5%, \(P < 0.01; n = 5\)) but was not changed in the group treated with Kir2.4 siRNA (100.4 ± 2.0%; \(n = 5\)). The change of proliferative capacity in EPCs under Ba\(^{2+}\) blockage conditions and the knockdown system suggested that Kir is an essential channel for the regulation of the proliferative activity and replicative capacity of EPCs. Also, the fact that specific inhibition of Kir2.1 through siRNA induced the increase in the number of EPCs.

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Fig. 5. Change in membrane potential by blockage of inward rectifier potassium channel (Kir) in EPCs. A: representative images and traces showing change in fluorescence intensity in 5, 60, and 140 mM KCl. Scale bar, 20 \(\mu\)m. Each up-down arrow is the point of measurement. B: summary of the membrane potential measurement in bath solution including 5, 60, and 140 mM KCl. Black circles show values for individual cells and the red circle shows the average of cells (n = 10). C: representative traces showing the increase of fluorescence at baseline, 10 \(\mu\)M BaCl\(_2\), and 140 mM KCl. Each up-down arrow is the point of measurement. D: summary of the change of membrane potential in bath including 5 mM KCl (baseline), 10 \(\mu\)M BaCl\(_2\), and 140 mM KCl. Black circles show values for individual cells and the red circle shows the average of cells (n = 9). The solution used in all experiments contained 0.3 \(\mu\)M DiBAC\(_3\).
provides support that Ba$^{2+}$ blocked Kir under our experimental conditions.

**DISCUSSION**

In the present study, using electrophysiological techniques, we recorded whole cell currents and confirmed the existence of Kir in EPCs. It was not until 1997 that researchers began to make efforts to establish definitive markers for EPCs and to evaluate the beneficial effects of EPCs in vascular disease models such as myocardial ischemia, cerebral atherosclerosis, and cerebral hemorrhage (9, 23). For a number of years, although EPCs were used to investigate cell therapy and differentiation, direct information regarding the expression and functional role of ion channels in EPCs has not been well documented. Also, except for a report concerning functional Kir in porcine bone marrow progenitor cells (27), there is no study on the expression and role of Kir in EPCs. Our results provide us not only with new information for establishing the characteristics of EPCs, but with a novel perspective about the relationship between expression of channels and functions in EPCs. This paper is the first to characterize the function of ion channels in EPCs from an electrophysiological point of view.

For this experiment, late EPCs, but not early EPCs, derived from human umbilical cord blood were examined because of the fact that late EPCs have been widely used from therapeutic research to clinical applications and in vitro culture systems.

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**Fig. 6.** The expression of several Kir subtypes in EPCs. A: PCR products showing mRNA expression of Kir subtypes (Kir2.1, Kir2.2, Kir2.4, Kir3.1, Kir3.4, Kir4.1, Kir6.1, and Kir6.2), human ether-a-go-go-related gene 1 (hERG1), which has inwardly rectifying properties, and GAPDH as PCR control. The mRNA from human heart was commercially used as a positive control for each primer, and water (no template) was used as a negative control ($n = 4$). B: protein expression of Kir2.1 (48 kDa), Kir2.4 (48 kDa), Kir3.1 (56 kDa), Kir6.2 (40 kDa), and GAPDH (38 kDa), detected using Western blotting ($n = 3$). C: immunofluorescence images showing the wide distribution of Kir2.1 (green) and DAPI staining (blue) for nuclear labeling ($n = 3$). Scale bar, 20 μm.
Fig. 7. Tube formation on Matrigel by blockade of Kir in EPCs. A: representative photographs showing the tubes formed in EGM-2-MV media containing 0 (control), 1, and 10 μM BaCl2 on Matrigel. Scale bar, 200 μm. B: bar graph showing the increase of complete tubes made by EPCs under each condition (1 μM, 18.1 ± 2.7; 10 μM, 11.2 ± 2.2, **P < 0.01; n = 4, respectively). C: bar graph showing the area (in mm2) of complete tubes made by EPCs under each condition. **P < 0.01, relative to control group.

have been well established with experiments in our laboratory (14, 15, 50). The electrophysiological properties of EPCs were shown as two types of currents containing inwardly rectifying currents and outwardly rectifying currents, similar to those of human umbilical vein endothelial cells (HUVECs; 47), which implies that the distribution and expression of channels in EPCs may not be different from HUVECs. Also, several studies have reported that the molecular biological and physiological characteristics of EPCs are similar to those of endothelial cells (14, 15, 47).

Generally, Kir has been well established as a key channel for the stability of membrane potential in cells. In particular, it was already reported that Kir maintains the RMP and is involved in the regulation of nitric oxide synthesis in endothelial cells (35) as well as the modulation of a host of signaling events. In our electrophysiology experiment, the amplitude of inwardly rectifying potassium currents in EPCs was increased depending on potassium concentration and extracellular application of submillimolar Ba2+ and Cs+ inhibited inward rectifying potassium currents, both results of which made sure that Kir exists in EPCs. Also, the fact that Kir is related to the formation of membrane potential was validated by DiBAC3 (voltage-sensitive dye), used as an alternative method for the visualization of the change in membrane potential. Using DiBAC3, we were able to record RMP by applying the calibration procedure based on the equilibrium potential of K+ and observe the relative elevation of membrane potential under Kir blockade by Ba2+. Although we did not fully exclude the possibility that Ba2+ may affect other channels, when we recorded the whole cell currents with Ba2+, any direct effects on other channels were not detected. Recently, several reports concerning the relation of membrane potential and cellular functions have demonstrated that adipogenic and osteogenic differentiation is mediated by the change of membrane potential in mesenchymal stem cells (40) and that developmental and cancerous cells tend to be depolarized and mitotically active (41). Moreover, lacking functional Kir displays active proliferation in response to injury (6). These reports support our assumption that the change of membrane potential by Kir blockage in EPCs may be considered as underlying mechanisms in our functional assay.

Kir is composed principally of seven subfamilies. The Kir2 and 4 subfamilies, as strong inward rectifier potassium channels, which are known to be expressed abundantly in the heart, are sensitive to voltage, phosphorylation, and other second messengers (29, 32, 33). Our RT-PCR data demonstrated that Kir2.1, 2.2, 2.4, and 4.1 are expressed in EPCs (Fig. 6A) and the protein expression of Kir2.1 and Kir2.4 (Fig. 6B) is detected using each antibody, thereby implying that the majority of inwardly rectifying K+ currents recorded in EPCs may pass through Kir2.1, 2.2, and 2.4. In addition to the Kir2 subfamily, mRNA and protein expression of the Kir3 and Kir6 subfamilies were determined in EPCs (Fig. 6, A and B). The Kir3 subfamily is a G protein-activated strong inwardly rectifying K+ channel and is expressed in the heart, brain, and endocrine tissue (29). Many reports have demonstrated that EPCs express G protein-coupled receptors, known to bind to ligands such as endothelium-I and angiotensin II associated with EPC homing, differentiation, and proliferation (12, 16, 25, 30, 33, 37, 39). We also found that EPCs express the Kir6 subfamily (Kir6.1), which is regulated by intracellular ATP. Recently, there is increasing evidence that proliferation and invasion of EPCs are controlled by the increase of extracellular glucose level (5, 44, 52). Finally, we evaluated the expression of hERG1, known as Kv11.1, which can pass inwardly rectifying currents.

Before the start of our functional study, we were still concerned about selectivity of Ba2+. However, although Ba2+ has the likelihood to pass through nonselective cation channels or calcium channels as well as to affect other potassium channels, most researchers have used low concentration of
Ba²⁺ (10 μM) as a Kir blocker in functional assays (26, 49). Also, based on our data, in our siRNA experiment, it was determined that specific siRNA approach to knock down Kir2.1 and 2.4 may relieve concerns about the specificity of Ba²⁺.

Generally, cell-to-cell networks have been shown to be indispensable for the differentiation of EPCs into endothelial cells, cell proliferation, and the operation of endothelial functions including the formation of vessels and the secretion of a variety of vascular growth factors. Our data showed that Ba²⁺ induced a reduction in the number of tubes formed by EPCs on Matrigel, which indicates that Kir is an important channel for EPCs to form networks and interact with each other. Interestingly, we observed different results between number and area of tubes perhaps because incomplete tubes were excluded from the total. Next, we evaluated changes in the proliferative capacity of EPCs under different Ba²⁺ concentrations and conditions using siRNA to understand the manner in which Kir regulates the proliferation of EPCs. In contrast to a recent report that the blockage of calcium-activated K⁺ channels and volume-sensitive chloride channels results in the reduction of proliferation in mouse mesenchymal stem cells (43), our results revealed that blockage of Kir by Ba²⁺ increases the cell number and the percentage of S phase in relation to DNA synthesis and replication in EPCs (Fig. 8, B and C). We believe that different proliferative roles of each channel are attributed to the diversity of fundamental functions and cellular responsibility of channels in cells. Finally, specific knockdown of Kir2.1, known to show voltage-dependent strong inward rectification, induced the increase of proliferation in EPCs. Although the increase of proliferative activity by siRNA2.1 was not as much as the increase of proliferation by Ba²⁺, these data...
implied that the membrane potential of EPCs treated with siRNA2.1 might be changed. However, the possibility that other subtypes of Kir would be also associated with the functions of EPCs still remains.

In conclusion, EPCs represent a novel candidate in regenerative vessel therapy and participate in postnatal vasculogenesis in a variety of physiological and disease conditions. In addition, under these conditions, several factors including VEGF, erythropoietin, and granulocyte-macrophage colony-stimulating factor regulate the number of EPCs in the circulation and trigger the endothelial function of EPCs in an injury site by binding to their respective receptors (2, 11, 48). Eventually, these series of responses are mediated by the activation of ion channels or receptors on the membrane, responding to the change of electrical potential and ligands. Here, we show that EPCs exhibit several Kir subtypes such as Kir2.3, 3.x, and 6.x, which may be modulated by the electrical activity, several ligands such as cytokines, and intracellular ATP level. Our observation may provide a novel foundation to understand the role and cellular mechanisms of EPCs in pathogenesis. Furthermore, we will further investigate functions of other channels as well as Kir in EPCs and manipulate their functional effects in EPCs using pharmacological and genetic modification. EPCs can be used as a more efficient candidate as stem cell-based therapy for vascular disease patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


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