BIOMOLECULES
& THERAPEUTICS

Modulation of Human Cardiac Progenitors via Hypoxia-ERK Circuit Improves their Functional Bioactivities

Seok Yun Jung[1], Sung Hyun Choi[2], So Young Yoo[1], Sang Hong Baek[2],* and Sang Mo Kwon[1],*

[1]*Laboratory of Vascular Medicine and Stem Cell Biology, Department of Physiology, School of Medicine, Pusan National University, Yangsan 626-870,* [2]*Laboratory of Cardiovascular Disease, Division of Cardiology, School of Medicine, The Catholic University of Korea, Seoul 137-040, Republic of Korea*

Abstract

Recent accumulating studies have reported that hypoxic preconditioning during *ex vivo* expansion enhanced the self-renewal or differentiation of various stem cells and provide an important strategy for the adequate modulation of oxygen in culture conditions, which might increase the functional bioactivity of these cells for cardiac regeneration. In this study, we proposed a novel priming protocol to increase the functional bioactivity of cardiac progenitor cells (CPCs) for the treatment of cardiac regeneration. Firstly, patient-derived c-kit[+] CPCs isolated from the atrium of human hearts by enzymatic digestion and secondly, pivotal target molecules identified their differentiation into specific cell lineages. We observed that hCPCs, in response to hypoxia, strongly activated ERK phosphorylation in *ex vivo* culture conditioning. Interestingly, pre-treatment with an ERK inhibitor, U0126, significantly enhanced cellular proliferation and tubular formation capacities of CPCs. Furthermore, we observed that hCPCs efficiently maintained the expression of the c-kit, a typical stem cell marker of CPCs, under both hypoxic conditioning and ERK inhibition. We also show that hCPCs, after preconditioning of both hypoxic and ERK inhibition, are capable of differentiating into smooth muscle cells (SMCs) and cardiomyocytes (CMs), but not endothelial cells (ECs), as demonstrated by the strong expression of α-SMA, Nkx2.5, and cTnT, respectively. From our results, we conclude that the functional bioactivity of patient-derived hCPCs and their ability to differentiate into SMCs and CMs can be efficiently increased under specifically defined culture conditions such as short-term hypoxic preconditioning and ERK inhibition.

Key Words: Human cardiac progenitor, Hypoxia preconditioning, U0126

INTRODUCTION

Stem cell-based therapies offer an alternative therapeutic solution to treat a number of ischemic heart diseases. Accumulating evidence demonstrated experimentally that various cell types have regenerative potential: skeletal myoblasts (Murry *et al.*, 1996; Taylor *et al.*, 1998), fibroblasts (Galli *et al.*, 2005), SMCs (Li *et al.*, 1999), fetal myocytes (Soonpaa *et al.*, 1994; Koh *et al.*, 1995), embryonic stem cells (ESCs) (Cao *et al.*, 2006), bone marrow-derived cells (BMCs) (Orlic *et al.*, 2001; Kawamoto *et al.*, 2003; Urbich *et al.*, 2005; Iwasaki *et al.*, 2006; Kawamoto *et al.*, 2006), induced pluripotent stem cells (iPSCs) (Sturzu and Wu, 2011), and cardiac stem/progenitor cells (CSCs or CPCs) (Bearzi *et al.*, 2007). Very recently, the isolation and *ex vivo* expansion of CPCs from ischemic hearts provide new therapeutic strategies for myocardial regenera-

tion and repair. Some researchers had reported that c-kit-positive progenitors among CSCs have robust regenerative potential (Linke *et al.*, 2005). C-kit-positive hCSCs can be efficiently isolated from small tissue samples of normal or failing hearts (Cesselli *et al.*, 2011), expanded *in vitro* (Bearzi *et al.*, 2007), and differentiated into cardiac, endothelial, and smooth muscle lineages in *in vitro* culture conditions and *in vivo* murine disease models (Bearzi *et al.*, 2007). Several groups recently attempted to establish well defined *ex vivo* expansion protocols so that patient-derived resident cardiac stem cells with regenerative properties could be expanded *in vitro*, in order to provide a sufficiently large number of functional human cardiac stem cells (hCSCs) for their therapeutic applications because the number of patient-derived CSCs is limited. Although it is a general concept that one of the cell-based cardiac regeneration strategies is injection of autologous CPCs

into ischemic regions (Bearzi *et al.*, 2007), consensus and molecular understanding of high-quality autologous *ex vivo*-expanded progenitor cells to regenerate injured myocardium has been not fully defined. Thus, both understanding of the complex and dynamic molecular mechanisms and identification of culture conditions involving a variety of cells and combinational growth factors, cytokines, and genes have been the focus of extensive investigations (Tang *et al.*, 2009; D'Amario *et al.*, 2011; Goichberg *et al.*, 2011; Tang *et al.*, 2011).

Tissue hypoxia has been reported to be a physiological master regulator for cardiac regeneration by triggering a variety of downstream signal molecules of vascular endothelial growth factor (VEGF) or stromal-derived factor-1 (SDF-1) (Yan *et al.*, 2012). In general, the physiologic oxygen pressure in the human body varies according to the organs; 1% in cartilage, 1-7% in bone marrow, and 10-13% in arteries, lungs, and liver (Kofoed *et al.*, 1985; D'Ippolito *et al.*, 2006). However, in the *ex vivo* culture system, the oxygen concentration is almost 20%, which is much higher than the physiologic oxygen pressure in the body. Thus, the physiologic oxygen pressure can provide a variety of effects on either tissue formation and/or functions. For example, low oxygen pressure in cardiac tissue cultures demonstrated positive effects on the bioactivitiy of CSCs/CPCs (Li *et al.*, 2011). In contrast, in connective tissue, high oxygen conditions efficiently induced bone formation, whereas low oxygen conditions induced cartilage differentiation (Markway *et al.*, 2010). In addition, it has also been reported that reduced oxygen tension enhances proliferation of some cell types. Cellular proliferation capacities significantly increased in bovine pericytes (Brighton *et al.*, 1992) and rat calvarial osteoblasts (Tuncay *et al.*, 1994) in tightly regulated culture conditions under low oxygen tension. A recent study has reported that hypoxia (1-5% O_2) also enhanced the self-renewal of murine neural stem cells (MSCs) (Horie *et al.*, 2008), ESCs (Ezashi *et al.*, 2005), and CSCs (Li *et al.*, 2011), while some experiments showed that hypoxia induced differentiation (Lennon *et al.*, 2001; Chen *et al.*, 2007).

ERK signal pathway has different effects on growth, prevention of apoptosis, cell cycle arrest and induction of drug resistance in cells of various lineages (McCubrey *et al.*, 2007). Accumulating reports have clearly shown that oxidative stress induced ERK1/2 activation in a variety of cell types (Jimenez *et al.*, 1997; Conde de la Rosa *et al.*, 2006) and MEK1 and 2 inhibitor, U0126 blocked oxidative stress-induced ERK1/2 activation (Lee *et al.*, 2005). By specific inhibition of activated ERK signaling under low oxygen status, *ex vivo* expanded stem cells might have more proliferative capacities and improved bioactivities.

In the current study, we established a novel priming protocol of freshly isolated patient-derived c-kit (+) cardiac progenitors from the human atrium by enzymatic digestion via identifying a pivotal target molecule for their differentiation into specific cell lineages. We found that our expanded hCPCs had a priming effect regarding functional bioactivity and differentiation potentials of hCPCs into ECs, SMCs, and CMs via preconditioning by both hypoxia and ERK inhibition.

MATERIALS AND METHODS

Human cardiac progenitor cell isolation and culture

Human heart tissue obtained from infants who had a con-

genital heart defect. After heart surgery, waste heart piece minced and were isolated by enzymatic digestion method. Isolated hCPCs plated in Ham's F-12 medium supplemented with 10% FBS, 10 ng/ml recombinant human fibroblast growth factor-basic (hFGF-b) (Peprotech, Rocky Hill, NJ, USA), 5 mU/ml recombinant human erythropoietin (R&D), 0.2 mM L-glutathione (Sigma, MO, USA), and 1% antibiotics. Cardiac cells were labeled with a c-kit monoclonal antibody (Santa Cruz, CA, USA) and c-kit-positive hCPCs were selected by using FACS Aria (BD, California, USA) or were labeled with a CD117 microbead (Miltenyi Biotec, Germany) and c-kit-positive hCPCs were selected by using MACS separator (Miltenyi Biotec, Germany) according to the manufacturer's instructions. The medium was changed twice per week. The Ethics Review Board of the Pusan National University Hospital of Yangsan, Gyeongsangnam-do, Korea, approved the protocols. The experimental study was conducted in accordance with the Declaration of Helsinki.

Hypoxia and hypoxic preconditioning

To study the effect of hypoxia or hypoxic preconditioning on hCPCs, hCPCs were incubated in a Modular Incubator Chamber (IB Science, Daejeon, Korea) that maintained a gas mixture composed of 93% N_2, 5% CO_2, and 2% O_2. Hypoxic preconditioning was performed by incubating cells for 15-30 min at 37°C under hypoxic conditions (2% O_2).

Western blot analysis

hCPCs were cultured in normoxic and hypoxic conditions for different time courses. Whole cell lysates were prepared using RIPA buffer. Protein extracts were separated by 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto PVDF membranes (Millipore, MA, USA) using a Bio-Rad transferring unit (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 30 min at room temperature. Then, the membranes were incubated overnight at 4°C with the respective primary antibody, i.e., rabbit anti-HIF-1α, mouse anti-β-actin (1:1,000, Santa Cruz) and rabbit anti-p-ERK (1:1,000, Cell Signaling, MA, USA) prepared in 5% skimmed milk. After this, the membranes were washed thoroughly with TBS-T and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The signal was detected using radiographic films.

Cell proliferation assay

In order to examine the difference in proliferative ability of hCSCs among normoxia, H/P, and H/P+U0126, cells from passage 6-8 were seeded at a density of $4×10^3$ cells/well in 96-well culture plates with growth medium. After 5 days of culture, we used the EZ-Cytox enhanced cell viability assay kit (DaeilLab Service, Seoul, Korea) to assess cell viability. The absorbance was measured at 420-480 nm using a microplate reader (Sunrise™ Remote Control; TECAN, Salzburg, Austria).

FACS analysis

To investigate how hypoxia or ERK inhibitor pretreatment affects the subpopulation of c-kit-positive stem cells, hCPCs expanded under normoxia and hypoxia were harvested, washed in PBS, and incubated for 1 h at 37°C in an incubator

with the primary antibody (rabbit anti-human c-kit; Santa Cruz) mixed in PBS containing 3% FBS and 1 mM EDTA. After incubation, cells were washed twice and incubated with an anti-rabbit Alexa 488 antibody for 30 min at 4°C. The expression of c-kit was quantitatively measured using FACSCalibur and analyzed with CellQuest software (BD Biosciences).

Tube formation assay

To assess the capability of hCPCs to form capillary-like structures, cells were seeded at a density of 1×10^4 cells/well with conditioned medium (100 μl) in 96-well culture plates coated with growth factor-reduced Matrigel (60 μl/well) (BD Biosciences) for 30 min at 37°C. Cells were cultured for 5 h at 37°C in an incubator with 5% CO_2.

Differentiation into vascular cells

To induce differentiation into ECs and SMCs, hCPCs were treated with differentiation medium for 10 days; medium was changed every 2-3 days. Differentiation medium consisted of DMEM/high glucose (Welgene, Daegu, Korea) supplemented with 20% FBS (GenDePOT, Barker, USA), 1% antibiotics, and recombinant hFGF-b (30 ng/ml) (Peprotech), or recombinant human platelet-derived growth factor-BB (hPDGF-BB) (40 ng/ml) (Peprotech).

Myogenic differentiation

To induce myogenic differentiation, hCPCs were treated with myogenic medium for 4 weeks; medium was changed daily. Myogenic medium consisted of MEM/EBSS (Hyclone) supplemented with 2% FBS, 1% antibiotics, and 10 nM dexamethasone (Sigma-Aldrich).

Immunofluorescence staining

hCPCs grown on cover slides were washed 2 times with PBS, fixed in 4% paraformaldehyde for 5 min, permeabilized with 0.5% triton X-100 (w/v in PBS) for 5 min, and incubated at room temperature with PBS containing 3% BSA to block non-specific binding. Then, hFGF-b-induced cells were incubated with primary antibodies, i.e., goat anti-platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31) and rabbit anti-c-kit (1:100, Santa Cruz). hPDGF-BB-induced cells were incubated with primary antibodies, i.e., mouse anti-α-SMA (1:1,000, Sigma) and rabbit anti-c-kit. Dexamethasone-induced cells were incubated with primary antibody, i.e., rabbit anti-c-kit, rabbit anti-Nkx 2.5 (1:100, Santa Cruz), rabbit anti-Gata4 (1:100, Santa Cruz), mouse anti-MHC (1:200, Abcam), or mouse anti-Troponin T (1:200, Abcam). Cells were incubated overnight at 4°C with primary antibodies. After washing, cells were incubated with secondary antibodies (anti-goat Alexa488, anti-rabbit Alexa594, and anti-mouse Alexa488). Images were obtained by confocal microscopy (Olympus, NY, USA).

RESULTS

Isolation and characterization of hCPCs

To establish an *ex vivo* expansion protocol of freshly isolated patient-derived c-kit+ CPCs from the human atrium and to identify pivotal target molecules for their differentiation into specific cell lineages, we isolated c-kit+ CPCs by enzymatic digestion and MACS sorting, and subsequently expanded them at a low density until passage 6, as previously published

(Bearzi *et al.*, 2007). As shown in Fig. 1A, the percentage of cells with c-kit receptor expression was approximately 31.44-62.3%. Consistent with a previously reported, typical hCPC phenotype, immunostaining analysis demonstrated that most adherent cells were positive for c-kit, Nkx2.5, and Gata4 (Fig. 1B, C).

Hypoxic preconditioning of *ex vivo*-expanded hCPCs involves upregulated ERK signaling

To investigate whether hypoxic preconditioning of *ex vivo*-expanded hCPCs affects the expression of hypoxia-inducible factor (HIF)-1α and mitogen-activated protein kinase (MAPK) proteins, hCPCs were incubated under low oxygen conditions (2%) for different defined time points (0, 15, 30, 60, 120 min).
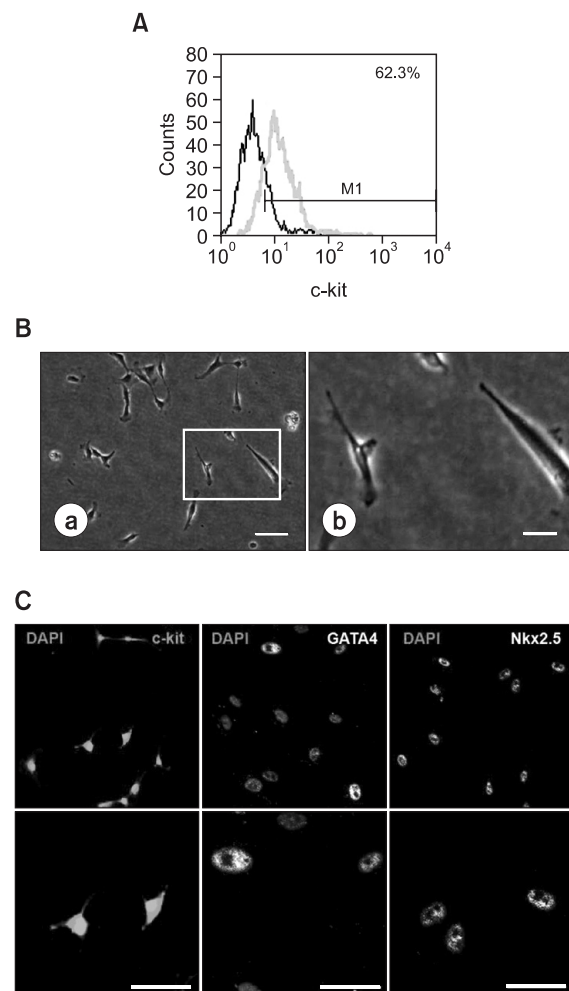
Fig. 1. Characterization of human cardiac progenitor cells (hCPCs). (A) Flow cytometry analysis showed that from P1 to P6, an average of 62.3% of human cardiac-derived progenitor cells was c-kit-positive. (B) Morphology of hCPCs. After culturing cells obtained from enzymatically digested human cardiac atrium (a) the progenitor cells sorted using c-kit-conjugated microbeads appeared like fibroblasts (b). (C) Immunostaining for cardiac progenitor cell markers demonstrated that hCPCs were positive for c-kit (red) and cardiac transcription factors, GATA4 and Nkx2.5 (green). c-kit was detected in the cell cytoplasm, and Nkx2.5 and GATA4 were detected in the cell nuclei. Scale bar represents 100 μm.
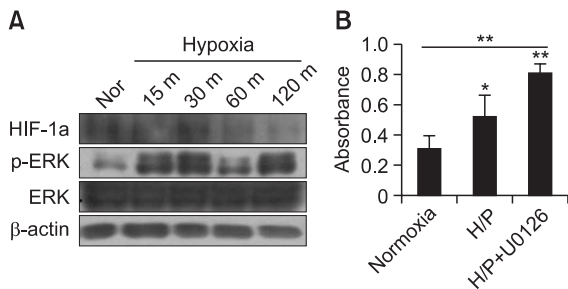
As shown in Fig. 2A, we observed a significant increase in the expression of HIF-1α and phosphorylation of ERK at early time points (15-30 min).

Hypoxic preconditioning and ERK signaling in *ex vivo*-expanded hCPCs modulate cellular proliferation
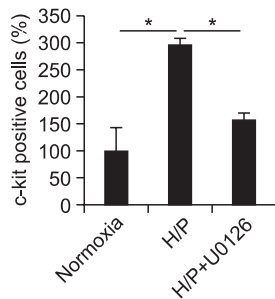
The proliferation capacity of hypoxia-preconditioned hCPCs cultured under low oxygen conditions (2%) for 5 days was evaluated by performing the EZ-Cytox cell proliferation assay. As shown in Fig. 2B, the proliferation ability of hCPCs in hypoxic preconditioning was significantly higher than that of the normoxia group. When we blocked hypoxia-induced phosphorylation of ERK in hCPCs by treatment of the cells with U0126, a specific ERK inhibitor, we observed that specific ERK inhibition in hCPCs under hypoxia conditions strongly increased cellular proliferation of hCPCs as compared to hypoxic preconditioning alone (Fig. 2B), implying that ERK activity may be negatively linked with cellular proliferation in hypoxia-preconditioned hCPCs.

Hypoxic preconditioning and ERK signaling regulate the expression of c-kit receptor in hCPCs

Therefore, we next attempted to analyze the expression of the c-kit receptor, a typical marker of hCPCs. We cultured our *ex vivo*-expanded hCPCs at specific experimental conditions of normoxia, hypoxia (H/P), and hypoxia with U0126 treatment (H/P+U0126). After 7 days, we observed that hypoxic conditioning significantly enhanced the c-kit expression ratio, but markedly reduced the percentage of c-kit-expressing cells by treatment with a specific ERK inhibitor, suggesting that ERK activity in hypoxic preconditioning might play a positive role in the maintenance of the stem cell-like character of hCPCs (Fig. 3).
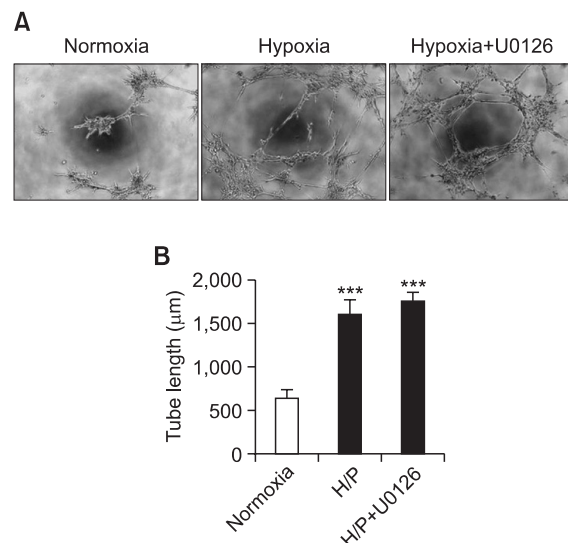
Enhanced tubular forming capability of hCPCs in hypoxic preconditioning and ERK inhibition

To test the possibilities of hCPCs for vascular structure formation by hypoxia and ERK inhibition, we assessed the tubular forming capacity of the above-defined experimental groups via tubular formation on Matrigel. As shown in Fig. 4, both H/P and H/P+U0126 groups showed significantly increased formation of vessel-like structures compared to the normoxia group.

Differentiation potential of hCPCs into smooth muscle cells in hypoxic preconditioning and ERK inhibition

We investigated whether our expanded hCPCs might efficiently differentiate into 3 cell lineages, i.e., ECs, SMCs, and CMs, in the above experimental groups. In order to differentiate into ECs, hCPCs were treated with a well-defined EC differentiating cytokine, hFGF-β (30 ng/ml). After treatment of hCPCs with hFGF-β for 5-10 days, we observed a decrease in c-kit-expressing cells and an increase in CD31-expressing cells. However, we did not find any significant difference in the ratios of CD31-expressing cells among the 3 groups (Fig. 5), suggesting that ERK inhibition might not affect the EC differentiation potential of hCPCs.

To differentiate into SMCs, hCPCs were treated with hP-

Fig. 2. Extracellular signal-regulated kinase (ERK) protein expression under hypoxia and proliferation activity after hypoxic preconditioning. (A) hCPCs were cultured under either normoxia or hypoxia at different time courses. Western blot analysis showed HIF-1α and p-ERK were up-regulated by hypoxia after 15 and 30 min. (B) Proliferation ability of hCPCs after hypoxic preconditioning (H/P) was significantly increased. The proliferation ability of hCPCs under hypoxia and in the presence of U0126 (ERK inhibitor) (H/P+U0126) was significantly higher than that of H/P hCPCs. Data were shown as mean ± SD (n=4). *$p<0.05$, **$p<0.01$.

Fig. 3. Hypoxia maintained stemness. After 7 days of culture under normoxia or hypoxia, the percentage of c-kit-positive cells was determined by FACS. hCPCs highly maintained expression of the stem cell marker c-kit under H/P condition compared with normoxia and H/P+U0126 condition. *$p<0.05$.

Fig. 4. Capacity of tube formation. hCPCs were cultured on Matrigel for 5 h after exposure to either normoxia, H/P, or H/P+U0126. (A) Representative tubular hCPCs are shown (×100 magnification). (B) The capacity of hCPCs to form tubes was analyzed by measuring tube length using the image J program. Tube formation capacity of hCPCs under H/P and H/P+U0126 was significantly higher than that under normoxia condition. ***$p<0.001$.

DGF-BB (40 ng/ml), a typical SMC differentiation-inducing cytokine. After treatment for 5 days, H/P and H/P+U0126 groups displayed a significant increase in the number of cells expressing α-SMA, a smooth muscle cell marker, at earlier time points than the normoxia group. Importantly, we observed that hCPCs of the H/P+U0126 group showed remarkably lower differentiation potential into SMCs compared to the H/P group (Fig. 6). It seems that ERK activity in hypoxic preconditioning may be involved in the differentiation of CPCs into SMCs.

Myogenic differentiation potential of hCPCs in hypoxic preconditioning

To assess the differentiation potential of CPCs into CMs by ERK inhibition in hypoxic preconditioning, hCPCs were treated with dexamethasone (10 nM) daily. After 2 weeks of treatment, we observed a reduced relative ratio of c-kit-expressing cells. As shown in Fig. 7 we observed that Nkx2.5, a cardiac-specific transcription factor, and cardiac troponin T (cTnT), a marker for CMs, were significantly upregulated in hypoxic preconditioning compared to normoxic condition. Interestingly, there was no difference in the expression levels of the cardiac transcription factor Gata4 and the CM marker cardiac myosin heavy chain (MHC) among the 3 experimental groups (Fig. 7).

DISCUSSION

Although resident human cardiac stem cells have been reported to contribute significantly to vascular repair at sites of ischemia and are capable of differentiating into ECs, SMCs, and CMs (Bearzi *et al.*, 2007), it is more important to obtain

hCPCs of better quality and higher quantity to achieve the best clinical outcomes in the treatment of myocardial infarction. To improve the quality and quantity of hCPCs, Tao-sheng Li *et al.* attempted long-term *ex vivo* expansion of human cardiac-derived cells (CDCs) under physiological low-oxygen conditions (5% O_2) (Li *et al.*, 2011). They obtained functionally superior hCDCs. Those cells have better qualities with regard to high proliferation ability, low chromosomal abnormality, resistance to oxidative stress, cell engraftment, and differentiation. Herein, we investigated whether the bioactivity of hCPCs
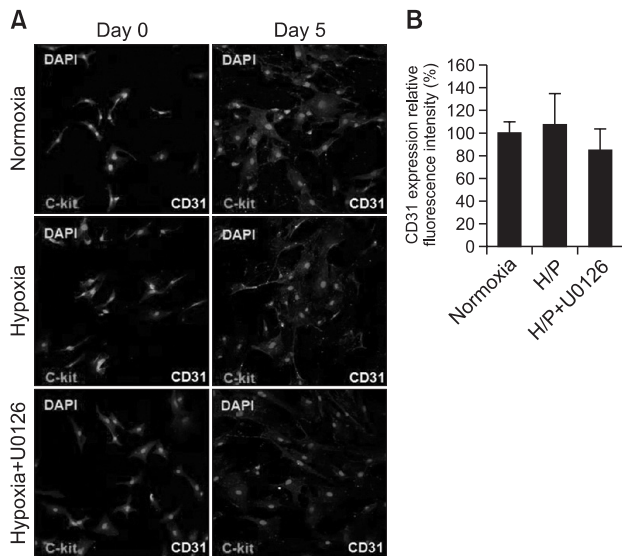
Fig. 5. Human cardiac progenitor cell (CPC) differentiation into vascular cells. hCPCs differentiated into endothelial cells (ECs) by bFGF treatment for 5 days after pretreatment with either hypoxia or U0126. (A, B) Cells were stained for c-kit (red) or CD31 (green). Nuclei were stained with DAPI (blue). Expression of the endothelial cell marker CD31 (green) following bFGF-induced EC differentiation was not different among normoxia, H/P, or H/P+U0126 groups. EC differentiation was evaluated by quantitative analysis of the staining for endothelial cell marker CD31 (green). Scale bar represents 100 μm.
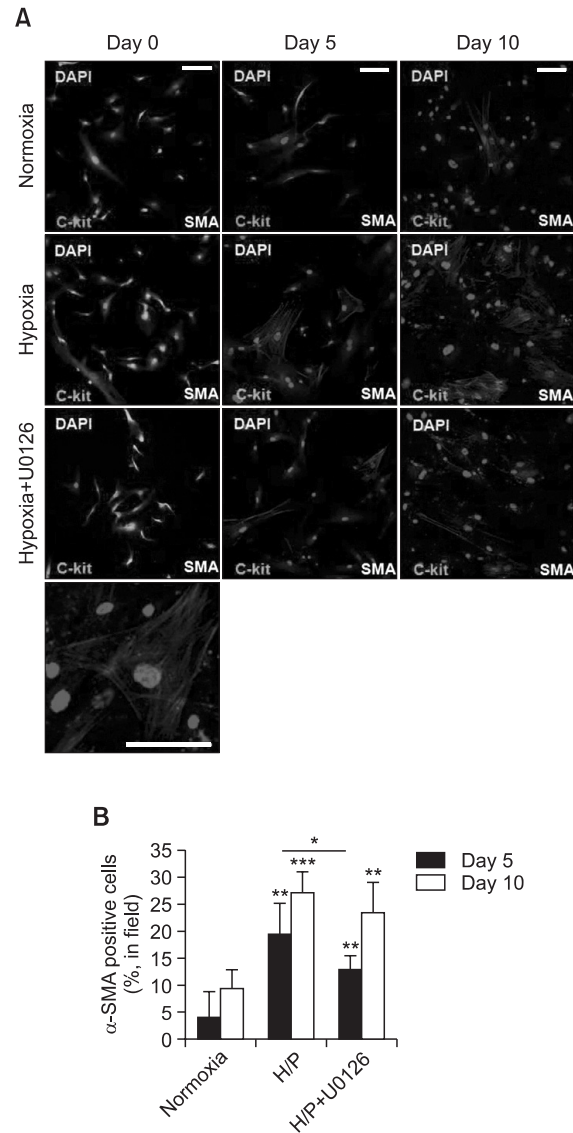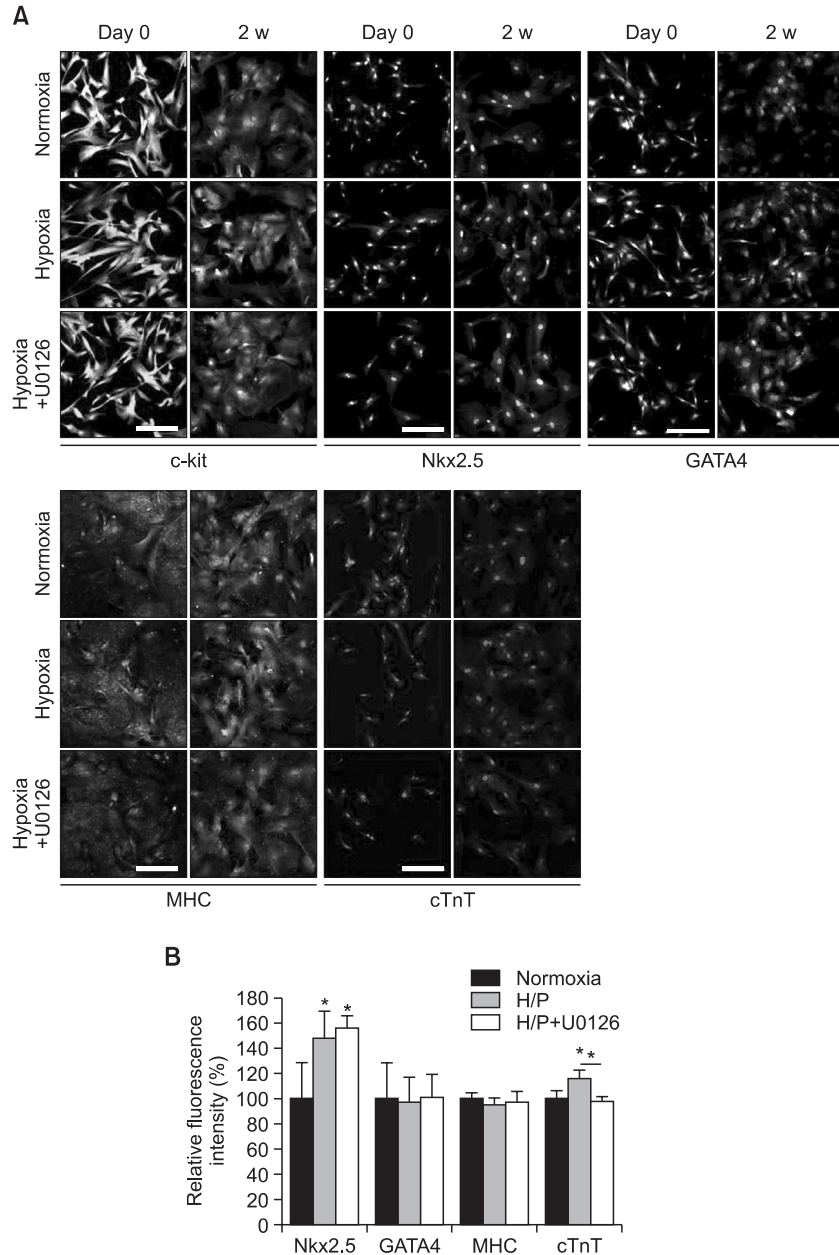
Fig. 6. Human cardiac progenitor cell (CPC) differentiation into vascular cells. hCPCs differentiated into smooth muscle cells (SMCs) by PDGF-BB treatment for 10 days. (A) Cells were stained for c-kit (red) or alpha-smooth muscle actin (α-SMA, green). Nuclei were stained with DAPI (blue). (B) H/P or H/P+U0126 groups showed enhanced PDGF-BB-induced SMC differentiation as compared to the normoxia group. SMC differentiation was evaluated by quantitative analysis of the staining for the smooth muscle cell marker α-SMA (green). Scale bar represents 100 μm. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

is enhanced when hCPCs were cultured in the presence of specific target modulators under low oxygen pressure condition during *ex vivo* expansion of patient-derived hCPCs.

We first demonstrated that hypoxic preconditioning during *ex vivo* expansion of hCPCs facilitates short-term activation of ERK (Fig. 2A). The ERK signaling cascade is well defined as a central MAPK pathway that plays a role in the regulation of various cellular processes such as proliferation, differentiation, development, learning, survival and, under some conditions, also apoptosis (Shaul and Seger, 2007). Thus, we expected

that robust ERK activation by hypoxic preconditioning might enhance the bioactivity of hCPCs. As expected, hypoxic preconditioning enhanced cellular functions such as proliferation and formation of tubes and vascular networks. In particular, it is noteworthy that hypoxic preconditioning of hCPCs in the presence of an ERK inhibitor, U0126, significantly increased functional activities as compared to hypoxic preconditioning in the absence of U0126 (H/P) (Fig. 2B, 4), suggesting that the physiological activity of hCPCs under hypoxic conditions increases in a ERK-dependent manner. According to Li *et al.*,

Fig. 7. Human cardiac progenitor cell (CPC) differentiation into cardiomyocytes. hCPCs differentiated into cardiomyocytes (CMs) within 14 days. (A) Cells were stained for CM-specific lineage markers, i.e., c-kit, Nkx2.5, GATA4, MHC, and cTnT (all green). Nuclei were stained with DAPI (blue). (B) During cardiomyogenic differentiation by dexamethasone, c-kit expression had become weaker in all groups. However, Nkx2.5 and cTnT expression were significantly higher in the H/P condition than in the normoxic condition. CM differentiation was evaluated by quantitative analysis of CM makers. Scale bar represents 100 μm. *$p<0.05$.

culturing cells at low-oxygen pressure can mimic the stem cell niche condition and, thus, might preserve the increased stemness of hCSCs (Li *et al.*, 2011). In the present study, we first observed that hCPCs significantly maintained the expression level of a stem cell marker, c-kit receptor, under preconditioning by both hypoxia and ERK inhibition (H/P+U0126) (Fig. 3). In the study, *ex vivo* expanded hCPCs exposed to hypoxia for 15 min (2% O_2) in hypoxia chamber, followed by reoxygenation (21% O_2). Hypoxia-reoxygeneration can generate oxidative stress and induce cellular apoptosis. It is likely that ERK inhibition by U0126 seems to inhibit partially ERK-mediated apoptosis in early stage, but it remain to be unresolved.

Another notable finding of the present study was that in the H/P+U0126 group, *ex vivo*-expanded hCPCs demonstrated higher potential to differentiate into SMCs and CMs, but not into ECs. Accumulating evidence shows that hypoxia and hypoxic preconditioning seems to promote cellular proliferation and osteogenic differentiation of hMSCs (Volkmer *et al.*, 2010; Hung *et al.*, 2012) and to facilitate cardiac differentiation of human cord blood-derived MSCs by regulation of ERK signaling (Qian *et al.*, 2012). In addition, Ong LL *et al.* reported that hypoxic preconditioning of human bone marrow CD133+ cells increases the endothelial differentiation potential (Ong *et al.*, 2010). In case of fibroblasts, hypoxia could up-regulate ERK1/2 phosphorylation and significantly up-regulated α-SMA protein level and gene promoter activity, while inhibition of ERK1/2 had no effect on the activation of the α-SMA gene (Short *et al.*, 2004). On the basis of previously published data together with ours, it seems controversial how hypoxic preconditioning and ERK inhibition together affect cell differentiation and regulate specific lineage differentiation, which, therefore, remains to be addressed in future studies, in particular with regard to molecular differential cascades.

Taken together, *ex vivo*-expanded hCPCs under conditions of both hypoxic preconditioning and ERK inhibition have a priming effect on multiple bioactivities, including cellular proliferation, vascular network forming ability, and differentiation potential into SMCs and CMs. We believe that our finding paves the way to further investigate novel targets for the establishment of CPC expansion protocols, which will contribute to overcome the current obstacles in cardiac regeneration and repair by supplying hCPCs of a better quality.

ACKNOWLEDGMENTS

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