• Basic Research •

Procollagen C-proteinase enhancer 1 promotes physiologic retinal angiogenesis *via* regulating the process of collagen

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Abstract

● **AIM:** To investigate the role of procollagen C-proteinase enhancer 1 (PCPE1) in retinal angiogenesis and relevant mechanisms.

● **METHODS:** The *Pcolce1*-knockout (KO) mice were used to explore the effect of PCPE1 on retinal angiogenesis *in vivo*. *Pcolce1* siRNA were designed, cell count kit 8 (CCK8) assays and tube formation assays were performed to investigate the cell proliferation and tube formation abilities of retinal microvascular endothelial cells (hRMECs) *in vitro*. Mouse embryo fibroblasts (MEF) cells were isolated and cultured to analyze the effect of PCPE1 on enhancing procollagen cleavage.

● **RESULTS:** *In vivo* studies showed that the retinal vascular density of *Pcolce1*$^{-/-}$ mice was significantly lower than that of the control group. Furthermore, silencing of *Pcolce1* inhibited cell proliferation and tube formation abilities of hRMECs *in vitro*. Additionally, much more procollagen was found in *Pcolce1*$^{-/-}$ MEF cells, compared to wild type MEF cells.

● **CONCLUSION:** PCPE1 may promote physiological retinal angiogenesis by regulating the processing of collagen, which may provide a potential therapeutic target of retinal vascular disease.

● **KEYWORDS:** retinal diseases; angiogenesis; procollagen C-proteinase enhancer 1; collagen
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INTRODUCTION

Angiogenesis refers to the formation of new blood vessels from existing capillaries, which plays a crucial role in both physiological and pathological processes, such as wound healing, tumor growth and so on. Retinal vascular diseases, including retinopathy of prematurity (ROP) or oxygen-induced retinopathy (OIR) model, diabetic retinopathy (DR), and age-related macular degeneration (AMD), are sight-threatening diseases which mainly due to abnormal retinal vascular development[1-3]. To describe the pathogenesis of these diseases and find the ways of prevention and treatments, many recent studies have focused on retinal angiogenesis[4].

Collagen synthesis is of great importance in many physiological functions, which is closely related to angiogenesis. Collagen I and III are crucial components for blood vessel formation[5-6], and collagen I and III are abundant in extracellular matrix (ECM) of retinal vasculature[7-8]. Procollagen C-proteinase enhancer 1 (PCPE1), encoded by *Pcolce1*, was found to be a glycoprotein which was responsible for potentiating the activity of procollagen C-proteinase, thereby prompting the deposition of collagen[9]. Kanaki *et al*[10] and Masuda *et al*[11] respectively showed that PCPE1 could regulate the growth of vascular smooth muscle cells and tumor. Newman *et al*[12] demonstrated that *Pcolce1* was important for endothelial cell lumen formation partly by enhancing the stiffness of the ECM, which indicated it played a possible role in

angiogenesis. And there were studies suggested that PCPE1 may act as an anti-angiogenetic factor during the recovery process of alkali-injured cornea[13-14]. Therefore, PCPE1 has been reported as a novel factor that might be related to angiogenesis, while its specific role and mechanisms in retinal angiogenesis remain unclear.

To determine the role of PCPE1 in retinal angiogenesis and possible mechanisms, we investigated the retinal vascular density of *Pcolce1*$^{-/-}$ and wild type (WT) mice retina *in vivo*, and the angiogenic property change of human retinal microvascular endothelial cells (hRMECs) after silencing *Pcolce1 in vitro*. Results showed that PCPE1 had the ability to promote retinal angiogenesis. Furthermore, procollagen α1(I) cleavage assays demonstrated that the process of procollagen was involved in retinal angiogenesis, and it may provide a potential target for the treatments of retinal vascular diseases.

MATERIALS AND METHODS

Ethical Approval All animal experiments were approved by the Animal Care Committee of Shanghai Jiao Tong University and conformed to the protocol of the Care and Use of Experimental Animals. We conducted all animal experiments adhering to the tenets of the Declaration of Helsinki or the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.

Human Retinal Microvascular Endothelial Cells Culture The hRMECs were purchased from X-Y Biotechnology (Shanghai, China), and cultured in endothelial cell medium (ScienCell Research Laboratories, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS, ScienCell), 1% penicillin-streptomycin (PS, ScienCell) and 1% endothelial cell growth supplement (ScienCell) at 37℃ in 5% CO_2 atmosphere. The culture medium was refreshed every 2-3d. For subculture, remove the medium, and rinse hRMECs with phosphate buffered saline (PBS) three times, and then digested with 0.25% trypsin supplemented with 0.02% ethylenediaminetetraacetic acid.

Transfection of Small Interfering RNA Three *Pcolce1* small interfering RNA (siRNA; si-*Pcolce1* #1, 2, and 3) and one negative control (NC) siRNA (si-NC) were purchased from RIBOBIO (Guangzhou, China) and the sequences were listed in Table 1. Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, USA) was used for transfecting the siRNA into hRMECs in 6-well plate following manufacturer's instructions, and cells were replaced with fresh medium 6h after transfection. RNA and protein were harvested from transfected cells at 24 and 48h after transfection. Cells with *Pcolce1* expression knockdown were used to investigate the effects of *Pcolce1* silencing on angiogenesis *via* cell proliferation and tube formation assays.

Table 1 Oligonucleotide sequences of siRNAs used in this study

si-RNA	Oligo sequence (5'-3')
si-*Pcolce1* #1	GAGTGCATCTGGACCATAA
si-*Pcolce1* #2	CAGGTTACGTGGCAAGTGA
si-*Pcolce1* #3	GTGCCTCCCTGAAGTTTTA

Quantitative Real-Time Polymerase Chain Reaction Analysis To evaluate the relative expression level of mRNA in mouse retina or hRMECs, total RNA was extracted and used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. TRIzol Reagents (Invitrogen, Carlsbad, USA) were used for RNA extraction following the protocol, then dissolved RNA was quantified by Nano Drop micro spectrophotometer (Thermo Fisher, Waltham, USA) and 1 μg of RNA was reverse-transcribed into 20 μL of cDNA using transcriptase reagent kit (Vazyme, Nanjing, China). Applied Biosystems QuantStudio 7 Flex-1 (Thermo Fisher) was used for qRT-PCR with a 10 μL reaction system, including 5 μL of ChamQSYBR Color qPCRMaster Mix (Vazyme), 4.5 μL of cDNA template, 0.2 μL of Rox Reference Dye (Vazyme), and 0.3 μL of primers. Relative gene expressions were analyzed by △△Ct and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The primer sequences involved were listed in Table 2.

Western Blot Analysis Total protein was extracted from mouse retina or hRMECs with radio immunoprecipitation assay lysis buffer supplemented with protease inhibitors (Thermo Fisher), and quantified using BCA protein assay (Thermo Fisher). Samples were then separated by 12.5% acrylamide sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto the polyvinylidene fluoride membranes, followed by blocking with 5% non-fat milk for 1h at room temperature (RT). After washing for three times with Tris buffered saline Tween (TBST) solution, the membranes were incubated with primary antibodies, including PCPE1 (1:600, Santa Cruz Biotechnology, CA, USA), heat shock protein 90 (HSP90, 1:1000, Abcam, Cambridge, UK), at 4°C overnight and then with anti-mouse or anti-rabbit horseradish peroxidase-labeled secondary antibodies (1:5000, Epizyme Biotech, Shanghai, China) for 1h at RT. Membranes were visualized using Omni-enhanced chemiluminescent reagent (EpiZyme) and exposed in e-BLOT Touch Imager (e-BLOT, Shanghai, China). HSP90 was used as a loading control.

Cell Count Kit 8 Assay Cell count kit 8 (CCK8) assay was used to evaluate cell proliferation. hRMECs in 3-5 passage were used for CCK8 assay. Cells transfected with siRNA were seeded in a 96-well plate with a density of 2×10^3 cells/well. According to manufacturer's instruction, cells cultured for 0, 24, 48, and 72h were then added with 10 μL CCK8

Table 2 Primer sequence of qRT-PCR assays used in this study

Primer name	Forward primer (5'-3')	Reverse primer (5'-3')
h-GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
h-Pcolce1	TCTCATTCCGAGTCTTCGACC	GTCCCACAAAAGCGTCCGA
m-GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG
m-Pcolce1	AACCTCCTTTCTGGGCCCATTCCT	GGAGCTCGTTCCCTTCAGAAGAGA

qRT-PCR: Quantitative real-time polymerase chain reaction.

solution (Dojindo Laboratories, Kumamoto, Japan) each well and incubated for 1h. Finally, the optical density (OD) values were measured at the wavelength of 450 nm and the cell viability were accessed according to the equation: cell viability=[(*As*-*Ac*)/(*Ab*-*Ac*)]×100%. *As* stands for OD value of wells with cells transfected with siRNA, medium, and CCK-8 solution. *Ac* stands for OD value of wells without cells but with medium and CCK-8 solution, and *Ab* stands for OD value of wells containing cells, medium, and CCK-8 solution.

Tube Formation Assay hRMECs in 3-5 passage were used for tube formation assay. Matrigel (Corning, NY, USA) was melted at 4°C and coated into a 48-well plate with 150 μL/well for 1h at 37°C. Cells were then seeded into the pre-coated 48-well plate with a density of 6×10^4 cells/well. Images were photographed at 2, 4, 6, and 8h after incubation to assess the tube-like structures. The tube formation potential was assessed with Image J software based on total number of branches and total tube length.

Animals The *Pcolce1*-knockout (KO) mice (C57BL/6J) were designed and purchased from Cyagen Bioscience Inc (Guangzhou, China). Mice were housed on a 12h light/dark cycle environment and fed with rodent chow diet. Genotypes were identified by PCR amplification on genomic DNA extracted from mousetails.

Retinal Whole Mount Preparation Mice were euthanized postnatally at 14d after birth (P14). Eyes were enucleated and fixed in 4% paraformaldehyde at 4℃ for 48h. Put the eyeball into PBS solution under the dissecting microscope and remove the anterior segments after cutting off the cornea, then be very careful to separate the retina from retinal pigment epithelium and sclera. It's notable to sever the optic nerve head from retina, rather than forcibly pull it out, aiming to maintain the integrity of retinal whole mount. Wash retina in distilled water for three times (30min each) and then left it in the distilled water overnight at 4°C. Then incubate it in 3% Trypsin 1:250 (Macklin, Shanghai, China) at 37℃ for 2h and wash it in distilled water to isolate retinal blood vessels. Make four radial incisions in the intact isolated retina to allow it lie flat. Finally, attach the retina onto the slide with eyecup facing up and let it dry overnight at RT.

Immunofluorescence Rehydration the retinal whole mount in PBS for 15min, and blocking the nonspecific sites by incubating in PBS supplemented with 10% goat serum and 0.5% Triton for 1h at RT. Then, the retinal whole mount was exposed to a 1:100 dilution of antibody CD31 (Bio-Rad, Hercules, California, USA) overnight at 4°C. Rinse the sample in PBS for 3 times (10min each) and dry it at RT for 10min. Finally, placed one drop of anti-fading mounting medium on the retina and covered it with a coverslip. The images were taken using immunofluorescent microscope (Olympus, Tokyo, Japan) and evaluated by Image J software.

Mouse Embryo Fibroblasts Cells Isolation Mouse embryo fibroblasts (MEF) cells were isolated from *Pcolce1*$^{-/-}$ and WT mice embryos as described previously[15]. Pregnant mice were sacrificed at E12.5-14.5 and soaked with 75% ethanol solution for sterilization. Then, dissect out the uterus and wash it in PBS, organs and brain were extracted for genotype identification, carcass was removed to 6 cm dish containing PBS individually. After washing for twice, the carcass was minced finely, and centrifuged for 15min at 15 000 rpm. Then remove the supernatant and add trypsin-EDTA (Thermo Fisher) for digestion reaction for 30min. Cell suspension was filtered by a 100 μm filter and centrifuged for MEF cells collection. Finally, resuspend the cells pellet to a concentration of 2×10^5 cells/mL for further cell culture. After cultured for 24h, cells were replaced with fresh medium. When they reached confluence, froze them with a concentration of 3×10^6 cells/mL at -80℃ for subsequent experiments. MEF cells in 1-3 passage were used for following experiments.

Analysis of Processing of Endogenous Type I Collagen in Cultured Mouse Embryo Fibroblasts Cells MEF cells isolated from WT and *Pcolce1*$^{-/-}$ mice were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 1% PS. For collagen samples collection, cells reached 80% confluence were washed with PBS and switched to FBS-free DMEM medium which supplemented with 25 μg/mL soybean trypsin inhibitor and 50 μg/mL ascorbate for another 24h incubation at 37℃. Meanwhile, collect the conditioned medium with protease inhibitors [1 mmol/L para aminobenzamidine, 1 mmol/L N-ethylmaleimide, and 0.2 mmol/L phenylmethylsulfonyl fluoride (all were purchased from Sigma)] and ammonium sulfate, then stir

the mixed medium gently at 4℃ for collagen components precipitation. Cell layers were scraped into 2× SDS loading buffer after washed twice with PBS, and then boiled for collagen sample preparation. Samples were finally electrophoresed on 7.5% acrylamide SDS-PAGE gels, and anti-α1(I) C-telopeptide polyclonal antibody LF67 (made in our lab from rabbits), were used at 1:1000 dilution for Western blot (WB) assays. Blots were quantified with Image J software.

Statistical Analysis Data were presented as mean±standard deviation (SD). Two-independent specimen *t*-test was utilized for comparison between two groups, and differences between groups were considered as significant with a *P*-value less than 0.05. Statistical analysis was performed using GraphPad Prism software version 8.4.0.

RESULTS

***Pcolce1* Knockdown Decreased Cell Proliferation and Tube Formation of hRMECs** Previous studies indicated that PCPE1 was an anti-angiogenesis factor, and *Pcolce1*-KO mice showed more intracorneal neovascularization than WT mice after alkali corneal burn[13-14]. To determine the role of PCPE1 in cell angiogenesis, three siRNAs (si-*Pcolce1* #1, 2, and 3) were designed and transfected into hRMECs according to manufacturer's information. The silencing efficiency of the siRNAs were evaluated by qRT-PCR and WB, and results showed that the PCPE1 expression reduced to approximately 30% in si-*Pcolce1* #1, which revealed the highest knockdown efficiency and was selected for following experiments (Figure 1A-1B). CCK8 assays were used to quantify cell proliferation abilities, results revealed that si-*Pcolce1* stimulation led to a significant decrease in cell proliferation of hRMECs (Figure 1C). The angiogenesis potential of hRMECs was evaluated by tube formation assay, which showed that *Pcolce1* silencing resulted in decreased angiogenesis potential (Figure 1D-1E).

Retinal Vascular Density Decreased in *Pcolce1*$^{-/-}$ Mice Compared to Wild Type Mice Functional assays in hRMECs indicated a pro-angiogenic effect of *Pcolce1*, which contradicted to the previous studies. To assess whether *Pcolce1* acts as an anti-angiogenesis factor or pro-angiogenesis factor in retinal angiogenesis, *Pcolce1*$^{-/-}$ mice were obtained and bred for retinal whole mounts preparation. Equal number of *Pcolce1*$^{-/-}$ mice and WT mice aging of 14d were collected, retina whole mounts were made and incubated with anti-CD31 antibodies which marked the epithelium cells. CD31 immunofluorescent results showed a significant lower density of retinal vascular of *Pcolce1*$^{-/-}$ mice than that of WT mice (Figure 2), indicating that *Pcolce1* KO leads to less retinal angiogenesis, which was consistent with our previous results.

Pro-collagen in *Pcolce1*$^{-/-}$ Mice From both Medium and Embryo Fibroblasts As previously demonstrated that *Pcolce1* could enhance the procollagen C-proteinase (PCP) activity of bone morphogenetic protein 1 (BMP1), which can cleave C-propeptides from the procollagen precursors to form mature collagen[16]. To further investigate whether PCPE1 can enhance the cleavage activity of BMP1 and result in more mature collagen produce, *in vitro* procollagen α1(I) cleavage assays were performed in MEF cells. Conditioned medium and cell layers of MEF cells isolated from *Pcolce1*$^{-/-}$ mice and WT mice embryos were harvested for WB. A polyclonal antibody LF67 was introduced to detect different forms of processing products: procollagen precursor, processing intermediates [pC/pN-α1(I)], and fully processed mature collagen α1(I) chain. As can be seen, in WT MEF cells, the mature collagen α1(I) chain was detected as the most abundant in the culture medium which contains about 60%, followed by pC/pN-α1(I) and procollagen-α1 precursor. Compared to WT MEF cells, procollagen α1(I) processing was markedly decreased in *Pcolce1*$^{-/-}$ MEF medium, the most abundant form of processing chains was pC-α1(I) and pN-α1(I) chains which accounting for approximately 91% in total, while the mature α1(I) chains were merely found in the culture medium. Similar results were also shown in cell layer of MEF cells (Figure 3). Due to the defects of collagens process which contributes to the angiogenesis, thus much less of mature type I collagens leads to the decreased retinal vascular density in *Pcolce1*$^{-/-}$ mice.

DISCUSSION

Retinal vessel can be counted as the most studied vessel in the entire body, and study retinal vasculature can further understand the pathogenetic mechanisms of retinal vascular diseases. As ROP, AMD and DR are vision-threatening and may lead to poor quality of life[17], great efforts are made to search for new factors and mechanisms in retinal angiogenesis, which may provide novel targets for intervention and treatments. PCPE1 has been reported to be involved in angiogenesis, but its role in retinal angiogenesis has not been studied. In this study, *in vivo* and *in vitro* experiments were established to investigate the role of PCPE1 in retinal angiogenesis and the possible mechanism. *Pcolce1*$^{-/-}$ mice showed less retinal vasculature than that of WT mice at P14. *In vitro* experiments revealed that *Pcolce1* knockdown inhibited cell proliferation and decreased the tube formation abilities of hRMECs. Further procollagen α1(I) cleavage assays showed that *Pcolce1*$^{-/-}$ MEF cells were less efficient in enhancing mature collagen deposition than WT MEF cells, thus led to the decreased retinal vascular density in *Pcolce1*$^{-/-}$ mice.

Studies showed that PCPE1 can regulate the growth of vascular smooth muscle cells (SCM) and tumor[10-11]. Previous study found that TGF-β1, a cell proliferation regulator, can

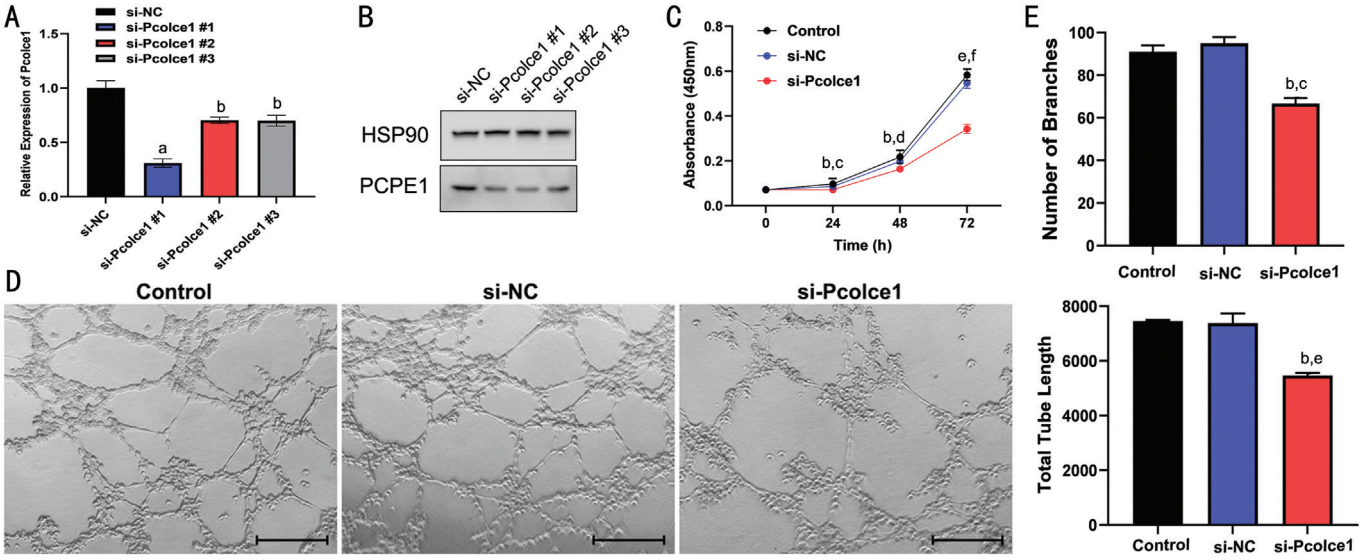

Figure 1 Silencing of *Pcolce1* in hRMECs decreased cell proliferation and angiogenesis hRMECs: Human retinal microvascular endothelial cells. [a]*P*<0.001 *vs* si-NC, [b]*P*<0.01 *vs* si-NC, [c]*P*<0.01 *vs* control, [d]*P*<0.05 *vs* control, [e]*P*<0.0001 *vs* control, [f]*P*<0.0001 *vs* si-NC.

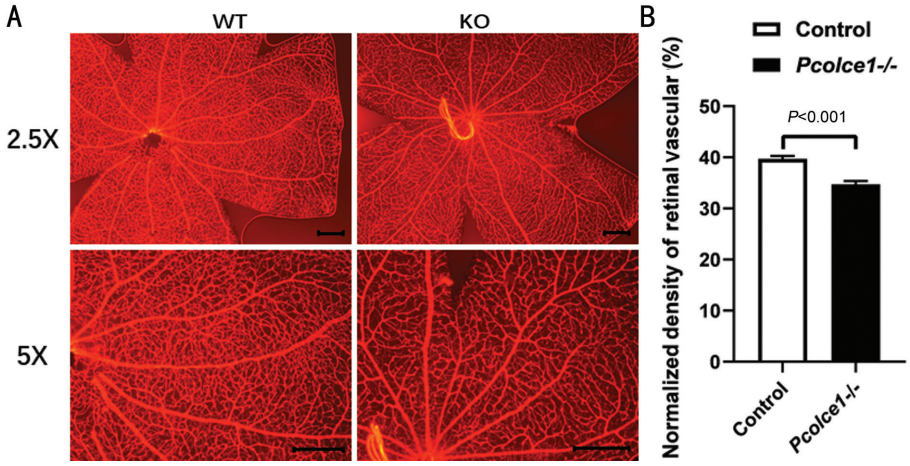

Figure 2 Evaluation of retinal vascular density at 14d after birth (P14).

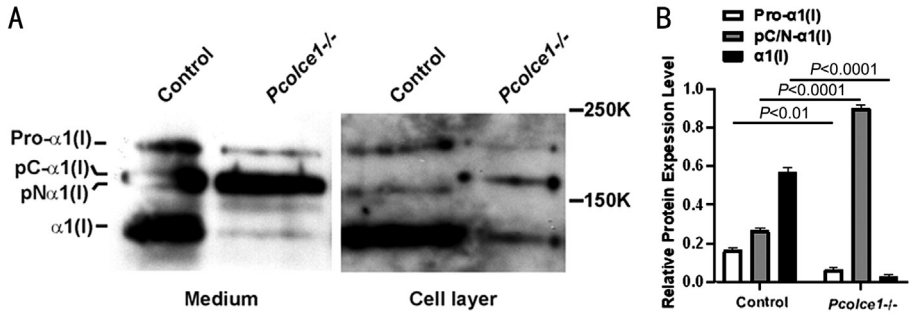

Figure 3 Type I procollagen processing efficiency of *Pcolce1*$^{-/-}$ and WT MEF mouse embryo fibroblasts cells were detected by procollagen α1(I) cleavage assays WT: Wild type; MEF: Mouse embryo fibroblasts.

increase the level of *Pcolce1*. Reduced SCM cell proliferation could lead to increased *Pcolce1* mRNA, which may serve as a compensatory mechanism, suggesting *Pcolce1* promotes cell proliferation. In this study, CCK8 results showed that *Pcolce1* knockdown inhibited cell proliferation, which was consistent with the previous study. Findings in another study suggested that *Pcolce1* gene played an important role in vessel lumen formation partly by enhancing the stiffness of the ECM[12], which was consistent with our results that PCPE1 enhanced the tube formation ability of hRMECs. All these findings indicated a promoting role of *Pcolce1* in retinal angiogenesis.

As one of the crucial contents of ECM, fibrillar collagen types I and III are derived from the procollagens containing

amino- and carboxyl-terminal peptides, which can be divided by two different proteases, procollagen N-proteinases and PCP, respectively[18]. PCP can remove the C-propeptides from procollagen, and the release of C-propeptides is regarded as the critical step in turning the soluble procollagen into insoluble fibrillar collagen[19]. PCPE1 is found to be a secreted glycoprotein which is responsible for potentiating the cleavage activity of BMP1, the same protein of PCP, up to 20 folds[20-21]. On the N-terminal of PCPE1, there are two complement/Uegf/BMP-1 (CUB) domains, which are thought to be the crucial functional binding regions of PCPE1 and related to the protein interactions[22]. It also contains a netrin-like (NTR) domain on the C-terminal, possessing the ability to suppress matrix metalloproteinases[23]. Studies revealed that many proteins contain the NTR domain, among which, secreted frizzled-related proteins act in synergy with PCPE1 to enhance the cleavage activity of BMP1[24-25]. Therefore, PCPE1 has pleiotropic effects in promoting collagen deposition. PCPE1 works *via* binding specifically to the type I procollagen C-propeptides *via* the calcium binding motif in CUB domain, so as to render the substrate a more suitable conformation to be cleaved by BMP1[16]. More specifically, the ability of *Pcolce1* to prompt collagen deposition can be only through its effect on BMP1[26], and PCPE1 only stimulates the activity of BMP1 when the substrates are fibrillar procollagen types I and III[27]. In this study, further procollagen α1(I) cleavage assays in MEF cells showed the same results with previous studies, which indicated that PCPE1 can potentiating the mature collagen production. The ECM structure contains instructive signals for migrating cells and plays a role of adhesive substrate in immature retinal astrocytes development[28-29]. Retinal astrocytes can not only induce VEGF expression and further stimulate retinal blood vessel development, but also influence retinal vasculature *via Fat1*-induced astrocyte proliferation and maturation[30-31]. Taking together, *Pcolce1* may promote retinal angiogenesis *via* promoting collagen deposition.

Contrary to the results of this study, previous study revealed *in vitro* that the N-terminal domains of PCPE1, CUB1CUB2 fragment, exhibited significant anti-angiogenetic capacities. Nevertheless, the entire PCPE1 protein and its C-terminal NTR domain exerted weaker anti-angiogenetic abilities. The same study also demonstrated that the NTR domain of PCPE1 could interact with endostatin, one anti-angiogenic factor[13]. In another study, *Pcolce1*$^{-/-}$ cornea exerted a more active intracorneal neovascularization effect than WT cornea after alkali injury. According to aortic ring culture *ex vivo*, the number of new blood vessel sprouts in *Pcolce1*$^{-/-}$ aortic rings was markedly richer than that in WT aortic rings, what is more, the effect of recombinant PCPE1 on inhibiting growth of angiogenic sprouts was dose-dependent[14]. All these results suggested PCPE1 may have the function to inhibit angiogenesis. The different effects of PCPE1 on angiogenesis may due to the different targeted organs or the different pathways between physiological and pathological angiogenesis. There were many factors involved in normal retinal angiogenesis and pathological retinal neovascularization, like ROP and DR. Some of them played the same role in both processes. For example, high level of VEGF resulted in retinal vasculature, while reducing VEGF induced by hyperoxia treatment in OIR model led to neovascular tufts[32]. Erythropoietin (EPO) deficiency decreased retinal vascular stability, and intravitreal injection of EPO siRNA effectively suppressed retinal neovascularization[33-34]. However, retinal astrocytes which modulate retinal blood vessel development *via* providing template guidance for vascular network and VEGF production have divergent roles under different conditions[35-36]. Conditional knockout VEGF in astrocytes had no effect on normal retinal vessel development, while in the OIR mouse model, VEGF induced by astrocytes was critical for hypoxia-derived neovascularization[37]. Therefore, the divergent roles of PCPE1 in physiologic and pathogenetic retinal vasculature were accessible and further studies are needed to verify the specific mechanisms.

In conclusion, PCPE1 is a promoting factor in normal retinal angiogenesis, and its effect on retinal angiogenesis is partly achieved by regulating collagen synthesis. Therefore, our results elucidate the role of PCPE1 in retinal angiogenesis and provide a new therapeutic target of retinal vascular diseases.

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