European Review for Medical and Pharmacological Sciences 2017; 21: 1820-1827

# Hypoxia increases expression of CXC chemokine receptor 4 via activation of PI3K/Akt leading to enhanced migration of endothelial progenitor cells

T. DAI[1], Y. HU[2], H. ZHENG[3,4]

[1]Department of Cardiology, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, Shandong, P.R. China
[2]Health Care & VIP Ward, The Affiliated Qingdao Hiser hospital of Qingdao University, Qingdao Hospital of Traditional Chinese Medicine, Qingdao, Shandong, P.R. China
[3]Department of Cardiovascular Diseases, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou, P.R. China
[4]Department of Cardiovascular Diseases, Hangzhou Xiasha Hospital, Hangzhou, P.R. China

*Tao Dai* and *Yong Hu* are co-first authors

**Abstract. – OBJECTIVE: Recruitment of endothelial progenitor cells (EPCs) to the ischemia has recently been suggested as an important mechanism of neovascularization. Although tissue ischemia can mobilize EPCs from bone marrow, the effects of hypoxia on the migration of EPCs are little known.**

**MATERIALS AND METHODS: In this study, migratory function of EPCs was examined by a modified Boyden chamber technique. The expression of CXCR4 was detected by reverse transcription PCR and flow cytometry assay. The protein expressions of ERK1/2 and PI3K/Akt signaling pathways were detected by Western blotting.**

**RESULTS: Migration of EPCs in response to the chemokine Stomal-derived factory-1α (SDF-1α) was much enhanced by hypoxia. The enhanced migration can be blocked by the CXCR4 antagonist AMD3100 and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, whereas mitogen-activated protein ERK1/2 kinase inhibitor PD98056 had no significant effect on enhanced migration induced by hypoxia. The expression of CXCR4 markedly increased under hypoxic conditions. A molecular analysis of these events indicated that augmented CXCR4 expression was regulated by the PI3K/Akt signal transduction pathway.**

**CONCLUSIONS: These results indicated that exposure of EPCs to hypoxia resulted in a significant up-regulation of CXCR4 expression by PI3K/Akt activation, leading to enhancing chemotaxis behavior.**

*Key Words:*
Endothelial progenitor cells, Hypoxia, Akt, CXCR4 migration.

## Introduction

Tissue hypoxia occurs in various disease states such as ischemic cardiovascular diseases and tumor development[1]. In response to these hypoxic conditions, mammalian cells have developed adaptive systems allowing them to survive to moderate or even severe hypoxia. This process involves an increase in neovascularization that ultimately relieves tissue hypoxia and contributes to wound healing[2-4]. Numerous reports[5,6] suggest that endothelial progenitor cells (EPCs) participate in neovascularization either by incorporating into the neovasculature or secreting proangiogenic factors. Further investigations indicated that these processes are mediated by interactions between EPCs and mature endothelial cells through the expression of adhesion molecules, and the release of chemokines, such as vascular endothelial growth factor (VEGF) and Stromal-derived factor-1α (SDF-1α). The chemotactic effect of SDF-1α on hematopoietic progenitor cells has been shown to be mediated via the chemokine receptor 4 (CXCR4). The chemokine receptor CXCR4 is a seven-transmembrane-domain G-protein-coupled receptor[7,8]. Recent researches[9] indicated that CXCR4 is essential for migration and homing of hematopoietic stem cells. Up-regulation of CXCR4 expression is required for the induction of SDF-1α-dependent chemotaxis in CD34+ progenitor and other several cell types[10-13]. The phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated protein kinases 1 and


*Corresponding Author:* Hao Zheng, MD; e-mail: zhenghao@medmail.com.cn

2 (ERK1/2) pathways modulate cell proliferation, migration and survival[14,15]. Previous studies[16,17] had suggested that ERK1/2 and PI3K/Akt are activated by hypoxia in many cell types such as rat nucleus pulposus cells and mesenchymal stem cells and play an important role in a variety of cell responses. However, the pathways involved in hypoxia-induced CXCR4 expression of EPCs are still unknown. In present work, the activity of ERK1/2 and PI3K/Akt in EPCs were examined in association with the chemokine receptor expression under hypoxic conditions. All data demonstrated that exposure of EPCs to hypoxia resulted in the activation of PI3K/Akt and ERK1/2. Hypoxia also enhanced CXCR4-dependent EPCs migration and up-regulated CXCR4 expression. Analysis with specific inhibitors revealed that Akt activation, but not ERK1/2 activation, was required for hypoxia-induced up-regulation of CXCR4 expression and enhanced chemotaxis behavior.

## Materials and Methods

### Reagents

Anti-ERK1/2, anti-phospho-ERK1/2, anti-Akt, anti-phospho-Akt, anti-HIF-1 were purchased from Cell Signaling (Beverly, MA, USA). Anti-β-actin monoclonal antibody was from Santa Cruz (Santa Cruz, CA, USA). Anti-hCXCR4 monoclonal antibody for flow cytometry was from R&D Systems (Minneapolis, MN, USA). Recombinant human SDF-1α and PI3K/Akt inhibitors, LY294002, and MEK inhibitors, PD98059 were from Cell Signaling (Beverly, MA, USA). Medium 199 was from Sigma (St. Louis, MO, USA). EGM-2 MV was from Cambrex Bio Science (Walkersville, MD, USA).

### Isolation of Mononuclear Cells and Cell Culture

Endothelial progenitor cells (EPCs) were cultured according to previously described techniques[15]. Briefly, mononuclear cells (MNCs) were isolated by density centrifugation (Histopaque 1077, Sangean Biotechnology Co. Ltd., Shanghai, China). After purification with 3 washing steps, $10^5$-$10^6$ MNCs were plated on fibronectin coated 6-well plate or cell culture flask. Cells were cultured in endothelial cell basal medium-2 supplemented with EGM-2 MV single aliquots consisting of 20% fetal bovine serum (FBS), (Jincheng Hayes Pharmaceutical Co. Ltd, Jincheng, Shanxi, China), vascular endothelial growth factors (VEGF), (Jincheng Hayes Pharmaceutical Co. Ltd, Jincheng, Shanxi, China), fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1, and ascorbic acid. After 4 days in culture, nonadherent cells were removed by washing with phosphate buffer saline (PBS), new medium was applied, and the culture was maintained through day 7. EPCs were characterized as adherent cells double positive for DiLDL-uptake and lectin binding under a laser scanning confocal microscope. They were further documented by demonstrating the expression of VE-cadherin, CD34 and AC133 by flow cytometry (data not shown).

### Hypoxia Treatment

For the hypoxic experiments, cell cultures were placed in a hypoxic atmosphere (modular incubator chamber, Billups-Rothenburg, Inc., Del-Mar, CA, USA) and flushed with a gas mixture of 2% $O_2$/5% $CO_2$/93% $N_2$. The airtight chamber containing the cell cultures was incubated for periods of up to 24 h at 37ºC.

### Migration Assay

Migratory function of EPCs, which is essential for angiogenesis, was examined using a modified Boyden chamber technique. A total of $2\times10^4$ EPC per group was isolated, resuspended in 500 μL M199. After being incubated for 30 min at 37ºC, with or without AMD3100 (5 μg/mL) or the ERK inhibitor PD98059 (20 nm/ml) or the PI3K inhibitor LY294002 (20 nm/ml), the cells were seeded in the upper chamber of a modified Boyden chamber with 8-μm-pore-size membranes (Qiling Medical Equipment Factory, Jiangsu, China). Recombinant human SDF-1α (100 ng/mL) was diluted in serum-free M199 medium and placed in the lower chamber, in a volume of 200 μL. Migration was allowed to proceed in either normoxia (5% $CO_2$/95% air) or hypoxia (2% $O_2$). After 24 h incubation at 37ºC, the lower side of the filter was washed with phosphate buffered saline (PBS) and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and were counted manually in 5 random microscopic fields by a blinded investigator.

### Western Blot Analysis

EPCs were cultured in culture flasks and growth arrested in serum-free medium 199 for 12 h. After stimulation with hypoxia (2% $O_2$)

for various periods of time, cells were washed twice with ice-cold phosphate buffered saline (PBS) and scraped and collected. Cell protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Thermo Fisher, Shanghai, China) on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated overnight with primary antibody in the appropriate dilution, before incubation for 1 h with a secondary anti-rabbit antibody conjugated to horseradish peroxidase (1:5000). Proteins were then visualized using enhanced chemiluminescence solution from Amersham and X-ray film.

### RT-PCR Analysis

Total RNA was isolated from cells using the TRIzol method (Life Technologies, Carlsbad, CA, USA). RNA was converted into cDNA using murine leukemia virus reverse transcriptase (Life Technologies, Carlsbad, CA, USA). The transcribed cDNA was then used for polymerase chain reaction (PCR) amplification to estimate the expression of CXCR4. Two specific primers matching the published sequences were used to identify and amplify CXCR4 (sense primer: 5'-CTT CTA CCC CAA TGA CTT GTG G-3'; antisense primer: 5'-AAT GTA GTA AGG CAG CCA ACA G-3'). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a reference. PCR products were resolved by electrophoresis on 2.0% agarose gels and visualized by ethidium bromide.

### Flow Cytometry

EPCs were serum-starved and exposed to either normoxia or hypoxia for 24 h. Adherent cells were first harvested using phosphate buffered saline (PBS). Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 20 min and washed twice with phosphate buffered saline (PBS). After washing, cells ($1 \times 10^5$) were incubated for 1 h at 4°C with 200 μL phosphate-buffered saline containing 0.2% bovine serum albumin (PBS-BSA) with Mab against CXCR4 (MAb173). After washed three times with PBS-BSA to remove unbound antibody, they were stained with the secondary antibody for 30 min at 4°C. The cells were then washed and resuspended in PBS-BSA, kept at 4°C, and analyzed using Beckman Coulter FACS Calibur (Miami, FL, USA).

### Statistical Analysis

Data were expressed as mean ± SEM for 3-6 individual experiments. Statistical analysis between two groups was performed using unpaired Student's *t*-test and differences of groups were compared by the method of one-way ANOVA. LSD test was used to post-hoc test ANOVA. Probability values were considered significant at $p < 0.05$.

## Results

### Hypoxia Enhances the Migratory Ability of Endothelial Progenitor Cells in Response to SDF-1α

To assess the effect of hypoxia on the migratory function of endothelial progenitor cells, a modified Boyden chamber assay was used. A total of $2\times10^4$ cells per group were treated with serum-free M199 medium for 12 h and isolated, then resuspended in 500 μL M199. After being incubated in normoxic (21% $O_2$) or hypoxic (2% $O_2$) conditions for 24 h, migrated cells adhering to the undersurface of the filters were stained with 4',6-diamidino-2-phenylindole (DAPI) (Figure 1A, B). There was a significant increase in migration to SDF-1α under hypoxic conditions ($p < 0.05$). To confirm the role of CXCR4, AMD3100, a CXCR4 antagonist was used, which inhibited EPC migration toward SDF-1α by 60% (Figure 1).

### Hypoxia Increases Akt and ERK1/2 Phosphorylation in Endothelial Progenitor Cells

Although many inhibitor studies have suggested that activation of Akt and ERK1/2 are involved in hypoxia-mediated responses, there is little direct evidence for increases in Akt and ERK1/2 activities under hypoxic conditions in endothelial progenitor cells. We performed time-course experiments, in which quiescent EPCs were exposed to hypoxia for different times. Phosphorylation of Akt-Ser473 and ERK1/2-Tyr202/Thr204 were analyzed. Akt phosphorylation slightly increased after 2 h and stayed at a steady level for up to 12 h before reaching maximal levels after 24 h of hypoxic exposure (Figure 2A). The active phosphorylated form of ERK1/2 increased after 12 h of incubation under hypoxia and peaked at 24 h (Figure 2B). On the other hand, no significant changes occurred in total Akt and ERK1/2 protein expression over the course of the investigation. To investigate whether the hypoxic treatment was adequate, HIF-1a expression was

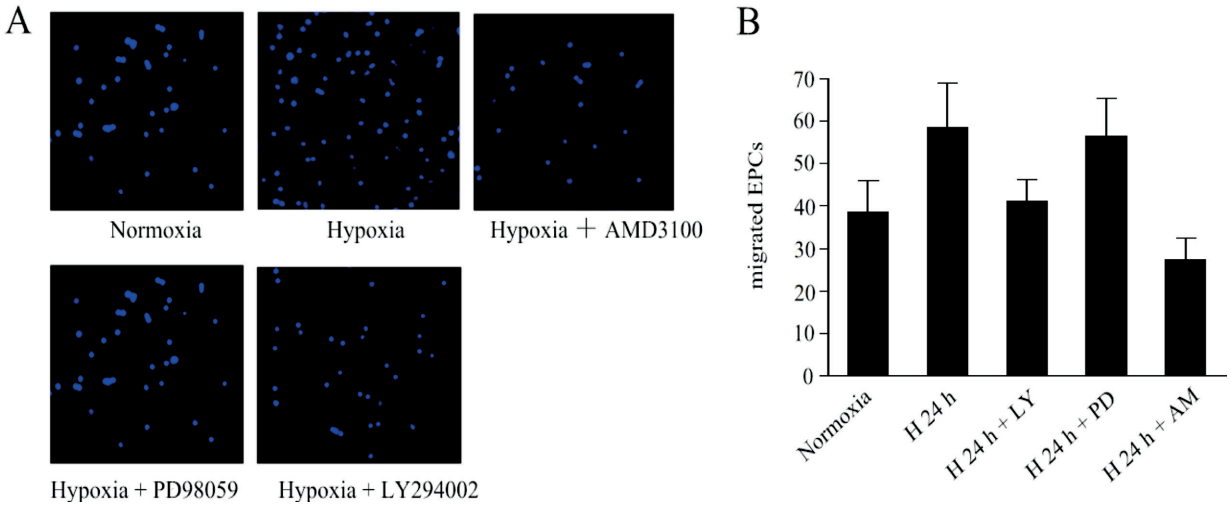


**Figure 1.** Hypoxia-induced increase of migration of human endothelial progenitor cells to SDF-1α (100 ng/mL). Quiescent cells were exposed to hypoxia (2% $O_2$) for 24 h. Migratory function was examined using a modified Boyden chamber technique. ***(A)*** DAPI staining was performed to determine number of migrated cells. ***(B)*** Quantification of hypoxia-induced increased of migration of human endothelial progenitor cells to SDF-1α (100 ng/mL). Experiments were repeated 3 times; data are expressed as means ± SEM. $^{*}p < 0.01$ *vs*. normoxia, $^{\#}p < 0.01$ *vs*. Hypoxia 24 h.

determined by Western blotting. HIF-1α began to appear at 2 h, and stayed at a steady level for up to 24 h in EPCs (Figure 2C). These observations suggested that hypoxia activated these pathways in endothelial progenitor cells.

### *SDF-1α-induced Chemotaxis of Endothelial Progenitor Cells in Hypoxia Requires Akt Activity*

Because these pathways are an important component of the metastatic pathways in endothelial progenitor cells, we want to determine whether the PI3-kinase and ERK pathways modulate migration of EPCs in response to SDF-1α under hypoxic conditions. Therefore, serum-starved EPCs were pretreated with either LY294002 or PD98056 for 30 min, and then exposed to hypoxia for a further 24 h in the presence of SDF-1α (100 ng/mL). The data revealed that PI3-kinase inhibitors strongly inhibited hypoxia-induced chemotaxis, whereas the MEK1/2 inhibitor, PD98059, had no effect on the migration (Figure 1). These results suggested that the PI3-kinase signaling pathway is required for hypoxia-induced migration of EPCs.

### *Hypoxia Promotes Up-Regulation of CXCR4 Expression in Endothelial Progenitor Cells, and CXCR4 Expression is Regulated by Akt Activation*

Hypoxia can up-regulate the expression of CXCR4 in many cell lines. Endothelial progenitor cells were exposed to a hypoxic environment and the changes in CXCR4 expression were examined. As shown in Figure 3, it revealed that the expression of CXCR4 mRNA was strongly elevated in hypoxia-exposed EPCs and LY294002 significantly decreased the expression of CXCR4 mRNA by hypoxia, whereas PD98059 did not exhibit any inhibitory effect. Next, it was determined whether the increase in CXCR4 mRNA correlated with an increase in protein levels of CXCR4. The results indicated that EPCs exposed to hypoxia show a significant increase in CXCR4 protein levels (Figure 4) and increased protein expression is decreased by LY294002. These results further suggested that PI3K signaling might be required for enhanced CXCR4 expression under hypoxic conditions.

## Discussion

In this study, we have shown that exposure of EPCs to hypoxia resulted in a significant up-regulation of CXCR4 expression, which might be associated with the activation of PI3K/Akt, leading to enhancing chemotactic behavior. Recent studies have shown that tissue hypoxia is a fundamental mechanism mediating stem and progenitor cell recruitment and retention at tissue repair. HIF-1 is a dimeric transcription factor that mediates cellular responses to hypoxia. Activated HIF-1 binds to the hypoxia-response element and initiates transcription of a broad range of genes that involve in cell

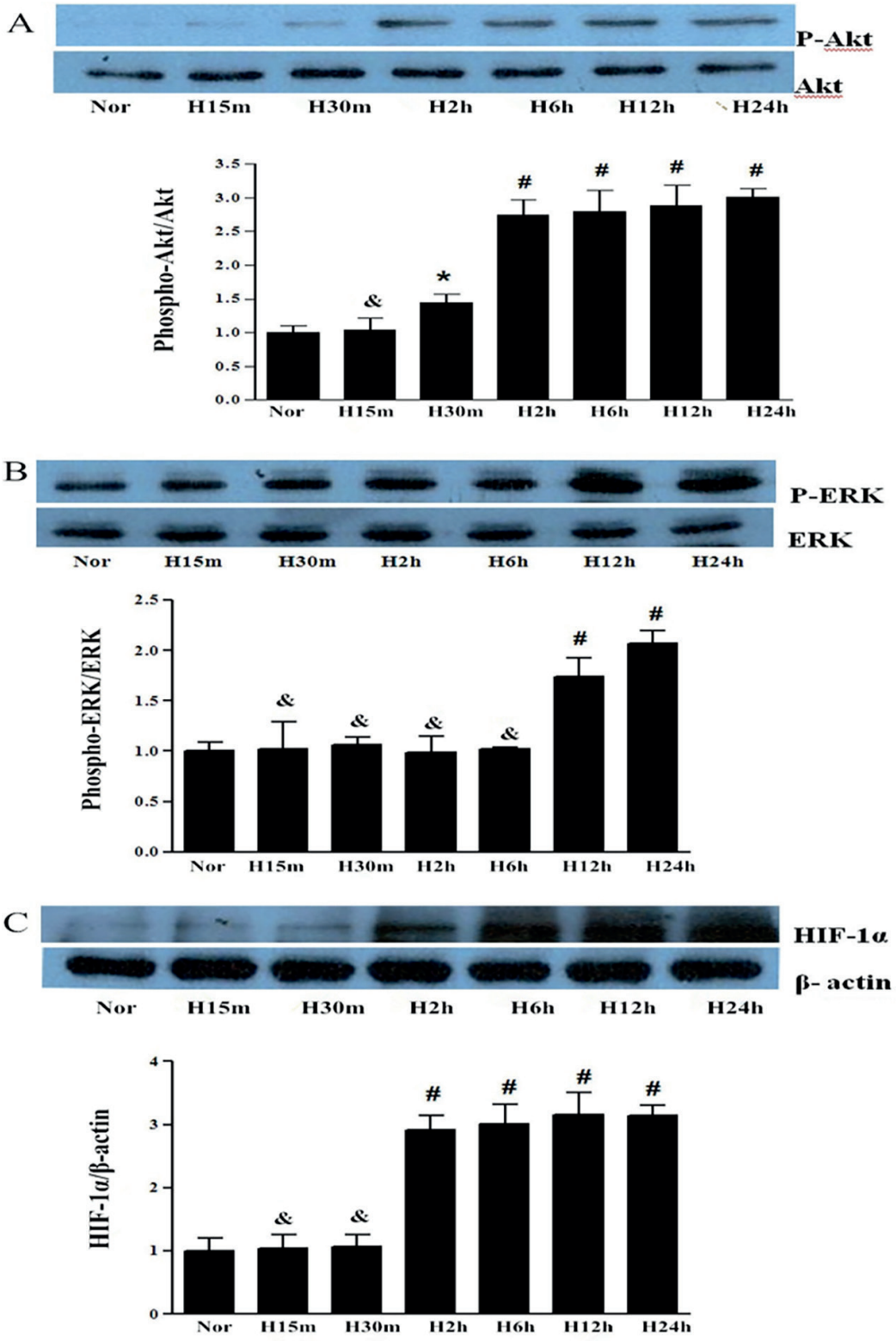


**Figure 2.** Hypoxia induces Akt and ERK1/2 phosphorylation in endothelial progenitor cells. Quiescent cells were exposed to hypoxia (Hy) for the indicated times. Equivalent amounts of total cell protein (50 μg) were subjected to Western blot analysis. ***(A)*** Hypoxia induced Akt phosphorylation in endothelial progenitor cells ***(B)*** Hypoxic conditions resulted in activation of ERK1/2. ***(C)*** Western blot analysis of HIF-1α expression in endothelial progenitor cells cultured in hypoxia. Data are means ± SEM from 3 independent experiments. $^{\&}p > 0.05$ vs. normoxia; $^{*}p < 0.05$ *vs.* normoxia; $^{\#}p < 0.01$ *vs.* normoxia.

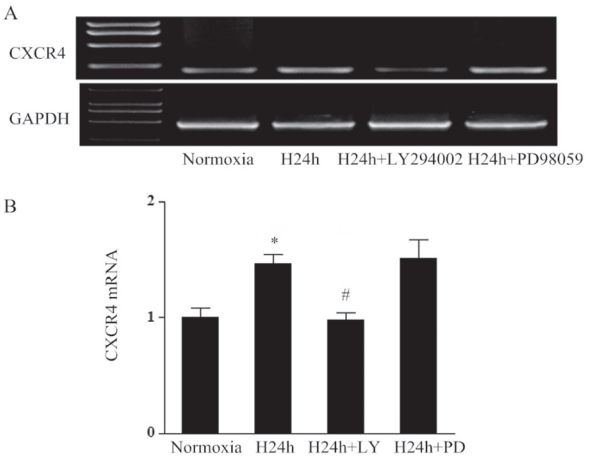


**Figure 3.** Hypoxia-induced increase of migration of human endothelial progenitor cells to SDF-1α (100 ng/mL). Quiescent cells were exposed to hypoxia (2% $O_2$) for 24 h. Migratory function was examined using a modified Boyden chamber technique. ***(A)*** DAPI staining was performed to determine number of migrated cells. ***(B)*** Quantification of hypoxia-induced increased of migration of human endothelial progenitor cells to SDF-1α (100 ng/mL). Experiments were repeated 3 times; Data are expressed as means ± SEM. $*p < 0.01$ *vs*. normoxia, $^{\#}p < 0.01$ *vs*. hypoxia 24 h.

differentiation and stem cell homing[18]. Hypoxia through HIF-1α stimulates the endothelial expression of SDF-1α and consecutively results in homing of CXCR4-positive progenitor cells to areas of hypoxia[19-21]. However, the influence of hypoxia on the function of EPCs is still unknown. Akita et al[22] have reported that hypoxia can enhance migration function of EPCs in response to VEGF. In this study, we observed that hypoxia also can enhance migration function of EPCs to SDF-1α. These results showed that hypoxia following vascular injury might be associated with increased EPCs migration. CXCR4 is a G-protein-coupled 7-transmembrane and selectively binds the CXC chemokine stromal cell-derived factor 1α (SDF-1α). CXCR4 is notably expressed on hematopoietic stem cells and has previously been shown to play a key role in their migration and proliferation. The overexpression of CXCR4 can improve human stem cell motility, retention and multiline age repopulation, which is beneficial for *in vivo* navigation and expansion of hematopoietic progenitors[23]. It was reported that EPCs from patients with CAD displayed impaired CXCR4 signaling for therapeutic integration of EPC into the vascular and then the importance of CXCR4 for human coronary heart disease (CAD) was underscored. Consistent with this notion, numerous reports suggested that blockade of CXCR4 by monoclonal antibody or AMD3100 significantly attenuate revascularization[24,25]. Therefore, understanding the molecular mechanisms regulating CXCR4 expression and activation in various CXCR4-positive cells may be necessary to develop therapeutic strategies to treat patients with cardiovascular diseases[26]. CXCR4 expression is endogenously regulated by tissue environmental factors such as cytokines, chemokines, stromal cells, adhesion molecules, myocardial ischemia and proteolytic enzymes[10]. Hypoxia mediates selective up-regulation of CXCR4 in human monocytes, macrophages, endothelial cells and cancer cells[27]. It was observed that hypoxia can augment the expression of CXCR4 in EPCs. This provided evidence to support the concept that tissue hypoxia may be a fundamental mechanism governing stem and progenitor cell recruitment and retention[19]. Recent studies[28,29] have shown that many intracellular signaling pathways are activated in response to hypoxia such as HIF-1α, NF-κB, MAPKs and PI3K/Akt signaling pathways. MAPKs and PI3K/Akt signaling pathways have been shown to mediate the cell migration induced by chemokines or cytokines in different cell types. In this study, hypoxia induced a time-dependent activation of ERK 1/2 and PI3K/Akt activation

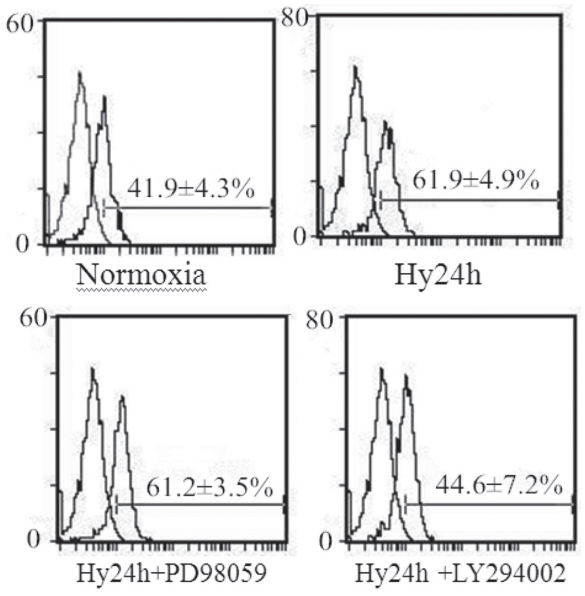


**Figure 4.** Expression of CXCR4 in endothelial progenitor cells under hypoxic conditions is analyzed by PR-PCR. ***(A)*** Hypoxia can up-regulate the expression of CXCR4 mRNA in EPCs. The enhanced expression can be inhibited by LY294002 (20 ng/ml). GAPDH was used as an internal control. ***(B)*** Quantitative data were expressed as percent of basal level. Data are means ± SE from 3 independent experiments. $*p < 0.01$ *vs*. normoxia, $^{\#}p < 0.01$ *vs*. Hypoxia 24 h.

in human EPCs. Notably, the activation of PI3K/Akt does not seem to be a general cellular response to hypoxia, because Akt phosphorylation is not observed in other cell lines. It seems to be a response restricted to specific cell types. In addition to EPCs, Akt phosphorylation is observed in human microvascular endothelial cells and rat aortic endothelial cells under hypoxic conditions[30]. A further indication was that inhibitors of PI 3-kinase abrogated increased expression of CXCR4 induced by hypoxia. The similar results were found in non-small cell lung cancer cells. Xia et al[31] has shown that CXCR4 expression was regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mTOR signal transduction pathway, activation of hypoxia inducible factor (HIF)-1α under hypoxic conditions. In the present study, downstream target of Akt leading to up-regulation CXCR4 expression has not been defined. Several reports[32-34] have shown that the activation of the PI3K/Akt pathway can increase translation of HIF-1α mRNA and protein stabilization under hypoxia. Thus Akt-dependent upregulation of HIF-1α may be important. Further investigations are required to elucidate our speculation.

## Conclusions

Recruitment of endothelial progenitor cells (EPCs) to the ischemia is an important mechanism of neovascularization. Thus, hypoxic pretreatment could up-regulate CXCR4 expression and enhance chemotaxis behavior by PI3K/Akt pathway may improve the functional activity of EPC and augment homing to ischemic tissue in patients with cardiovascular diseases.

### Acknowledgement

The project supported by National Natural Science Foundation of China (30900615) and by Natural Science Foundation of Zhejiang Province (Y2090173).

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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