SDF-1α/CXCR4 signaling promotes capillary tube formation of human retinal vascular endothelial cells by activating ERK1/2 and PI3K pathways *in vitro*

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Abstract. The purpose of this study is to address the effect and mechanism of stromal cell-derived factor-1 (SDF-1) α/chemokine (C-X-C motif) receptor 4 (CXCR4) signaling on capillary tube formation of human retinal vascular endothelial cells (HRECs). The expression of CXCR4 in HRECs was quantified by reverse transcription (RT-PCR) and western blotting. The effects of SDF-1α/CXCR4 signaling in capillary tube formation and migration of HRECs was examined using three-dimensional Matrigel assay and wound scratching assay respectively *in vitro*. Cell proliferation of HRECs was examined using cell counting kit (CCK)-8 assay in the presence of different concentrations of SDF-1α protein. The effect of SDF-1α/CXCR4 signaling in HREC expression of VEGF, basic fibroblast growth factor (bFGF), IL-8 and intercellular cell adhesion molecule (ICAM)-1 was examined using RT-PCR and western blotting. RT-PCR and western blot analysis revealed CXCR4 was expressed in HRECs. The number of intact capillary tubes formed by HRECs in the presence of SDF-1α was markedly more compared with a PBS treated control group. However, it was reduced with treatment with an CXCR4 antagonist. Wound scratching assay showed a significant increase in the number of migrated HRECs under SDF-1α stimulation and the number was reduced with treatment with an CXCR4 antagonist. RT-PCR and western blotting showed that SDF-1α significantly promoted VEGF, bFGF, IL-8 and ICAM-1 expression in HRECs. The proliferation of HRECs in the presence of SDF-1α was promoted in a dosage-dependent manner. SDF-1α/CXCR4 signaling can increase HREC capillary tube formation through promoting HREC migration, proliferation and expression of VEGF, bFGF, IL-8 and ICAM-1.

Introduction

Retinal pathological neovascularization is a primary characteristic of neovascular age-related macular degeneration, retinopathy of prematurity, retinal vein occlusion and diabetic retinopathy and it is the main cause of refractory blindness worldwide (1,2). Typical choices for treatment of retinal neovascularization involve vitrectomy, retinal laser photocoagulation and cryotherapy; however, they are invasive and the functional results are typically suboptimal (3-6). Vascular endothelial growth factor (VEGF) and its receptors have been shown to be pivotal for the production and advancement of neovascular eye diseases; they have therefore become the ideal targets for anti-angiogenesis therapy (7). However, anti-VEGF agents can also induce local and systemic side effects (8). Therefore, to create novel therapeutic targets, it is necessary to obtain fuller knowledge of the mechanisms of ocular neovascularization (6).

The pathogenesis for ocular vascular diseases is associated with hypoxia, chronic inflammation and high level of angiogenic factors such as VEGF, platelet-derived growth factor-B (PDGF-B) and stromal cell-derived factor-1 (SDF-1)α (9). VEGF is pivotal in retinal neovascularization prompted by hypoxia-induced retinal injury. Hypoxia in the retina causes compensatory alterations in blood flow, the overexpression of cytokines and angiogenesis (10,11). Currently, the lack of efficacious anti-VEGF treatments could be due to the impacts of this treatment on the HIF pathway-mediated expression of other pro-angiogenic factors, including PDGF-B, insulin-like growth factor 1, erythropoietin and SDF-1 (12,13).

SDF is part of the CXC subfamily of chemokines and it was first cloned from murine bone marrow and described as a pre-B cell growth stimulating factor (14). The chemokine receptor, chemokine (C-X-C motif) receptor 4 (CXCR4),

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was first cloned as an orphan chemokine receptor and it was determined to be expressed on numerous cell types, including monocytes, lymphocytes and hematopoietic and endothelial progenitor cells (15-18). CXCR4 regulates numerous activities, including chemotaxis, adhesion, proliferation and survival (19). Moreover, CXCR4 is also detected in endothelial cells, which suggests a possible role for SDF-1α/CXCR4 cell signaling in angiogenesis (20). Considerable evidence suggests that SDF-1α/CXCR4 signaling is involved in the process of pathological neovascularization (21-29). Jin *et al* (21) report SDF-1α is implicated in revascularization of ischemic hind limbs through recruitment of CXCR4+ hemangiocytes. Our previous studies found that CXCR4 expressed on leukocytes, such as monocytes, stimulates monocytes chemotaxis, resulting in recruitment of leukocytes to inflammatory sites; SDF-1α-treated mice exhibited enhanced alkali-induced corneal neovascularization through enhanced intracorneal progenitor cells infiltration and increased macrophage VEGF expression (22,23).

CXCR4/SDF-1α signaling is pivotal in the progression of a few types of ocular neovascularization, such as corneal neovascularization, diabetic retinopathy and oxygen-induced ischemic retinopathy (22-29), but the precise mechanism of its effects in ocular neovascularization still needs further exploration. In the present study, HREC bio-functions were examined in SDF-1α recombinant protein or CXCR4 antagonist treated groups and was compared with control group *in vitro*. The expression of angiogenic factors and transcription factors in HRECs were detected and compared. The present study provided the definitive evidence of critical role of SDF-1α/CXCR4 signaling in HREC behavior of tube formation, proliferation and migration.

Materials and methods

Reagents and antibodies. CXCR4 antagonist (AMD3100 octahydrochloride, cat. no. 3299/50) was purchased from Tocris Bioscience. Recombinant human SDF-1α (CXCL12) protein (cat. no. 350-NS-050) was purchased from R&D Systems. CCK-8 kit was purchased from Dojindo Laboratories, Inc. Trypsin-EDTA was purchased from MilliporeSigma. Rabbit anti-bFGF, VEGF, IL-8 and ICAM-1 antibodies were purchased from Santa Cruz Biotechnology, Inc. Primers were synthesized by GeneScript. Total RNA extraction kit and reverse transcription kit were purchased from Qiagen Sciences, Inc. D2000 DNA Ladder (cat. no. M1060) was purchased from Solarbio. Gelred nucleic acid stain (cat. no. SCT123) was purchased from Sigma-Aldrich. Matrigel was purchased from Becton, Dickinson and Company. Dulbecco's modified Eagle medium (DMEM) was purchased from HyClone (Cytiva). Fetal bovine serum (FBS) was purchased from PAA laboratories (Cytiva). Mouse anti-human GAPDH antibody (cat. no. AF0006, 1:1,000), HRP-labeled goat anti-mouse IgG(H+L) (cat. no. A0216, 1:1,000) and HRP-labeled goat anti-rabbit IgG(H+L) (cat. no. A0208, 1:1,000) was purchased from Beyotime Institute of Biotechnology. APC-conjugated mouse anti-human CD106 antibody (cat. no. ab103173) and Alexa Fluor 700-conjugated mouse anti-human CD54 antibody (cat. no. ab275944) were purchased from Abcam. Rabbit anti-Erk 1,2 monoclonal antibody (cat. no. orb178404; Clone B20-U;

1:5,000), Rabbit anti-phosphorylated (p-) ERK 1,2 monoclonal antibody (cat. no. orb178405; Clone G15-B; 1:5,000), rabbit anti-PI3K P85 (phospho-Tyr467) polyclonal antibody (cat. no. orb14998, 1:5,000) and rabbit anti PI3K polyclonal antibody (cat. no. orb1089274, 1:1,000) were purchased from Biorbyt Ltd. Human retinal vascular endothelial cells (HRECs; cat. no. YS0884) were purchased from Yaji Biological Technology Co., Ltd.

Cell culture and treatment of HRECs. The HRECs were cultivated with DMEM (HyClone; Cytiva) containing 10% (v/v) FBS, 100 μg/ml streptomycin and 100 U/ml penicillin (HyClone; Cytiva) and incubated in an incubator under humid, 5% CO_2 and 37°C conditions (30). The HRECs were exposed to PBS treated control group, SDF-1α groups, in which 10, 50, 100 and 200 ng/ml of recombinant human SDF-1α protein were added and CXCR4 antagonist groups, to which SDF-1α protein (200 ng/ml) combined with CXCR4 antagonist were added (1 nmol/ml). The HRECs were passaged by trypsinization at ~90% confluence and subcultured in either 6-well or 96-well plates with the SDF-1α protein and/or the CXCR4 antagonist for either 12 h or 24 h depending on the assay conditions. The cells cultured were all at 37°C.

Cell migration assay. Cell horizontal migration ability was detected by wound healing assay for assessment of the effects of SDF-1α/CXCR4 signaling on the migration of HRECs, as described in detail previously (31). The cells were seeded in a 6-well plate and scratched with a 100 μl pipette tip to obtain scratches of a constant width when cells reached ~80% confluence. After scratching, the well was gently washed twice with PBS to remove the detached cells. Fresh serum-free medium (DMEM) was then added into each well. The cells were then treated with human recombinant SDF-1α protein or SDF-1α protein plus CXCR4 antagonist in the experimental wells, whereas the control wells were treated with PBS. Images were captured of the cells invading the wound line at 0, 12 and 24 h with Olympus TMS inverted phase contrast microscope (Olympus Corporation) and measured distances traveled by the cells from the wound edge to the cell-free space to calculate the migration rate.

Cell proliferation assay. HREC proliferation was analyzed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) (32). The cells were diluted and seeded in a 96-well plate at a density of 8,000 cells/well in 100 μl of DMEM with 10% FBS and different concentrations of SDF-1α protein or SDF-1α protein plus CXCR4 antagonist. After incubation at 37°C for 24 h, 10 μl CCK-8 was added to each well and incubated at 37°C for 2 h in an incubator. Subsequently, absorbance was measured at a wavelength of 450 nm using a microplate reader (Thermo Multiskan EX plate reader; Thermo Fisher Scientific, Inc.).

Tube formation assay. A tube formation assay was performed to assess the effect of SDF-1α/CXCR4 signaling on HRECs. In brief, Matrigel (50 μl/well; Becton, Dickinson and Company) was applied to a 96-well plate and plate was put into a 37°C incubator for 30 min. Then, HRECs were seeded onto the gel and kept for 6 h at 37°C condition. Image-Pro Plus 6.0

Table I. Sequences of primers used for reverse transcription PCR.

Primers	Sequence (5'→3')	Product Size (bp)	Annealing temperature (˚C)	PCR Cycle (sec)
CXCR4	F: TGTCCATTCCTTTGCCTCTTTTG	1,020	57	37
	R: GTCCACCTCGCTTTCCTTTG			
VEGF	F: CTTGCTGCTCTACCTCCACC	118	60	40
	R: GCAGTAGCTGCGCTGATAGA			
bFGF	F: CAAGCGGCTGTACTGCAAAA	100	60	40
	R: TAGCTTGATGTGAGGGTCGC			
IL-1β	F: GCAGAAGTACCTGAGCTCGC	109	60	40
	R: CCTGGAAGGAGCACTTCATCT			
IL-6	F: CAATAACCACCCCTGACCCA	106	60	40
	R: AAGCTGCGCAGAATGAGATG			
IL-8	F: GGTGCAGTTTTGCCAAGGAG	117	60	40
	R: GTGTGGTCCACTCTCAATCACT			
ICAM-1	F: CCAGGAGACACTGCAGACAG	100	60	40
	R: CTTCACTGTCACCTCGGTCC			
IL-18	F: TGACCAAGGAAATCGGCCTC	117	60	40
	R: GCCATACCTCTAGGCTGGCT			
TNF-α	F: GCTGCACTTTGGAGTGATCG	119	60	40
	R: CTACAGGCTTGTCACTCGGG			
MCP-1	F: GATCTCAGTGCAGAGGCTCG	105	60	40
	R: TCAGCACAGATCTCCTTGGC			
ZO-1	F: TCAAAGGGAAAGCCTCCTGA	108	60	40
	R: ATACTGCGAGGGCAATGGAG			
VE-cadherin	F: CTTCACCCAGACCAAGTACACA	113	60	40
	R: ACTTGGTCATCCGGTTCTGG			
GAPDH	F: CAAATTCCATGGCACCGTCA	108	60	40
	R: GCATCGCCCCACTTGATTTT			

F, forward primer; R, reverse primer.

(Media Cybernetics, Inc.) was used for imaging, followed by statistical analyses on tube number. Each experiment was conducted in triplicate.

Reverse transcription-quantitative (RT-q)PCR. RT-qPCR was used to analyze the transcript levels of CXCR4, VEGF, bFGF, IL-1β, IL-6, IL-8, ICAM-1, IL-18, TNF-α, monocyte chemotactic protein 1, zonula occludens-1 and VE-cadherin [33]. The total RNA was extracted from HRECs ($2x10^5$ cells) using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA concentration and the absorbance values on A260 and A280 nm were measured by Nanodrop Nd-1000 spectrophotometer (Invitrogen; Thermo Fisher Scientific, Inc.) and the OD260/OD280 ratio of RNA was between 1.8 and 2.0, which could be used as a template for reverse transcription. Thereafter, complementary (c)DNA was generated via RT reaction by using a PrimeScript first strand cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Subsequent qPCR was performed using SYBR green reagent (Takara Biotechnology Co., Ltd.) on an ABI 7000 PCR instrument (Thermo Fisher Scientific, Inc.). The 20 μl PCR reaction mixture consisted of 10 μl SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.), 2 μl cDNA template, 0.8 μl Primer (0.4 μl each forward and reverse) and 7.2 μl dH$_2$O. The primer pairs used are listed in Table I. All primers used were purchased from Genescript. PCR was performed by initial denaturation at 95˚C for 10 min, followed by 40 cycles of 10 sec at 95˚C and 20 sec at 50˚C and a final extension of 25 sec at 72˚C. The relative mRNA levels were measured by quantification cycle values using the $2^{-\Delta\Delta Cq}$ method [34]. Data display the average of triplicate experiments.

Western blot analysis. Immunoblotting analysis was adopted for the detection of HRECs expression levels of VEGF, bFGF, IL-8, ICAM and ERK1/2 as well as associated phosphorylated signaling proteins of ERK1/2 and PI3K. 6-well plates were used to culture the HRECs ($2.5x10^5$ cells) in DMEM containing 10% FBS. The cell medium was replaced with the media without serum for another 24 h once 95% cell confluence was achieved. Then, the starved cells were incubated for another 24 h in serum-free DMEM with human recombinant SDF-1α protein or SDF-1α protein plus CXCR4 antagonist. The treated cells were washed twice using chilled PBS. Then, protein lysate (Beyotime Institute of Biotechnology) was added to each well, prior to collecting the proteins on ice. Lysate protein concentrations were evaluated using the BCA method (Beyotime Institute of Biotechnology). SDS-PAGE was performed using in-house produced 10% gels. Equal

amounts of protein (50 μg) were loaded per lane. The separated proteins were transferred onto PVDF membranes (0.45 μm) purchased from MilliporeSigma and were blocked with 5% skimmed milk dissolved in 1X TBS containing 0.3% Tween-20 for 1.5 h at room temperature to inhibit endogenous reactions. The membranes were then incubated with the blocking buffer-diluted primary antibodies overnight at 4°C. After rinsed the following day using a Tris-HCl (pH 7.4) buffer (20 mM) as well as Tween-20 (0.1%), membranes were incubated again at room temperature with the corresponding secondary antibodies bound to horseradish peroxidase for 1 h. The protein blots were promptly visualized using a 1 Tanon-5200 Multi-imaging System after treatment using an enhanced chemiluminescence (ECL) kit obtained from Tanon Science and Technology. The relative protein levels were quantified by ImageJ (version 1.5, National Institutes of Health). The experiments were performed in triplicate.

Flow cytometry. Flow cytometry was performed to identify the feature of HRECs. The HRECs were seeded in 60 mm wells (1x10⁶ cells) cultured with DMEM containing 10% FBS. When 95% cell confluence was achieved, the cells were harvested by trypsinization for staining. After washing twice using chilled PBS, the cells (1x10⁶ per 100 μl) were co-stained with 10 μl APC-conjugated mouse anti-human CD106 and Alexa Fluor 700-conjugated mouse anti-human CD54 antibodies or IgG isotype as control for 30 min at 4°C. After washing with PBS, the cells were analyzed with Beckman coulter FC500 Flow Cytometer. Data were analyzed using FlowJo 7.6 (Tree Star). The experiments were performed in triplicate.

Statistical analysis. SPSS 20.0 (IBM Corp.) was employed to perform all data analyses. All data were depicted as mean ± standard error (number of observations). Comparisons between variables were carried out by a two-tailed unpaired Student's t test. Comparisons among multiple datasets were performed by one-way analysis of variance (ANOVA) and Bonferroni correction was used to adjust P-values for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

The expression of CXCR4 in HRECs. First, the expression of CXCR4 in HRECs was examined. CXCR4 mRNA and protein expression were detected in HRECs. The expression of CXCR4 in HRECs suggested the possible involvement of the SDF-1α/CXCR4 interactions in the biological function of HRECs. Additionally, the expression of CD106 and CD54 in HRECs was examined using flow cytometry to identify the characteristic of HRECs (Fig. 1).

Effects of SDF-1α/CXCR4 signaling on cell proliferation. To determine the effects of SDF-1α/CXCR4 signaling on vascular endothelial cell bio-function, the effect of SDF-1α/CXCR4 signaling in cell proliferation of HRECs *in vitro* was assessed. After incubation with SDF-1α or SDF-1α plus CXCR4 antagonist for 24 h, cell viability was evaluated. HRECs incubated with SDF-1α showed a significant increasing in cell
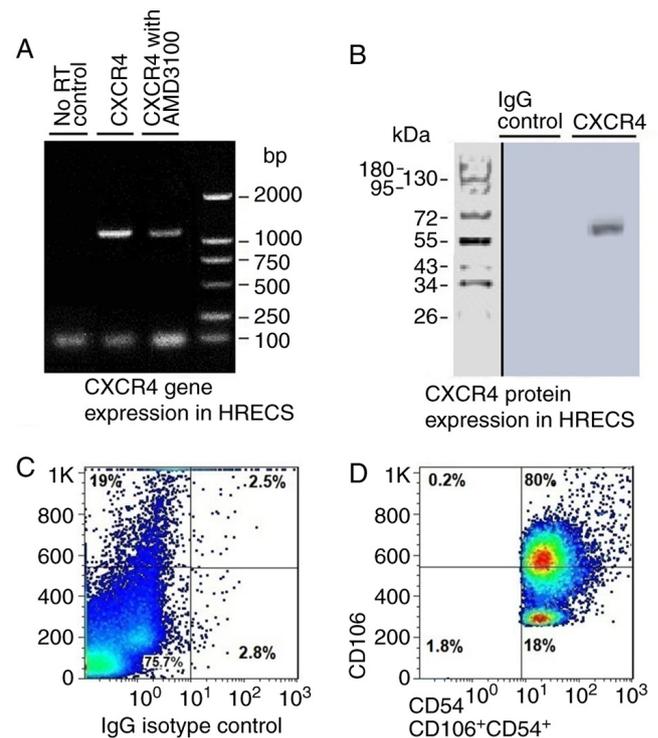
Figure 1. CXCR4 gene and protein expressions in HRECs. (A) Semi-quantitative RT-PCR to evaluate mRNA expression of CXCR4. HRECs were harvested to extract total RNAs. RT-PCR was performed using the obtained total RNAs. (B) Western blotting was used to evaluate protein expression of CXCR4. HRECs were harvested to extract the total protein (n=3). (C) IgG isotype control performed by flow cytometry. (D) Flow cytometry to examine the expression of CD106 and CD54 in HRECs. CXCR, chemokine (C-X-C motif) receptor; HREC, human retinal endothelial cell; RT-PCR, reverse transcription-PCR.
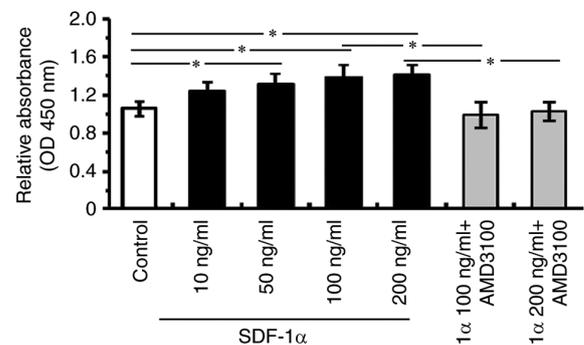
Figure 2. The effects of SDF-1α/CXCR4 signaling on HREC proliferation. CCK-8 assays revealed that cell proliferation in the SDF-1α groups was significantly enhanced compared with the control. However, the proliferation of HRECs was significantly suppressed by AMD3100 compared with 100 ng/ml SDF-1α group and 200 ng/ml SDF-1α group. Mean ± standard error of the mean (n=3). *P<0.05. SDF, stromal cell-derived factor; CXCR, chemokine (C-X-C motif) receptor; HREC, human retinal endothelial cell, OD, optical density.

proliferation compared with the control, while HRECs incubated with CXCR4 antagonist after precondition with SDF-1α showed a significant reduction in cell proliferation compared with 100 ng/ml or 200 ng/ml SDF-1α groups (Fig. 2). Optical density (OD) value quantification demonstrated that
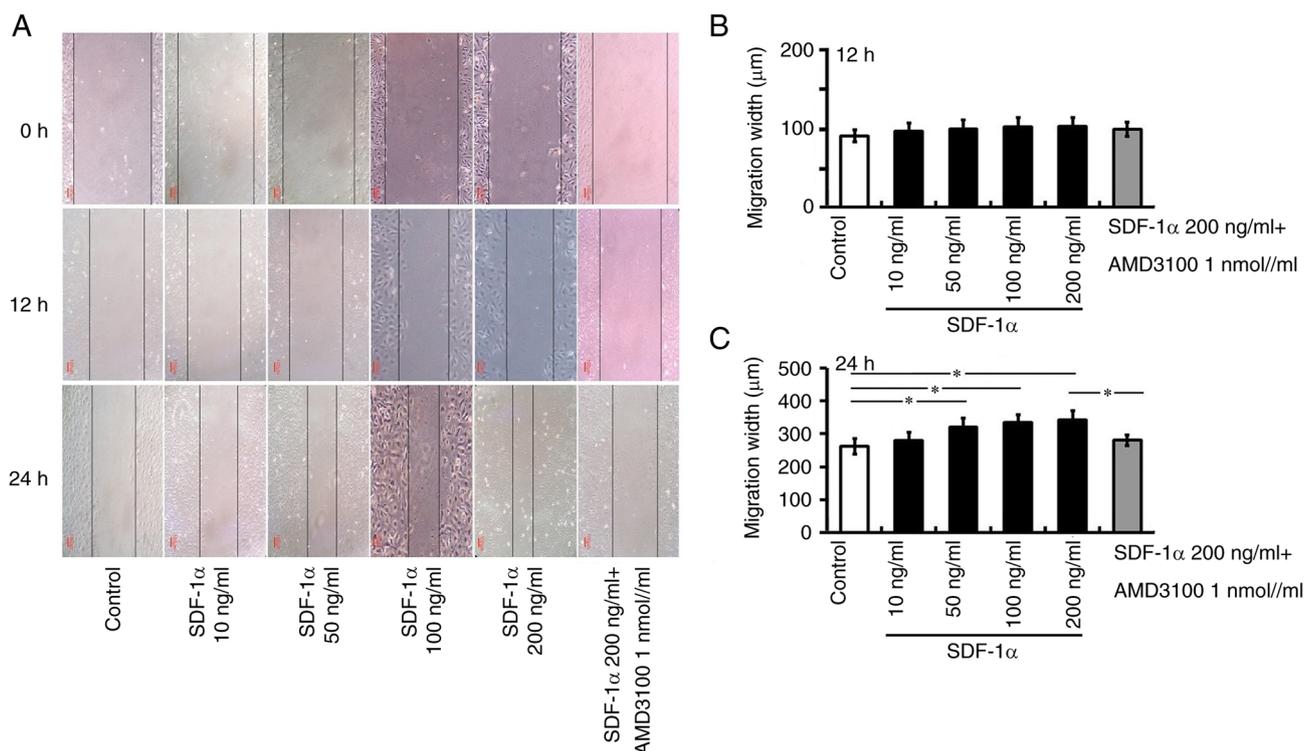
Figure 3. The effects of SDF-1α/CXCR4 signaling on cellular migration of HRECs. (A) Representative results for the effects of SDF-1α/CXCR4 signaling on cell migration using wound scratching assay. Magnification, x200. Data of cell migration were quantified in width at (B) 12 h (non-significant) and (C) 24 h after wound scratching. All values represent mean ± standard error of the mean (n=3). *P<0.05. SDF, stromal cell-derived factor; CXCR, chemokine (C-X-C motif) receptor; HRECs, human retinal endothelial cells.

SDF-1α/CXCR4 signaling was capable of promoting cell proliferation. These data indicated that an enhancement in proliferation of HRECs after SDF-1α stimulation was responsible for the promotion effect of SDF-1α/CXCR4 signaling on tube formation of HRECs *in vitro*. In addition, the elevating of cell proliferation of HRECs peaked in the 200 ng/ml SDF-1α group when compared with other groups including 10, 50 and 100 ng/ml SDF-1α groups as well as 500 ng/ml SDF-1α group or other higher concentration groups, so the dose of 200 ng/ml SDF-1α was chosen as the intervention concentration to treat HRECs in subsequent protein examining experiment and three-dimensional Matrigel vascular tube formation assay.

Effects of SDF-1α/CXCR4 signaling on cell migration. The effects of SDF-1α/CXCR4 signaling on HREC migration have yet to be reported, to the best of the authors' knowledge. To evaluate whether SDF-1α/CXCR4 signaling affects the process of the migration of HRECs, a scratch wound assay was performed *in vitro* to measure the migration property of HRECs in different concentration of SDF-1α or SDF-1α combined with CXCR4 antagonist. As shown in Fig. 3, compared with control group, a significantly accelerate wound closure was shown in the group treated with SDF-1α and the wound almost closed at 24 h after injury. However, the wound area was still wide in HRECs with CXCR4 antagonist treatment after precondition with SDF-1α at 24 h. The quantitative data of the migration distance were shown in Fig. 3B and C. Those data showed that SDF-1α/CXCR4 signaling had the potential of promoting the migration property of HRECs.

Effects of SDF-1α/CXCR4 signaling on tube formation of HRECs. To determine whether SDF-1α/CXCR4 signaling plays a role in the process of tube formation of HRECs, HREC cell line was seeded in Matrigel-coated 96-well plates. After incubation for 12 h, the cells were able to form tubes. HRECs incubated with SDF-1α showed a significant increasing in tube formation compared with control cells (Fig. 4). After incubated with SDF-1α and CXCR4 antagonist, the number of tubes formed was significantly reduced. Tube formation quantification and statistical analysis demonstrated SDF-1α/CXCR4 signaling was able to promote HREC tube formation.

Enhanced angiogenic factors, p-ERK1/2 and p-PI3K expression in SDF-1α treated HRECs. The balance between angiogenic and anti-angiogenic elements establishes the results of angiogenesis operations in different situations (35). Therefore, the mRNA and protein expression of angiogenic factors in HRECs were next determined. Among the angiogenic associated factors, such as VEGF, bFGF, IL-8 and ICAM-1, which were detected, the mRNA expression of *VEGF, bFGF, IL-8* and *ICAM-1* were increased in SDF-1α treated cells compared with control groups (Fig. 5). VEGF, bFGF, IL-8 and ICAM-1 protein expression also revealed that VEGF, bFGF, IL-8 and ICAM-1were increased by the treatment with SDF-1α compared with PBS-treatment (Fig. 6). These analyses indicated that the SDF-1α treatment elevated the expression of the angiogenic factors, VEGF, bFGF, IL-8 and ICAM-1 and as a result, overturned the balance to encourage angiogenesis.

The present study also examined p-ERK1/2 and p-PI3K expression in HRECs. PI3K and ERK1/2 activation are
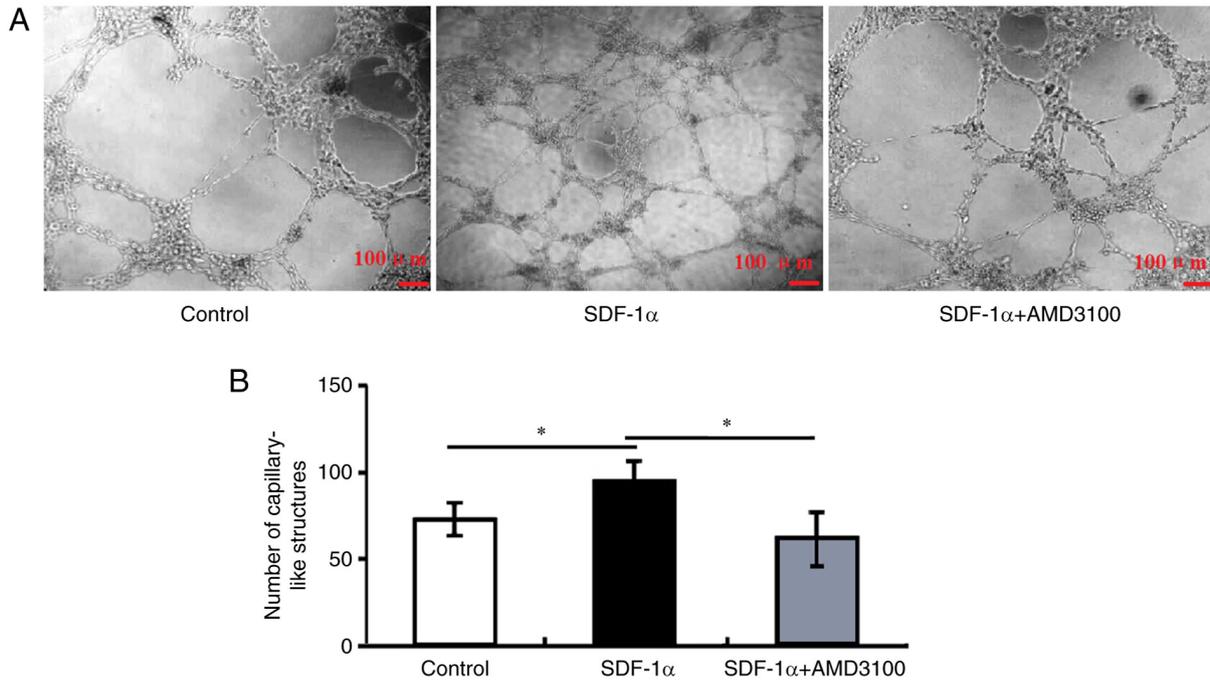
Figure 4. The effects of SDF-1α/CXCR4 signaling on the capillary-like tube formation of HRECs. (A) The representative results for HREC capillary like structure formation. The tube formation assays showed that CXCR4 significantly promoted the formation of capillary-like structures. Magnification, x200. (B) The plot of statistical analysis for numbers of capillary-like structures of HRECs. All values represent mean ± standard error of the mean (n=3). *P<0.05. SDF, stromal cell-derived factor; CXCR, chemokine (C-X-C motif) receptor; HRECs, human retinal endothelial cells.

integral components of pro-angiogenic signaling pathway and promotes endothelial migration and proliferation. The present study sought to determine whether SDF-1α/CXCR4 signaling had effects on cell migration and proliferation through activation of ERK1/2 and PI3K in HRECs. It was found that p-ERK1/2 and p-PI3K expression were markedly increased in SDF-1α treated HRECs (Fig. 7). These results suggested that SDF-1α induces ERK1/2 and PI3K activation and therefore promoted angiogenesis.

Discussion

The authors have previously documented that SDF-1α-treated mice showed improved alkali-induced corneal neovascularization via amplified intracorneal progenitor cell infiltration and elevated VEGF expression by macrophages, while SDF-1α neutralizing antibody- or CXCR4 antagonist-treated mice demonstrated impeded experimental alkali-induced corneal neovascularization via downregulated VEGF and C-Kit expression (22,23). The results provided evidence that SDF-1α/CXCR4 signaling is implicated in corneal neovascularization and its potential of pro-angiogenesis may be through indirect effects of promoting VEGF secreting by intracorneal macrophages and C-Kit positive progenitor cell migration. In addition, various evidence indicate that SDF-1α/CXCR4 signaling may have direct effects on vascular endothelial biofunction (36-39). However, further exploration on the mechanism of these direct effects is required. In order to delineate the direct effects of SDF-1α/CXCR4 signaling on vascular endothelial function of proliferation, migration and tube formation, the present study performed an *in vitro* study using HRECs to evaluate SDF-1α/CXCR4 signaling directed pro-angiogenesis efficacy.

The present study showed that SDF-1α/CXCR4 signaling has the ability to increase tube formation of HRECs by promoting HREC proliferation and migration and VEGF, bFGF, IL-8 and ICAM-1 production of HRECs. These results indicated that SDF-1α/CXCR4 signaling has pro-angiogenesis property not only through activating cell types of monocytes/macrophages but also through activating vascular endothelial migration, proliferation and pro-angiogenic cytokine secretion. Thus, the data verified the hypothesis that SDF-1α/CXCR4 signaling has an important role in angiogenesis through indirect and direct pathways, which had not been confirmed in our previous study (22,23).

Endothelial migration and proliferation are initial steps for angiogenesis (40). Any effects on these two steps may subsequently have an impact on vascular tube formation (40). Various studies indicate that VEGF, bFGF, IL-8 and ICAM-1 are involved in vascular endothelial migration and proliferation. VEGF and bFGF as well as other pro-angiogenic cytokines promote the process of vascular endothelial migration and proliferation while ADAMTS-1 and TSP-1 inhibit these processes (41,42). SDF-1/CXCR4 signaling promotes angiogenesis through multiple pathways, including recruiting macrophages, c-Kit positive cells and stroma cells and elevating expression level of pro-angiogenic factors by macrophages and stroma cells (43). The present study also examined the effects of SDF-1/CXCR4 signaling on HREC migration and proliferation (22,23). Consistent with the hypothesis of the present study, in SDF-1α stimulating groups, both HRECs migration width and proliferation rate were greater compared with those in the PBS treated group, while in the CXCR4 antagonist-treated groups, HRECs migration width
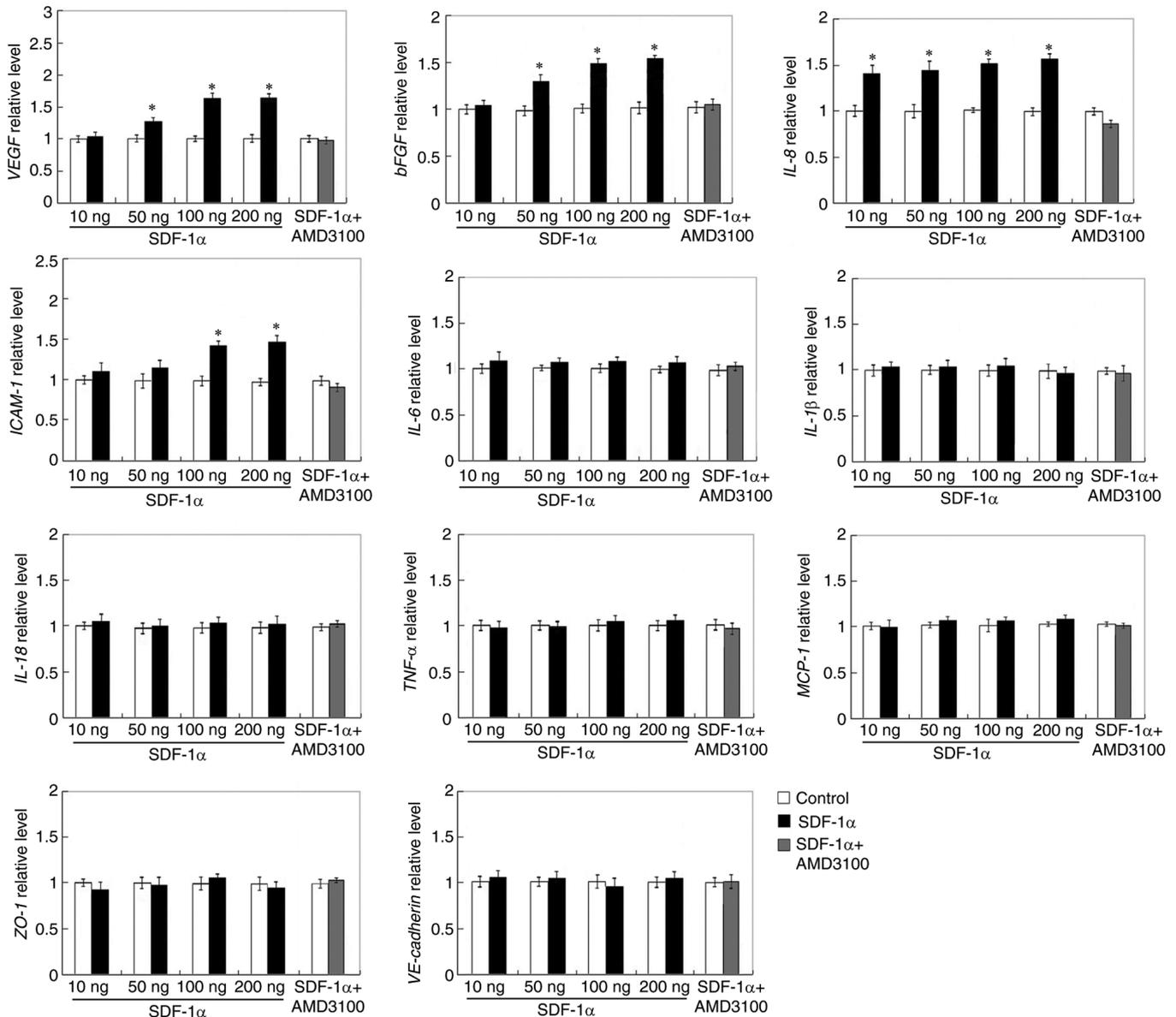
Figure 5. Effect of SDF-1α/CXCR4 signaling on angiogenic gene expression of HRECs. Statistical analysis of ratios of target genes to GAPDH of control groups (open bars), SDF-1α groups (black bars) and CXCR4 antagonist groups (grey bars) were determined by reverse transcription-PCR. All values represent mean ± standard error of the mean (n=3). *P<0.05 vs. control. SDF, stromal cell-derived factor; CXCR, chemokine (C-X-C motif) receptor; bFGF, basic fibroblast growth factor; ICAM, intercellular cell adhesion molecule; MCP-1, monocyte chemotactic protein 1; ZO-1, zonula occludens-1.

and proliferation rate were reduced more compared with the SDF-1α treated groups. This indicated that directly promoting endothelial migration and proliferation would be another crucial pathway for SDF-1/CXCR4 signaling implicated in the process of angiogenesis.

The process of angiogenesis is precisely modulated by a series of pro- and anti-angiogenic molecules under physiologic condition, while under pathologic condition, the expression balance upset, serious consequences, such as neovascularization, may occur (44). Angiogenic factors, such as VEGF and bFGF have strong efficacy in stimulating blood vessel formation (45). These factors are expressed by various cells, including fibroblasts, macrophages, neutrophils and also by vascular endothelial cells themselves (46). The present study detected the mRNA and protein expression of VEGF, bFGF, IL-8, ICAM-1 and other cytokines in HRECs

and the results showed that the expression of VEGF, bFGF, IL-8 and ICAM-1 in SDF-1α treated cells were significantly higher compared with control cells. It indicated that SDF-1/CXCR4 signaling is implicated in the process of angiogenesis by altering pro-angiogenic milieu and thereby causing neovascularization (47,48). These results are consistent with other reports (49-51), which report that SDF-1α promotes pro-angiogenic cytokine expression in endothelial cells. The previous reports and the results of the present study imply that SDF-1/CXCR4 signaling would be a candidate for treating vascularization diseased by blocking or silencing the signaling.

To explore the mechanisms of how SDF-1/CXCR4 signaling mediated HREC capillary tube formation, the present study also evaluated the influence of SDF-1/CXCR4 signaling on signal expression of PI3K/Akt and ERK1/2.
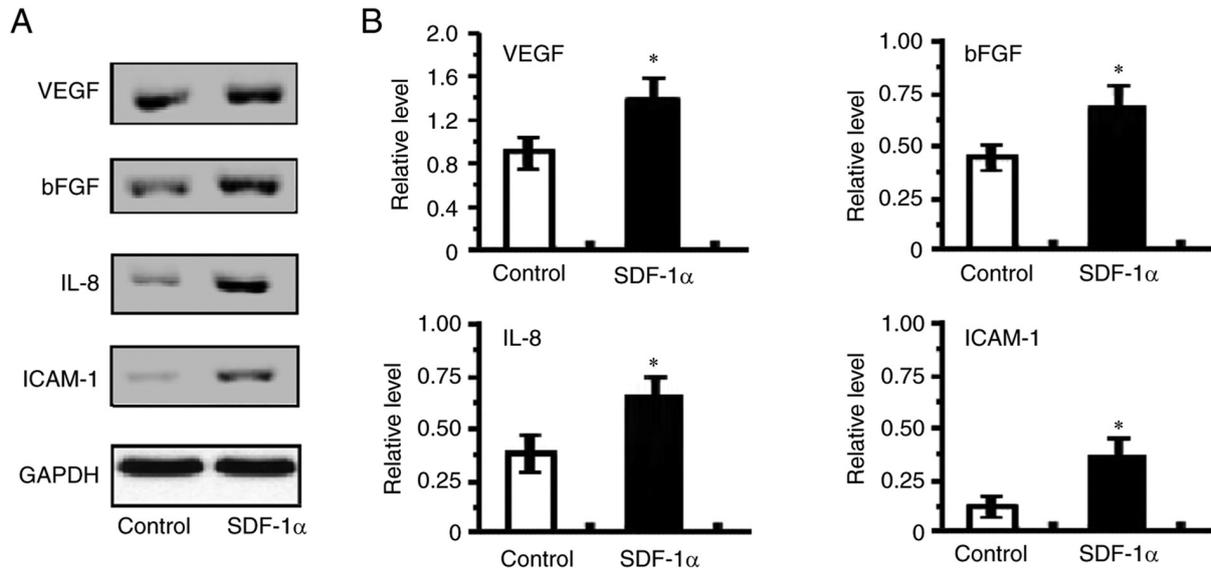
Figure 6. Effect of SDF-1α/CXCR4 signaling on VEGF, bFGF, IL-8 and ICAM-1 expression of HRECs. (A) Protein extracts were obtained and subjected to western blotting. Representative results of VEGF, bFGF, IL-8 and ICAM-1 expression of HRECs were determined. (B) Statistical analysis of ratios of VEGF, bFGF, IL-8 and ICAM-1 to GAPDH protein. All values represent mean ± standard error of the mean (n=6-8 animals). *P<0.05 vs. control. SDF, stromal cell-derived factor; CXCR, chemokine (C-X-C motif) receptor; HRECs, human retinal endothelial cells; bFGF, basic fibroblast growth factor; ICAM, intercellular cell adhesion molecule.
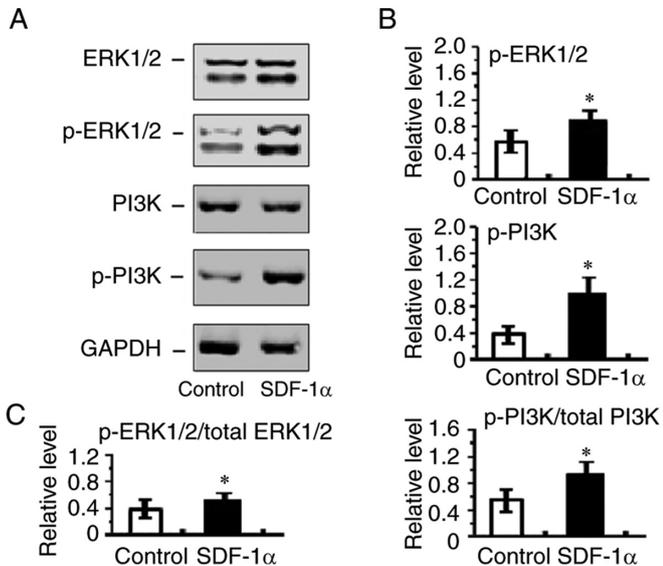
Figure 7. Effect of SDF-1α/CXCR4 signaling on the protein expression of ERK1/2 and PI3K pathway. (A) Representative results of ERK1/2, p-ERK1/2, PI3K and p-PI3K expression of HRECs were determined using western blotting. SDF-1α/CXCR4 signaling significantly increased the protein expression of ERK1/2 and PI3K pathway. (B) Statistical analysis of ratios of p-ERK1/2 and p-PI3K to GAPDH protein. (C) Statistical analysis of ratios of p-ERK1/2/total ERK1/2 and p-PI3K/total PI3K. All values represent mean ± standard error of the mean (n=3). *P<0.05 vs. control. SDF, stromal cell-derived factor; CXCR, chemokine (C-X-C motif) receptor; p-phosphorylated; HRECs, human retinal endothelial cells.

Several signaling pathways are involved in the process of angiogenesis. Activation of PI3K/Akt and ERK1/2 in endothelial cells is a crucial intracellular signaling step for angiogenesis (52,53). Barbero *et al* (54) report that SDF-1/CXCR4 axis are capable of activating various signaling pathways, including PI3K/Akt and ERK1/2, in the process

of tumor development and promote tumor vascular growth through these activated signaling pathways (55). Lin *et al* (56) report that SDF-1/CXCR4 signaling can promote tumor cell proliferation and migration by activating PI3K/Akt signaling. Based on these studies, the present study examined whether SDF-1/CXCR4 signaling promoted HRECs proliferation, migration or capillary tube formation through PI3K/Akt and ERK1/2. The present study found that SDF-1/CXCR4 signaling promoted the expression of active p-PI3K and p-ERK1/2, suggesting that SDF-1/CXCR4 signaling had pro-angiogenesis property via activating PI3K/Akt and ERK1/2 signaling.

In conclusion, the findings of the present study illustrated a novel mechanism of SDF-1/CXCR4 signaling effects on the process of neovascularization. It promoted HRECs capillary tube formation by promoting cell proliferation and cell migration. The effects may work by enhancing cytokine expression, such as VEGF and bFGF, and promoting these functions of HRECs via activating PI3K/Akt and ERK1/2 signaling. These results could provide a theoretical basis for the possibility of suppressing ocular neovascularization by inhibiting SDF-1/CXCR4 signaling using anti-SDF-1 antibody or anti-CXCR4 antagonist or other blocking agents.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GL, HW, XL and XY designed the study, led the experiments, prepared figures and wrote the manuscript. XY, XL, HW, YX, LC and ZC analyzed the data and prepared the figures. GL and PL conceived, designed and coordinated the study as well as drafted the manuscript. GL and PL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Campochiaro PA: Ocular neovascularization. J Mol Med (Berl) 91: 311-321, 2013.
2. Lee YM, Lee YR, Kim CS, Jo K, Sohn E, Kim JS and Kim J: Cnidium officinale extract and butylidenephthalide inhibits retinal neovascularization in vitro and in vivo. BMC Complement Altern Med 16: 231, 2016.
3. Multicenter trial of cryotherapy for retinopathy of prematurity. 3 1/2-Year outcome-structure and function. Cryotherapy for retinopathy of prematurity cooperative group. Arch Ophthalmol 111: 339-344, 1993.
4. Phelps DL: Retinopathy of prematurity. Pediatr Rev 16: 50-56, 1995.
5. Tsilimbaris MK, Kontadakis GA, Tsika C, Papageorgiou D and Charoniti M: Effect of panretinal photocoagulation treatment on vision-related quality of life of patients with proliferative diabetic retinopathy. Retina 33: 756-761, 2013.
6. Ameri H, Liu H, Liu R, Ha Y, Paulucci-Holthauzen AA, Hu S, Motamedi M, Godley BF, Tilton RG and Zhang W: TWEAK/Fn14 pathway is a novel mediator of retinal neovascularization. Invest Ophthalmol Vis Sci 55: 801-813, 2014.
7. Witmer AN, Vrensen GF, Van Noorden CJ and Schlingemann RO: Vascular endothelial growth factors and angiogenesis in eye disease. Prog Retin Eye Res 22: 1-29, 2003.
8. Yu W, Bai Y, Han N, Wang F, Zhao M, Huang L and Li X: Inhibition of pathological retinal neovascularization by semaphorin 3A. Mol Vis 19: 1397-1405, 2013.
9. Praidou A, Androudi S, Brazitikos P, Karakiulakis G, Papakonstantinou E and Dimitrakos S: Angiogenic growth factors and their inhibitors in diabetic retinopathy. Curr Diabetes Rev 6: 304-312, 2010.
10. Risau W: Mechanisms of angiogenesis. Nature 386: 671-674, 1997.
11. Gariano RF and Gardner TW: Retinal angiogenesis in development and disease. Nature 438: 960-966, 2005.
12. Zhang SX and Ma JX: Ocular neovascularization: Implication of endogenous angiogenic inhibitors and potential therapy. Prog Retin Eye Res 26: 1-37, 2007.
13. Fong GH: Mechanisms of adaptive angiogenesis to tissue hypoxia. Angiogenesis 11: 121-140, 2008.
14. Liekens S, Schols D and Hatse S: CXCL12-CXCR4 axis in angiogenesis, metastasis and stem cell mobilization. Curr Pharm Des 16: 3903-3920, 2010.
15. Federsppiel B, Melhado IG, Duncan AM, Delaney A, Schappert K, Clark-Lewis I and Jirik FR: Molecular cloning of the cDNA and chromosomal localization of the gene for a putative seven-transmembrane segment (7-TMS) receptor isolated from human spleen. Genomics 16: 707-712, 1993.
16. Nomura H, Nielsen BW and Matsushima K: Molecular cloning of cDNAs encoding a LD78 receptor and putative leukocyte chemotactic peptide receptors. Int Immunol 5: 1239-1249, 1993.
17. Aiuti A, Webb IJ, Bleul C, Springer T and Gutierrez-Ramos JC: The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med 185: 111-120, 1997.
18. Jo DY, Rafii S, Hamada T and Moore MA: Chemotaxis of primitive hematopoietic cells in response to stromal cell-derived factor-1. J Clin Invest 105: 101-111, 2000.
19. Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A and Springer TA: A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med 184: 1101-1109, 1996.
20. Pablos JL, Amara A, Bouloc A, Santiago B, Caruz A, Galindo M, Delaunay T, Virelizier JL and Arenzana-Seisdedos F: Stromal-cell derived factor is expressed by dendritic cells and endothelium in human skin. Am J Pathol 155: 1577-1586, 1999.
21. Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, Avecilla ST, Heissig B, et al: Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. Nat Med 12: 557-567, 2006.
22. Liu G, Lu P, Li L, Jin H, He X, Mukaida N and Zhang X: Critical role of SDF-1α-induced progenitor cell recruitment and macrophage VEGF production in the experimental corneal neovascularization. Mol Vis 17: 2129-2138, 2011.
23. Liu GQ, Lu PR, Li LB and Zhang XG: Inhibited experimental corneal neovascularization by neutralizing anti-SDF-1α antibody. Int J Ophthalmol 5: 7-12, 2012.
24. Sonmez K, Drenser KA, Capone A Jr and Trese MT: Vitreous levels of stromal cell-derived factor 1 and vascular endothelial growth factor in patients with retinopathy of prematurity. Ophthalmology 115: 1065-1070.e1, 2008.
25. Butler JM, Guthrie SM, Koc M, Afzal A, Caballero S, Brooks HL, Mames RN, Segal MS, Grant MB and Scott EW: SDF-1 is both necessary and sufficient to promote proliferative retinopathy. J Clin Invest 115: 86-93, 2005.
26. Bhutto IA, McLeod DS, Merges C, Hasegawa T and Lutty GA: Localisation of SDF-1 and its receptor CXCR4 in retina and choroid of aged human eyes and in eyes with age related macular degeneration. Br J Ophthalmol 90: 906-910, 2006.
27. Lima e Silva R, Shen J, Hackett SF, Kachi S, Akiyama H, Kiuchi K, Yokoi K, Hatara MC, Lauer T, Aslam S, et al: The SDF-1/CXCR4 ligand/receptor pair is an important contributor to several types of ocular neovascularization. FASEB J 21: 3219-3230, 2007.
28. Sengupta N, Afzal A, Caballero S, Chang KH, Shaw LC, Pang JJ, Bond VC, Bhutto I, Baba T, Lutty GA and Grant MB: Paracrine modulation of CXCR4 by IGF-1 and VEGF: Implications for choroidal neovascularization. Invest Ophthalmol Vis Sci 51: 2697-2704, 2010.
29. Lee E and Rewolinski D: Evaluation of CXCR4 inhibition in the prevention and intervention model of laser-induced choroidal neovascularization. Invest Ophthalmol Vis Sci 51: 3666-3672, 2010.
30. Chen Z, Liu G, Xiao Y and Lu P: Adrenomedullin22-52 suppresses high-glucose-induced migration, proliferation, and tube formation of human retinal endothelial cells. Mol Vis 20: 259-269, 2014.
31. Liu G, Zhang W, Xiao Y and Lu P: Critical Role of IP-10 on reducing experimental corneal neovascularization. Curr Eye Res 40: 891-901, 2015.
32. Chao TI, Xiang S, Chen CS, Chin WC, Nelson AJ, Wang C and Lu J: Carbon nanotubes promote neuron differentiation from human embryonic stem cells. Biochem Biophys Res Commun 384: 426-430, 2009.
33. Lu P, Li L, Liu G, van Rooijen N, Mukaida N and Zhang X: Opposite roles of CCR2 and CX3CR1 macrophages in alkali-induced corneal neovascularization. Cornea 28: 562-569, 2009.
34. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

35. Farnoodian M, Wang S, Dietz J, Nickells RW, Sorenson CM and Sheibani N: Negative regulators of angiogenesis: Important targets for treatment of exudative AMD. Clin Sci (Lond) 131: 1763-1780, 2017.

36. Mao L, Huang M, Chen SC, Li YN, Xia YP, He QW, Wang MD, Huang Y, Zheng L and Hu B: Endogenous endothelial progenitor cells participate in neovascularization via CXCR4/SDF-1 axis and improve outcome after stroke. CNS Neurosci Ther 20: 460-468, 2014.

37. Li B, Bai W, Sun P, Zhou B, Hu B and Ying J: The effect of CXCL12 on endothelial progenitor cells: Potential target for angiogenesis in intracerebral hemorrhage. J Interferon Cytokine Res 35: 23-31, 2015.

38. Tu TC, Nagano M, Yamashita T, Hamada H, Ohneda K, Kimura K and Ohneda O: A chemokine receptor, CXCR4, which is regulated by hypoxia-inducible factor 2α, is crucial for functional endothelial progenitor cells migration to ischemic tissue and wound repair. Stem Cells Dev 25: 266-276, 2016.

39. Wang YB, Liu YF, Lu XT, Yan FF, Wang B, Bai WW and Zhao YX: Rehmannia glutinosa extract activates endothelial progenitor cells in a rat model of myocardial infarction through a SDF-1 α/CXCR4 cascade. PLoS One 8: e54303, 2013.

40. Griffioen AW and Molema G: Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol Rev 52: 237-268, 2000.

41. Hsu YP, Staton CA, Cross N and Buttle DJ: Anti-angiogenic properties of ADAMTS-4 in vitro. Int J Exp Pathol 93: 70-77, 2012.

42. Lawler PR and Lawler J: Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. Cold Spring Harb Perspect Med 2: a006627, 2012.

43. Kryczek I, Wei S, Keller E, Liu R and Zou W: Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis. Am J Physiol Cell Physiol 292: C987-C995, 2007.

44. Martínez A: A new family of angiogenic factors. Cancer Lett 236: 157-163, 2006.

45. Uno K, Hayashi H, Kuroki M, Uchida H, Yamauchi Y, Kuroki M and Oshima K: Thrombospondin-1 accelerates wound healing of corneal epithelia. Biochem Biophys Res Commun 315: 928-934, 2004.

46. Sakaguchi I, Ikeda N, Nakayama M, Kato Y, Yano I and Kaneda K: Trehalose 6,6'-dimycolate (Cord factor) enhances neovascularization through vascular endothelial growth factor production by neutrophils and macrophages. Infect Immun 68: 2043-2052, 2000.

47. Edelman JL, Castro MR and Wen Y: Correlation of VEGF expression by leukocytes with the growth and regression of blood vessels in the rat cornea. Invest Ophthalmol Vis Sci 40: 1112-1123, 1999.

48. Lai CM, Spilsbury K, Brankov M, Zaknich T and Rakoczy PE: Inhibition of corneal neovascularization by recombinant adenovirus mediated antisense VEGF RNA. Exp Eye Res 75: 625-634, 2002.

49. Newey SE, Tsaknakis G, Khoo CP, Athanassopoulos T, Camicia R, Zhang Y, Grabowska R, Harris AL, Roubelakis MG and Watt SM: The hematopoietic chemokine CXCL12 promotes integration of human endothelial colony forming cell-derived cells into immature vessel networks. Stem Cells Dev 23: 2730-2743, 2014.

50. Smadja DM, Bièche I, Uzan G, Bompais H, Muller L, Boisson-Vidal C, Vidaud M, Aiach M and Gaussem P: PAR-1 activation on human late endothelial progenitor cells enhances angiogenesis in vitro with upregulation of the SDF-1/CXCR4 system. Arterioscler Thromb Vasc Biol 25: 2321-2327, 2005.

51. Hamed S, Egozi D, Dawood H, Keren A, Kruchevsky D, Ben-Nun O, Gilhar A, Brenner B and Ullmann Y: The chemokine stromal cell-derived factor-1α promotes endothelial progenitor cell-mediated neovascularization of human transplanted fat tissue in diabetic immunocompromised mice. Plast Reconstr Surg 132: 239e-250e, 2013.

52. Fernández JG, Rodríguez DA, Valenzuela M, Calderon C, Urzúa U, Munroe D, Rosas C, Lemus D, Díaz N, Wright MC, *et al*: Survivin expression promotes VEGF-induced tumor angiogenesis via PI3K/Akt enhanced β-catenin/Tcf-Lef dependent transcription. Mol Cancer 13: 209, 2014.

53. Karar J and Maity A: PI3K/AKT/mTOR pathway in angiogenesis. Front Mol Neurosci 4: 51, 2011.

54. Barbero S, Bonavia R, Bajetto A, Porcile C, Pirani P, Ravetti JL, Zona GL, Spaziante R, Florio T and Schettini G: Stromal cell-derived factor 1alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. Cancer Res 63: 1969-1974, 2003.

55. Wu D, Guo X, Su J, Chen R, Berenzon D, Guthold M, Bonin K, Zhao W and Zhou X: CD138-negative myeloma cells regulate mechanical properties of bone marrow stromal cells through SDF-1/CXCR4/AKT signaling pathway. Biochim Biophys Acta 1853: 338-347, 2015.

56. Lin ML, Lu YC, Chen HY, Lee CC, Chung JG and Chen SS: Suppressing the formation of lipid raft-associated Rac1/PI3K/Akt signaling complexes by curcumin inhibits SDF-1α-induced invasion of human esophageal carcinoma cells. Mol Carcinog 53: 360-379, 2014.