Hypoxic preconditioning enhances bone marrow mesenchymal stem cell migration via Kv2.1 channel and FAK activation

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Submitted 12 January 2010; accepted in final form 4 May 2011

Hypoxic preconditioning enhances bone marrow mesenchymal stem cell migration via Kv2.1 channel and FAK activation. Am J Physiol Cell Physiol 301: C362–C372, 2011. First published May 11, 2011; doi:10.1152/ajpcell.00013.2010.—Transplantation using stem cells is emerging as a potential regenerative therapy after ischemic attacks in the heart and brain. The migration capability of transplanted cells is a critical cellular function for tissue repair. Based on our recent observations that hypoxic preconditioning (HP) has multiple benefits in improving stem cell therapy and that the potassium Kv2.1 channel acts as a promoter for focal adhesion kinase (FAK) activation and cell motility, the present investigation tested the hypothesis that HP treatment can increase BMSC migration via the mechanism of increased Kv2.1 expression and FAK activities. BMSCs derived from green fluorescent protein-transgenic mice were treated under either normoxic (N-BMSC) or hypoxic (0.5% O2) (HP-BMSC) conditions for 24 h. Western blot analysis showed HP selectively upregulated Kv2.1 expression while leaving other K+ channels unaffected. Compared with normoxic controls, significantly larger outward delayed rectifier K+ currents were recorded in HP-BMSCs. HP enhanced BMSC migration/homing activities in vitro and after intravenous transplantation into rats subjected to permanent myocardial infarction (MI). The HP-promoted BMSC migration was inhibited by either blocking K+ channels or knocking down Kv2.1. Supporting a relationship among HP, Kv2.1, and FAK activation, HP increased phosphorylation of FAK397 and FAK576/577, and this effect was antagonized by blocking K+ channels. These findings provide novel evidence that HP enhances the ability of BMSCs to migrate and home to the injured region; this effect is mediated through a regulatory role of Kv2.1 on FAK phosphorylation/activation.

therapy is limited by the fact that very few transplanted cells survive and home to the injured tissues, and functional recovery often is inadequate (5, 19, 46). A previous study showed that only 1.5% of injected stem cells accumulate at infarcted myocardial tissue 2 h after intracoronary infusion; the poor survival and low homing rate of transplanted cells severely restrict the clinical potential of this therapeutic approach (21). Taking advantage of the less-invasive nature, many stem cell transplantation protocols employ intravenous or intracoronary routes of injection. Since the ischemic tissue has low or no blood flow, successful migration toward the site of injury is a critical step in cell engagement for tissue repair. Aimed to target these issues, our recent investigations tested hypoxic preconditioning of stem cells before transplantation. We showed in embryonic stem cell-derived neural progenitor cells and BMSCs that the survival and migration of hypoxic preconditioned cells were greatly improved both in vitro and after transplantation into the ischemic heart and brain (15, 19, 45).

Cell migration to the injury region is an inherently inefficient process (21) and its enhancement should potentiate the effects of stem cell therapy. To achieve this, an understanding of the intracellular signaling pathways underlying BMSC motility is crucial. It was shown in many cell types that cell migration requires participation of adhesion proteins such as integrins and activation of the focal adhesion kinase (FAK) (13, 33). A recent study demonstrated that BMSCs genetically modified to overexpress integrin-linked kinase (ILK) showed greater survival and adhesion ability both in vitro and after transplantation into rats subjected to occlusion of the left coronary artery (41). Whether FAK plays a role in BMSC migration and/or mediates the hypoxic preconditioning enhanced migration remains to be tested.

K+ channels are a heterogeneous family of membrane proteins that mediate K+ efflux and control a cell’s intrinsic electrical excitability (32). The ion channel-mediated K+ efflux, resulting in increased local extracellular K+ concentrations, has been proposed as a mechanism that stimulates or guides cell migration (8, 25). Consistently, K+ channel blockers such as tetraethylammonium (TEA) and 4-aminopyridine inhibit cell migration (17, 39, 42, 49). Our investigation has shown that the Kv2.1 K+ channel can act as an “adhesion protein,” form a complex with FAK, and promote the activation of FAK in both neuronal and nonneuronal cells (51). The interaction of Kv2.1 with FAK enhances FAK576/577 and FAK397 phosphorylation, significantly increases cell adhesion and directional migration in wound healing processes both in vitro and in animal models (51). Taken together, these data

Bone marrow mesenchymal stem cells (BMSCs) are multipotent progenitors capable of differentiating into a variety of cell types, including cardiomyocytes, vascular endothelial cells, and neuronal cells such as neurons and astrocytes (36). BMSC transplantation is an emerging and promising therapy for the treatment of ischemia-induced injuries in the heart and brain, improving functional outcomes after injury (35, 58). Animal studies and clinical trials have shown that BMSCs can contribute to the repair of ischemic heart and brain tissues via enhanced trophic support and/or cell replacement (18, 47, 54, 58). Currently, however, the efficiency and efficacy of BMSC
suggest that the Kv2.1 protein is a potent promoter for cell migration via increasing activation of FAK.

Several types of K⁺ currents, including a Ca²⁺-activated K⁺ channel current (I_{KCa}), a delayed rectifier K⁺ current (I_K), an inward rectifier K⁺ current (I_{K+i}), and transient outward K⁺ currents (I_o), have been identified in rat BMSCs. Specifically, K⁺ channel molecular subfamily members of Kv2.1, Kv1.2, Kv1.4, Kv3.1, and KCNN4 have been detected in these cells (40, 43). The physiological and pathophysiological function of these channels in BMSCs is not well defined. It was suggested, nonetheless, that delayed rectifier and KCa channels played an important role in maintaining membrane potential and participated in modulation of proliferation in rat BMSCs (10). Whether a K⁺ channel contributes to any other function of BMSCs has not been reported thus far.

A major problem in stem cell transplantation therapy is the massive cell death of transplanted cells after implantation. In general, 70–90% of transplanted cells die within a few days (29, 52, 56). Various attempts have been made to increase cell survival after transplantation. For example, we and others have shown that overexpression of the anti-apoptotic gene bcl-2 or bcl-XL enhances cell viability after transplantation into the ischemic brain or damaged spinal cord (24, 52). Trophic factors such as vascular endothelial growth factor (VEGF) coapplied with transplanted cells may have protective effects and augment therapeutic benefits (3, 38). In our recent investigations, we tested a new strategy of preconditioning cells before transplantation with sublethal hypoxia. This pretreatment substantially enhances cells’ tolerance to both necrosis and apoptosis. The survival rate of hypoxic preconditioned

Fig. 1. Characterization of bone marrow mesenchymal stem cells (BMSCs) from green fluorescent protein (GFP)-transgenic mouse. Surface marker characterization of isolated BMSCs under culture condition using flow cytometry analysis. A: normal BMSCs in culture. These cells were adherent to the bottom of the plastic dishes. The majority of BMSCs were in spindle or triangular-like shape. Blue color is nuclear staining of Hoechst 33342 and green is GFP. Bar = 50 μm. B: BMSCs were identified by fluorescence-activated cell sorting using flow cytometry. Cells isolated from the bone marrow of rats expressed the stem cell marker CD90. C and F: flow cytometry confirmed that these cells lacked expression of the hematopoietic marker CD34 (C) or CD45 (D) but expressed CD105 (E) and CD73 (F) compared with their isotype controls, respectively. G and H: BMSCs subjected to hypoxia preconditioning (0.5% O2 for 24 h) remained to be CD34 and CD45 negative. N = ≥3 assays of different cultures.
neural progenitor cells and BMSCs is approximately doubled compared with normoxic control cells (19, 34, 45). In addition to promoting cell survival, hypoxia may stimulate migration activities of endothelial progenitor cells and augment the efficacy of therapeutic neovascularization (1, 12, 50). In the present investigation, we tested the hypothesis that hypoxic preconditioning could augment migration capability of BMSCs and benefit tissue repair in the ischemic heart. To understand the mechanism of this promotion, we examined the possibility based on our previous investigations that the Kv2.1 channel in the mechanism of this promotion, we examined the possibility.

**MATERIALS AND METHODS**

**BMSC cultures from green fluorescent protein transgenic mouse.** BMSCs were isolated and harvested as previously described (4). Briefly, BMSCs were flushed from the femurs and tibias of green fluorescent protein (GFP) transgenic mice using a 25-gauge needle. Mononuclear cells were suspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and plated in flasks. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h, nonadherent cells were discarded, and adherent cells were washed three times with PBS. Fresh complete medium was added and replaced every 4 days. Each discarded, and adherent cells were washed three times with PBS. Fresh complete medium was added and replaced every 4 days. Each primary culture was subcultured 1:2 when BMSCs grew to ~80% confluence. Passage 4–10 cells were used in this investigation.

**Flow cytometry of cell phenotype identification.** To identify that the isolated cells in our investigation were BMSCs, we performed flow cytometry to exam the cell surface markers CD34, CD45, CD73, CD90, and CD105, respectively (eBioscience, San Diego, CA or BD Pharmingen, Rockville, MD). Cells (0.5–1 × 10⁶) were incubated in 100 μl of buffer (PBS with 1% BSA and 1 with 5 μl of CD34-FITC, CD45-FITC, CD73-FITC, CD90-FITC, and CD105-PE antibodies for 1 h, respectively). Next, the cells were washed five times with wash buffer, and then 300 μl of wash buffer was added to the cells. Cell fluorescence was analyzed by flow cytometry in a FACS Calibur instrument (Becton Dickinson, Franklin Lakes, NJ), and the data were analyzed using FACS Diva software (Becton Dickinson). All experiments were done in triplicate using an isotype control.

**Osteogenic, adipogenic, and chondrogenic differentiation.** Passage 4–10 BMSCs were seeded on coverslips in six-well plates and cultured in complete medium until confluency. For osteogenic differentiation, the medium was replaced with a calcification medium containing DMEM/10% FBS specialized for osteogenic differentiation (Stem Cell Technologies) and incubated for 21 days. These coverslips were fixed with formalin for 15 min and stained with fresh 0.5% alizarin red solution for 2 h. For adipogenic differentiation, at confluency, the cells were switched to an adipogenic medium (DMEM/10% FBS supplemented for adipogenic differentiation; Stem Cell Technologies) and further cultured up to 21 days with the medium being changed on every 3–4 days. At day 21, the adipogenic cultures were fixed with 10% formalin for at least 15 min and stained with fresh 0.3% Oil Red-O solution for 2 h. After staining was completed, the cultures were washed three times with sterile diH₂O and counter stained with hematoxylin. Differentiation into bone lineage and fat cells was identified under a light microscope for specific staining.

Chondrogenic differentiation was performed on BMSCs from passage 3–5. BMSCs cells (5 × 10⁴) were resuspended in a 15 ml Falcon tube at 200 g for 10 min. The MSC medium was removed, and 2 ml of chondrogenic medium (DMEM w/10% FBS supplemented with chondrogenic supplements; HyClone, Thermo Scientific, Rockford, IL) were added to the cells. The cap of the conical tube was loosened and the tube was placed in the incubator at 37°C with 5% CO₂. Two days later a ball of cells formed in the bottom of the tube. Chondrogenic medium was replaced every 2–4 days. We removed our pellet and sliced and stained with alizarin red staining. Image A was a positive control of osteogenic cells. B: negative control of BMSC in MSC media only. C and D: positive alizarin red staining of osteogenic differentiated BMSCs (×20 magnifications). F–J: BMSCs were seeded in chondrogenic medium for 21 days (F). Negative control cells were BMSCs in DMEM with FBS only and hematoxylin (blue) staining (G); Oil Red (adipocytes) was applied to slides to detect fat lipids in differentiated cells (H) (×10 magnification). J: ×20 magnification. H, N ≥ 3 assays of different cultures. F–J: BMSCs were seeded in chondrogenic media for 19 days, and J shows pellet (arrow) before slicing, K and L: pellet after slicing and staining with Alcian blue and nuclear fast red for negative cells, Bar = 50 μm and 100 μm for ×10 and ×20 in K and L, respectively.
Even so, the low oxygen (0.5% O₂) used in our experiments is justified to be “hypoxic” to these cells.

Kv2.1 targeting shRNA constructs and lentivirus particle packaging.

The short hairpin RNA (shRNA)-expressing vector, pLKO.1-TRC cloning vector (10878), pLKO.1-TRC control vector (10879), envelope vector pMD2.G, and packaging vector psPAX2 were obtained from Addgene (Cambridge, MA). The specific Kv2.1 shRNA were designed and generated according a protocol from Addgene. Briefly, paired DNA oligos targeting specific sequences in Kv2.1 gene (GC-CTTGAGCTAGAACAAA, S2; CGCCTTCACCTCTATTCT-CAA).

S3 from Operon Biotechnologies (Huntsville, AL) were annealed and subcloned into the pLKO.1-TRC cloning vector via AgeI and EcoRI restriction sites. This and all constructs were sequence verified on both strands. Lentivirus particles were packaged according to a protocol from Addgene, using the envelope vector pMD2.G, the packaging vector psPAX2, and HEK293 cells. The medium was changed 24 h later, and lentivirus particles in the cell culture supernatant were collected after 48 h.

Infected target cells. BMSCs were seeded and incubated at 37°C, 5% CO₂ overnight. At ~70% confluency, 1.5 ml of filtered lentiviral supernatant was added, and 24 h later, the fresh medium was exchanged. Empty vector was used as transfection controls. To select for infected cells, puromycin (1 µg/ml) was added, and then fresh puromycin-containing medium was exchanged every 3–4 days.

Wound healing assay. Cell migration was assessed using an in vitro wound healing assay as follows. BMSCs (3 × 10⁴) were seeded in a six-well plate, and the proliferation inhibitor mitomycin-c (0.25 mg/ml) was added. The cell layer was scratched along the central vertical line using a 200-ml pipette tip, and each well was washed six times with a serum-free medium. Photo images at the same location were taken immediately after and 12 h after wound formation. The cells within the wound area were then counted as migrating cells (57).

Transwell assay. Migration of BMSCs were also assessed using transwell chambers with polycarbonate membrane filters of 6.5 mm diameter and 8 µm pore size (Becton Dickinson, Franklin Lakes, NJ). Cells (5 × 10⁴) in 200 µl of media (DMEM with 1% FBS) were added to the upper compartments, and lower compartments were filled with DMEM with 10% FBS as a source of chemoattractants. Transwell chambers were incubated for 12 h at 37°C and 5% CO₂ to allow cell migration. Cells on the top surface of the filters were then wiped off with cotton swabs, and cells that had migrated into the lower compartment, attaching to the lower surface of the filter, were stained with crystal violet. To count migrated BMSCs, five fields per samples were randomly chosen and photographed at ×10 magnification. Measurements were performed at least three times with different samples.

Fig. 3. Hypoxic preconditioning increased Kv2.1 channel expression and associated currents. Expression of several K⁺ channels was detected using Western blot analysis. A: Western blot analysis of the protein levels of Kv2.1, Kv1.5, and Kv1.4 in normoxic (N)-BMSCs and hypoxic (HP)-BMSCs after 24 h normoxia (21% O₂) and hypoxia (0.5% O₂) treatment, respectively. Mouse ß-actin was used as the loading control. B: densitometry analysis for comparisons of the relative expression levels of different K⁺ channels in N-BMSCs and HP-BMSCs; N = 6. *P < 0.05 compared with N-BMSCs. C: Western blot of Kv2.1 protein levels in BMSCs under normal condition (N-CTL) and after hypoxic preconditioning with Kv2.1 short hairpin RNA (shRNA) (N-sh-Kv2.1, H-sh-Kv2.1) or control vector (H-V-CTL) transfection. The shRNA-expressing vector effectively kept the Kv2.1 level low in HP-treated BMSCs (H-sh-Kv2.1). D: patch clamp whole cell recording showed the outward K⁺ current in naïve control cells, HP-treated cells, empty vector control, and HP-treated cells subjected to Hp-Kv2.1 knockdown (HP-sh-Kv2.1). The member holding potential was −70 mV, and currents were triggered by stepping the membrane potential to +40 mV for 400 ms. With the use of this protocol, there was only small outward I_K current detectable in N-BMSCs. Sizable outward I_K current was recorded in HP-BMSCs but not in Kv2.1 knockdown cells. E: bar graph summarizes the patch clamp experiments showing low current density of the outward current in normal BMSCs and its marked increases in HP-treated BMSCs with and without the control vector. Kv2.1 knockdown kept the current density low after the 24-h HP treatment. N = 6 or 10 in each group. *P < 0.05 vs. normal controls. *P < 0.05 vs. vector controls.
**Patch clamp whole cell recording.** Outward-delayed rectifier K⁺ currents were recorded using the whole cell recording configuration (55). Briefly, cells in 35-mm dishes were detached with tryplified and then incubated for 30 min. BMSCs on the stage of an inverted microscope were patched and whole cell currents were recorded using an EPC-7 amplifier. Series resistance compensation was routinely applied during recording. Current and voltage signals were collected using the data acquisition/analysis program PULSE (HEKE, Lambrecht, Germany). Currents were digitally sampled at 0.33 kHz and filtered at 3 Hz by a three-pole Bessel filter. The extracellular solution contained (in mM) 115 NaCl, 2.5 KCl, 2.0 MnCl₂, 10 HEPES, 0.1 BAPTA, 10 D-glucose, and tetrodotoxin (TTX; 0.1 μM). The electrode solution contained (in mM) 120 KCl, 1.5 MgCl₂, 1.0 CaCl₂, 2.0 Na₂-ATP, 1.0 BAPTA, and 10 HEPES. Solution pH was adjusted to 7.3.

**Immunoprecipitation and Western blot analysis.** Cultured cells were lysed with modified RIPA buffer [50 mM HEPES, pH 7.3, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, and protease inhibitor cocktail (Roche, Nutley, NJ)] for 30 min, followed by centrifugation at 14,000 g for 30 min. Protein concentration of each sample was determined using the bicinchoninic acid assay (Sigma, St. Louis, MO). Immunoprecipitation was performed as previously described (51). Briefly, cell lysates were incubated with specific antibodies overnight at 4°C, followed by incubation with protein G (Upstate, Charlottesville, VA) for 2 h. The immune complexes were washed three times with modified RIPA buffer without sodium deoxycholate and SDS. Samples were separated via standard SDS-PAGE and then electroblotted onto PVDF membranes for subsequent immunoblotting according to standard protocol. After being blocked with 7% nonfat milk protein in Tris-buffered saline with 0.2% Tween-20 (TBS-T) for 2 h at room temperature, the membranes were incubated overnight at 4°C with specific primary antibodies. Blots were washed three times with TBS-T and incubated with AP-conjugated secondary antibodies for 2 h at room temperature. Specific signals were detected using a colorimetric assay (BCIP/NBT).

**Myocardial infarction model.** All animal experiments and surgical procedures were approved by the University Animal Research and Use Committee and met the National Institutes of Health standards. Wistar rats were subjected to general anesthesia with 4% chloral hydrate (4 mg/kg ip) and ventilated with room air by using a small animal ventilator (Vetronics, Lafayette, IN). Myocardial infarction (MI) was induced by ligation of the left anterior descending coronary artery with a 6 – 0 silk suture (31). Successful performance of coronary artery occlusion was verified by the blanching of the myocardium distal to the coronary ligation.

**Cell preparation and transplantation.** BMSCs from GFP-transgenic rats were cultured for 10–15 days. The GFP facilitates tracking cells after transplantation. Before transplantation, BMSCs in the hypoxic preconditioning group (HP-BMSCs) were treated for 24 h with sublethal hypoxia (0.5% O₂) followed by reoxygenation in 21% O₂ for 2 h. For transplantation, similar numbers of N-BMSCs or HP-BMSCs (3 x 10⁴) were suspended in 1.5 ml of serum-free medium. The viability of cells under this condition was confirmed using TUNEL and/or trypan blue staining. Twenty hours after left anterior descending coronary artery ligation, suspensions containing BMSCs were injected via the tail vein. Rats were randomly divided into two groups of 10 rats that received N-BMSCs and HP-BMSCs, respectively.

**Immunofluorescence staining.** Tissues were fixed with 10% formalin for 10 min. After 5 min of permeabilization with 0.2% Triton X-100, tissues were blocked with 10% BSA for 1 h at room temperature and then incubated with specific antibodies overnight at 4°C. After being washed three times with PBS, tissues were incubated with Alexa Fluor 488 anti-goat IgG (1:200, Molecular Probes, Carlsbad, CA) for 1 h at room temperature. Tissues were stained for 5 min with Hoechst 33342 (1:20,000) and then mounted and analyzed using fluorescence microscopy.

**Cell counting.** Cell counts were performed using a design-based stereological method. For counting GFP-positive cells in heart sections, every tenth heart section (100 μm apart) across an entire region of interest was counted. Six fields per section were randomly chosen under fluorescence at ×40 magnification. The latter was repeated in four separate sections per heart. Counts are expressed as the average number of cells or vessels per field.

**Statistical analysis.** The Student’s two-tailed t-test was used for comparison of experimental groups. Multiple comparisons were done using Tukey’s test for multiple pair-wise examinations. Data were expressed as means ± SE. Differences were considered to achieve statistically significance if P values were ≤0.05.

**RESULTS**

**Characterization of BMSCs.** Typical BMSCs showed either small spindle-shaped or triangular-shaped morphology, adherent to the bottom of plastic culture dishes (Fig. 1A). Flow cytometry analysis revealed that these cells expressed CD73, CD90, and CD105 surface markers but were CD34 and CD45 negative (Fig. 1). The expression of CD73, CD90, and CD105 was consistent with characteristic surface markers of undifferentiated BMSCs (Fig. 1, B–F) (11). The lack of expression of CD34 and CD45 suggested that the cell population was depleted of hematopoietic stem cells during subcultivation by plastic adherence. We noticed that there were some CD73- and CD105-negative cells, suggesting a heterogenic component among these cells. After 10–15 days in culture and subjection to hypoxic preconditioning (0.5% O₂ for 24 h), cells still expressed CD105 and remained to be CD34 and CD45 negative (Fig. 1, G and H).

![Fig. 4. The mediator role of Kv2.1 in hypoxia-promoted migration of BMSCs. The relationship between Kv2.1 channel and cell migration was studied using Kv2.1 knockdown and migration assay. A: cell migration was compared using transwell assay. Phase contrast images show normal control BMSCs, HP-BMSCs (H-sh-Kv2.1), N-Vector control, H-vector control, and H-sh-Kv2.1 channel blocker TEA (5 mM), and elevated K⁺ (25 mM KCl) that diminish the driving force for K⁺ efflux.](http://ajpcell.physiology.org/content/301/8/810/fig4)

![Fig. 4. The mediator role of Kv2.1 in hypoxia-promoted migration of BMSCs. The relationship between Kv2.1 channel and cell migration was studied using Kv2.1 knockdown and migration assay. A: cell migration was compared using transwell assay. Phase contrast images show normal control BMSCs, HP-treated BMSCs with empty vector (H-Vector control) cells, and Kv2.1 knockdown HP-BMSCs (H-sh-Kv2.1) 12 h after the test. B: summarized data from experiments in A. The sublethal hypoxia treatment significantly promoted BMSC migration. The hypoxia-promoted cell migration was abolished by knocking down Kv2.1, K⁺ channel blocker TEA (5 mM), and elevated K⁺ (25 mM KCl) that diminish the driving force for K⁺ efflux.](http://ajpcell.physiology.org/content/301/8/810/fig4)
To further identify the cells in our investigation, isolated cells were tested for the multipotency, as suggested by Dominici et al. (11). Cells were induced in vitro using the adipogenic and osteogenic specific media. After 3 wk of osteogenic stimulation, the cells were stained positive to Alazirin red and showed calcium formation of an osteocyte phenotype (Fig. 2, A–D). When plated with the adipogenic media for 3 wk, cells showed positive adipogenic formation upon Oil Red O staining that is specific for fat lipids (Fig. 2, E–I). We also pelleted BMSCs, sliced pellets formed on day 19, and stained them with 3% Alcian blue. There was positive chondrogenic formation upon Alcian blue staining, suggesting that these cells could differentiate into chondrogenic cells (Fig. 2, J–L). The cell surface markers and multipotency of these cells are thus consistent with the characteristics of bone marrow mesenchymal stem cells (11).

Effect of hypoxia on potassium channels. We showed in a previous investigation an important role of the potassium Kv2.1 channel in regulating cell adhesion and migration (51). Since hypoxia is one of the major regulatory signals affecting K⁺ channel expression (27), we analyzed protein expression levels of some important K⁺ channels in BMSCs by Western blot analysis. BMSCs cultured under normoxia (21% O₂; N-BMSCs) expressed Kv2.1, Kv1.5, and Kv1.4 channel proteins but at low levels (Fig. 3, A and B). Hypoxia preconditioning (0.5% O₂, 24 h) significantly increased the expression of the Kv2.1 channel in HP-BMSCs, whereas Kv1.5 and Kv1.4 expression remained unchanged (Fig. 3B).

To understand potential roles of the enhanced expression of the Kv2.1 channel in BMSCs, the shRNA technique was used to knockdown the Kv2.1 channel expression in N-BMSCs and HP-BMSCs. In cells subjected to Kv2.1 knockdown (Kv2.1-shRNA), the Kv2.1 expression was virtually undetectable in both cells (Fig. 3C). Using whole cell voltage clamp recordings, we observed that N-BMSCs exhibited small delayed rectifier K⁺ currents (175 ± 50 pA, n = 5), while hypoxia-preconditioned cells had much larger outward K⁺ currents and significantly increased current density (around fourfolds increases; Fig. 3, D and E). These findings suggested that hypoxic preconditioning upregulated the Kv2.1 expression as well as the associated delayed rectifier currents. In Kv2.1 knockdown cells, the outward rectifier current remained to be small after hypoxic preconditioning (Fig. 3, D and E). As a control, transfection with the empty vector did not affect hypoxia-induced Kv2.1 upregulation and showed no disruption.

Fig. 5. The role of Kv2.1 in directed migration of BMSCs. BMSC directed migration toward the injured region was examined in BMSCs transfected with empty control vector or sh-Kv2.1 in an in vitro wound healing test. A and B: phase contrast images of the scratched region in cultured dishes of vector control and sh-Kv2.1-BMSCs. At 12 h after wound formation, fewer sh-Kv2.1-BMSCs moved into the central region of wound area compared with control cells. C: phase contrast images of vector control and sh-Kv2.1-BMSCs in transwell assays at 12 h time point. D: summarized data of wound healing assay significantly fewer sh-Kv2.1-BMSCs were counted in the wound area then control cells. E: summarized data of transwell assay. Kv2.1 knockdown reduced cell migration in this three-dimensional test. N = 5 in each assay; *P < 0.05 when compared with controls.

Fig. 6. HP promoted BMSC migration and homing to the ischemic heart after transplantation. Immunohistochemical staining of heart sections after myocardial infarction (MI) and BMSC injections. Transplanted BMSCs were isolated from GFP-transgenic rats; the green fluorescent color was used to identify exogenous cells. Hoechst staining (blue) was used to show all cells in the region. A: GFP-positive cells (green) indicate that the injected BMSC migrated into the ischemic heart 72 h after MI. There were much more GFp-positive cells in the sections from HP-BMSC transplanted animals. The Hoechst-positive (blue) but GFP-negative cells are likely endogenous cells in the region. B: summarized data of GFP-labeled cells. Hypoxic preconditioning significantly increased migration/homing of HP-BMSCs to the ischemic heart. N = 6, *P < 0.05 when compared with N-BMSC.
on hypoxia-increased outward membrane currents (Fig. 3, D and E).

Effects of hypoxia and Kv2.1 channel modulation on BMSC migration in vitro and in vivo. To understand whether hypoxia preconditioning could affect migration activity of BMSCs, we used an in vitro three-dimensional assay of transwell migration test. After 12 h in the transwell, significantly more HP-BMSCs migrated to the lower compartment compared with the movement of N-BMSCs (Fig. 4A). In the transwell assay, Kv2.1 knockdown prevented the hypoxia-promoted BMSC migration (Fig. 4, A and B). When HP-BMSC cultures were exposed to the K+ channel blocker TEA (5 mM) or a high K+ (25 mM KCl) medium during transwell migration assays, the increased migration activity of HP-BMSCs was abolished (Fig. 4B). These data suggested that not only the Kv2.1 expression but also the K+ channel activity was important in BMSC migration.

We next tested the role of Kv2.1 in the directed migration of BMSCs toward injury areas in an established in vitro model of wound healing test. BMSCs transfected with empty vector or Kv2.1-shRNA were plated onto culture dishes and subjected to a scratch-generated wound region crossing the center of the dish. The Kv2.1 channel knockdown in BMSCs decreased the migration of cells into the central area (Fig. 5, A, B, and D). This observation was confirmed in the three-dimensional transwell migration assay (Fig. 5, C and E). These in vitro findings suggested that Kv2.1 knockdown attenuated directional migration of BMSCs.

We then evaluated whether hypoxic preconditioning could enhance migration and homing activities of intravenously injected BMSCs after cardiomyocyte ischemia. N-BMSCs and HP-BMSCs from GFP-transgenic mice were introduced via tail vein injection 24 h after initiation of permanent MI. Three days later, animals were euthanized, and migration/homing of implanted cells to the ischemic lesion was assessed by counting the number of GFP-positive cells. In the HP-BMSC transplantation group, there were significantly more GFP-positive cells in the ischemic lesion compared with N-BMSC transplantation group (Fig. 6).

Kv2.1 expression potentiates Kv2.1-FAK interaction and FAK phosphorylation. We have shown in a recent investigation that interaction between Kv2.1 and FAK promoted FAK phosphorylation that increased FAK activation and cell adhesion/migration (51). To examine whether hypoxic preconditioning increased Kv2.1-FAK interactions in BMSCs, immunoprecipitated protein complexes from N-BMSCs and HP-BMSCs were analyzed. Compared with N-BMSCs, formation of the Kv2.1-FAK complex significantly increased in HP-BMSCs (Fig. 7). Consistent with their effects on cell migration, TEA and high K+ medium markedly attenuated the Kv2.1-FAK interaction in BMSCs (Fig. 7).

We also explored the possibility that the interaction between Kv2.1 and FAK augmented FAK phosphorylation that is the underlying mechanism of FAK activation and FAK-mediated migration. In Western blot analysis, phosphorylation levels of FAK576/577 and FAK397, two crucial phosphorylation sites for...
FAK activation, were noticeably higher in HP-BMSCs compared with N-BMSCs, while cotreatment with either TEA or KCl prevented the phosphorylation of FAK proteins (Fig. 8).

DISCUSSION

The data presented here demonstrates that hypoxic preconditioning promotes migration of BMSCs in vitro and increases homing of intravenously injected BMSCs to infarct zone of the heart in an MI model of rats. Hypoxia could enhance BMSC migration by increasing the expression of CXCR4 (2, 20). Unique to our study is the evidence for a novel mechanism involving the increased formation of Kv2.1-FAK complex and FAK activation behind HP potentiation of BMSC migration. We show that: 1) hypoxic preconditioning enhances the Kv2.1 expression in BMSCs that is correlated to increased FAK phosphorylation and BMSC migration capacity; and 2) knockdown of Kv2.1 or inhibiting K⁺ channel activities significantly reduces migration of naïve BMSCs and antagonizes the effect of hypoxia preconditioning on directed migration into the injury area in the wound healing test. The data suggests a novel relationship among hypoxic preconditioning, Kv2.1 channel upregulation, FAK phosphorylation/activation, and BMSC migration or homing to the injured heart.

Several studies have reported hypoxia-mediated effects on K⁺ channels, suggesting that the effect varies depending on cell types and the severity of hypoxia. For example, Haddad’s group demonstrated that a relatively severe oxygen deprivation (PO₂ = 10–20 mmHg) induced membrane depolarization in mammalian central neurons mediated by a marked inhibition of large-conductance Ca²⁺-activated K⁺ channel (28). Chronic hypoxia inhibits Kv1.2 and Kv2.1 gene expression in rat vascular smooth muscle cells of pulmonary artery (48). Under the condition of sublethal hypoxia of preconditioning treatment, most studies demonstrate upregulation of K⁺ currents, such as outward ATP-sensitive K⁺ currents in isolated cardiomyocytes (27). In the present investigation, a sublethal hypoxia upregulates the Kv2.1 expression and outward delayed rectifier currents in BMSCs, this is in line with the general effect of mild hypoxia on K⁺ channels. Gene regulations induced by hypoxic/ischemic preconditioning are transient events, lasting for hours and up to several days depending on the preconditioning protocol (9, 37). The hypoxic upregulation of Kv2.1 expression is most likely also a transient event. The transient Kv2.1 increase and cell migration enhancement should be sufficient for cell homing to the injury region within hours or the first couple days after transplantation.

The mechanism by which hypoxia upregulates or activates the Kv2.1 channel in BMSCs is unclear. Previous studies have shown that the ATP-sensitive K⁺ channel activity is associated with endogenous factors, such as adenosine, nitric oxide, and intracellular H⁺ concentration (14). K⁺ channel activities may relate to H⁺ concentration, such a mechanism could be involved here (23). The release of endogenous mediators and/or an alteration in the endogenous microenvironment during hypoxic preconditioning may therefore be involved in the activation of the Kv2.1 channel in BMSCs. The mechanisms for the protein level increases remain to be elucidated.

Adhesion and directed migration are cellular activities involved in homing of transplanted cells to the injury region. While the successful migration by stem cells to the sites of injury is crucial for cell-based therapies (53), the underlying mechanisms that regulate migration and homing toward injury sites remain unclear. Currently available evidence suggests that the release of inflammatory chemotactic agents by damaged tissues, as well as the expression of specific integrins by BMSCs, may play a role in cell migration (26). Some studies have shown that a subpopulation of BMSCs expresses the stromal-derived factor (SDF)-1 chemokine receptor CXCR4, and these BMSCs may therefore home to the injured tissue in response to SDF-1 expression (6, 30). Interestingly, relatively fewer BMSCs actually express CXCR4, and blocking CXCR4 only caused marginal inhibition of BMSC homing to the bone marrow. These results suggest that other mechanisms are involved in this process. The present investigation provides a possible mechanism that involves hypoxia/ischemia induced upregulation of a K⁺ channel that can regulate FAK activation and promote BMSC migration and homing to an injured region. It should be pointed out that although the regulatory role of Kv2.1 in directed migration of tissue repair was dem-

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Fig. 8. Kv2.1 promoted FAK phosphorylation. FAK⁵⁷⁶/⁵⁷⁷ and FAK³⁹⁷ phosphorylation was examined by Western blot analysis. A: total phosphorylated FAK and phosphorylation levels of FAK⁵⁷⁶/⁵⁷⁷ and FAK³⁹⁷ in N-BMSCs and HP-BMSCs were measured. Mouse β-actin was used as the loading control. Coapplied TEA (5 mM) and 25 mM KCl attenuated phosphorylation levels of FAK. B and C: densitometry analysis for comparisons of the relative phosphorylation levels in N-BMSCs and HP-BMSCs plus HP-BMSCs subjected to TEA or KCl. N = 6, *P < 0.05 compared with N-BMSCs.
onstrated not only in vitro but also in a rat model of corneal traumatic injury model (51), more in vivo tests will be necessary to confirm the specific role of Kv2.1 in the regulation of BMSC activities in the ischemic heart. In addition, it is well known that systemically administrated BMSCs shows distributions in multiple organs, although without attracting signals the distribution to noninjured organs may be low (16). It can be expected that promoting directional cell migration toward the injured region would not increase BMSC deposition in other organs; this prediction, however, needs to be specifically examined in future investigations.

Among a number of genes and environmental cues regulating the adhesion/migration/homing cascade, FAK is a well-established key regulator for the chain of reactions. Phosphorylation of FAK is essential for FAK activation and the regulation of cell spreading and migration (13, 33). Activated upon cell attachment to the extracellular matrix FAK, a tyrosine kinase, binds to several proteins to regulate cell adhesion and migration. In our previous investigations, we revealed that the Kv2.1 channel affects cell migration via regulating FAK phosphorylation in neuronal and nonneuronal cells (51). We found in the previous investigation that the Kv2.1 channel can form a protein-protein complex with FAK stimulated by fibronectin/integrin and acts as a promoter for phosphorylation of FAK397 and FAK576/577. Both are key phosphorylation sites for FAK activation. The NH2 terminus-containing residue L45 of Kv2.1 is required for this process (51). A similar mechanism was demonstrated with the human ether-a-go-go-related gene (Herg) channels and Kv1.3 channel that showed direct interaction with β1 integrin to modulate adhesion-dependent signaling in SH-SY5Y neuroblastoma cells (7). Since phosphorylation of the FAK397 and FAK576/577 residues leads to increased FAK catalytic activity, the Kv2.1-mediated regulation may provide a unique voltage-dependent mechanism responsive to both membrane activities and microenvironmental changes (51). Consistent with our previous work and hypothesis, the current study demonstrates similar results in an important subtype of stem cells BMSCs. Increased levels of Kv2.1 in HP-BMSCs potentiated FAK phosphorylation, whereas a K+ channel blocker or knocking down the Kv2.1 channel decreased phosphorylation of FAK. The blocking effect of TEA and high K+ on increased BMSC migration agrees with previous investigations that K+ channel-mediated K+ efflux and the local K+ concentration gradient across the plasma membrane may be important for the migration process (8, 25). It is well known, however, that TEA is widely used as a pharmacological tool to identify and block different K+ channels and is not a specific K+ channel subtype inhibitor. In this regard, shRNA experiments provide stronger and consistent evidence for the involvement of Kv2.1 channel in the migration activity of BMSCs. Since cell adhesion and migration are critical cellular capabilities in development and tissue repair, this mechanism may be of significance in a broad spectrum of clinical settings involving these cellular events.

We show in BMSC cultures that hypoxia-promoted cell migration is attenuated in a high K+ (25 mM KCl instead of 5 mM) extracellular medium. Elevation of extracellular K+ concentrations has been applied for different purposes in electrophysiological, cell biological, and cytotoxicity experiments. The main consequences of raising extracellular K+ concentration are membrane depolarization and activation of voltage-gated ion channels that may lead to increased Ca2+ influx into the cells. Meanwhile, raising extracellular K+ diminishes the K+ gradient across the plasma membrane, which directly attenuates the driving force for K+ efflux. Membrane depolarization activates more voltage-gated K+ channels, and cell migration was expected to increase. However, high K+ medium consistently attenuated cell migration, suggesting the depolarization or presumed Ca2+ changes were not strong enough to overcome the inhibitory effect, i.e., reducing the driving force for K+ efflux. This idea concurs with the previous proposition that the local K+ concentration gradient acts as a signal or trigger for cell migration (8, 25).

In the ischemic heart, we tracked transplanted BMSCs that were prelabeled with Hoechst or carried the fluorescent gene GFP. Previous research discovered that cell fusion might be accountable for the expression of neural lineage markers in cocultured and transplanted BMSCs (44). This issue raised a concern over the mechanism of transdifferentiation of BMSCs. It was possible that cell fusion might occur in our experiments so endogenous cells such as myocytes, glial cells, and macrophages could appear to be Hoechst or GFP positive. We could not exclude that fusion occurred to a few cells; however, this should not affect the conclusion of this investigation because it aimed to detect how many intravenously injected BMSCs could reach the ischemic heart even if they might fuse with endogenous cells after their arrival. Whether the possible fusion might affect the outcome of BMSC transplantation therapy is an interesting question that requires a separated investigation.

In conclusion, our study suggests the possibility that, in some other cell types, the Kv2.1 channel plays an important role in directed migration and homing of BMSCs. Hypoxic preconditioning increases Kv2.1 expression and enhances the channel activity that subsequently augments FAK phosphorylation/activation and cell migration, resulting in increased homing of transplanted BMSCs to the injured region. A better understanding of the role of Kv2.1 channel as well as the interaction between Kv2.1 and FAK may provide a novel means for promoting migration/homing of BMSCs after transplantation that can promote tissue repair in the cell-based therapy.

GRANTS
This work was supported by National Institutes of Health Grants NS-37372, NS-045155, and NS-045810 to S. P. Yu and L. Wei and by the American Heart Association Established Investigator Award to L. Wei.

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

REFERENCES


