Original Research

Essential role of HDL on endothelial progenitor cell proliferation with PI3K/Akt/cyclin D1 as the signal pathway

Qiuhua Zhang, Hongchao Yin, Peimao Liu, Hua Zhang and Mingpeng She

Department of Pathology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College, #5, Dong Dan San Tiao, Beijing 100005, China
Corresponding author: Mingpeng She. Email: mingpengs0251@yahoo.cn

Abstract

High-density lipoprotein (HDL) is known as an important factor in vascular wall remodeling that also affects gene expression in cell proliferation and differentiation. In this article, the role of HDL on endothelial progenitor cell (EPC) proliferation, angiogenesis and the signal pathway involved was studied, particularly the influence of HDL in strengthening the promoting effect of EPCs on wound healing of the arterial wall in hypercholesterolemic rats. Mononuclear cells isolated from rat bone marrow displayed characteristics of EPCs after cultivation. The role of HDL on EPC function and the signal pathway involved were studied by Western blotting, *in vitro* migration and 'tube' formation. Re-endothelialization and the number of circulating EPCs were compared between normal rats, hypercholesterolemic rats and hypercholesterolemic rats with HDL treatment. Results showed that HDL participated in the healing process by promoting EPC proliferation, migration and 'tube' formation. HDL activates cyclin D1 via phosphatidylinositol 3-kinase (PI3K)/Akt stimulation. Inhibition of PI3K/Akt via pharmacological or small interfering RNA approaches significantly attenuated HDL-induced EPC migration, proliferation and 'tube' formation. Results of experiments *in vivo* showed that HDL increased the number of circulating EPCs and promoted re-endothelialization in wound healing. These findings demonstrate for the first time that PI3K/Akt-dependent cyclin D1 activation plays an essential role in HDL-induced EPC proliferation, migration and angiogenesis.

Keywords: high-density lipoprotein, endothelial progenitor cells, proliferation

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Introduction

The integrity of endothelium and its restoration after injury is regarded as a key factor in preventing atherosclerosis progression and the maintenance of endothelium integrity is of primary importance. The restoration of endothelium is mostly considered to have been contributed to the neighboring endothelial cells or by the migration of smooth muscle cells from the media.[1] However, this notion was challenged by the discovery of endothelial progenitor cells (EPCs).[2] These cells, originating from the bone marrow, peripheral blood or vascular adventitia,[3] are able to be mobilized to the injury site, promoting endothelial proliferation and differentiation; their actual contribution to angiogenesis and the outcome are still controversial.[4,5] EPCs belong to a cell type of undifferentiated state.[6] Once transplanted into the injury site at the intima, the EPCs, aside from differentiating into endothelial or smooth muscle cells, may mostly stimulate the recipient's bone marrow, inducing a further proliferation of the progenitor cells. This process is also closely related to the local microenvironment, i.e. the 'niche' around them.

Emerging evidence has showed that the number and function of EPCs are correlated with the main risk factors of atherogenesis, e.g. hypertension, smoking, diabetes mellitus and physical inactivity.[7–9] Previous data indicated that incubation with drugs of the statin family *ex vivo* promoted EPC growth and inhibited cellular senescence.[10] Members of the statin family were noticed to be able to mobilize EPCs and accelerate re-endothelialization after a balloon-catheter denudation *in vivo*.[11,12] However, the underlying mechanism remains largely unknown. Since HDL is known to be active in anti-inflammation, antioxidation and protection of endothelial cells from damage,[13] it is important to detect whether hypercholesterolemia and HDL affect the bioactivity of EPCs, respectively, in the process of endothelium regeneration.

It is well known that the mesenchymal stem cells of bone marrow (BMSCs) bear the properties of multipotentiality,

immunomodulation and easy accessibility, and in addition, there are experimental evidences that BMSC transplantation will be beneficial to the repair of cell injury and cell loss.[14] In order to identify the promoting effect of HDL on endothelial progenitor cell proliferation *in vivo*, a hypercholesterolemic model with injury to the intima of the femoral artery was established in rats to identify the effect of HDL on wound healing. The *in vitro* experimental data also indicated that HDL promoted EPC proliferation most likely through the phosphatidylinositol 3-kinase (PI3K)/Akt/cyclin D1 signaling pathway.

Methods

Isolation and culture of rat EPCs

Collection and analysis of rat bone marrow EPCs (rEPCs) were carried out according to the Blood Donation Law of NIH. The mononuclear cells (MNCs) were isolated using density gradient centrifugation with Histopaque 1083 (Sigma, St Louis, MO, USA) at 400 g for 25 min.[15] After washing twice with phosphate-buffered saline (PBS), the cells (5×10^6/mL) were plated onto the bottom of the attached factor-coated flasks and cultured in M131 (Cascade Biologics) supplemented with microvascular growth supplements (Cascade Biologics, Portland, OR, USA) and 40 ng/mL vascular endothelial growth factor (VEGF) (Sigma). Non-adherent cells were removed on the fourth day and the cultivation was continued. The cultured cells were collected on the seventh day and used as the rEPC-enriched population.

To characterize the EPC phenotype, cells of the seventh day were incubated with antibodies, including anti-CD133 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat. No. SC-130127), anti-Flk-1 (Santa Cruz; Cat. No. SC-6251) and anti-CD45 (Santa Cruz; Cat. No. SC-504) as the primary antibodies, followed by incubating with the tetramethyl rhodamine iso-thiocyanate (TRITC)-conjugated, fluorescein isothiocyanate (FITC)- and Alexa Fluor-350-conjugated secondary antibodies. Images were captured using a fluorescence inverted microscope. The class of EPCs was defined as positive for CD133 and KDR, and negative for CD45. Using this conditioning culture medium, we consistently obtained more than 90% of EPCs. To confirm this definition, we also stained the cells with anti-CD34 antibody, followed by incubation with TRITC-conjugated secondary antibodies. Cells used for the subsequent experiments were obtained on the seventh day.

Preparation of HDL

HDL was prepared as described.[16] Blood samples were collected from healthy volunteers. HDL was prepared by sequential ultracentrifugation. The purity of HDL was assessed by gel electrophoresis and the protein content was determined using the Bradford method. HDL was finally collected by dialysis against a 10×1 L endotoxin-free 0.9% NaCl solution and sterilized by passing through a 0.22 μm filter before use.

Effect of HDL on rEPCs viability using MTT assay

A cell survival test for rEPCs was performed on the seventh culture day. The cells were harvested, washed three times with PBS and suspended in the medium at a concentration of 10^6 cells/mL. The medium (100 μL) was randomly distributed into each well of the 96-well microliter plates. After 24 h preculture in M131 without growth factors, cells were then incubated in different concentrations of HDL as follows: (1) 0; (2) 12.5; (3) 25; (4) 37.5; (5) 50; and (6) 62.5 μg/mL, and the total volume of each was adjusted to 100 μL, respectively. After incubation at 37°C and 5% CO_2 for 24 h, 10 μL of 5 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and the cells were incubated for a further four hours. The supernatant was discarded and the cells were co-cultured with 150 μL of dimethyl sulfoxide for another four hours. The cell density was measured using an enzyme-linked immunosorbent assay reader (spectophotometry) at 570 nm ($n = 5$ in each group).

A similar procedure was followed for cells incubated with 37.5 μg/mL of HDL (based on the above result of HDL effect on rEPC proliferation) at different intervals, namely: (1) 0; (2) 2; (3) 4; (4) 8; (5) 16; and (6) 24 h, respectively. A MTT assay and densitometer were adopted for cell proliferation detection. Each sample was repeated five times.

[^{3}H]Thymidine incorporation by rEPCs

rEPCs were quiescent for 24 h, then treated with or without HDL (37.5 μg/mL for 24 h and pulse labeled with 1 μCi/mL [^{3}H]thymidine for another 16 h. Cells were then collected and suspended in a cold 20% (w/v) trichloroacetic acid, and the cell lysate mixture was filtered through a Whatman GF/C glass fiber filter. Filters collected were counted using a Beckman LS 5000TA liquid scintillation counter.

Small interfering RNA transfection

The small interfering RNA (siRNA) duplexes of Akt and scramble control were purchased from Cell Signaling Technology (SignalSilence® Control siRNA [Unconjugated]; Cat. No. 6568; Signal-Silence™ Akt siRNA, targets Akt1 and Akt2; Cat. No. 6211). rEPCs were cultured in six-well plates (2×10^5 cells/well) and transfected with 100 nmol/L siRNA using Lipofectamine 2000. After transfection, the cells were quiesced for 24 h and treated with different agents required.

Western blotting analysis

After appropriate treatments, rEPCs were rinsed with cold PBS and lysed in a lysis buffer on ice for 30 min. Cell lysates were removed to 1.5 mL Eppendorf tubes and centrifuged at 4°C, 12,000 rpm for 15 min. The protein component was analyzed by gel electrophoresis and then transferred to a nitrocellulose membrane. After a fat-free milk blocking for two hours at room temperature, the membrane was treated with anti-cyclin D1(Cell Signaling; Cat. No. 2926) or anti-phospho-Akt (Cell Signaling; Cat. No.4051), followed

by incubation with horseradish peroxidase-conjugated secondary antibodies. Antigen–antibody complexes were detected using a chemiluminescent reagent kit (GE Health Sciences, Piscataway, NJ, USA).

Cell migration assay

Migration of cultured rEPCs was studied as described.[17] To study the migration rate of the cultured rEPCs, a piece of membrane 10 mm in diameter and 8.0 μm in pore size (Nalge Nunc International, Rochester, NY, USA) was placed in a 24-well tissue culture plate (Costar; Corning, Corning, NY, USA). The undersurface of the porous membrane was coated with Matrigel at 4°C overnight before hand. rEPCs were cultured on the seventh day, kept quiescent for 24 h in medium 131, and then trypsinized and seeded into the upper chamber with a concentration of 10^5 cells/well. Vehicle or HDL (37.5 μg/mL) was added to the lower chamber at an indicated concentration. In the case of testing the effect of Akt siRNA on HDL-induced EPC migration, cells were first transfected with Akt siRNA (100 nmol/L) or scramble siRNA (100 nmol/L) and then treated with HDL. To test the effect of LY294002, a pharmacological inhibitor of the PI3K/Akt pathway, on HDL-induced rEPC migration, LY294002 (25 μmol/L), was added to both the upper and lower chambers 30 min before administration of HDL. After eight hours of incubation at 37°C, the non-migrated cells were removed from the upper surface of the membrane, and the cells adherent to the lower surface were fixed in methanol for 15 min. The membrane was then stained with 4′,6-diamidino-2-phenylindole in a Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and studied under a Nikon diaphot fluorescence microscope attached with a photometrics CH250 CCD camera (Nikon, Garden City, NY, USA). Cells were counted in five randomly selected squares per well and presented as the number of the migrated cells per field.

In vitro angiogenesis assay

Angiogenesis was assessed on the basis of a tube formation assay as described previously.[17] Twenty-four-well culture plates (Costar; Corning) were coated with growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) in a total volume of 260 μL and solidified for 30 min at 37°C. rEPCs on the seventh day were trypsinized and resuspended to a concentration of 5×10^4/mL. Two hundred microliters of the cell suspension were added into each well and HDL (37.5 μg/mL) or LY294002 (25 μmol/L) were added to the appropriate wells, respectively, and then the cells were incubated at 37°C for six hours. In the case of testing the effect of Akt siRNA on HDL-induced EPC tube formation, cells were first transfected with Akt siRNA (100 nmol/L) or scramble siRNA (100 nmol/L) and then treated with HDL. The appearance of 'tube' was observed under an inverted microscope (Model TS100, Nikon). Images were captured with a CCD color camera (Model KP-D20 AU; Hitachi, Tokyo, Japan) attached to the microscope and the 'tube' length was measured using the NIH Image J 1.31v Program.

Preparation of hypercholesterolemic rat model

Wistar rats ($n = 18$), aged 15–16 weeks, weighing 285–315 g, were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, Beijing, China. All animal procedures were performed according to the Administration Regulation for Laboratory Animal (Beijing, China). Based on the average weight and serum total cholesterol level, the rats were divided into three groups ($n = 6$ for each group). Rats of group I had the standard ration. Rats of both groups II and III received a cholesterol-enriched ration first (0.5 g/d/kg cholesterol, 0.15 g/d/kg cholic acid and 0.5 g/d/kg propylthiouracil), followed by a standard ration daily.[15] All the 18 rats were reared for four weeks and then received an injury of the femoral artery. Among these animals, nine were used for detecting re-endothelialization of the injured femoral arteries, and another nine animals were used for circulating cell analysis. HDLs, 18 mg/kg body weight, were given intravenously only to the rats of group III ($n = 3$), 16 h before, and 0 and 16 h after the catheter injury; therefore, altogether three doses. Instead of HDL, PBS was given to the rats of groups I (normal rats plus catheter injury, $n = 3$) and II (hypercholesterolemic rats plus catheter injury without HDL, $n = 3$). The femoral arteries were removed and fixed for pathological examination on the seventh day after the injury.

Establishment of femoral artery injury model

Wistar rats were anesthetized with 10% chloral hydrate. Two incisions, one along the mid-line of the neck and another one along the medial side of the left thigh, were made for exposing the arteries. A 2F Fogarty Arterial Embolectomy Catheter (Edwards Lifesciences, Memphis, TN, USA) was inserted into the left carotid artery downwards through the descending aorta to the left femoral artery, about 30 cm distant from the insertion site. After inflating 150 μL of air in the air sac, the catheter was then moved 'back and forth' gently along its long axis for three rounds, within a distance of 2 cm. The proximal end of the injured segment was clamped immediately after the surgery. The right femoral artery was used as the internal controls.

Fluorescence-activated cell sorting analysis of peripheral blood samples

Two milliliters of blood sample were collected from the canthus of each rat, respectively, 24 h after the operation on the neck and lower extremity. The blood sample was centrifugated with Ficoll for MNC isolation. The cells harvested were stained with the following procedures: mouse monoclonal antibody (McAb) anti-CD34 (Santa Cruz) or rabbit polyclonal Ab anti-Flk-1 (Dako, Glostrup, Denmark) for identifying EPCs; mouse McAb anti-vascular cell adhesion molecule-1 antibody (Novocastra Laboratories Ltd, Newcastle, UK) or mouse McAb anti-intercellular adhesion molecule-1 (Zeta Corporation, Sierrs Madre, CA, USA) to detect the adhesion ability. FITC-conjugated anti-mouse or rabbit immunoglobulin G antibody (Zeta Corporation) was used as the secondary antibody. The cells were analyzed under a fluorescence-activated cell sorter (BD FACSAria, San Jose, CA, USA) and BD FACSDiva software.

The total peripheral blood collected finally from the right carotid arteries of all of the nine animals on the seventh day after the operation was used for rEPC isolation and the isolated cells were cultured till the seventh day. The rEPCs were stained with rabbit polyclonal anti-endothelial nitric oxide synthase antibody (Santa Cruz) and FITC-conjugated antibody (Zeta Corporation), and analyzed under a fluorescence-activated cell sorter (BD FACSAria) and BD FACSDiva software.

Pathological and morphometric analysis of lesions after vascular injury

All the rats were killed under chloral hydrate. The femoral arteries were rinsed with PBS on site, followed by infusion with 4% formalin for 20 min, taken out and fixed with 10% buffered formalin, and embedded in paraffin through the routine procedure. Tissue blocks were taken from the left femoral arteries (about 2 cm in length) and serial paraffin sections were prepared (5 μm, hematoxylin–eosin stain, 5 serial sections in succession were prepared for each block and 3 blocks were taken for each artery). Tissue blocks were also taken from the right femoral artery as the self-control.

Statistical analysis

Results were given as mean $\pm$ standard error of mean. An unpaired *t*-test was used for comparisons between control and experimental groups. One-way analysis of variance (ANOVA) test and a *post hoc* test were used for comparisons in the three groups. $P < 0.01$ was considered to indicate statistical significance.

Results

Characterization of EPCs

On the third and fourth day of cultivation, the MNCs began to adhere to the wall of the fibronectin-coated

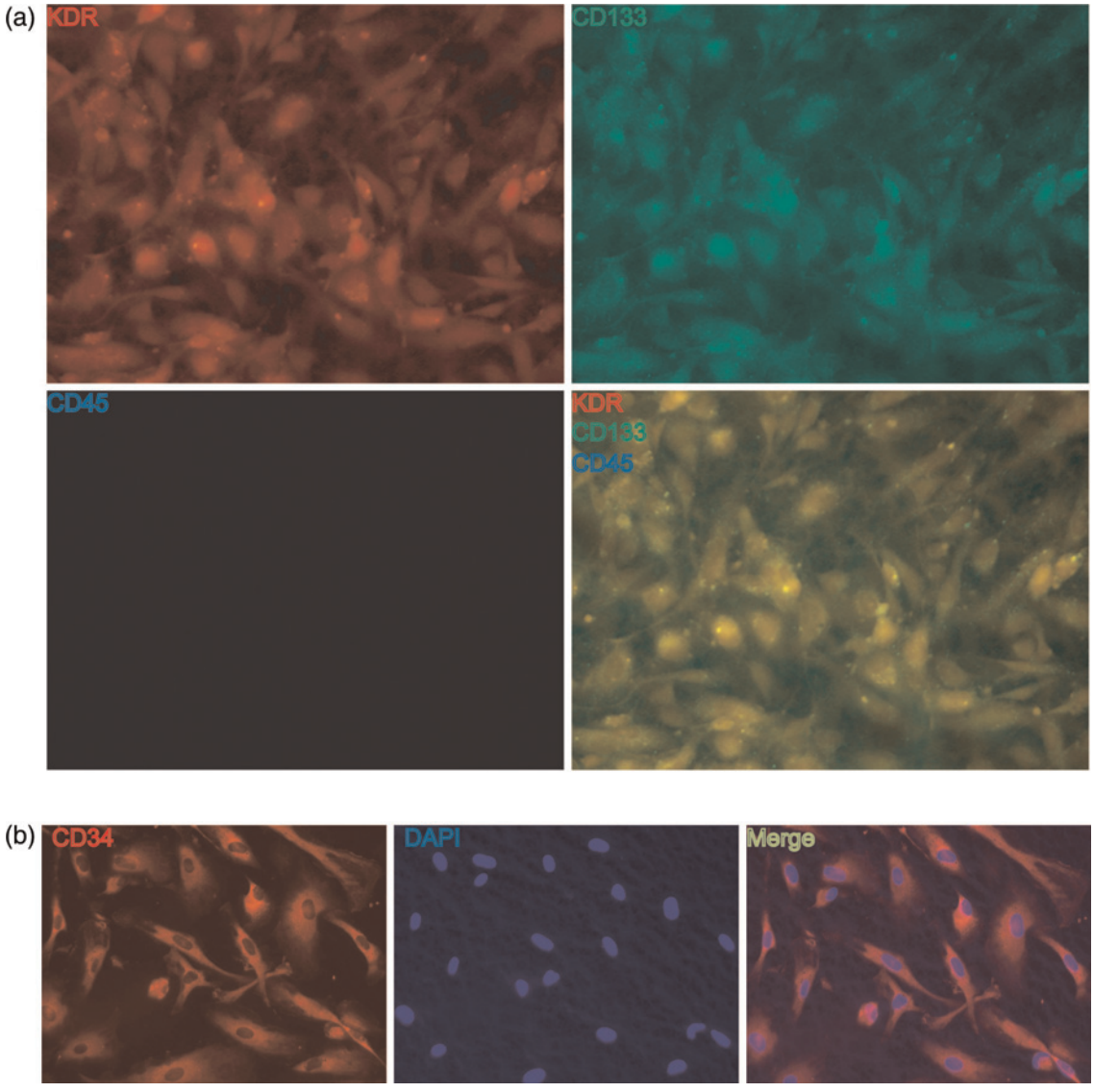

Figure 1 Characterization of EPCs. (a) Cells of the seventh culture day were defined by expressing the markers CD133 and KDR and being negative for CD45. (b) EPCs were reconfirmed by expressing CD34 (magnification $\times$400). EPC, endothelial progenitor cell

plates, became spindle in shape and developed to cell clusters later, with round cells in the center and cobblestone-shaped cells on the periphery. After incubation for seven days in EGM, the cells became elongated and were characterized by the expression of CD133 and KDR and being negative for CD45 (Figure 1a), as reported by Ashara and Zhang, respectively.[2,15] These cells exhibited a monolayer in pattern, expressed the endothelial cell marker CD34 (Figure 1b) and proliferated through multiple passages.

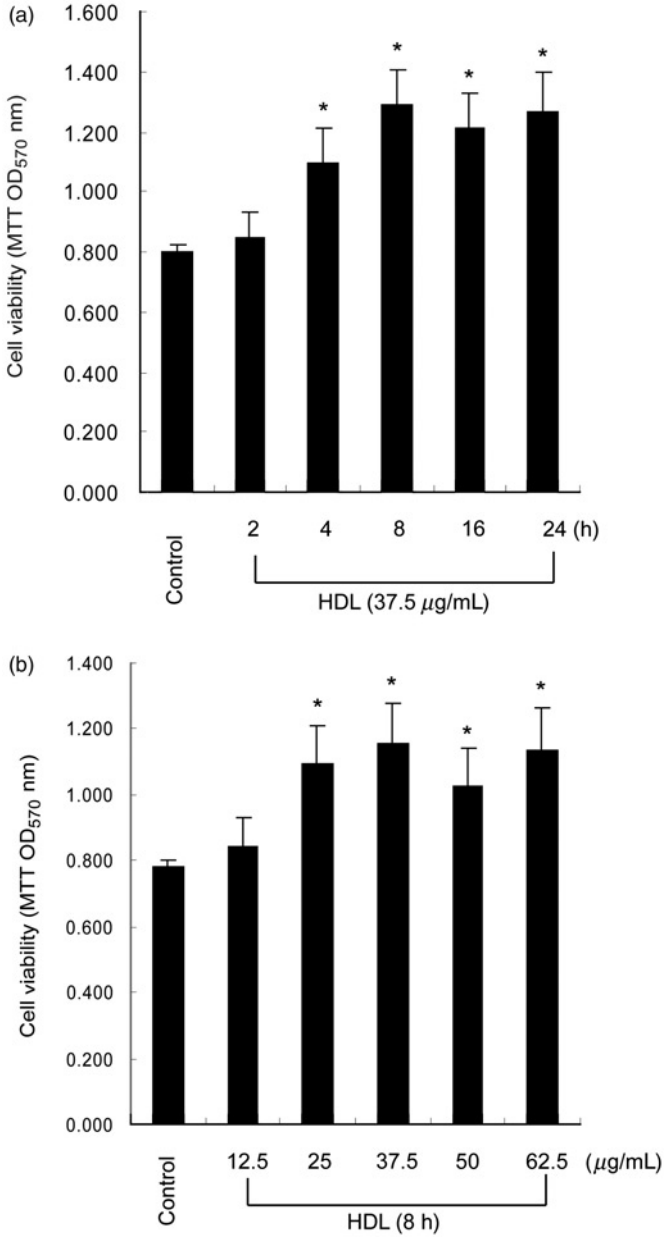

Figure 2 HDL-induced proliferation of rEPCs, which is time- and dose-dependent. (a) Time-dependent: rEPC proliferation by using MTT assay at the prescribed intervals under a concentration of 37.5 μg/mL HDL. Mean $\pm$ SEM ($n = 5$ experiments per time point). (b) Concentration-dependent: rEPC proliferation by exposure to HDL for eight hours (OD_{570}). Mean $\pm$ SEM ($n = 5$ experiments for each concentration). $^*P < 0.01$ versus control group value. HDL, high-density lipoprotein; rEPC, rat bone marrow endothelial progenitor cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM, standard error of mean; OD, optical density

rEPCs viability influenced by HDL

MTT is a method to show cell motility and growth. MTT viability tests was used to show that the effect of HDL in promoting rEPCs and their metabolic activity was dose-dependent with a concentration range from 12.5 to 62.5 μg/mL and was time-dependent under a concentration of 37.5 μg/mL (Figure 2). In addition, data of [^{3}H]thymidine incorporation assay also indicated that HDL was capable of inducing rEPC proliferation (Figure 3).

Effect of HDL on rEPC proliferation through PI3K/Akt pathway

To detect the effect of HDL on Akt activation and cyclin D1 expression, Western blotting was applied to reveal phosphorylation occurring in Akt and cyclin D1. Figures 4a and b show that HDL could promote phosphorylation of Akt and cyclin D1 expression and were time-dependent, almost reaching the highest point at the fourth hour. It also showed that this effect was dose-dependent, increasing remarkably at a concentration of 37.5 μg/mL.

The result also indicated that the induction effect of HDL on rEPC proliferation was mostly passing through the PI3k/Akt pathway. Figure 4c demonstrates that both cyclin D1 protein level and Akt activity decreased after LY294002 (10 μmol/L) inhibition, as compared with that of the HDL group. When LY294002 concentration was increased to 25 μmol/L, the outcome was even more remarkable. Figure 4d also shows a downregulation of

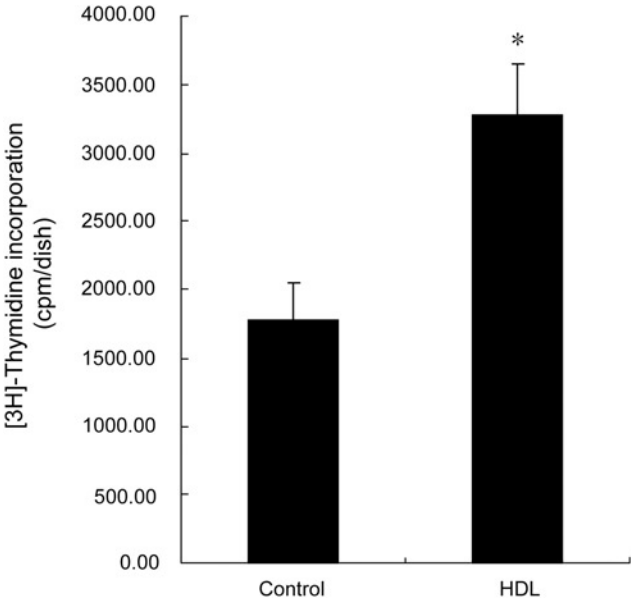

Figure 3 Influence of HDL on rEPC DNA synthesis. Quiescent rEPCs were treated with or without HDL (37.5 μg/mL) for eight hours; cell proliferation assayed using [^{3}H]thymidine incorporation. (Each column represents the quantitative analysis of three independent experiments.) $^*P < 0.01$ versus control group value. HDL, high-density lipoprotein; rEPC, rat bone marrow endothelial progenitor cell

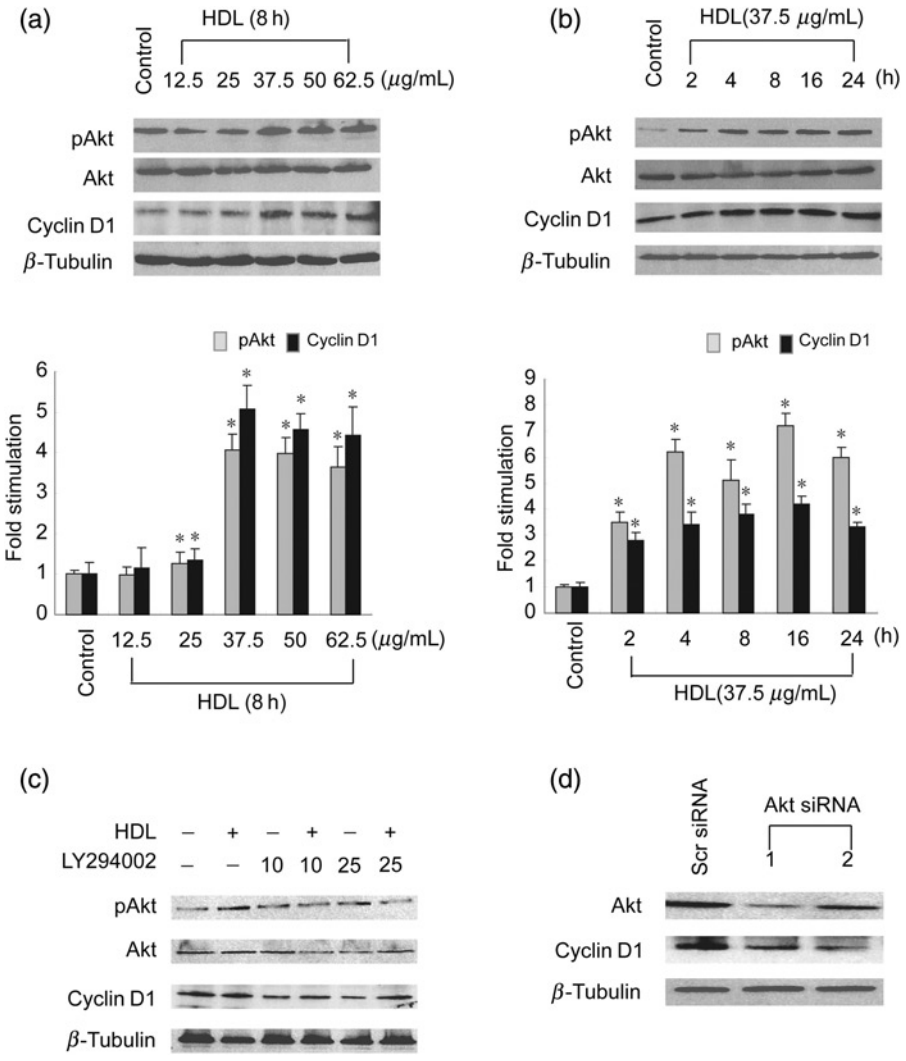

Figure 4 HDL is capable of inducing Akt phosphorylation and cyclin D1 expression. The effects were time- and dose-dependent. (a) Quiescent rEPCs, co-cultured with or without HDL at various concentrations for eight hours. (b) Quiescent rEPCs treated with or without HDL (37.5 μg/mL) and then co-cultured for eight hours. Cell extracts were prepared and equal amounts of protein from the control and experimental groups were analyzed, respectively, by Western blotting for Akt activity and cyclin D1 expression. (c) Quiescent rEPCs, co-cultured with or without HDL (37.5 μg/mL) accompanying the presence or absence of LY294002 (10 or 25 μmol/L) for eight hours. Western blotting showed increase of Akt phosphorylation and cyclin D1 expression after HDL. (d) rEPCs transfected with 100 nmol/L Akt-specific siRNA and non-silencing siRNA were used as the control. Western blotting showed a decrease of Akt and cyclin D1 levels after Akt-specific siRNA application, respectively. β-Tubulin was used as the control. HDL, high-density lipoprotein; rEPC, rat bone marrow endothelial progenitor cell; siRNA, small interfering RNA

Akt activity by Akt siRNA accompanied with a decline of cyclin D1 protein level, which indicates that the participation of Akt is important in regulating cyclin D1 expression in rEPCs to respond to HDL activation.

LY294002 and Akt siRNA mediates HDL-induced rEPC migration

To examine the involvement of PI3K/Akt pathway in HDL-induced cell migration, rEPCs were treated with HDL alone or HDL plus different inhibitors, respectively. As shown in Figure 5, the promoting effect of HDL on rEPC migration, denoted by counting the number of cells attached on the under surface of the membrane and expressed as the percentage of figures of the controls, was increased and was statistically significant ($P < 0.01$). Nevertheless, participation of either LY294002 or Akt siRNA was capable of inhibiting the promoting effect of HDL on rEPC migration (Figure 5).

Effect of LY294002 and Akt siRNA on HDL-induced rEPC tube formation

To examine the role of PI3K/Akt signaling in HDL-treated rEPCs, there were robust elongated tube structures formed when rEPCs were treated with HDL (37.5 μg/mL), and only a few incompleted tubes obtained when rEPCs were cultivated as the control. The diameters or lengths of the tubes observed in the HDL group were significantly increased by approximately two-fold over that of the controls. However, this effect of HDL on rEPCs was reversed

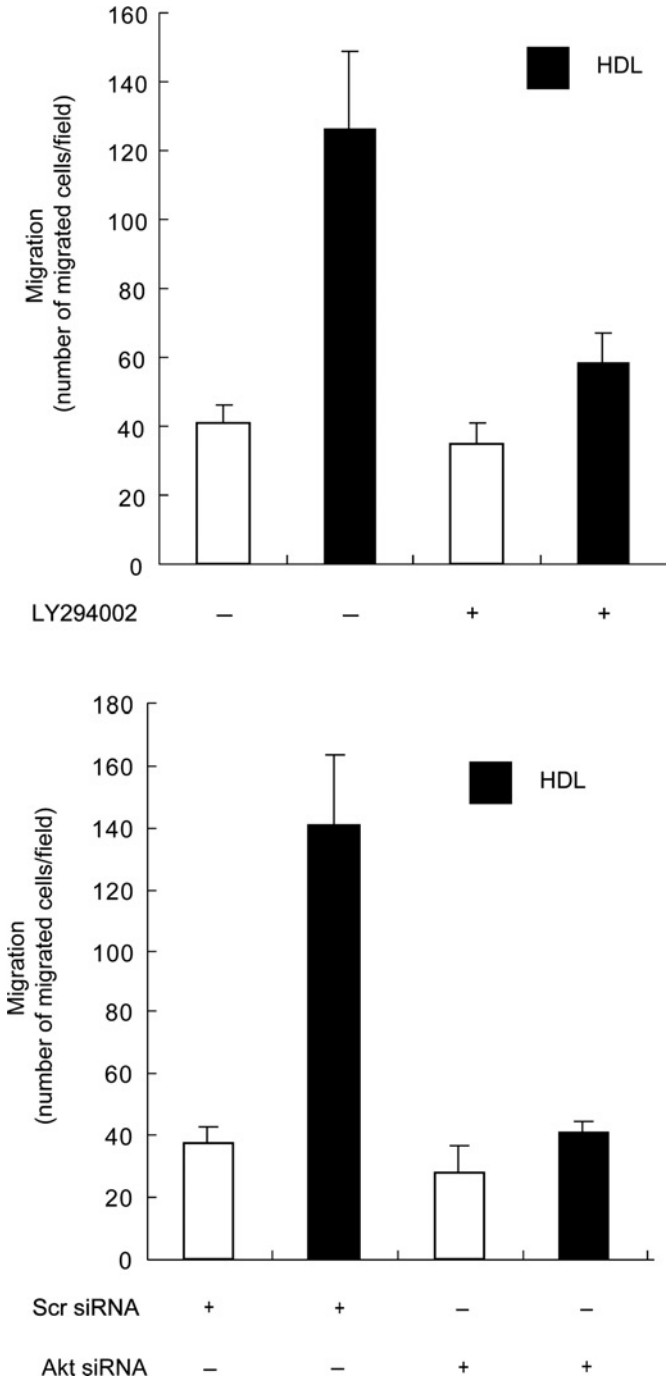

Figure 5 Administration of either LY294002 or Akt siRNA was capable of inhibiting transmembrane migration of rEPCs induced by HDL. Cells were cultured for six hours, respectively. Results were expressed as mean ± standard deviation. Each column represented the mean result of three separate experiments. $^{*}P < 0.01$ versus control; $^{**}P < 0.01$ versus HDL alone. HDL, high-density lipoprotein; rEPC, rat bone marrow endothelial progenitor cell; Scr, scramble; siRNA, small interfering RNA

by blocking the Akt pathway with either LY294002 or Akt siRNA (Figure 6). When one-way ANOVA was used, there was a statistical significance seen between the different groups. It indicated that the development of HDL-induced tube formation in rEPCs requires active PI3K/Akt signaling (Figure 6).

Rat serum TC after high cholesterol mixed ration

After receiving either a standard ration or a high cholesterol mixed ration for four weeks, there was no remarkable difference in the body weight among rats in all three groups (304.50 ± 10.93 versus 302.17 ± 19.03 g or versus 307.51 ± 9.33 g; $n = 6$), but serum total cholesterol level was remarkably elevated in the rats of groups II and III (225.05 ± 12.87 or 225.26 ± 3.41 mg/dL versus 97.90 ± 3.93 mg/dL of the control; $P < 0.01$; $n = 6$).

Circulating EPCs in rats under different environments

Data from fluorescence-activated cell sorting (FACS) analysis (Figure 7) showed that the amount of MNCs, including $CD34^+$ or Flk-1^+ cells in the peripheral blood of hypercholesterolemic rats, was lower than that of the normal control rats ($1.60 \pm 1.56\%$ versus $3.10 \pm 0.72\%$ for $CD34^+$ cells; $3.70 \pm 0.61\%$ versus $8.37 \pm 2.29\%$ for Flk-1^+ cells; $n = 3$, $P < 0.05$). However, the total number of MNCs, including $CD34^+$ or Flk-1^+ cells, was higher in the hypercholesterolemic rats after HDL administration ($3.97 \pm 0.15\%$ for $CD34^+$ cells and $14.03 \pm 1.36\%$ for Flk-1^+ cells; $n = 3$ for each group). Data showed that EPCs isolated from hypercholesterolemic rats collected on the seventh culture day had a significant decrease of endothelial nitric oxide synthase expression (Figure 7c) than that of the control rats ($21.33 \pm 1.06\%$ versus $57.33 \pm 13.15\%$; $n = 3$, $P < 0.05$), and this was restored after HDL administration ($21.33 \pm 1.06\%$ versus $69.87 \pm 1.78\%$; $n = 3$, $P < 0.05$). The expression of ICAM-1 from MNCs isolated from the peripheral blood of hypercholesterolemic rats with vessel injury was higher ($7.43 \pm 0.90\%$ versus $12.17 \pm 0.76\%$; $n = 3$, $P < 0.05$) than that of the control and was even higher after HDL administration ($18.00 \pm 2.26\%$; $n = 3$) (Figure 7d).

Effect of HDL on promotion of wound healing at the arterial intima

Microscopy of slides prepared from samples of femoral arteries of the experimental rats under a high power and oil-immersion fields showed that overall, only mild inflammatory cell infiltration, mainly the MNCs and lymphocytes, was obtained over the injury area seven days after the injury. There was no tissue necrosis. As shown in Figure 8a, re-endothelialization appeared in the control rats, including both the regenerated endothelium and newly formed neointima, but incomplete. The lining endothelium in the hypercholesterolemic rats as shown in Figure 8b was also incomplete and substantial neointima formation occurred. Figure 8c shows the pathological findings of the hypercholesterolemic rats plus HDL. There was a thin layer of fibrin over the surface of the regenerated endothelium. The lining endothelium was clear and more complete than that of group II, but still unable to cover the whole inner wall of the artery lumen. Both cell infiltration and neointima formation were not remarkable, indicating that the participation of HDL is beneficial for the regeneration process. Digitized images showed that the neointima formation of the injured arteries in rats of the HDL group was inhibited by over 80% than

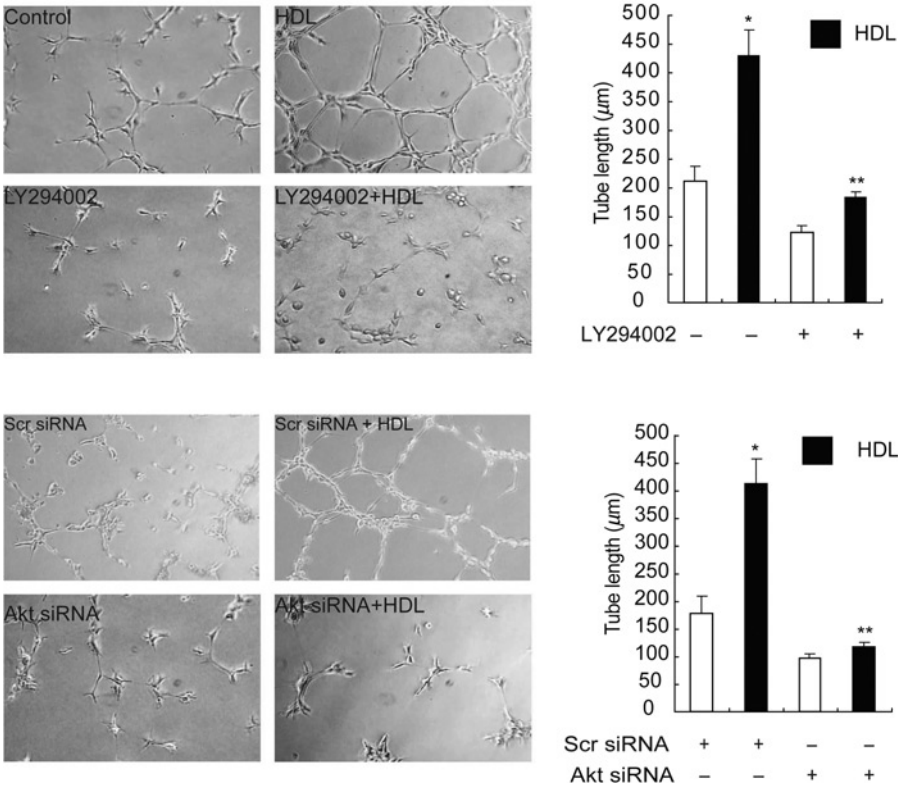

Figure 6 Tube formation assay of cultured rEPCs. Upper: EPCs were quiesced, incubated with or without LY294002 and subjected to HDL-induced tube formation. Lower: EPCs were transfected with Scr siRNA or AKt siRNA, quiesced, and subjected to HDL-induced tube formation. $^{*}P < 0.01$ versus control; $^{**}P < 0.01$ versus HDL alone. HDL, high-density lipoprotein; rEPC, rat bone marrow endothelial progenitor cell; Scr, scramble; siRNA, small interfering RNA

that of the hypercholesterolemic group by intima/media ratio. It was likely that HDL influenced the development of newly formed endothelium, promoting re-endothelialization and leading to a well-developed neointima.

Discussion

Recently, studies on the effect of HDL on endothelial progenitor cells have been performed and published in the literature.[18–20] HDL can enhance EPC incorporation in allografts, stimulate re-endothelialization and attenuate transplant arteriosclerosis. HDL also augments EPC function to maintain vascular homeostasis and promotes collateral development.[21] In this study, the role of HDL in regulating EPC proliferation and function was further detected. Our previous work had demonstrated that HDL or apolipoprotein A is associated in wound healing, atherosclerosis and re-stenosis following percutaneous transluminal coronary angioplasty. In this paper, data of *in vivo* FACS results also showed that HDL promotes EPC proliferation.

HDL enables promotion of migration and 'tube' formation of EPCs, which indicates the possession of an angiogenesis ability of these cells in wound healing. Interestingly, both the promoting effects of EPC proliferation and tube formation induced by HDL could be inhibited by the pharmacological inhibitor on siRNA-mediated depletion of gene level.

LY294002 has been identified as a highly specific inhibitor of PI3K. Studies with this compound have demonstrated its inhibiting ability and its involvement in a variety of processes in the cultured EPCs. We also used Akt siRNA to evaluate the role of Akt in HDL-dependent signaling pathway. These results strongly supported that the PI3k/Akt, a key pathway in vasculogenesis, mediates the promoting effects observed with HDL. This is a novel finding in HDL activation in EPCs.

Neovascularization does not exclusively rely on the proliferation and migration of local endothelial cells, but also includes the recruitment and differentiation of stem and progenitor cells.[22] Evidence of experimental result in our laboratory strongly suggested a role of EPCs, which considerably contribute to neovascularization in response to vascular injury.[15] EPCs are recruited and incorporated into the injured vessel wall to promote local wound healing at intima by direct cellular differentiation and/or by releasing of certain growth factors. Temporary ischemia locally seemed beneficial to the installation of a preconditioning environment for the transplanted EPCs undergoing cell differentiation and proliferation, and further stimulation to the bone marrow might provide more progenitor stem cells as well as more liberation of growth factors such as VEGF, platelet-derived growth factor and granulocyte colony-stimulating factor to facilitate re-endothelialization.[23–25] Data from our other experiment indicated that hypercholesterolemia could downregulate the promoting ability of EPCs on endothelial cell regeneration. In this study, seven days after endothelium denudation, only

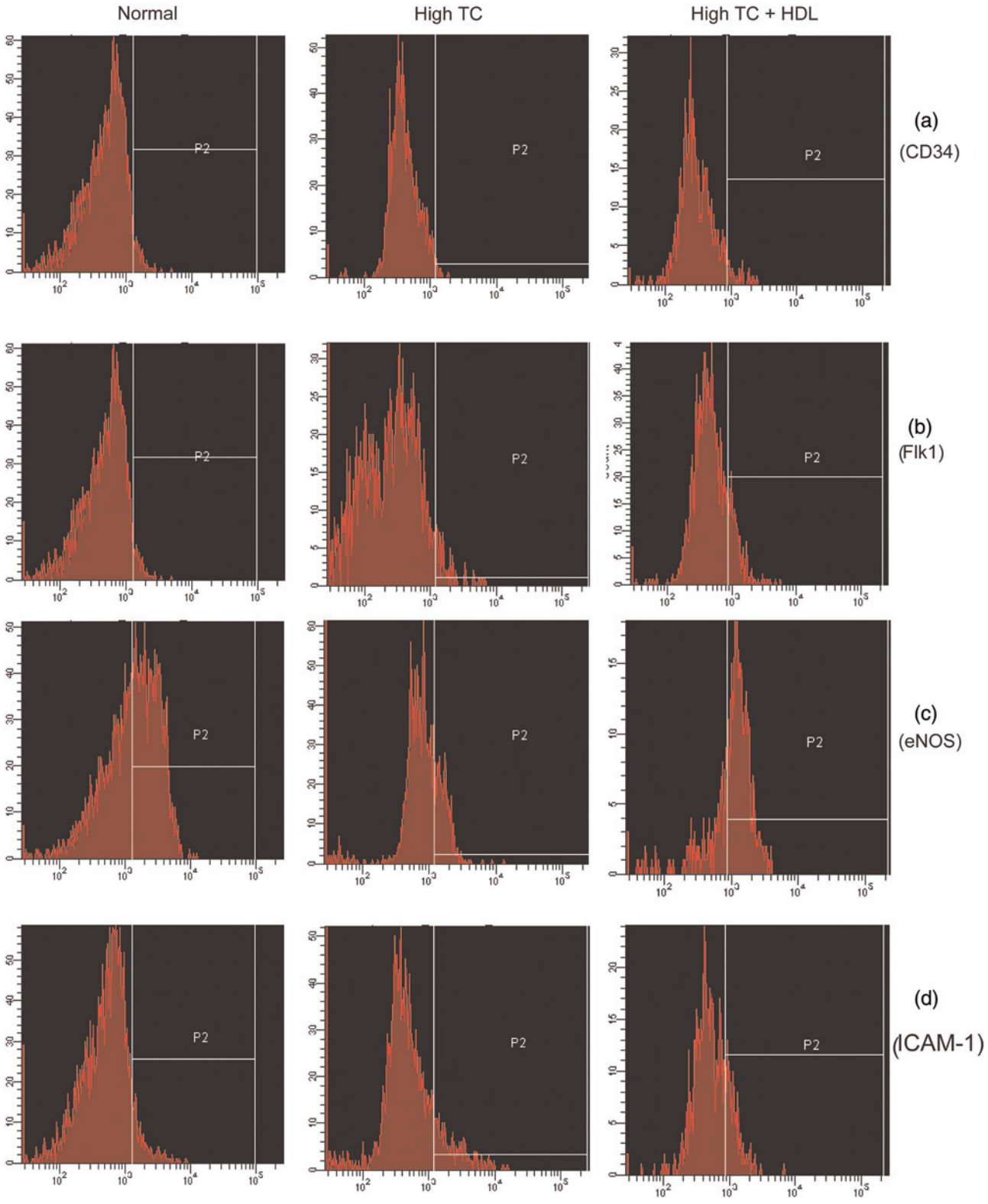

Figure 7 FACS analysis on distribution of mononuclear cells isolated from rats' circulating blood (Figures 6a, b and d) and EPCs harvested on the seventh culture day (Figure 6c). Cells were labeled with fluorescent antibodies against CD34 (Figure 7a), Flk-1 (Figure 7b), eNOS (Figure 7c) and ICAM-1 (Figure 7d). The P2 gate denotes the positive area ($n = 3$). FACS, fluorescence-activated cell sorting; EPC, endothelial progenitor cell; eNOS, endothelial nitric oxide synthase; ICAM-1, intercellular adhesion molecule-1 (A color version of this figure is available in the online journal)

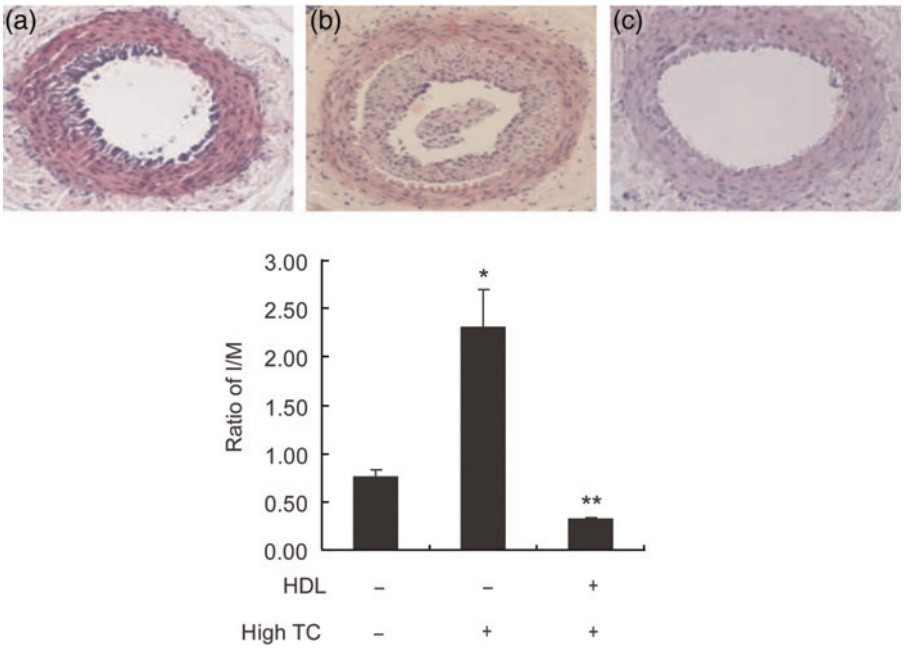

Figure 8 Effect of HDL on re-endothelialization after a balloon-catheter injury (HE stain, $n = 3$ in each group). (a). Control rats, (b) hypercholesterolemic rats and (c) hypercholesterolemic rats plus HDL. The bar graph at the bottom showed statistical analysis of intima/media ratio (I/M) in the control, hypercholesterolemic and hypercholesterolemia plus HDL groups. The area of I/M was evaluated with a software of CMIAS series analysis system and is shown as ratio of I/M. $^{*}P < 0.05$ compared with the normal group and $^{**}P < 0.05$ compared with hypercholesterolemia plus HDL group; $n = 3$. HDL, high-density lipoprotein; HE, hematoxylin–eosin (A color version of this figure is available in the online journal)

a mild proliferation of endothelial cells was obtained in the control rats; however, there was a thin layer of regenerated endothelium obtained accompanying a marked $CD34^{+}$ cell infiltration in the hypercholesterolemic plus HDL rats as compared with that of the hypercholesterolemic rats without HDL administration.

In summary, the present results provide the mechanism of HDL-induced proliferation in EPCs, particularly the involvement of Akt-cyclin D1, leading to their migration and angiogenesis. In addition, HDL could promote EPC proliferation in hypercholesterolemic rats.

Author contributions: QZ performed experiments on Western blot analysis, cell isolation and culture, MTT, DNA synthesis, EPC tube formation and migration, immunohistochemistry experiments, the rat artery balloon injury model and FACS, and wrote the paper; HY performed experiments on balloon injury model and interpreted results; PL performed immunohistochemistry experiments, HDL preparation and established the rat artery balloon injury model; HZ performed preparation of hypercholesterolemic rat model and immunohistochemistry experiments; MS designed the experiments, checked and interpreted the results, wrote and modified the article, and was responsible for application of grants to raise funds for the reagents, animals and facilities required.

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