MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2017; 23: 4665-4676
DOI: 10.12659/MSM.902470

Received: 2016.11.21
Accepted: 2017.03.03
Published: 2017.09.29

Hypoxic Preconditioning Enhances Biological Function of Endothelial Progenitor Cells via Notch-Jagged1 Signaling Pathway

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Source of support: This study was funded by the International Scientific and Technological Cooperation Project, Science and Technology Bureau, Wuhan, Hubei, China, funding number: 2013030409020110

Background: Hypoxic preconditioning may be a key influence on functions of endothelial progenitor cells (EPCs).

Material/Methods: To investigate the role and mechanism of the Notch-Jagged1 pathway on endothelial progenitor cells in hypoxic preconditioning, endothelial progenitor cells were randomly allocated into 5 groups: 1 Normoxic control group; 2 Hypoxic blank group; 3 Hypoxic+25 μM DAPT group; 4 Hypoxic+50 μM DAPT group; 5 Hypoxic+100 μM DAPT group. After reoxygenation, protein and mRNA levels of Jagged1 were measured by Western blot and quantitative RT-PCR. The MTT test was used to assess proliferation. ELISA was used to measure NO and VEGF secretion.

Results: Hypoxic preconditioning treatment significantly upregulated both protein and mRNA levels of Jagged1 in endothelial progenitor cells. It also enhanced proliferation ability and elevated secretion of NO and VEGF. Furthermore, after blocking the Notch pathway by using DAPT, Jagged1 expression and EP proliferation, migration, and secretion of NO and VEGF were decreased in a dose-dependent manner.

Conclusions: Our results suggest the Notch-Jagged1 pathway enhances EPCs proliferation and secretion ability during hypoxic preconditioning.

MeSH Keywords: Cell Hypoxia • Receptors, Notch • Stem Cells

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Background

Ischemic heart diseases (IHD) are the leading cause of global morbidity and mortality [1]. Myocardial infarction (MI) is a type of IHD that causes irreversible damage to the myocardium and progressive loss of function, eventually leading to heart failure. Although many therapies attempt to mitigate myocardial damage, the most potent endogenous protective measure against MI is ischemic preconditioning (IPC). Accumulating lines of evidence indicate IPC has beneficial effects for treating MI *in vivo* [2].

First described in 1984, hypoxia is a classic element of ischemia, and hypoxic preconditioning may play an important role in ischemia preconditioning [3]. Hypoxia can be upregulated by expression of SDF-1/CXCR4/CXCR7 to promote migration, adhesion, and viability of mesenchymal stem cells (MSCs) after limb ischemia [4]. Tang et al. demonstrated cardiac progenitor cells exhibit better migration to serious hypoxia after hypoxic preconditioning in myocardial infarction [5], and hypoxic preconditioning also can promote the survival of cardiac progenitor cells via attenuating mitochondrial damage to prevent cell apoptosis [6]. Therefore, hypoxic preconditioning may have an important effect on the functions of stem cells in various diseases.

Because progenitor cells reside in bone marrow and have been identified in peripheral blood, EPCs have been shown to have powerful biological effects under physiology and pathological conditions [7], such as supporting the cellular reconstruction of damaged tissues mobilizing from BM, such as ischemic limbs and infarcted myocardium [8], and regulating the angiogenic process in tumors [9]. Dysfunction of EPCs was induced by angiogenesis to promote the progression of cardiovascular syndrome [10]. Increased expression of EPCs can immediately mobilize and home to the impaired myocardium upon IPC, as seen by an increased number of EPCs in cardiac lumen, and reduced infarct size preserved capillary density following I-R injury [11].

Although it has been established that circulating molecules such as SDF-1α [12,13], VEGF [14], GM-CSF [15], and IL-8 [16] are involved in EPC mobilization, the mechanism remains unclear [17].

Due to the powerful biology of EPCs, we aimed to observe the effects changed under hypoxia preconditioning of EPCs and to discover the mechanism involved.

Material and Methods

EPCs preparation

Human umbilical cord blood for the experiment was obtained from the Department of Gynecology and Obstetrics of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. After childbirth, we collected 50 ml samples of umbilical cord blood. Before conducting the experiment, we acquired authorized protocols by the Medical Affairs Office, and signed endorsed informed consent was obtained from each donor.

Culture of EPCs

EPCs were isolated using a previously described method [18]. Density gradient centrifugation was used to separate mononuclear cells from human umbilical cord blood. Lymphocyte separation solution Ficoll-1077-1 (Tian Jin Hao Yang Biological Manufacture Co., Ltd., China) was added to human umbilical cord blood. Then, the mixed solution was centrifuged at 1500 rpm for 20 min. After washing twice, cells were collected and cultivated on a petri dish at a concentration of 6×10^7 cells and then cultured in an endothelial basalmedium-2 (EGM-2, Lonza Group Ltd, SWISS), followed by addition of EGM-2 SingleQuots, including growth factors, cytokines, supplements, and 5% FBS (Catalog No: CC-4176, Lonza Group Ltd, SWISS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, USA). We used an incubator with 5% CO_2 and 21% O_2 to incubate cells at 37°C. Cells that were not attached to the well were discarded after 3 days and the medium was replaced every 2 days. The attached cells were then analyzed by fluorescence-activated cell sorting (FACS) on day 15.

Identification of EPCs

Based on results of our previous experiment [18], cell suspensions were prepared. With the treatment of buffered 0.14 M NH4Cl, cells were stained with antibodies for 30 min at 4°C. Then cells were washed 3 times in PBS (Gibco, USA) followed by fixation by 4% paraformaldehyde. Finally, cells were analyzed by using the FACS CaliburTM (BD Immunocytometry Systems, CA). The antibodies used in FACS analyses included FITC-conjugated anti-CD34 antibody (catalog No. 130-0980142, Miltenyi Biotech, Bergish-Gladbach, Germany), PE-conjugated anti-human CD133 antibody (catalog No.130-080-801, Miltenyi Biotech, Bergish-Gladbach, Germany), and Alexa Fluor 647-conjugated anti-human VEGFR2 antibody (catalog No. 359910, BD Immunocytometry Systems, CA).

Hypoxic preconditioning of EPCs

EPCs were cultured in a hypoxia chamber incubator (catalog No. 51030388; Thermo Fisher Scientific, USA) at 37°C in 3% O_2, 5% CO_2, and 92% N_2 atmosphere for 25 min, then were reoxygenated in a normal incubator for 30 min. Cells were then cultured in a hypoxia chamber incubator for 5 h and reoxygenated for 24 h, and these EPCs are referred to as hypoxia-preconditioned EPCs (HP-EPCs). Normoxia-preconditioned (for 24 h in 95% air, 5% CO_2) EPCs (NP-EPCs) were used as a control.

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N-S-phenyl-glycine-t-butyl ester (DAPT) was the classic inhibitor of Notch

Several different concentrations (25 μmol/L, 50 μmol/L, and 100 μmol/L) of gamma-secretase inhibitor (Notch pathway inhibitor) DAPT (D5942, SIGMA-ALDRICH, USA) were added to the medium in advance to investigate the function of Notch signal in hypoxic preconditioning. After culturing for 24 h, the medium containing DAPT was removed and fresh medium was added.

Evaluation of cellular biological functions

Assessment of cellular proliferation

After trypsinization, EPCs were collected and 2×10^3 EPCs was suspended in 100 μl of culture medium and cultured on 96-well dishes for 24 h. After incubation in different environments, cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Beyotime Institute of Biotechnology, China) at 37°C in the dark for 4 h. The supernatant was then removed. After 100 μl of formazan was added, we detected the optical density value (OD) at 570 nm, with an ELISA plate reader (model 550, BioRad, USA).

Transwell migration assay for evaluation of cellular migration ability

A Transwell chamber (Corning-Costar Corp. USA) was used to assess the migration ability of EPCs in a special 24-well plate with polycarbonate filters (8-μm pores) and a double chamber. After being trypsinized, EPCs were collected and 2×10^4 EPCs were seeded in the upper chambers. Endothelial basal medium-2 with 10% bovine serum was added to the lower chamber. After incubation at 37°C for 24 h, phosphate-buffered saline was used to wash the lower chamber. Migrated cells in the lower chamber were photographed and counted under a microscope in 7 randomly selected microscopic fields.

Measurement of NO and VEGF secretion by ELISA analysis

NO and VEGF levels released from EPCs were assessed by using a commercially available NO colorimetric assay kit (Enzo Life Sciences, Inc., USA) and VEGF colorimetric assay kit (Neo Bioscience Technology, China) according to the instructions.

Western blot analysis

Western blot analysis for Jagged1 was performed. Cells were lysed in RIPA buffer and sonicated for 30 min on ice. The lysate was then centrifuged at 20000 rpm for 20 min. The protein concentrations were detected by the BCA method (Beyotime Institute of Biotechnology, China). Proteins (50 μg) were run on 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were probed with primary antibodies: Jagged1 (ab9536: Abcam, USA) 1: 1000 and β-actin 1: 20000 (sc-81178: Santa Cruz Biotechnology Inc., USA). Immune-positive proteins bands were detected by horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Thermo Fisher Scientific, USA).

Quantitative real-time PCR

Total RNA was extracted from cells. Complementary DNA was prepared by using a reverse transcription kit from Takara (Osaka, Japan). PCR amplification was performed using an SYBR PrimeScript kit (SYBR Premix EX Taq, Takara, Japan) in the ABI PRISM 7300 real-time PCR system. GAPDH was used as a control reference. The primers used in PCR were: 5'-CC TGAAGGGGTGCGGTATAT-3'and 5'-GGAGTTGACACCATCGATGC-3'for Jagged1; 5'-AATCCCATCACCATCTTCCAG-3'and 5'-GAGCCCC AGCCTTCTCCAT-3' for GAPDH.

Statistical analysis

We used SPSS 19.0 to analyze the data. Data are presented as mean ± standard deviation (SD). The statistical differences compared were calculated by *t* test for 2 groups and ANOVA for multiple groups. We considered that a P-value less than 0.05 indicated a significant difference.

Results

Cells demonstrated the characteristics of EPCs

In the experiment, we found the cells were adherent within 4 days (Figure 1A). The number of adherent cells increased over time. Classical EPCs colonies appeared after 7 days (Figure 1B). Our research used cells cultured for 20 days (Figure 1C). Microscopy pictures were taken at 400×. Flow cytometry revealed staining with CD34 (4.05%) (Figure 2A), CD133 (2.42%) (Figure 2B), and VEGFR2 (6.62%) (Figure 2C).

Hypoxic preconditioning enhances the proliferation, migration, and secretion of EPCs

The effect of hypoxic preconditioning on proliferation, migration, and secretion of EPCs was studied. As shown in Figures 3 and 4 (microscopy pictures were taken at 400×), in the hypoxic preconditioning group, the proliferation and migration of EPCs was obviously enhanced compared to the normoxia group. Figures 5 and 6 show that NO and VEGF secretion markedly increased in the hypoxic preconditioning group.

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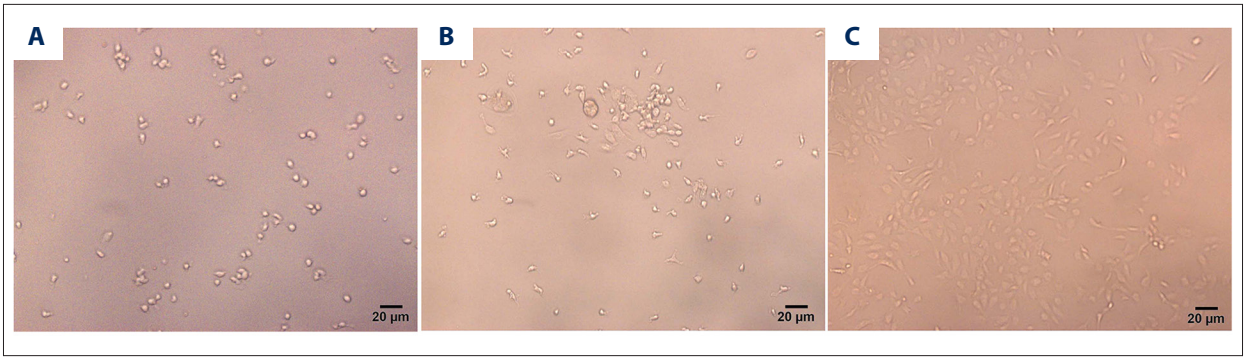

Figure 1. Morphology and characterization of EPCs from umbilical cord blood-derived mononuclear cells. (**A**) Four days after culturing, non-adherent cells were removed and adherent EPCs exhibited a spindle-shaped, endothelial cell-like morphology; (**B**) A typical colonies of EPCs were defined as central clusters of round cells surrounded by spindle-shaped cells after 7 days; (**C**) After being cultured for 20 days, we obtained classic EPCs, which we used for the next experiments. The images were collected using a Zeiss Axiovert 2 inverted microscope (Carl Zeiss, NY) with a 5 *CP-ACHROMAT/0.12 NA objective. Image was captured with a SPOT RT color camera (Diagnostic, Sterling Heights, MI), and microscopy pictures were taken at 400×.

Notch signaling improves biological function of EPCs in hypoxic preconditioning

The hypoxic preconditioning model was constructed after pretreatment with DAPT for 24 h. Then, we determined the proliferation, migration, and secretion of EPCs in each group. As shown in Figure 7, after being pretreated with 100 μmol/L DAPT, the proliferation of EPCs was decreased. Increasing concentration of DAPT reduced cell migration (Figure 8), and microscopy pictures were 400×. The secretion of NO was decreased with the increasing DAPT concentration (Figure 9), and the secretion of VEGF was obviously decreased in the group pretreated with 100 μmol/L DAPT (Figure 10).

Activation of Notch/Jagged1 signaling pathways mediates the effects of hypoxic preconditioning on EPCs

To investigate the internal mechanisms of Notch signaling effects involved in EPCs biological functions changes in IPC, we explored the protein and mRNA expressions of Jagged1 of EPCs, which is one of the ligands of Notch. Hypoxic preconditioning significantly increased the protein and mRNA expression of Jagged1 (Figure 11), and Notch signaling blocker DAPT markedly downregulated the protein and mRNA level of Jagged1 (Figure 12). These data further suggest that the Notch/Jagged1 plays an important role in the IPC-induced protective effects.

Discussion

Our study shows that hypoxia preconditioning increased the proliferation, migration, and secretion of endothelial progenitor cells *in vitro* by inducing the expression of Jagged1, which was regulated by the Notch signaling pathway.

A recent study showed that the hypoxic culture environment also could enhance the generation of the parturition embryonic stem cells and the "induced pluripotent stem cells (iPSCs)", which are a new type of pluripotent stem cell induced by human adult somatic cells [19]. Thus, the hypoxia environment may be an important influence on the proliferation and differentiation of stem cells. We found hypoxia preconditioning effectively induced the proliferation, migration, and secretion of endothelial progenitor cells, which is consistent with these previous studies.

One of the significant signaling pathways implicated in stem cell differentiation is Notch signaling. The Notch signaling pathway is an evolutionarily conserved intercellular transduction pathway that regulates diverse cellular processes in stem cells, ranging from differentiation and proliferation to apoptosis [20,21]. In vertebrates, 4 different Notch receptors (Notch 1 to 4) and 5 ligands, and Jagged 1 and 2, complete with Delta-like 1, 3, and 4 have been identified.

Kwon et al. [22] found that Notch ligand Jagged1 appears to determine the differentiation of BM cells into EPCs. Deficient Jagged1 resulted in reduced expression of endothelial genes in BM cells and lower proliferative, migratory, and survival ability of BM-EPCs.

A Gamma-secretase inhibitor (Notch pathway inhibitor), DAPT, was widely used for investigating the Notch signaling pathway [23,24]. Our findings suggest that, under hypoxia preconditioning, the Notch-Jagged1 pathway may play an important role in promoting the proliferation and secretion of EPCs.

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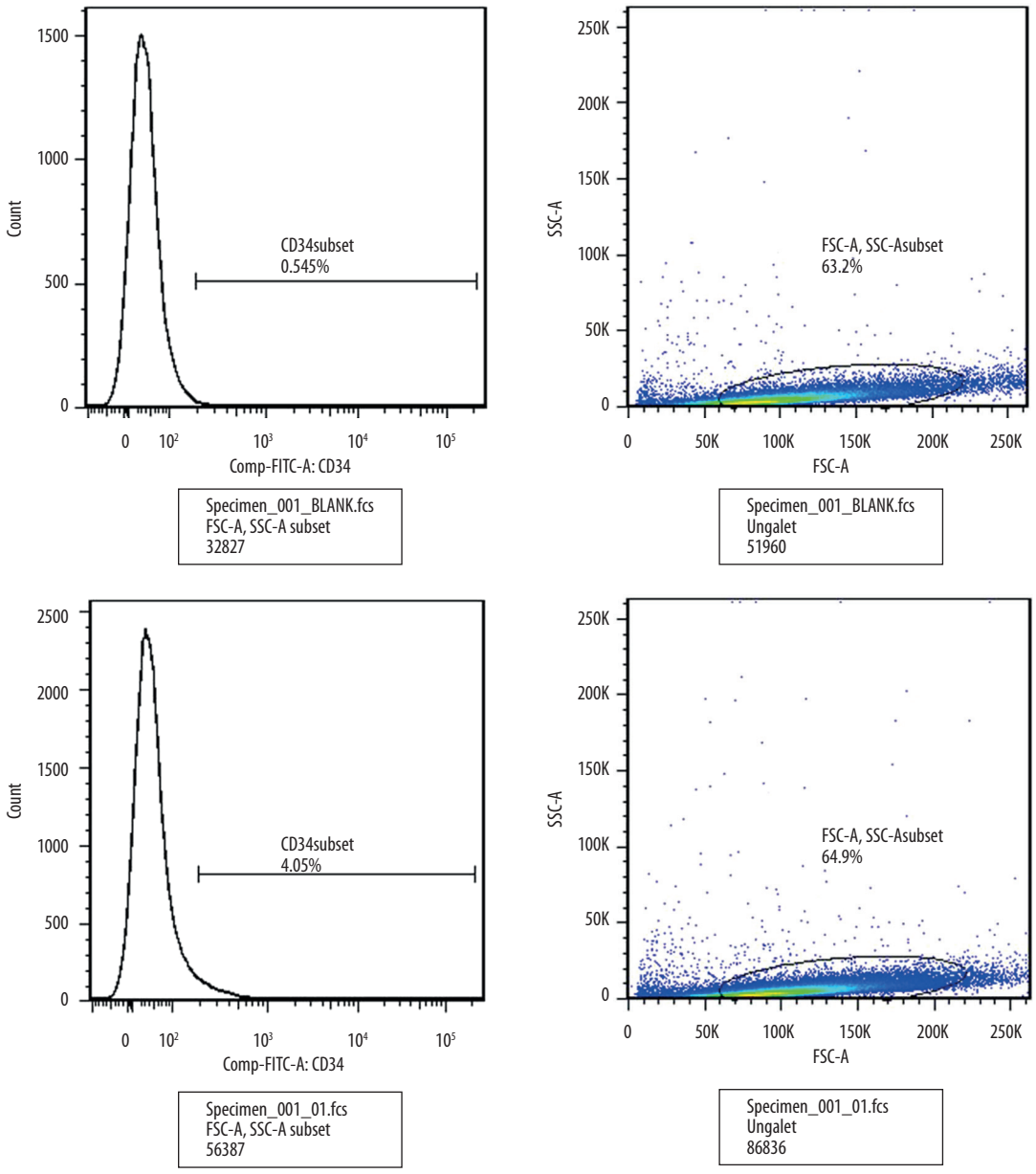
A



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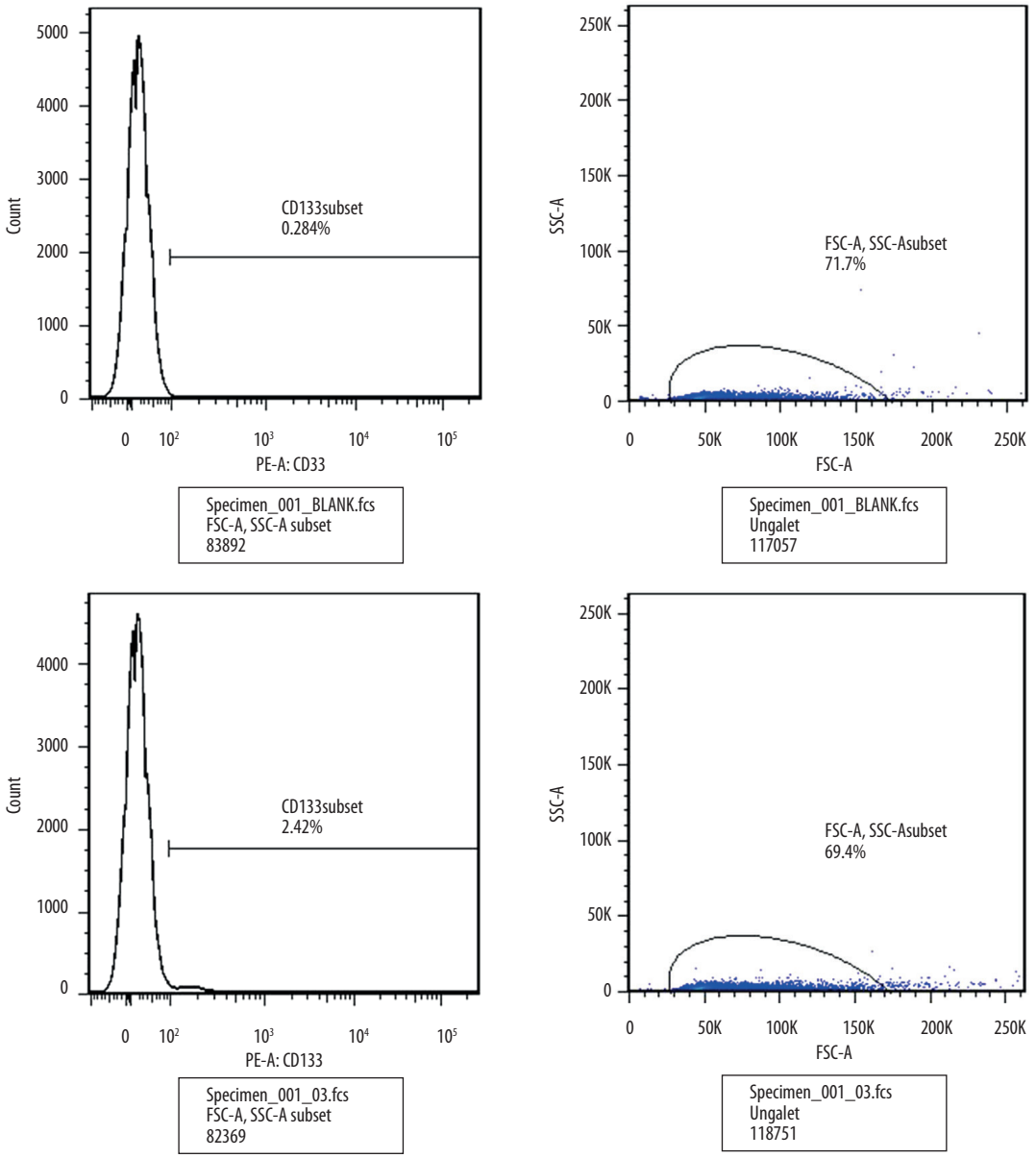

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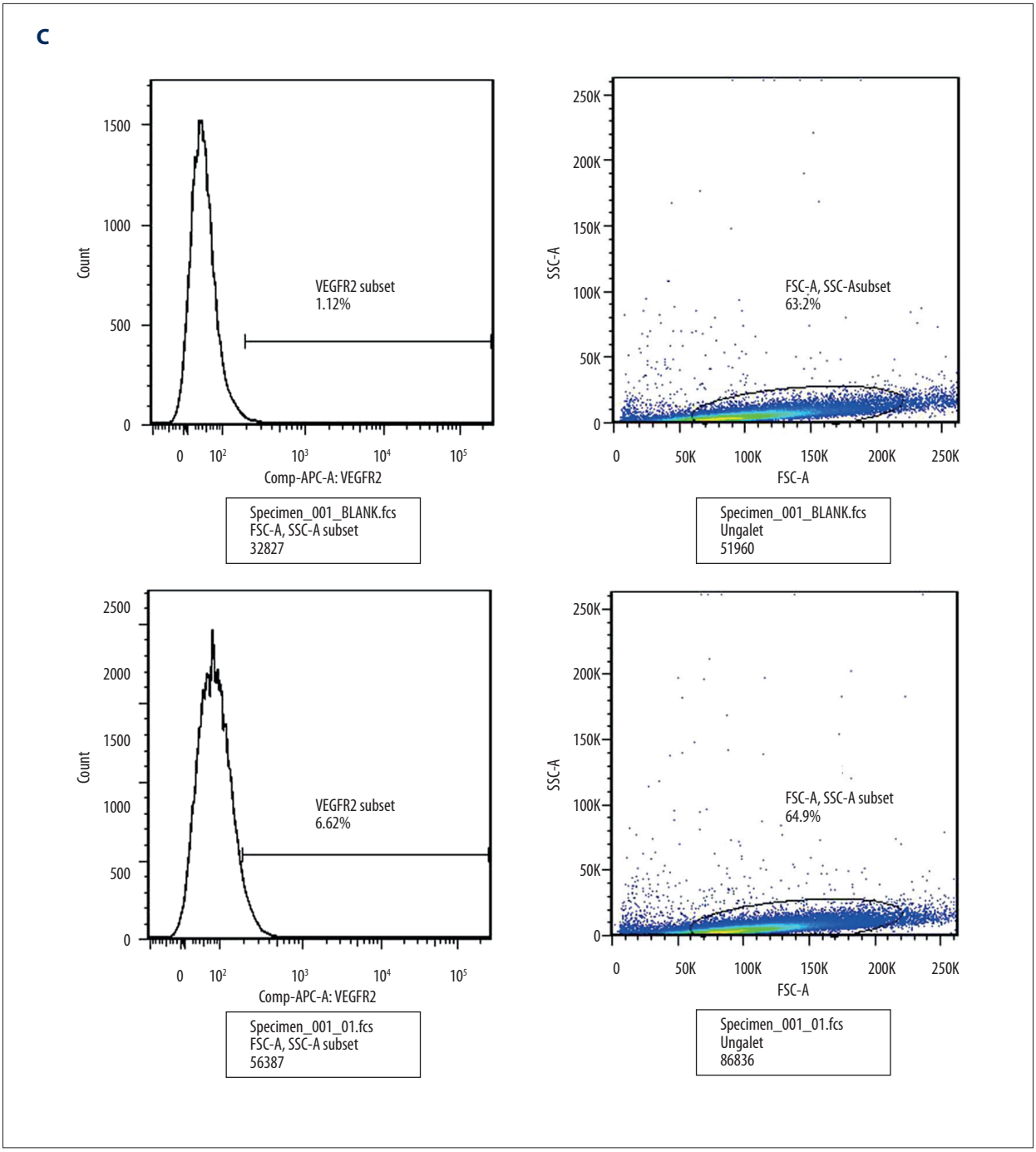

Figure 2. EPCs were further confirmed by demonstrating the expression of well-established cell surface markers CD34 (**A**), CD133 (**B**), and VEGFR2 (**C**) by FACS.

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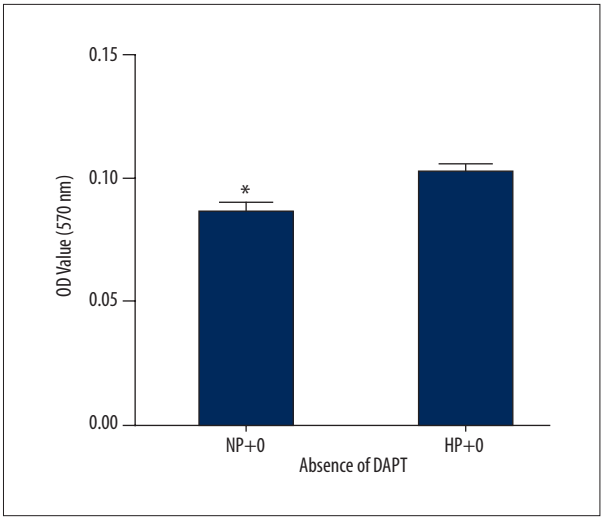

Figure 3. The effect of hypoxic preconditioning on proliferation of EPCs. In the hypoxic preconditioning group, the proliferation of EPCs was obviously enhanced. Data are presented as mean ±SD, n=3, * P<0.01, NP+0 *vs.* HP+0 (without DAPT).

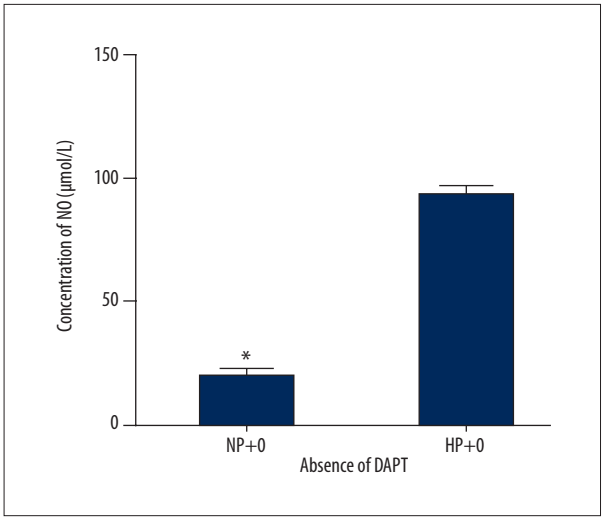

Figure 5. The effect of hypoxic preconditioning (HP) on NO secretion of EPCs. NO secretion of EPCs markedly increased in the hypoxic preconditioning (HP) group. Data are presented as mean ±S.D., n=3, * P<0.01, NP+0 *vs.* HP+0 (without DAPT).

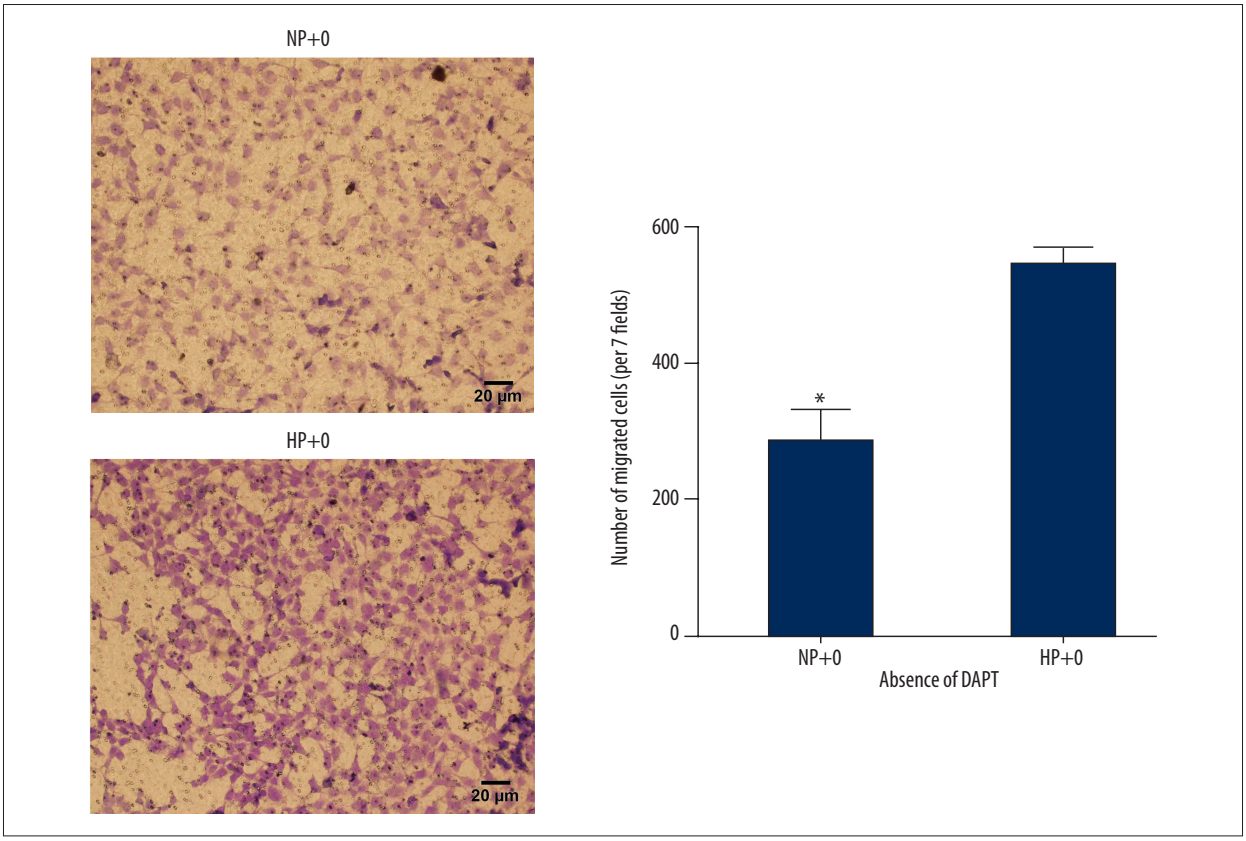

Figure 4. The effect of hypoxic preconditioning on migration of EPCs. The migration of EPCs in the hypoxic preconditioning (HP) group was obviously enhanced. Microscopy pictures were taken at 400×. Data are presented as mean ±SD, n=3, * P<0.01, NP+0 *vs.* HP+0 (without DAPT).

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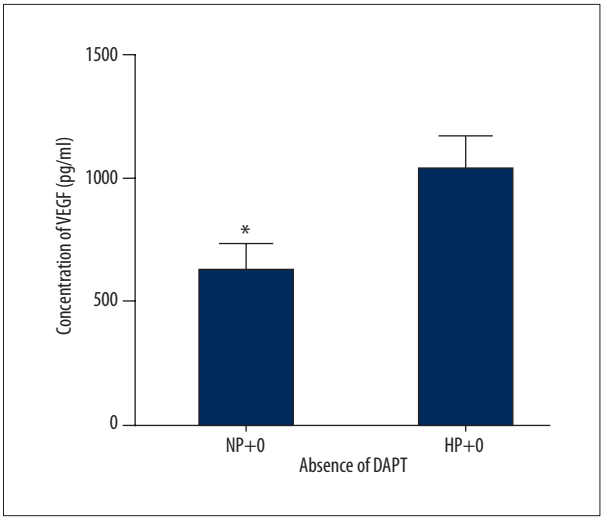

Figure 6. The effect of hypoxic preconditioning (HP) on VEGF secretion of EPCs. VEGF secretion of EPCs markedly increased in hypoxic preconditioning (HP) group. Data are presented as mean ±S.D., n=3, * P<0.01, NP+0 *vs.* HP+0 (without DAPT).

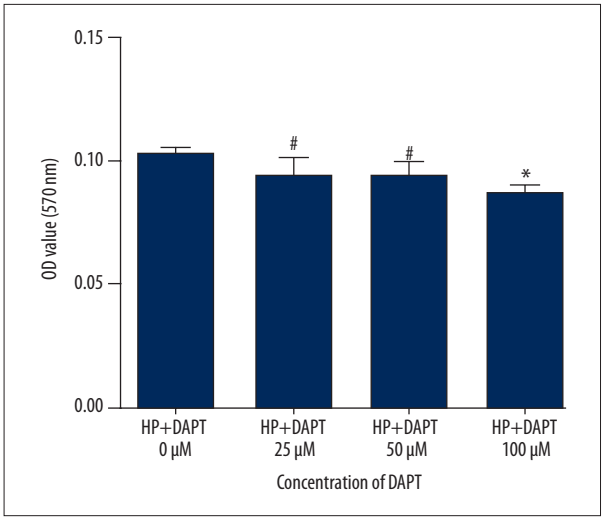

Figure 7. The hypoxic preconditioning (HP) model was constructed after pretreated with DAPT for 24 h. Then, we determined the proliferation of EPCs in each group. After pretreatment with 100 μmol/L DAPT, the proliferation of EPCs was decreased. Data are presented as mean ±SD, n=3. * P<0.05, HP+DAPT 0 μM *vs.* HP+DAPT 100 μM. # P>0.05, HP+DAPT 25 μM *vs.* HP+DAPT 0 μM, HP+DAPT 50 μM, HP+DAPT 100 μM; HP+DAPT 50 μM *vs.* HP+DAPT 0 μM, HP+DAPT 100 μM.

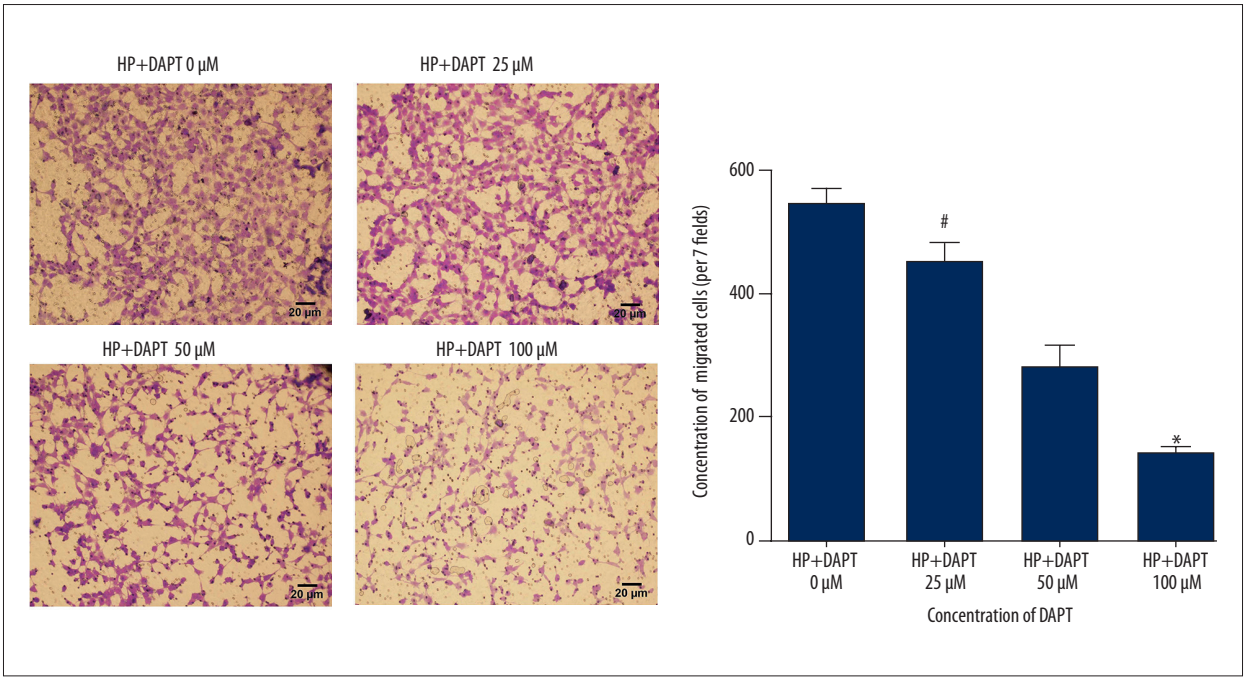

Figure 8. The hypoxic preconditioning (HP) model was constructed after pretreatment with DAPT for 24 h. Then, we determined the migration of EPCs in each group. We found that with increasing concentration of DAPT, the number of migration cells was reduced (Figure 8). Microscopy pictures were taken at 400×. Data are presented as mean ±SD, n=3. * P<0.01, HP+DAPT 0 μM *vs.* HP+DAPT 50 μM, HP+DAPT 100 μM; HP+DAPT 25 μM *vs.* HP+DAPT 50 μM, HP+DAPT 100 μM; HP+DAPT 50 μM *vs.* HP+DAPT 100 μM. # P<0.05, HP+DAPT 0 μM *vs.* HP+DAPT 25 μM.

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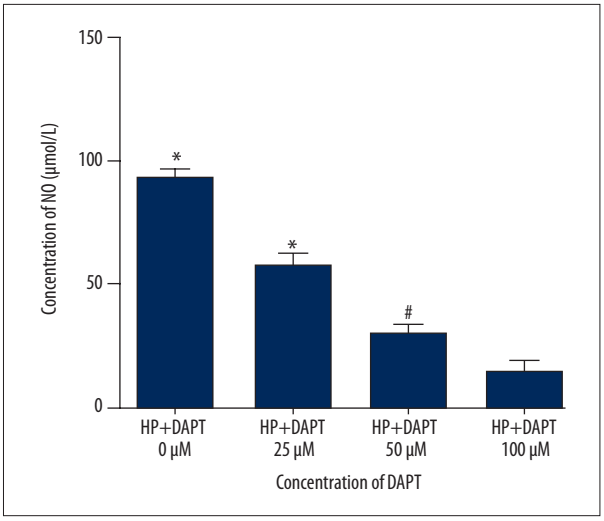

Figure 9. Hypoxic preconditioning (HP) model was constructed after pretreatment with DAPT for 24 h. Then, we determined the NO secretion of EPCs in each group. The secretion of NO decreased with increasing DAPT concentration. Data are presented as mean ±SD, n=3. * P<0.01, HP+DAPT 0 μM *vs.* HP+DAPT 25 μM, HP+DAPT 50 μM, HP+DAPT 100 μM.; HP+DAPT 25 μM *vs.* HP+DAPT 50 μM, HP+DAPT 100 μM. # P<0.05, HP+DAPT 50 μM *vs.* HP+DAPT 100 μM.

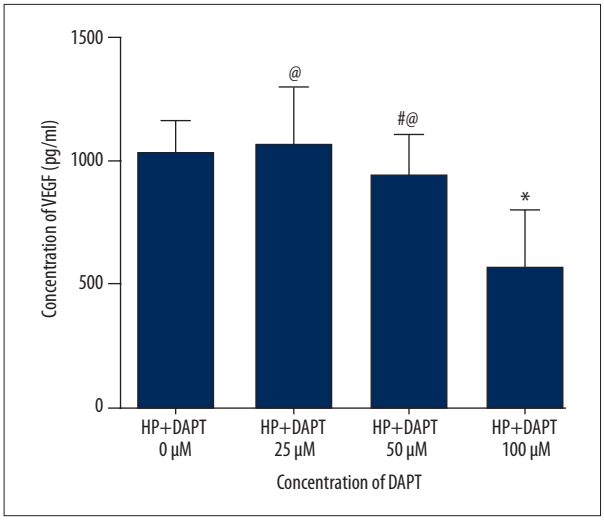

Figure 10. The hypoxic preconditioning (H) model was constructed after pretreatment with DAPT for 24h. Then, we determined the VEGF secretion of EPCs in each group. The secretion of VEGF was obviously decreased in the group pretreated with 100 μmol/L DAPT. Data are presented as mean ±SD, n=3. * P<0.01, HP+DAPT 0 μM *vs.* HP+DAPT 100 μM; HP+DAPT 25 μM *vs.* HP+DAPT 100 μM. # P<0.05, HP+DAPT 50 μM *vs.* HP+DAPT 100 μM. @ P>0.05, HP+DAPT 0 μM *vs.* HP+DAPT 25 μM, HP+DAPT 50 μM; HP+DAPT 25 μM *vs.* HP+DAPT 50 μM.

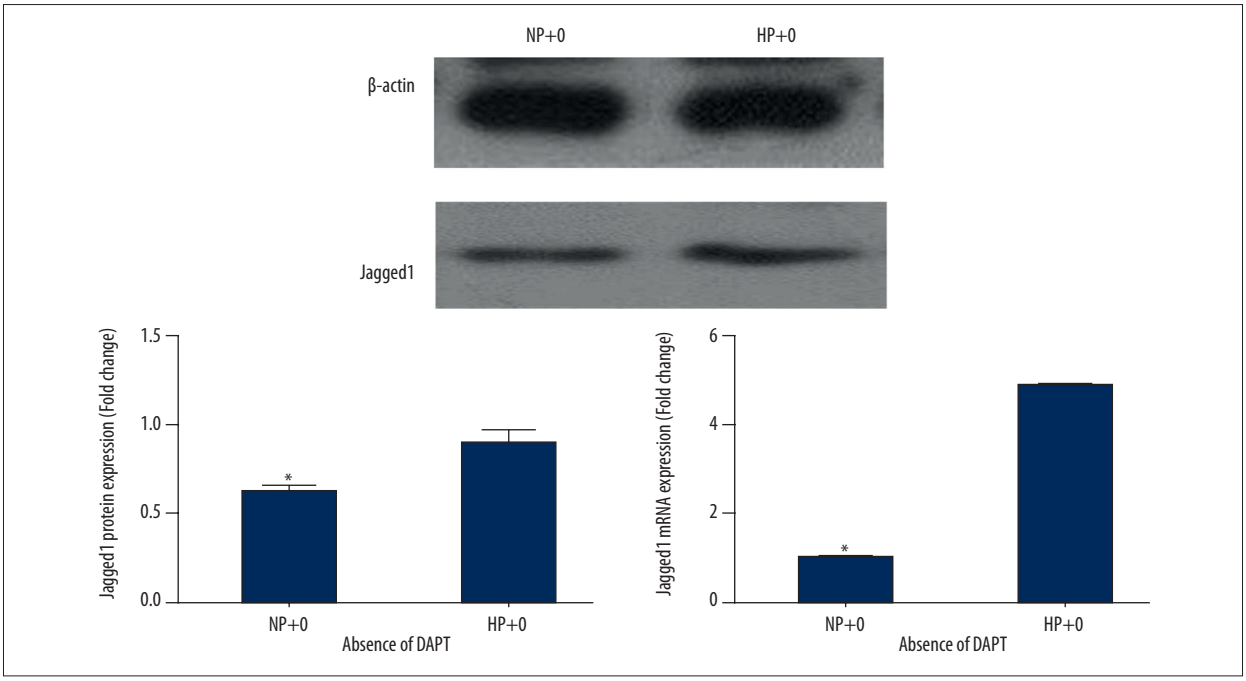

Figure 11. To investigate the preliminary molecular mechanisms accounting for the Notch signaling effects on biological functions of EPCs in IPC, we investigated the protein and gene expressions of Jagged1 of EPCs under hypoxia preconditioning (HP). Jagged1 is one of the ligands of Notch. As shown in Figure 11, hypoxic preconditioning (HP) significantly increased the protein and mRNA expression of Jagged1, compared with normoxia preconditioning (NP). Data are presented as mean ±SD, n=3. * P<0.05, NP+0 *vs.* HP+0 (without DAPT).

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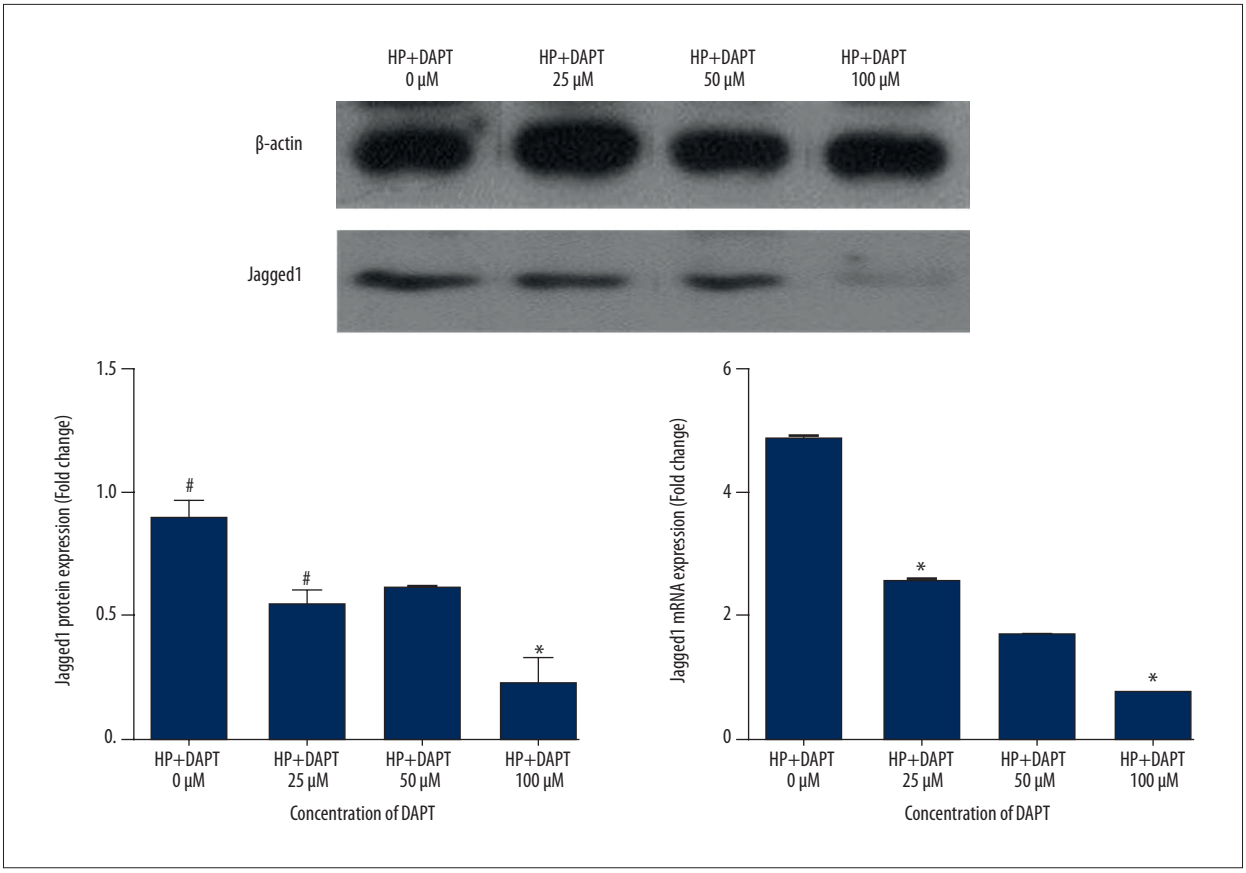

Figure 12. To investigate the preliminary molecular mechanisms accounting for the Notch signaling effects on biological functions of EPCs in IPC, we investigated the protein and gene expressions of Jagged1 of EPCs under hypoxia preconditioning (HP) with different concentration of DAPT. Jagged1 is one of the ligands of Notch. The Notch signaling blocker DAPT markedly downregulated the protein and mRNA levels of Jagged1. Data are presented as mean ±SD, n=3. * P<0.05, HP+DAPT 0 μM *vs.* HP+DAPT 25 μM, HP+DAPT 50 μM, HP+DAPT 100 μM, HP+DAPT 25 μM *vs.* HP+DAPT 100 μM.

Conclusions

In this study, we showed that hypoxia preconditioning could change the biological function of EPCs, possibly via increasing Jagged1 expression. The Notch signal pathway blocker DAPT markedly decreased the expression of Jagged1 and downregulated the proliferation, migration, and secretion functions of EPCs.

Due to the low quantity of peripheral EPCs, newer treatments that enhance the biological function of EPCs are greatly needed. Notch could be a new target of genetic modification in EPCs. We propose that injecting EPCs processed with hypoxic preconditioning may be an effective therapeutic treatment for ischemic myocardial disease. Increasing the expression of Notch could strengthen the biological function of EPCs. However, excessive activation of Notch signaling could induce vessel hyperplasia and malignant transformation. More research is needed to explore the role of genetically modifying Notch on EPCs.

Acknowledgement

We want to sincerely thank Prof. Zhi-Ping Liu, who is the assistant professor of Department of Internal Medicine-Division of Cardiology University of Texas Southwestern Medical Center, she provided valuable advice in creating this experiment. Thanks also go to Prof. Li Zhou, staff of Wuhan Union hospital for helping collecting the umbilical cord blood to make the experiment come true.

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