European Review for Medical and Pharmacological Sciences 2019; 23: 7098-7109

Apelin-13 promotes late endothelial progenitor cells differentiation by regulating Krüppel-like factor 4

M. YU[1], H.-J. FENG[2], A.M.E. ABDALLA[3], Y.-F. TENG[4], Q. LI[4]

[1]Department of Vascular Surgery, General Hospital of Ningxia Medical University, Yinchuan, China
[2]Department of Vascular Surgery, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[3]Department of Biomedical Engineering, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China
[4]Department of Vascular Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Miao Yu and Haijun Feng are co-first authors and contributed equally to this work

Abstract. – OBJECTIVE: The endothelial progenitor cells (EPCs) differentiation plays an essential role in angiogenesis. The purpose of this study is to determine the potential mechanisms of apelin-13 in EPCs differentiation.

MATERIALS AND METHODS: The rats EPCs were isolated from the bone marrow and identified by DIL-acLDL and FITC-UEA-1 staining. EPCs were divided into four groups: the negative control group, the Krüppel-like factor 4 (KLF4) upregulation group, the KLF4 downregulation group, and the apelin-13 group. The EPCs differentiation was evaluated by cell migration, proliferation, and the expressions of surface markers CD31, CD34, CD133, and VEGFR-2 on mRNA and the protein levels, as well as immunofluorescence.

RESULTS: In the KLF4 up-regulation and apelin-13 groups, the EPCs number of G1, S, and G2/M phases decreased and suggested that KLF4 and apelin-13 were closely related to the EPCs proliferation. EPCs showed stronger migration ability with the elevation of KLF4 and apelin-13 while declined migration was detected in KLF4 siRNA transfected EPCs. The surface markers expressions on mRNA and the protein levels increased in the KLF4 upregulation group, and in the apelin-13 group there were similar results, as well as increased KLF4 expression.

CONCLUSIONS: The upregulation of apelin-13 significantly increased the expressions of EPCs surface markers, which were involved in early EPCs differentiated into late EPCs and influenced the cells migration and proliferation. Taking the elevation of KLF4 which performed similar effects of apelin-13, we believe that apelin-13 activates or synergizes with KLF4 to promote this process.

Key Words:
Endothelial progenitor cells, Apelin-13, Krüppel-like factor 4, Cell differentiation.

Introduction

Millions of patients worldwide are suffering from ischemic diseases, including myocardial infarction, stroke, and critical limb ischemia (CLI) with a high risk of amputation[1-3]. It is estimated[4-6] that up to 40% of CLI patients with symptoms have almost no treatment options, and the patients who have severe underlying medical illness are left untreated and are in hope of conservative care to achieve limb salvage. Cell therapy as a treatment for CLI patients was first introduced in 2000, and Tateishi-Yuyama et al[7] were the first to report the efficacy of intramuscular implantation of autologous bone marrow-derived mononuclear cells (BM-MNCs) in CLI patients. Since then, many clinical research centers have adopted this approach to ischemic patients and the efficacy from the long-term follow-up proven to be effective[4,8,9]. Endothelial progenitor cells (EPCs) are the key components in BM-MNCs to mediate new blood vessels formation and play an important role in the process of vascular remodeling under physiological and pathophysiological conditions,

Corresponding Authors: Qin Li, MD; e-mail: litchi103@163.com
Yunfei Teng, MD; e-mail: tyf224@163.com

and also participate in embryonic vasculogenesis. EPCs in the bone marrow can migrate to the ischemic or vascular injury sites, and differentiate into mature endothelial cells (ECs), promoting the formation of new blood vessels and repairing the ischemic injury[10-12].

It has been controversial for EPCs identification, since they shared a common precursor with other lineages, and the cells can be isolated/trans-differentiated from many sources, sharing similar phenotypic characteristics[13]. Until 1997 when Asahara et al[14] isolated EPCs in human peripheral blood, they were gradually recognized as a heterogeneous population. The EPCs with "spindle-shaped" morphology, up-taking acetylated low density lipoprotein (acLDL), as well as binding Ulex Europaeus Agglutinin I (UEA-1), are named early EPCs (eEPCs)[15] or circulating angiogenic cells[16]. However, if the EPCs are cultured for a longer period over 2 weeks, their morphology appears "cobblestone" and are termed as late EPCs (lEPCs)[15], or endothelial colony-forming cells[17]. Differentiation from eEPCs to lEPCs is an important step of mature ECs formation, but the specific mechanism has not been determined.

Apelin is identified as an endogenous ligand of the APJ receptor, a G protein-coupled receptor related to angiotensin receptor AT1[18]. The apelin-APJ regulatory system is widely distributed in various systems in human and exhibits different biological effects, and especially plays an important role in the maintenance and regulation of vascular function[19,20]. Apelin-13 is the proteolytic cleavage production of apelin preproprotein, which shows greater affinity for the APJ receptor and constitutes the principal endogenous ligand[21]. It is reported that apelin-13 can promote human embryonic stem cells to differentiate into cardiomyocytes by enhancing APJ[22], and it is also involved in human aortic vascular smooth muscle cells proliferation to aggravate atherosclerosis by activating AMPKα[23]. Apelin-13 is also found to effectively promote bone marrow mesenchymal stem cells survival and vascularization under hypoxic-ischemic conditions *in vitro*[24].

This study aimed to investigate the potential roles and underlying mechanisms of apelin-13 in EPCs differentiation. Our results revealed that apelin-13 and Krüppel-like factor 4 (KLF4) played a vital role in inducing the differentiation of eEPCs into lEPCs and thereby provided new insights into the EPCs mediated angiogenesis.

Materials and Methods

This study was approved by the Animal Care and Committee of Ningxia Medical University. All animal procedures were in line with the National Institutes of Health (NIH), Guide for the Care and Use of Laboratory Animals.

EPCs Isolation and Identification

Male Wistar rats (450 ± 10 g) were purchased from Hubei Provincial Center for Disease Control and Prevention (Wuhan, Hubei, China). The rat granulocyte colony-stimulating factor (rG-CSF, PeproTech Inc., Rocky Hill, NJ, USA) was applied for 3 consecutive days to mobilize EPCs from the bone marrow, and the bilateral femur bone marrow was isolated, cultured, and identified as previously described[25,26]. Briefly, the bone marrow cells were made into single-cell suspension and isolated by density gradient centrifugation (Cence, Changsha, China), suspended at a density of 2×10^6 cells/mL in Endothelial Cell Growth Medium-2 Bullet Kit (EGM-2 BulletKit, Lonza, Switzerland) medium, and then incubated in fibronectin-coated well plates at 37°C in an incubator (Heal Force, Shanghai, China) with 5% CO_2. After 7 days in culture, the adherent cells were treated with 10 μg/mL 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate labeled acLDL (DIL-acLDL, Molecular probes, Waltham, MA, USA) and 10 μg/mL fluorescein isothiocyanate labeled UEA-1 (FITC-UEA-1, Merck KGaA, Darmstadt, Germany). The nuclei were counterstained with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI, Roche, Basel, Switzerland) for 15 minutes at room temperature. The treated cells were washed with Phosphate-Buffer Saline (PBS) and then observed under a confocal fluorescence microscope (Carl Zeiss, Heidenheim, Germany).

EPCs Transfection

The cells were in 50%-70% confluence in adherent growth before they were transfected with KLF4 siRNA (RiboBio, Guangzhou, China) or pcDNA-3.1-Rat_KLF4 plasmid (ELK Biotechnology, Wuhan, China). The siRNA sequence of KLF4 was GCGCUACAAUCAUGGUCAAdTdT. The non-targeting scrambled siRNA (negative control siRNA, RiboBio, Guangzhou, China) or empty plasmid (ELK Biotechnology, Wuhan, China) was served as a negative control. We diluted the siRNA or plasmid to their specific concentration according to the instructions with 50 uL Opti-minimum essential medium (Opti-MEM) low

serum medium (Gibco, Massachusetts, USA) and mixed them gently. We added 1 μL of Lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA, USA) into 50 μL Opti-MEM low serum medium and incubate for 5 minutes at room temperature to make the transfection solution. The diluted siRNA or plasmid were mixed gently with the above solution and stood at room temperature for 20 minutes. We added 100 μL of transfected cells suspension to each well for incubation for 5 hours and replaced the transfected medium with EGM-2 to incubate the cells at 37°C in a 5% CO_2 incubator overnight. The effectiveness of transfection was measured 48 hours after transfection by Real Time-quantitative Polymerase Chain Reaction (qPCR), and the protein level was measured by Western blot.

EPCs Proliferation Assay

The cells in the logarithmic growth phase of each group were inoculated in 96 well plates (Corning Costar, Corning, NY, USA) at the density of 1×10^4/well, and the volume of each well was 200 μL. The blank, as well as the control group were set, and for each concentration there were three duplicate wells. The plate was incubated at 37°C with 5% CO_2 for 24/48/72/96/120 hours before CCK-8 assay was carried out. We replaced the former medium with 100 μL fresh culture medium which contained 10 μL of CCK-8 regent (Beyotime Biotechnology, Shanghai, China), and maintained the plate in an incubator for 2 hours before the microplate reader (Bio-Rad, Hercules, CA, USA) was used to measure the optical density (OD) at the wavelength of 450 nm. The cell growth curve was drawn and the relative proliferation rates were calculated.

Transwell Migration Assay

The migration assay was performed using 8 μm pore sized transwell plates (Corning Costar, Corning, NY, USA). The upper chambers were loaded with 2×10^4 EPCs in a 100 μL of Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA), and the lower chambers were loaded with 600 μL of EGM-2. These chambers were incubated at 37°C with 5% CO_2. After 24 hours of incubation, the non-migrated cells in the upper chambers were removed and the surfaces of the filters were gentle scraped and washed with PBS. Then, the adherent cells were fixed in 4% paraformaldehyde (Absin Bioscience Inc, Shanghai, China) for 20 minutes and stained by 1% crystal violet solution (Absin Bioscience Inc, Shanghai, China) for 30 minutes. The average number of migrated cells was determined by checking five random fields per well. All experiments were repeated in triplicate for each group.

Cell Cycle Analysis

The cells in the logarithmic growth phase were collected by centrifuge and pre-cooled 70% ethanol was mixed to fix the cells at 4°C overnight. The cells were washed and resuspended with 4°C PBS solution, and the cell suspension concentration of control and each experimental group were adjusted to 1×10^9/L. For each sample, 50 **μg**/mL propidium iodide (PI, Beyotime Biotechnology, Shanghai, China) staining solution was added and incubated at 4°C for 30 minutes in the dark for flow cytometry analysis (BD FACS Calibur, BD Bioscience, Franklin Lakes, NJ, USA) according to the instructions.

EPCs' Surface Markers Identification

Cytospin preparations were fixed on slides in 4% paraformaldehyde for 30 minutes and then permeabilized in 0.1% Triton X-100 (Absin Bioscience Inc, Shanghai, China) for 10 min at room temperature. We added 3% hydrogen peroxide solution (Merck KGaA, Darmstadt, Germany) to incubate the cells for 20 minutes in the dark, and used a bleaching shaker (Cence, Changsha, China) to wash the cells in PBS. Next, the cells were covered with 5% bovine serum albumin (BSA, Solarbio Life Sciences, Beijing, China) diluted primary antibodies at 4°C overnight. Then, the cells were incubated with specific secondary antibodies for 1 hour at room temperature. DAPI was applied for nuclei staining, and after washing with PBS, the cells were observed under a fluorescence microscope (Carl Zeiss, Heidenheim, Germany). The representative images of each molecule were shown. All confocal analyses were repeated multiple times, and at least 10 images were analyzed for each molecule.

Quantification of mRNA Expressions by Real Time-qPCR

The total RNA was extracted from the cell lines using TRIzol reagent (Invitrogen Life Sciences, Carlsbad, MA, USA), and the RNA concentrations were measured by a spectrophotometer (Life Technology, Waltham, MA, USA) to determine the purity. Complementary DNA (cDNA) was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA), with a maximum

content of 1 μg RNA. A Real Time-qPCR kit (SYBR® Premix Ex Taq™, TaKaRa Biomedical Technology, Otsu, Shiga, Japan) was used to perform amplification and quantification with the qPCR machine (StepOne™ Real-Time, Life technologies, Waltham, MA, USA) according to the instructions. The primers (Sangon Biotechnology, Shanghai, China) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CD31, CD34, CD133, KLF4, and vascular endothelial growth factor receptor 2 (VEGFR-2) were summarized in Table I. The expression of the target genes was normalized using the GAPDH gene as a control. The Real time-qPCR cycle parameters included initial denaturation at 95°C for 1 minute followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 45 seconds.

Western Blot Analysis

The total cellular proteins were extracted in the presence of the protease inhibitors. The protein concentration was determined by a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). The protein lysates were adjusted to 20 **μg** per sample, separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Beyotime Biotechnology, Shanghai, China) and transferred to a polyvinylidene difluoride (PVDF; Merck KGaA, Darmstadt, Germany) membrane at 300 mA, at 4°C. Then, the membranes were blocked with 5% skimmed milk in Tris-Buffered Saline (TBS, Absin Bioscience Inc, Shanghai, China) supplemented with 0.01% Tween 20 (TBST, Absin Bioscience Inc., Shanghai, China) for 2 hours and incubated overnight at 4°C with primary antibodies in TBST. Next, the membranes were washed by TBST and incubated with the appropriate secondary antibody for 1 hour at room temperature and then washed again with TBST. The detection of the immunoreactive bands was performed by enhanced chemiluminescence (ECL, Beyotime Biotechnology, Shanghai, China), and the membranes were imaged by the imaging system (Bio-Rad, Hercules, CA, USA). The density of each band was analyzed using the GelDoc software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

The data were presented as the mean ± SD. The differences between the groups were evaluated by One-way analysis of variance (ANOVA) with the Tukey's post-hoc test (GraphPad Prism 5 Software, La Jolla, CA, USA). The two-sided probability level of $p < 0.05$ was considered statistically significant. The differences with $p < 0.05$ are indicated as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Identification of EPCs

After seven days of cultivation, the isolated BM-MNCs were stained by DIL-acLDL and FITC-UEA-1 observed under a fluorescence microscope. The cells taking DIL-acLDL were emitting red while green for those taking FITC-UEA-1 as presented in Figure 1. Since it is widely accepted that EPCs could endocytose acLDL and bind UEA-1, the yellow cells in the merged figure were confirmed as the EPCs.

KLF4 Overexpression Enhanced EPCs Proliferation

In this study, KLF4 was screened for its ability to change the contents of flow cytometry cell cycle profiles when exogenously expressed. Compared with the control group (Figure 2A), the upregulation of KLF4 decreased the EPCs number of G1, S, and G2/M phases (Figure 2B). However, KLF4 downregulation group increased the number of cells in the G1 phase and decreased the numbers of cells in the S and G2/M phases (Figure 2C). The treatment by apelin-13 altered the flow cytometry cell cycle profiles in a similar manner as KLF4 overexpression (Figure 2D). The results suggested that the KLF4 and apelin-13 genes were significantly associated with the EPCs periodic distribution and promoted the EPCs proliferation.

Table I. The primer sequences of GAPDH, CD31, CD34, CD133, KLF4, and VEGFR-2.

Primer Name		Primer Sequences
GAPDH	Forward	CGCTAACATCAAATGGGGTG
	Reverse	TTGCTGACAATCTTGAGGGAG
CD31	Forward	GATCTCCATCCTGTCGGGTAAC
	Reverse	GTGTCATTCACGGTTTCTTCGT
CD34	Forward	CCACAGACTTACCCAACCGTC
	Reverse	CCTCGGATTCCTGAACATTTG
CD133	Forward	CTTCCTGCTGACCCTCATTGT
	Reverse	GATCGTTGAGCAGGTAGGGAG
KLF4	Forward	AGTCCCGAGGAACTGCTGAAC
	Reverse	GGCATGAGCTCTTGATAATGGAG
VEGFR-2	Forward	TTCATAATAGAAGGCGTCCAGG
	Reverse	GCATCATAAGGCAAGCGTTC

Overexpression of KLF4 Promoted Cell Migration

Cell migration is essential during EPCs differentiation, and these two events always occur concomitantly. It is generally believed that lEPCs has greater migration activity than eEPCs[27]. To assess the effect of KLF4 on EPCs migration, we performed a transwell migration assay. Compared with the control group (Figure 3A), the migrations were enhanced in the KLF4 upregulation group (Figure 3B) and the apelin-13 group (Figure 3D) by 40.8% and 19.7% respectively after 24 hours of cultivation. In the KLF4 downregulation group (Figure 3C), the migration rate was inhibited by 15.8%.

To further investigate whether the improvement of the migration ability was related to cell proliferation, CCK-8 assay was conducted. Briefly, the proliferation of KLF4 in the upregulation group and the apelin-13 group was significantly higher than that of the control group, and the KLF4 downregulation group showed a weaker proliferation than the control group (Figure 3F). In the first 24 hours after cultivation, the relative proliferation rate of KLF4 upregulation group, KLF4 downregulation group and the apelin-13 group were 4.8%, -5.7%, and 3% compared with the control, which was significantly lower than that of the migration rate. We believed that the migration manifestations of the cells in KLF4 upregulation group and apelin-13 group were more consistent with the characteristics of lEPCs.

Apelin-13 Promoted eEPCs Differentiation to lEPCs in Accordance with KLF4 Up-Regulation

The cell surface markers play an important role in the identification of cells by providing a specific target and we used these markers to identify

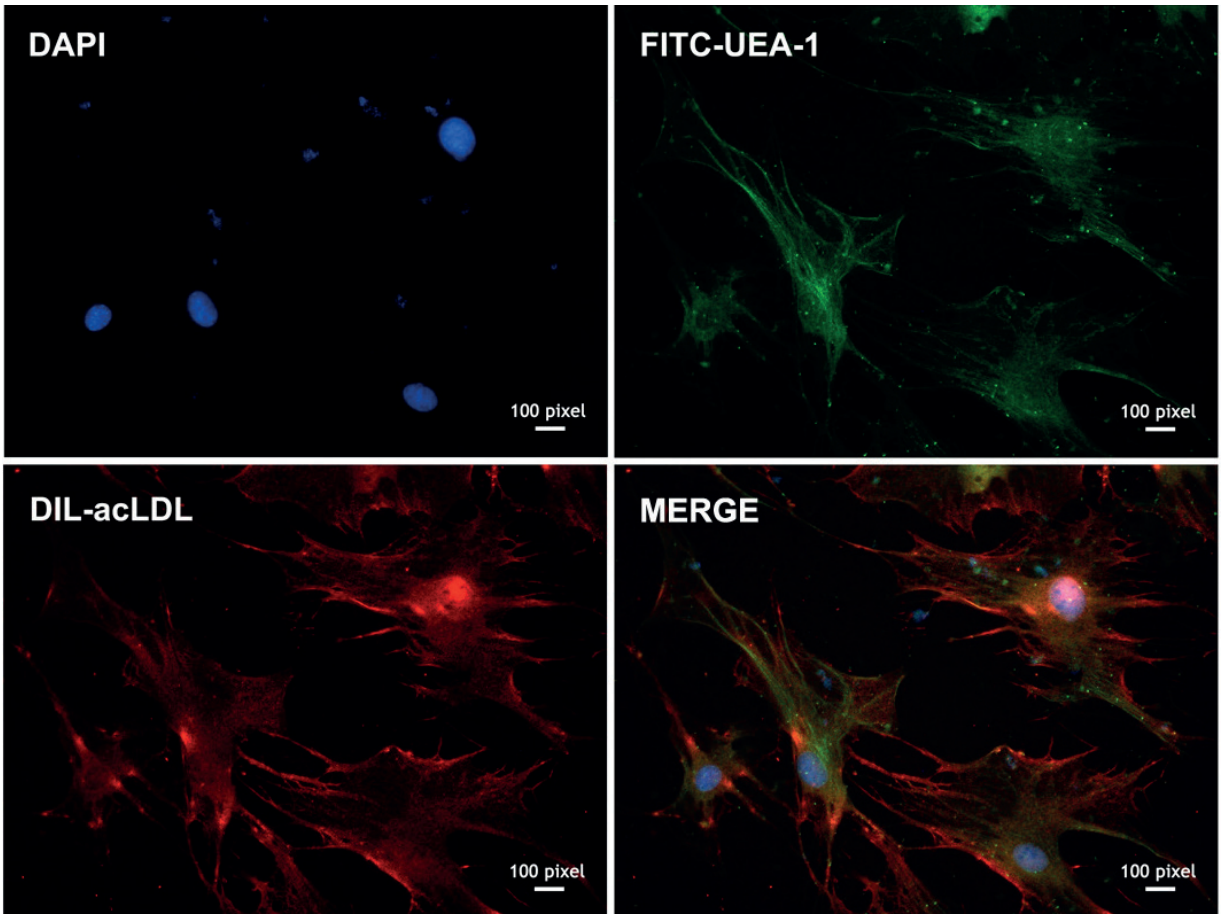

Figure 1. Identification of EPCs under fluorescence microscopy. The blue dots were EPCs' nuclei stained by DAPI. The red fluorescence was for DIL-acLDL and the green for FITC-UEA-1. The cells in the merged image labeled yellow fluorescence were EPCs. Magnification: ×600.

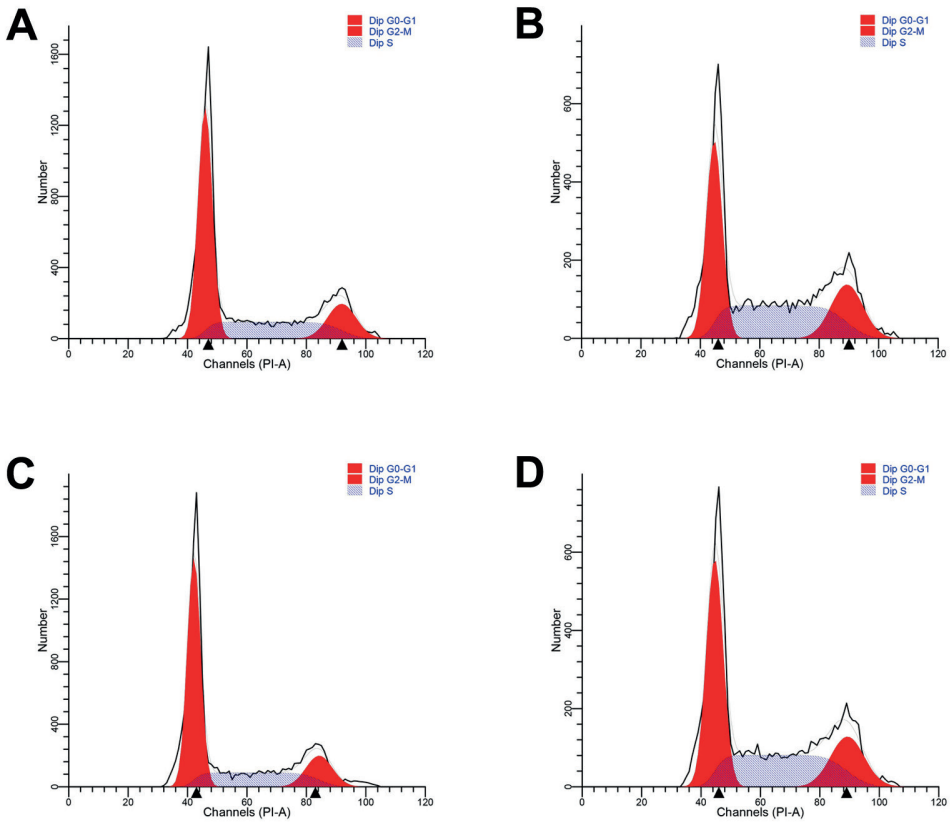

Figure 2. Cell cycle progressions in four groups were detected by flow cytometry. ***A,*** Cell cycle of EPCs in the negative control group. %G0-G1: 54.35 at 45.94; %S: 29.38; %G2-M: 16.27 at 91.87; %CV: 5.20. ***B,*** Cell cycle of EPCs in the KLF4 upregulation group. %G0-G1: 38.18 at 44.66; %S: 41.22; %G2-M: 20.60 at 89.32; %CV: 5.99. ***C,*** Cell cycle of EPCs in the KLF4 downregulation group. %G0-G1: 58.08 at 42.14; %S: 26.32; %G2-M: 15.63 at 84.28; %CV:5.29. ***D,*** Cell cycle of EPCs in the apelin-13 group. %G0-G1: 43.35 at 44.62; %S: 37.69; %G2-M: 18.96 at 89.23; %CV: 6.31. The X-axis represented for the DNA content and the Y axis represented for viable cells number. There were a significantly reduced %G0-G1 and increased %S in KLF4 upregulation group and apelin-13 group suggested active cell proliferation.

EPCs. Compared with the control group, the expressions of lEPCs markers CD31, CD34, CD133, and VEGFR-2 raised with the elevation of KLF4 showing the shining red immunofluorescence. Contrarily to the KLF4 upregulation, the expressions of CD31, CD34, CD133, and VEGFR-2 decreased in KLF4 downregulation group and the fluorescence intensity was significantly weakened (Figure 4). The surface markers of EPCs in the apelin-13 group showed a similar representation as if KLF4 upregulated and the expression of KLF4 was also increased.

To further quantify these expression changes, we conducted the Real Time-qPCR and Western blot analysis to determine the mRNA and protein levels in each group. For the mRNA expressions (Table II and Figure 5A), CD31, CD34, CD133, and VEGFR-2 in the KLF4 upregulation group and apelin-13 group were significantly higher than the control group ($p < 0.001$ and $p < 0.01$). Correspondingly, we found similar results on the protein expressions (Table III and Figure 5C), and there were distinctive differences of gray band in the Western blot image (Figure 5B) of each group.

Discussion

Differently from the classic progenitor cells, EPCs are more like stem cells with features of self-renewability, clonogenicity, and differentiation capacity[13]. Extensive research has reported that lEPCs are a proliferative subtype that forms tube-like structures and is directly participated in the vasculature. In contrast, eEPCs do not differentiate into mature ECs and indirectly promote

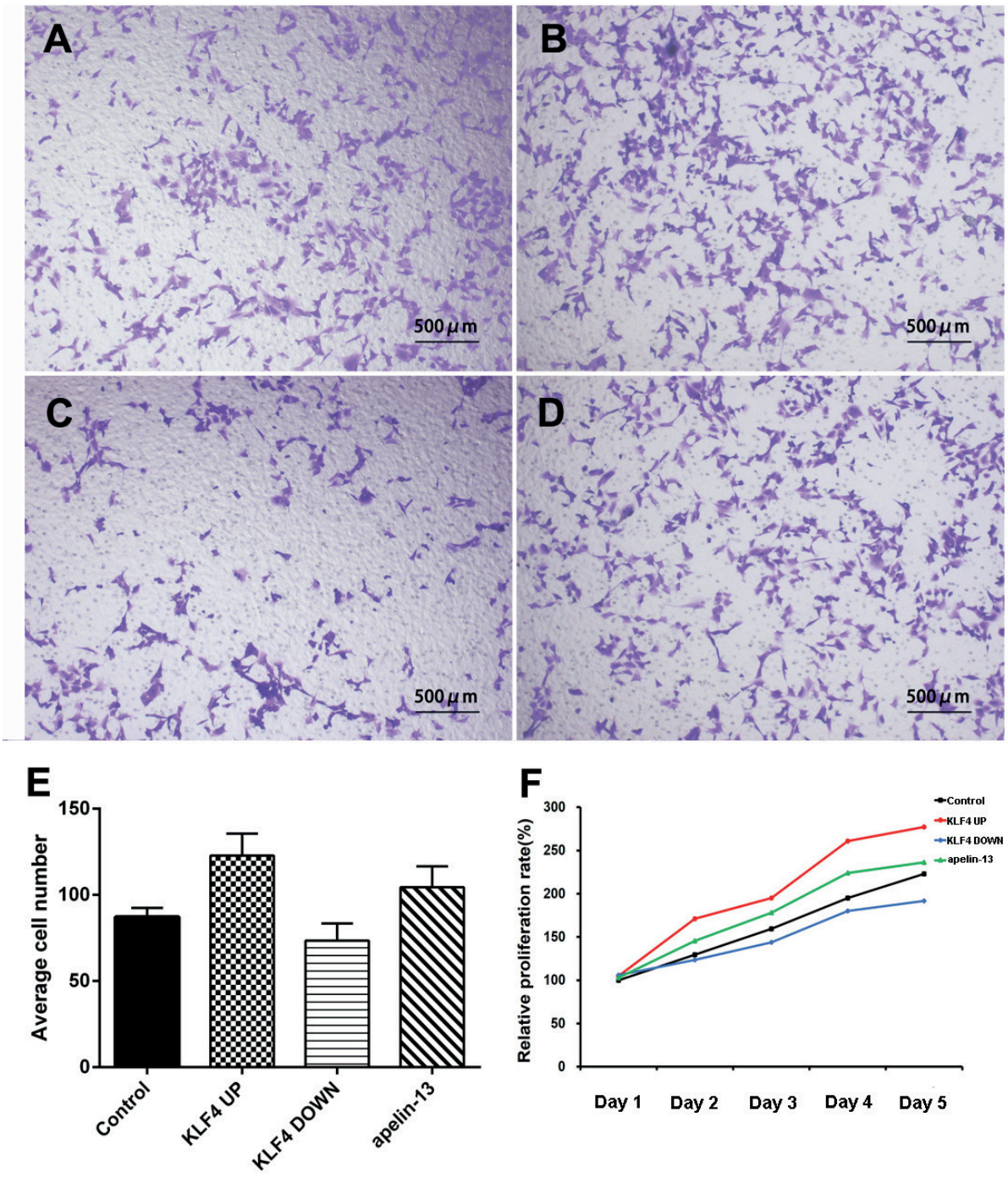

Figure 3. *In vitro* migration and proliferation assay of EPCs in the four groups. ***A,*** to ***D,*** The transwell migration assay of the negative control group, KLF4 upregulation group, KLF4 downregulation group, and apelin-13 group (magnification: ×40). The average cells numbers were 87.20±4.71, 122.80±11.44, 73.40±8.96, and 104.40±10.93, respectively. ***E,*** The histogram of the above results. Compared with the negative control group, the KLF4 upregulation group and apelin-13 group showed a significantly increased cell migration. ***F,*** The line chart of EPCs proliferation by CCK-8 assay, in which the results were consistent with the transwell migration assay. *$p<0.05$ as compared to negative control group, data represented the mean ± SD.

angiogenesis through paracrine mechanisms[15,28]. Based on these physiological differences, only lEPCs are thought to trans-differentiate into mature ECs following their differentiation from mononuclear cells. Although the contributions of EPCs to ischemia induction or tumor angiogen-

Table II. The relative mRNA levels of KLF4, CD31, CD34, CD133, and VEGFR-2 among the four groups. The data were represented as mean ± SD..

Marker	Control	KLF4 UP	KLF4 DOWN	apelin-13
KLF4	1	2.19±0.17	0.24±0.00	1.63±0.09
CD31	1	2.91±0.12	0.37±0.01	1.88±0.13
CD34	1	2.66±0.23	0.50±0.05	1.58±0.11
CD133	1	2.95±0.15	0.35±0.01	1.64±0.22
VEGFR-2	1	3.41±0.32	0.33±0.03	2.61±0.14

Table III. The protein levels of KLF4, CD31, CD34, CD133, and VEGFR-2 of the four groups. The data were represented as mean ± SD.

Marker	Control	KLF4 UP	KLF4 DOWN	apelin-13
KLF4	0.18±0.02	0.52±0.04	0.12±0.01	0.38±0.04
CD31	0.13±0.02	0.70±0.05	0.07±0.01	0.38±0.03
CD34	0.10±0.02	0.56±0.04	0.05±0.01	0.29±0.03
CD133	0.15±0.01	0.60±0.03	0.04±0.01	0.31±0.04
VEGFR-2	0.29±0.03	0.77±0.05	0.14±0.01	0.42±0.04

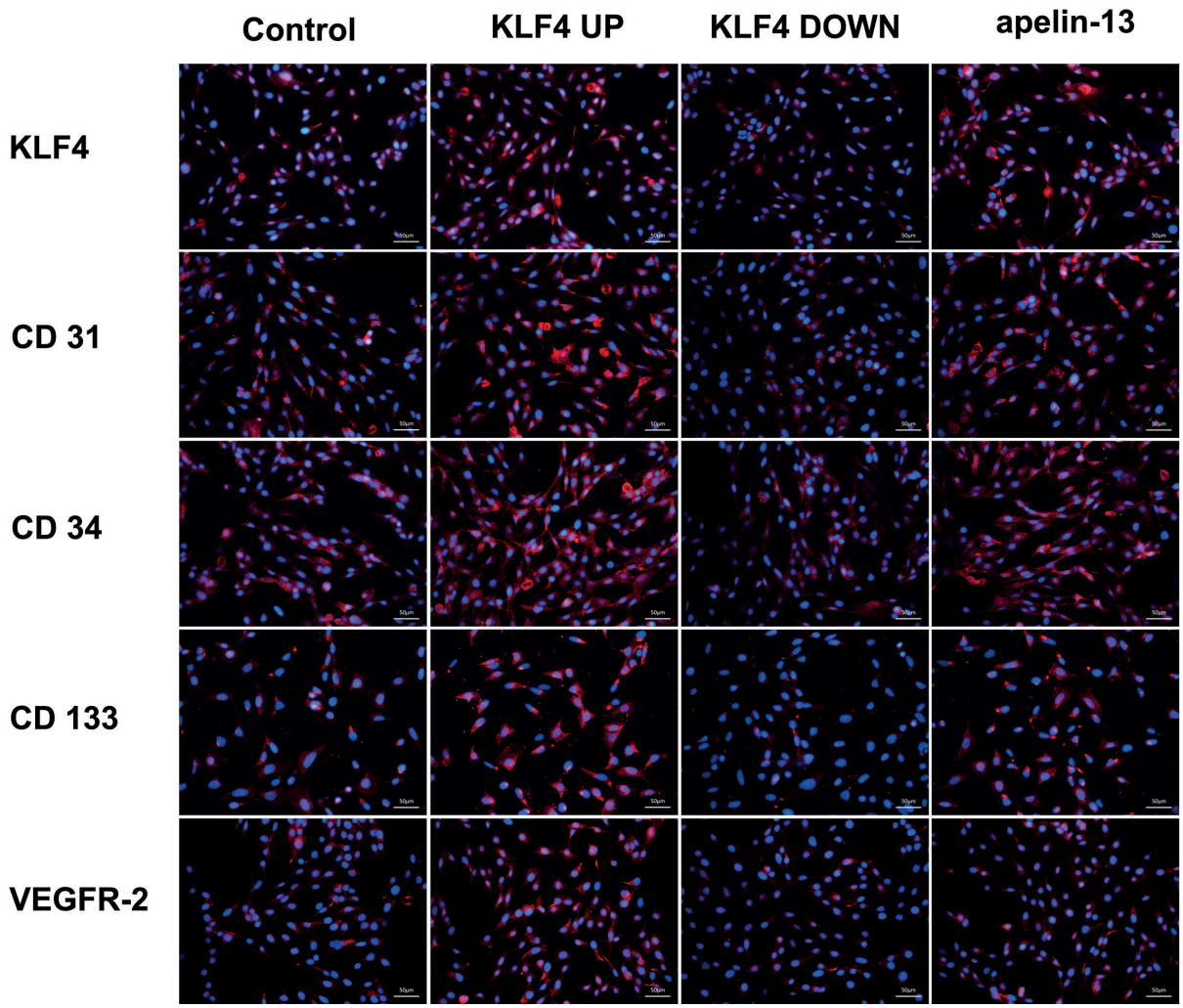

Figure 4. Immunofluorescence staining of KLF4, CD31, CD34, CD133, and VEGFR-2 in the four groups. The blue dots represented the nuclei and the red fluorescence from top to bottom were markers of KLF4, CD31, CD34, CD133, and VEGFR-2. Scale bar = 20 μm. Magnification: ×200.

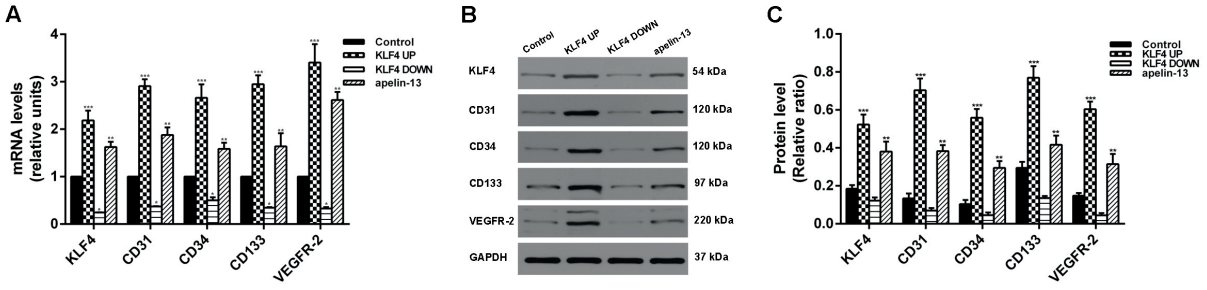

Figure 5. Expressions of KLF4, CD31, CD34, CD133, and VEGFR-2 on mRNA and protein level. ***A,*** Relative mRNA expressions of KLF4, CD31, CD34, CD133, and VEGFR-2 in four groups. ***B,*** Western blot assay indicated the expression of the above markers elevated in the KLF4 upregulation group and apelin-13 groups. ***C,*** Relative gray ratio of KLF4, CD31, CD34, CD133, and VEGFR-2 protein bands in the above Western blot. $^{**}p<0.01$ as compared to negative control group, $^{***}p<0.001$ as compared to negative control group.

esis are under intensive investigation, detailed molecular pathways of differentiation focusing on lEPCs are lacking.

KLF4 is a member of the Sp/KLF family with evolutionarily conserved zinc finger-containing transcription factors which regulates many biological processes, including cell growth, proliferation, and differentiation[29-31]. Previous studies[32,33] indicated that KLF4 is highly expressed in epithelial tissues such as the intestine and skin. However, the importance of KLF4 in stem cells has become a central issue in the last years[34,35]. In our study, we observed that the elevation of KLF4 directly causes the changes of EPCs surface markers, CD31, CD34, and VEGFR-2, and the elevation of these surface molecules expression is considered to be a feature of differentiation from eEPCs to lEPCs[13]. For CD133, conflicting reports existed on its expression of lEPCs[36-38], and in our study the increased expression of CD133 was detected on the surface of lEPCs as other markers after KLF4 and apelin-13 elevation. In addition, Boriushkin et al[39] showed that in the mouse retinal angiogenesis model, the overexpression of KLF4 in transgenic mice resulted in an increased number of vessels, as well as branching and tip cell filopodia density.

A series of experiments have been conducted to confirm that the cells-based therapy with apelin-13 and hyperbaric oxygen efficiently promote neovascularization *in vivo*[26]. In that study, we noticed the neovascular number of ischemic limbs in the apelin-13 with BM-MNCs transplantation group was twice than that of BM-MNCs transplantation group alone, and suggested that apelin-13 was closely related to ECs formation. In this study, we found that the elevation of KLF4 and apelin-13 contributed to increased expressions of EPCs surface markers, and when we raised the apelin-13 we detected an elevation of KLF4. Blann et al[40] found that when EPCs differentiated into ECs, the vascular endothelial growth factor (VEGF) activated PI3K/AKT pathway by phosphorylation by binding to its two tyrosine kinase receptors, VEGFR-1 and VEGFR-2, to regulate ECs activity and promote endothelial nitric oxide synthase (eNOS) expression which is closely related to the function of ECs. Wang Y. et al[41] have detected that KLF4 is a positive regulator of proliferation, migration, and tube formation in human retinal microvascular endothelial cells via upregulation of the VEGF levels. In our study, the VEGFR-2 expression was significantly increased by the elevation of KLF4 and apelin-13. Therefore, we suggested that apelin-13 regulate eEPCs to lEPCs differentiation by activating KLF4 or synergistically with it through the PI3K/AKT/eNOS pathway to promote ECs maturation.

In addition, the regulation of the cell cycle is one of the critical mechanisms in tissue specification, organ homeostasis and disease progression. These processes were finally regulated by the organization between proliferation and differentiation of specific stem cells or progenitors. It was reported that KLF4 suppresses the p53-dependent apoptotic pathway by directly inhibiting TP53 and suppressing the BAX expression[42,43]. However, Wang B. et al[44] showed that KLF4 induced apoptosis and inhibited tumorigenic progression in SK-BR-3 breast cancer cells. Together, KLF4 is considered to switch its role from anti-apoptotic to pro-apoptotic under certain conditions[45,46]. In this study, we noticed that under the circumstance of apelin-13 elevation, the expression of KLF4 increased while the EPCs number of G1, S, and G2/M phases in their cell cycle decreased, which

indicated an active cell proliferation. Considering that the KLF4 is context-dependent in cell proliferation and apoptosis, we believed apelin-13 contributed to the anti-apoptotic condition and promoted the EPCs differentiation process by KLF4.

Conclusions

We demonstrated that cell therapy is an important treatment for ischemic diseases, and the differentiation of EPCs is the key. We also showed that the elevation of apelin-13 significantly increased the expressions of EPCs surface markers that were involved in eEPCs differentiation into lEPCs and influenced the cell cycle. Considering that the increase of apelin-13 caused an elevation of KLF4, we believe that apelin-13 promoted this process by activating or synergizing with KLF4. This study provides valuable information to explore the mechanism of EPCs differentiation and may help in the development of more efficient treatment approaches in the future.

Acknowledgements

This study was supported by the Ningxia Higher Education Research Project (No. NGY2017085) and Hubei Provincial Natural Science Foundation of China 2017CFB122.

Conflict of Interests

The authors declare that they have no conflict of interests.

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