

An Inhibitory Aptamer Against PDGF-C Overcomes Anti-VEGF Refractoriness and Reduces Choroidal Neovascularization and Fibrosis

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PURPOSE. Wet age-related macular degeneration is a leading cause of irreversible vision loss, primarily due to choroidal neovascularization (CNV) and subsequent fibrosis. Although current anti-vascular endothelial growth factor A (anti-VEGF) therapies offer significant benefits, many patients exhibit limited or no response and develop drug resistance over time, necessitating the exploration of complementary or alternative therapeutics. This study aimed to identify and characterize a platelet-derived growth factor-C (PDGF-C)-targeting DNA aptamer and to evaluate its therapeutic potential for suppressing CNV and fibrosis, including in an anti-VEGF-refractory setting.

METHODS. A DNA aptamer against PDGF-C (α -PC aptamer) was identified using systematic evolution of ligands by exponential enrichment. Its binding to PDGF-C and inhibition of PDGF-C/platelet-derived growth factor receptor alpha (PDGFR α) interaction were assessed using surface plasmon resonance. The effects of the α -PC aptamer on PDGF-C-induced proliferation, migration, and PDGFR α , Akt, and extracellular-regulated kinase (ERK) signaling were examined in fibroblasts and human umbilical vein smooth muscle cells (HUVSMCs). In vivo efficacy was evaluated in a laser-induced CNV mouse model, including anti-VEGF refractory aged mice.

RESULTS. The α -PC aptamer specifically bound to PDGF-C and effectively blocked its binding to PDGFR α . The α -PC aptamer significantly inhibited PDGFR α , Akt, and ERK activation and suppressed PDGF-C-induced proliferation and migration of both fibroblasts and HUVSMCs. Importantly, in a laser-induced CNV mouse model, the α -PC aptamer markedly reduced neovascularization and fibrosis; it particularly retained efficacy in suppressing CNV in anti-VEGF refractory aged mice, where anti-VEGF treatment failed to do so.

CONCLUSIONS. These findings suggest that the α -PC aptamer represents a promising therapeutic agent for treating neovascular diseases, especially in patients refractory to anti-VEGF treatment.

Keywords: aptamer, PDGF-C, choroidal neovascularization, anti-VEGF refractoriness, fibrosis

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salmon sperm DNA, and Tween 20 to minimize non-specific interactions. Following washing, bound sequences were eluted by heating at 95°C for 10 minutes, and the resulting supernatant was collected for amplification.

We performed symmetric polymerase chain reaction (PCR) using a forward primer and a 5'-biotin-modified reverse primer to enable ssDNA separation using streptavidin resin.⁵⁰ The ssDNA was then desalted using PD-10 columns (Cytiva, Marlborough, MA, USA) for subsequent cycles. SELEX was performed for a total of five cycles. After enrichment, the resulting library was barcoded and subjected to next-generation sequencing (NGS; Sunnybrook Research Genomics Core Facility, Toronto, ON, Canada) using the Ion Torrent PGM (Thermo Fisher Scientific, Waltham, MA, USA). Data analysis was performed using the Galaxy platform (<https://usegalaxy.org/>) and FASTAptamer toolkit.⁵² The secondary stem-loop structures of the identified aptamers were predicted using the M-FOLD web server (<http://www.unafold.org/mfold/applications/dna-folding-form.php>) under default settings for DNA folding.^{53,54} The final DNA aptamers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) with high-performance liquid chromatography (HPLC)-grade purification.

Surface Plasmon Resonance Assay

The binding affinities and kinetic constants of the selected aptamers to the recombinant PDGF proteins were precisely determined using a surface plasmon resonance (SPR) assay on a Cytiva BIAcore 8000 system, as previously described.⁵⁵ Briefly, the aptamers were immobilized onto a Cytiva SA sensor chip (BR-1005-31) at around 370 resonance units (RU). The recombinant PDGF proteins including human PDGF-A (PDA-H5113; ACROBiosystems, Newark, DE, USA), human PDGF-B (PDB-H4112; ACROBiosystems), human PDGF-C core domain (10273-H08B; Sino Biological, Beijing, China), mouse PDGF-C core domain (mPDGF-C; 50458-M07Y; Sino Biological), and human PDGF-D core domain (1159-SB/CF; R&D Systems, Minneapolis, MN, USA) were diluted in HBS-EP+ (10-mM HEPES, 150-mM NaCl, 3-mM EDTA, and 0.05% Tween 20, pH 7.4) and used as analytes at various concentrations. Binding signals were monitored in real time. The sensor surface was regenerated with 3-M MgCl₂ for 30 seconds when shifting analyte concentration. Data analysis was performed using the BIAevaluation software (Biacore, Uppsala, Sweden). The kinetic constants, including the association rate (k_a) and the dissociation rate (k_d), were calculated by fitting the sensorgrams to a 1:1 binding model. The equilibrium dissociation constant (K_D) was determined as the ratio k_d/k_a . A competitive binding assay was conducted to investigate the inhibitory effect of the α -PC aptamer on the PDGF-C/PDGFR α interaction. Specifically, recombinant PDGFR α extracellular domain was immobilized onto a Cytiva Protein A-derivatized CM5 chip. The PDGF-C, pre-incubated with or without increasing concentrations (0 to 400 nM) of the α -PC aptamer, was then injected over the chip. The binding response between PDGF-C and the immobilized PDGFR α was monitored in real-time to assess the ability of the aptamer to block this interaction.

Aptamer PEGylation and Retention Assay

The aptamers were modified with 40-kDa polyethylene glycol (PEG; 9i Technology Co., Ltd., Shanghai, China) to enhance their stability. Aptamer retention in the cell

culture medium was determined using PCR analysis. Briefly, cultured NIH/3T3 cells were incubated with 300-nM α -PC aptamer (either unmodified or PEGylated) for various time intervals (0, 2, 4, 6, and 8 hours). At each time point, cell culture media were collected and subjected to PCR amplification using the forward primer (5'-CTACTGTGTGATGTCTGA-3') and reverse primer (5'-GTGTCTGATGTACTGTCCTC-3') to detect the remaining aptamers. The PCR products were then analyzed via gel electrophoresis to assess aptamer retention in the media.

Cell Culture

Human umbilical vein smooth muscle cells (HUVSMCs; 8020; ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in smooth muscle cell medium (1101; ScienCell Research Laboratories) supplemented with 2% (v/v) fetal bovine serum (0010; ScienCell Research Laboratories), 1% penicillin-streptomycin, and a smooth muscle cell growth supplement (1152; ScienCell Research Laboratories). Cells were used at passages below 7. The identity of HUVSMCs was verified through alpha-smooth muscle actin (α -SMA) staining (Supplementary Fig. S2). NIH/3T3 cells (BNCC100843; BNCC, Beijing, China) were authenticated by short tandem repeat (STR) profiling (Supplementary Table S1). The cells were cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM; C11995500BT; Thermo Fisher Scientific) containing 10% (v/v) fetal bovine serum (10099141C; Thermo Fisher Scientific) and 1% penicillin-streptomycin. Human retinal microvascular endothelial cells (HRECs) were purchased from HZbscience (HZ-H1095; Shanghai, China) and cultured in endothelial cell medium (1001; ScienCell Research Laboratories) supplemented with 5% (v/v) fetal bovine serum, 1% endothelial cell growth supplement, and 1% penicillin-streptomycin. Cells of passages 3 to 7 were used for experiments. All cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Cell Proliferation Assay

Cell proliferation was assessed using a Cell Counting Kit-8 (CCK8; K1018-3000T; APExBio Technology, Houston, TX, USA) in accordance with the manufacturer's instructions. HUVSMCs and NIH/3T3 cells were seeded at densities of 1×10^4 and 1.5×10^4 cells/well, respectively, in 48-well plates (CS016-0095; ExCell Bio, Shanghai, China). They were then incubated overnight prior to treatment with 50-ng/mL (3-nM) PDGF-C,^{17,51} either alone or in combination with the α -PC-PEG or control (Ctrl) aptamer (both at 300 nM), in serum-free medium for 24 to 72 hours. Subsequently, the cells were incubated with 20 μ L of CCK-8 reagent for 4 hours, and the absorbance at 450 nm was measured using a microplate reader (BioTek Instruments, Winooski, VT, USA).

Cell Migration Assay

Cell migration was examined using a previously described wound healing assay.²⁵ Briefly, cells were seeded at a density of 3×10^4 cells/well in a Culture-Insert 2 Well Plate (80209; Ibidi, Gräfelfing, Germany). After overnight incubation for cell attachment, the Culture-Insert was removed, and the cells were washed with serum-free medium. The cells were then treated with 50-ng/mL (3-nM) PDGF-C,^{17,51} either alone or in combination with the α -PC-PEG aptamer (300 nM) in serum-free medium for 24 hours. Cell migration was monitored from the moment of treatment (0 hour) until the end of

the experiment (24 hours), and images were captured at 5 $\times$ magnification using an Axio Observer A1 inverted microscope (Carl Zeiss Microscopy, Jena, Germany). Quantification of migrated cells was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Receptor Activation Assay and Western Blot

Cells were serum-starved overnight before being stimulated with 50-ng/mL (3-nM) PDGF-C, with or without the α -PC-PEG aptamer (300 nM) for 10 minutes. HRECs were serum starved for 6 hours before VEGF-A stimulation (50 ng/mL) for 10 minutes with or without the α -PC-PEG aptamer (300 nM). The cells were then washed with ice-cold phosphate buffered saline (PBS) and lysed using radioimmunoprecipitation assay (RIPA) buffer (R0010-100; Solarbio, Beijing, China) supplemented with Pierce Protease and Phosphatase Inhibitors (88668; Thermo Fisher Scientific). The cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (162-0177; Bio-Rad Laboratories, Hercules, CA, USA). Western blotting was performed using antibodies specific for phosphorylated PDGFR α (pPDGFR α Y849; ab79318; Abcam, Cambridge, UK), PDGFR α (3174; Cell Signaling Technology, Danvers, MA, USA), pAkt (4060S; Cell Signaling Technology), Akt (4691S; Cell Signaling Technology), extracellular-regulated kinase (ERK; 4696S; Cell Signaling Technology), phosphorylated ERK (pERK; 4370S; Cell Signaling Technology), phosphorylated VEGFR2 (pVEGFR2; 3770; Cell Signaling Technology), and VEGFR2 (9698; Cell Signaling Technology). The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and developed using Immobilon Western Chemiluminescent Substrate (WBKLS0500; Merck Millipore, Burlington, MA, USA). The bands were visualized using a G: BOX (Syngene, Bangalore, India) and quantified using ImageJ. Phosphorylated proteins were normalized to their corresponding total protein levels.

Quantitative Reverse Transcription PCR

Total RNA was extracted from dissected mouse retinas using the TRIzol reagent (Tiangen Biotech, Beijing, China). cDNA was synthesized using a FastKing RT Kit (Tiangen Biotech). Quantitative reverse transcription PCR (qRT-PCR) was performed in triplicate using SYBR Green qPCR Master Mix (Vazyme Biotech, Nanjing, China) on an ABI QuantStudio 6 Flex Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Primer sequences were as follows: *Pdgfa* forward 5'-ctcttgagatagactcctgtagg-3', reverse 5'-actctcttcctgcg aatgg-3'; *Pdgfb* forward 5'-catcgctcctttgatgatctt-3', reverse 5'-gtgctcgggtcatgttcaagt-3'; *Pdgfc* forward 5'-gcatgagagagttg tcatcat-3', reverse 5'-acttgcatatcatcttctg-3'; *Pdgfd* forward 5'-ttcccgacacgactaccaag-3', reverse 5'-tccttgaggatctcctgt-3'; *Actb* forward 5'-gccaacacagtgtgtctgg-3', reverse 5'-ggagc aatgatctgatcttc-3'. The relative mRNA levels were calculated using a $\Delta\Delta C_t$ method and normalized to *Actb*.

Animals

C57BL/6J mice 6 to 8 weeks old (young)^{56–58} or 15 months old (aged, anti-VEGF refractory model)⁵⁹ were purchased from GemPharmatech (Nanjing, China) and used in this study. The animals were housed in a standard experimental

facility under a 12-hour light/dark cycle and were provided ad libitum access to food and water. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Care and Use Committee at Zhongshan Ophthalmic Center, Sun Yat-Sen University in Guangzhou, China (approval nos. Z2022029, Z2024033).

Laser-Induced CNV and Fibrosis Model and Treatment

The laser-induced CNV mouse model was established as described previously.^{25,60} Briefly, mice were anesthetized with 0.5% pentobarbital sodium (50 mg/kg body weight). Their pupils were then dilated with topical tropicamide phenylephrine drops (Santen Pharmaceutical, Osaka, Japan). Four laser spots (75- μ m spot size, 75-ms duration, 90-mW power) were applied around the optic disk of each mouse eye using an OcuLight Infrared Laser System (810 nm wavelength; Iridex, Mountainview, CA, USA). PDGF-C expression in mouse retina following laser injury was determined by western blotting. Retinas were harvested and lysed. Lysates were analyzed using primary antibodies against PDGF-C (SAB1300229; Sigma-Aldrich, St. Louis, MO, USA), and the band intensities were normalized to β -actin (RM2001; RayBiotech, Peachtree Corners, GA, USA) as a loading control.

Intravitreal injections of the α -PC-PEG or Ctrl aptamer (100 pmol in 1 μ L per eye or as indicated in figure legends) were administered immediately post-laser, at 3 days post-laser, or once weekly starting immediately post-laser. Mice receiving intravitreal injections of PBS (1 μ L/eye) served as additional controls. The efficacy of the α -PC-PEG aptamer was also compared with that of VEGF blockade. For this comparison, CNV mice were treated with a neutralizing antibody against VEGF-A (5 μ g/ μ L per eye; AF-493-NA; R&D Systems). Normal IgG (5 μ g/ μ L per eye; AB-108-C; R&D Systems) was used as a control for the antibody treatment. CNV lesions were analyzed 7 days post-laser induction, and fibrosis was examined 21 days post-laser induction.

FITC-Dextran Leakage Assay

Vascular leakage in the CNV model was assessed as described previously with some modification.⁶¹ Briefly, 1 week after laser-induced CNV, the mice were anesthetized. FITC-dextran (40 kDa, 100 μ L of 25 mg/mL in PBS; Sigma-Aldrich) was injected via tail vein within 10 seconds. After 30 minutes, the eyes were enucleated, fixed, and eye cups dissected and flatmounted. Vascular leakage was assessed by detecting extravascular FITC signal using an Axio Imager Z2 upright microscope (Carl Zeiss Microscopy). The leakage areas were quantified using AxioVision SE64 software (Carl Zeiss Microscopy).

Immunofluorescence Staining

Mice were euthanized, and their eyes were harvested for immunostaining. Briefly, the eyes were fixed in 4% paraformaldehyde for 2 hours. After removal of the cornea, lens, and retina, the remaining eyecups were rinsed with PBS and permeabilized with 0.5% Triton X-100 (X100-100ML; Sigma-Aldrich) in PBS for 30 minutes. Following three PBS washes, the tissues were blocked with a solution of 5%

donkey serum (HQ30080-P100; Guangzhou Hongquan biot., Guangzhou, China) and 0.5% Triton X-100 in PBS for 1 hour at room temperature. The samples were then incubated overnight at 4°C with Alexa Fluor 488–conjugated Isolectin GS-IB4 (I21411; Life Technologies) or with primary antibodies against COL1 (14695-1-AP; Proteintech, Rosemont, IL, USA) and α -SMA (M0851; Dako Products, Santa, Clara, CA, USA), both diluted (1:200) in blocking buffer. After three PBS washes, the samples were incubated with Invitrogen Alexa Fluor 555–conjugated secondary antibodies (A31570; Thermo Fisher Scientific) and diluted 1:500 in blocking buffer for 1 hour at room temperature. Following three PBS rinses, the eyecups were flatmounted with the RPE facing upward. Images were captured using the Zeiss Axio Imager Z2 upright microscope, and CNV areas were analyzed using the AxioVision SE64 software, as previously described.²⁵ All image analyses were performed in a blinded manner.

Statistics

Prism 8.2.1 (GraphPad, Boston, MA, USA) was used for graph creation and statistical analyses. Statistical analysis was performed using a two-tailed Student's *t*-test or one-way ANOVA followed by Dunnett's or Sidak's post hoc tests for parametric multiple comparisons or Kruskal–Wallis test with Dunn's post hoc test for non-parametric comparisons. A difference was considered statistically significant for $P < 0.05$. Data are presented as the mean $\pm$ SEM from at least three independent biological samples or experiments. Eyes with massive intraocular hemorrhage or fused CNV spots upon laser injury were excluded from the study analysis and no other data were excluded.

RESULTS

Generation of an Anti-PDGF-C (α -PC) Aptamer That Inhibits PDGF-C Binding to PDGFR α

We and others have previously reported that PDGF-C is a potent angiogenic factor that plays a critical role in pathological angiogenesis, such as CNV.^{22,33,17,62–65} Aptamers have demonstrated therapeutic potential in several pathological conditions^{45–47,66} and possess advantages such as minimal immunogenicity, low manufacturing cost, and ease of chemical synthesis. Using a custom library consisting of approximately 1.1×10^{15} sequences of 74-nt-long ssDNA,⁵⁰ we performed SELEX followed by sequencing and identified a promising PDGF-C-binding DNA aptamer, termed the α -PC aptamer (Table). Secondary structural prediction using the M-FOLD software revealed that the α -PC aptamer exhibited a more complex hairpin-bulge architecture with multiple branching loops, and the control (Ctrl) aptamer displayed a simpler structure (Figs. 1A, 1B). SPR analysis demonstrated that the α -PC aptamer bound tightly to PDGF-C with a dissociation constant (K_D) of 20 pM ($k_a = 7.29 \times 10^7$ M⁻¹ s⁻¹, $k_d = 1.47 \times 10^{-3}$ s⁻¹, Figs. 1C, 1D). The aptamer exhib-

ited no detectable cross-reactivity with other PDGF family members (Figs. 1E–G, Supplementary Fig. S3). Importantly, in a competitive SPR assay, the α -PC aptamer effectively inhibited PDGF-C binding to its receptor PDGFR α ¹⁷ in a dose-dependent manner (Fig. 1H).

Polyethylene Glycol Modification of the α -PC Aptamer

PDGF-C is a secreted growth factor that exerts its biological effects extracellularly by binding to the PDGFR α receptor on the membrane of target cells, such as NIH/3T3 fibroblasts.¹⁷ Therefore, aptamer-based therapeutics targeting PDGF-C must ensure long-term stability in extracellular media (e.g., culture medium) to maintain effective neutralizing activity. As such, to enhance the stability of the α -PC aptamer, we conjugated it to PEG, a modification known to reduce aptamer degradation.^{67,68} Indeed, using cultured NIH/3T3 fibroblasts, we incubated the cells with either PEG-modified or unmodified α -PC aptamers and assessed the stability of them in the culture medium by PCR. The results demonstrated that the PEG-modified α -PC (α -PC-PEG) aptamer showed a markedly prolonged retention, remaining detectable for over 8 hours, compared to less than 2 hours for the unmodified aptamer (Figs. 2A, 2B). Given that fibroblasts play key roles in pathological angiogenesis and extracellular matrix (ECM) remodeling by producing large amounts of pro-angiogenic factors and ECM components (such as collagen),^{33,69–71} enhancing aptamer stability in this context is critical.

The α -PC-PEG Aptamer Blocks PDGF-C-Induced PDGFR α Activation and Downstream Signaling in Both Fibroblasts and Vascular Smooth Muscle Cells

We next tested whether the α -PC-PEG aptamer could inhibit PDGF-C-induced PDGFR α signaling in NIH/3T3 cells. Treatment with the α -PC-PEG aptamer markedly reduced PDGFR α phosphorylation following PDGF-C stimulation (Figs. 2C, 2D). The α -PC-PEG aptamer also suppressed PDGF-C-induced activation of the downstream signaling molecules Akt (Figs. 2E, 2F) and ERK (Figs. 2G, 2H) in a dose-dependent manner. At 300 nM, the α -PC-PEG aptamer completely blocked PDGFR α , Akt, and ERK phosphorylation, and this concentration was used in subsequent experiments. In addition to fibroblasts, vascular smooth muscle cells also promote angiogenesis by secreting various cytokines, such as VEGF, PDGF, and fibroblast growth factor (FGF).^{72,73} We therefore examined the inhibitory effect of the α -PC-PEG aptamer on human umbilical vein smooth muscle cells (HUVSMCs). Similar to the findings in fibroblasts, the α -PC-PEG aptamer significantly suppressed PDGF-C-induced PDGFR α phosphorylation (Figs. 3A, 3B) and reduced the downstream Akt (Figs. 3C, 3D) and ERK activation (Figs. 3E, 3F) in HUVSMCs. These results demonstrate

TABLE. SELEX-NGS Screened DNA Aptamer Specifically Targeting Human PDGF-C

Aptamer	Sequence (5'–3')
α -PC	CTACTGTGTGATGTCTGAGAGCAGATACCAGTGAACGGGGAGTCATCTGCACAGAGGACAGTACATCAGACAC
Control	CTACTGTGTGATGTCTGAGAGCAGCGTCTAAACGAACAAGCGAACCTATGCACAGAGGACAGTACATCAGACAC

The sequences of variable regions are indicated by bold italic.

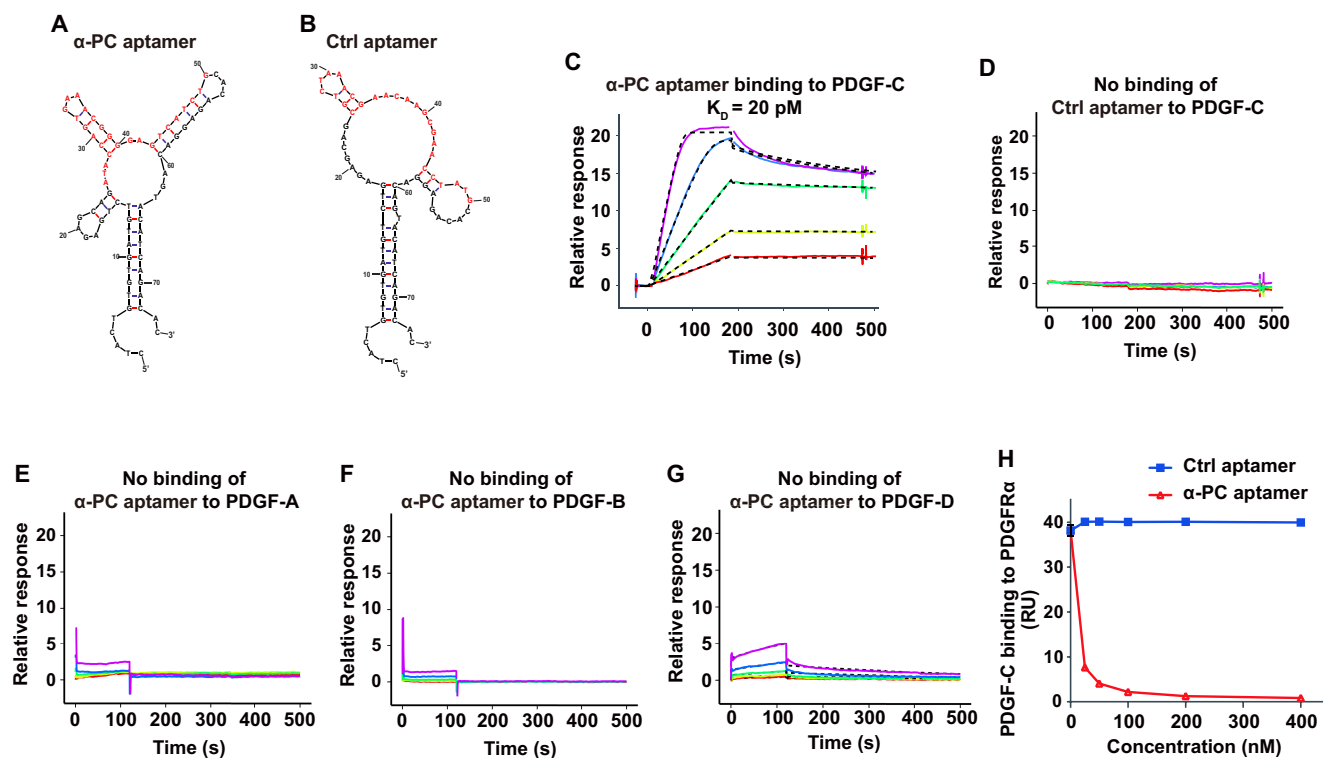


FIGURE 1. The α -PC aptamer binds to PDGF-C and inhibits PDGF-C binding to PDGFR α . (A, B) Predicted secondary stem-loop structures of the α -PC aptamer (A) and the Ctrl aptamer (B) determined by the M-FOLD analysis. The α -PC aptamer represented one of the highly enriched sequences identified by NGS after five rounds of SELEX against human PDGF-C. (C–G) SPR sensorgrams showing the binding kinetics and specificities of the α -PC aptamer to PDGF-C or other PDGF family members. Lines from bottom to top correspond to increasing concentrations of recombinant PDGF proteins (12.5, 25, 50, 100, and 200 nM). Binding kinetics of the α -PC aptamer to PDGF-C are shown in C, and no detectable binding of the Ctrl aptamer (negative control) to PDGF-C can be seen in D. There was no detectable binding of the α -PC aptamer to other human PDGF family members, including PDGF-A (E), PDGF-B (F) and PDGF-D (G). (H) Competitive binding assay based on SPR technology shows that the α -PC aptamer dose-dependently inhibited the binding of PDGF-C to PDGFR α .

that the α -PC-PEG aptamer can effectively inhibit the PDGF-C/PDGFR α signaling axis across multiple cell types.

The α -PC-PEG Aptamer Reduces PDGF-C-Induced Proliferation of Both Fibroblasts and Vascular Smooth Muscle Cells

To determine the functional impact of the α -PC-PEG aptamer-mediated inhibition of PDGF-C signaling, we assessed cell proliferation using a CCK-8 assay with NIH/3T3 fibroblasts and HUVMSCs. In NIH/3T3 cells, PDGF-C stimulation significantly promoted cell proliferation at both 24 and 48 hours, an effect that was completely abolished by the α -PC-PEG aptamer (Figs. 4A, 4B). Likewise, in HUVMSCs, PDGF-C markedly increased cell proliferation at 48 and 72 hours, which was strongly inhibited by the α -PC-PEG aptamer (Figs. 4C, 4D). These findings show that the α -PC-PEG aptamer can effectively block PDGF-C-driven proliferative responses in different cell types.

The Inhibitory Effect of the α -PC-PEG Aptamer Is Specific

Aptamers are known to exhibit high target specificities with minimal cross-reactivity, even toward homologous proteins with considerable sequence similarities.⁷⁴ To evaluate the specificity of the α -PC-PEG aptamer, we examined its effect

on PDGF-B-induced cell proliferation, as PDGF-B shares 34% sequence identity and 46% similarity with PDGF-C (Supplementary Figs. S3A, S3B). We found that the α -PC-PEG aptamer did not affect PDGF-B-induced proliferation of either NIH/3T3 cells (Figs. 4A, 4B) or HUVMSCs (Figs. 4C, 4D), demonstrating that the α -PC-PEG aptamer is highly specific for PDGF-C.

The α -PC-PEG Aptamer Inhibits PDGF-C-Induced Cell Migration

We subsequently examined whether the α -PC-PEG aptamer affects PDGF-C-driven cell migration, a crucial process in pathological neovascularization.^{71,75} Using a monolayer cell wound healing assay, we found that PDGF-C stimulation significantly enhanced the migration of NIH/3T3 cells (Figs. 5A, 5B), whereas co-treatment with the α -PC-PEG aptamer effectively suppressed this effect (Figs. 5A, 5B). Similarly, PDGF-C stimulation markedly increased the migration of HUVMSCs (Figs. 5C, 5D), and this effect was significantly inhibited by the α -PC-PEG aptamer (Figs. 5C, 5D). Notably, the α -PC-PEG aptamer did not affect PDGF-B-stimulated migration of either NIH/3T3 cells or HUVMSCs (Figs. 5A–D), further confirming that its inhibitory activity was selective for PDGF-C. These data thus support the potential of the α -PC-PEG aptamer in inhibiting pathological neovascularization.

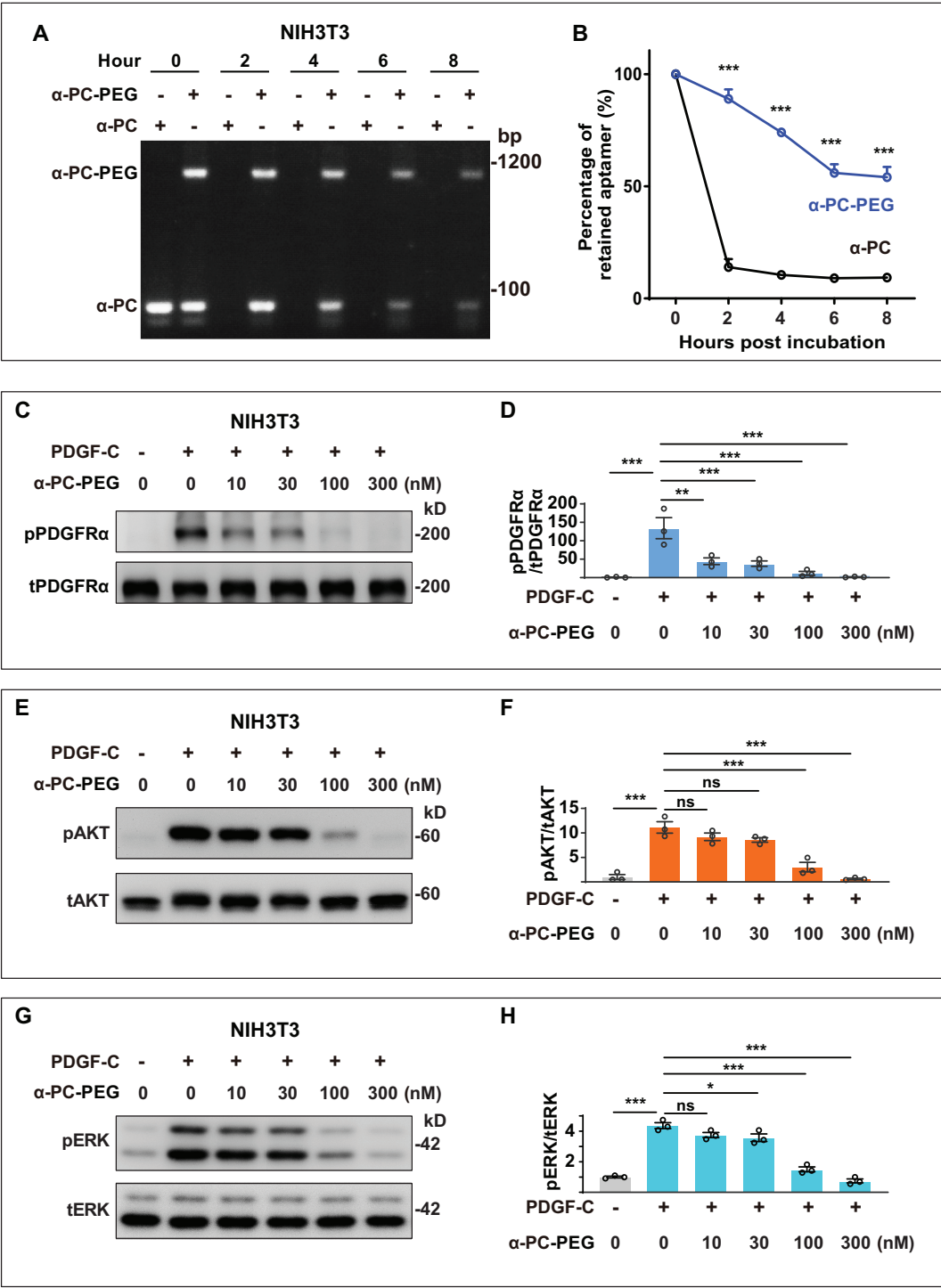


FIGURE 2. PEG modification of the α -PC aptamer and its inhibition on PDGF-C-induced PDGFR α activation and downstream signaling in NIH/3T3 cells. (A) NIH/3T3 cells were incubated with α -PC aptamer with or without PEG modification for the indicated times. Culture media were collected and analyzed by PCR and gel electrophoresis to assess aptamer stability and retention. (B) Quantification of retained aptamers based on integrated density of PCR products shown in A ($n = 2$ or 3). Two-way ANOVA followed by Sidak's multiple comparisons test revealed an adjusted $P < 0.001$ for the PEG-modified α -PC aptamer versus the unmodified aptamer at indicated time points. (C, D) Western blot analysis showing that the α -PC-PEG aptamer suppressed PDGF-C-induced PDGFR α phosphorylation in a dose-dependent manner in NIH/3T3 cells. Adjusted $P < 0.001$ for PDGF-C versus BSA; $P = 0.0014$, $P = 0.0008$, $P < 0.001$, and $P < 0.001$ for PDGF-C versus PDGF-C + 10-, 30-, 100-, or 300-nM α -PC-PEG aptamer, respectively. (E, F) Western blot analysis showing that the α -PC-PEG aptamer inhibited PDGF-C-induced phosphorylation of Akt (pAkt) (E, F) in a dose-dependent manner in NIH/3T3 cells. Adjusted $P < 0.001$ for PDGF-C versus BSA; $P = 0.30$, $P = 0.12$, $P < 0.001$, and $P < 0.001$ for PDGF-C versus PDGF-C + 10-, 30-, 100-, or 300-nM α -PC-PEG aptamer, respectively, for pAkt/tAkt (E, F). Adjusted $P < 0.001$ for PDGF-C versus BSA; $P = 0.089$, $P = 0.026$, $P < 0.001$, and $P < 0.001$ for PDGF-C versus PDGF-C + 10-, 30-, 100-, or 300-nM α -PC-PEG aptamer, respectively, for pERK/tERK ($n = 3$) (G, H). Data are represented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA with Dunnett's multiple comparisons test for panels C to H).

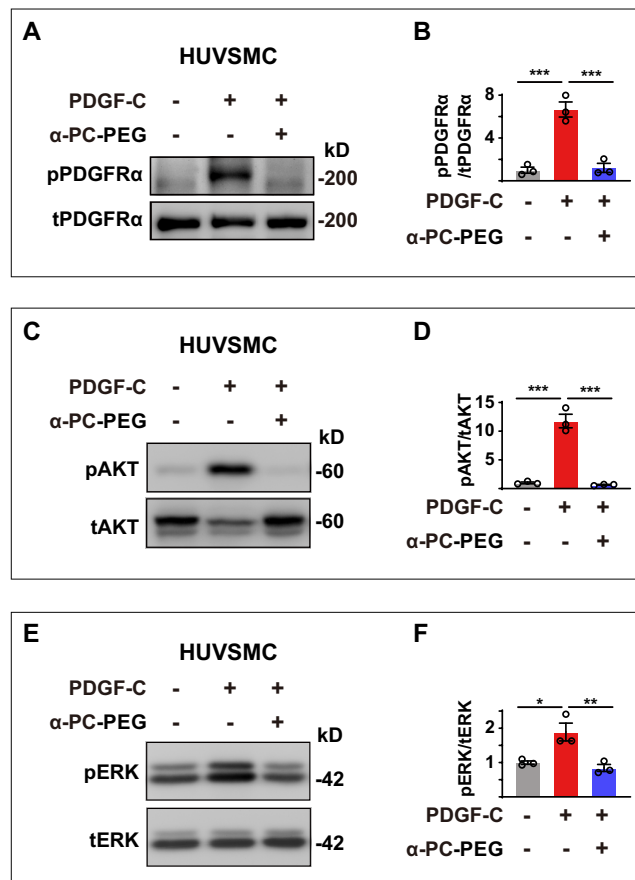


FIGURE 3. The α -PC-PEG aptamer blocks PDGF-C-induced PDGFR α activation and downstream signaling in HUVECs. (A, B) Western blots showing that the α -PC-PEG aptamer inhibited PDGF-C-induced PDGFR α phosphorylation in HUVECs. Adjusted *P* values were *P* < 0.001 for both PDGF-C versus BSA and PDGF-C versus PDGF-C + α -PC-PEG aptamer. (C–F) Western blots show that the α -PC-PEG aptamer suppressed PDGF-C-induced phosphorylation of Akt (pAkt) (C, D) and ERK (pERK) (E, F) in HUVECs. Adjusted *P* < 0.001 for both PDGF-C versus BSA, and PDGF-C versus PDGF-C + α -PC-PEG aptamer for pAkt/tAkt (C, D). Adjusted *P* = 0.0148 for PDGF-C versus BSA, and *P* = 0.007 for PDGF-C versus PDGF-C + α -PC-PEG aptamer for pERK/tERK (*n* = 3) (E, F). Data are represented as mean $\pm$ SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (one-way ANOVA with Dunnett's multiple comparisons test).

The α -PC-PEG Aptamer Binds to Mouse PDGF-C and Suppresses Choroidal Neovascularization in Young (2-Month-Old) Mice

In order to evaluate the effect of the α -PC-PEG aptamer in vivo using mouse models, we first examined whether it bound to mouse PDGF-C. SPR analysis demonstrated that the α -PC aptamer bound tightly to mouse PDGF-C with a high affinity (K_D = 33 nM, k_a = 1.72×10^5 M $^{-1}$ s $^{-1}$, k_d = 5.69×10^{-3} s $^{-1}$) (Fig. 6A), consistent with the high sequence conservation between human and mouse PDGF-C (more than 94% sequence identity and 97% similarity, Supplementary Fig. S3). We subsequently tested the therapeutic potential of the α -PC-PEG aptamer using a laser-induced CNV model and 2-month-old mice (Fig. 6B), a commonly used experimental setting in the field.^{56–58} The qRT-PCR analysis showed that PDGF-C mRNA level in the retina markedly increased as early as day 1 post-laser injury and continued

to increase through day 5 (fourfold versus baseline, *P* < 0.001) (Fig. 6C), but other PDGF family members showed no upregulation or even a slight decrease (Fig. 6C), suggesting a potential function of PDGF-C in CNV progression. Indeed, western blot analysis also confirmed a 1.9-fold increase of PDGF-C protein at day 3 post-laser injury (Figs. 6D, 6E).

A dose-response study showed that intravitreal injection of the α -PC-PEG aptamer reduced CNV sizes in a dose-dependent manner, with the maximal efficacy at 100 pmol (Supplementary Fig. S4), which was adopted in subsequent experiments. At this dose, the α -PC-PEG aptamer markedly suppressed CNV lesions by $\sim$ 44% (*P* = 0.004) (Figs. 6F–H), a magnitude comparable to the effect of anti-VEGF antibody treatment (39% reduction, *P* = 0.0014) (Figs. 6F–H). Together, these results suggest a therapeutic potential of the α -PC-PEG aptamer in suppressing CNV.

The α -PC-PEG Aptamer Inhibits CNV in Anti-VEGF Refractory Aged (15-Month-Old) Mice

Aged mice with CNV are known to be refractory to anti-VEGF therapy,⁵⁹ which may be relevant to the clinical observation that up to 40% of wAMD patients exhibit limited or poor response to anti-VEGF treatment.⁵ We therefore investigated whether the α -PC-PEG aptamer could inhibit CNV in aged mice (15 months old). We found that, although anti-VEGF treatment did not reduce CNV sizes in aged mice (Figs. 6I, 6J), the α -PC-PEG aptamer markedly suppressed CNV by approximately 35% (Figs. 6I, 6J). The α -PC-PEG aptamer did not affect VEGF-A-induced VEGFR2 activation (Supplementary Fig. S5). These findings highlight a unique ability of the α -PC-PEG aptamer to inhibit pathological neovascularization in anti-VEGF-refractory settings, supporting a valuable therapeutic potential of the α -PC-PEG aptamer in the treatment of anti-VEGF therapy-resistant neovascular diseases.

The α -PC-PEG Aptamer Reduces Established CNV Lesions and Attenuates Vascular Leakage

To determine whether the α -PC-PEG aptamer can also reduce established CNV, we administered the aptamer at day 3 post-laser when CNVs are already formed.^{76–78} Notably, treatment with the α -PC-PEG aptamer at this time point still significantly reduced CNV areas by about 40% (Figs. 7A, 7B), and markedly decreased FITC-dextran leakage by about 68% (Figs. 7C, 7D), demonstrating that the aptamer not only suppresses neovascularization but also ameliorates vascular leakage in laser-induced CNV model.

The α -PC-PEG Aptamer Suppresses Subretinal Fibrosis in CNV

Given previous reports implicating PDGF-C in fibrotic remodeling across multiple tissues, we next sought to determine whether the α -PC-PEG aptamer could mitigate subretinal fibrosis, a major cause of irreversible visual impairment.⁷⁹ We employed a laser-induced CNV fibrosis mouse model (Fig. 8A).⁸⁰ At 21 days post-laser injury, mouse choroidal wholemounts were collected to assess the levels of α -SMA and type I collagen (COL1), two established fibrosis markers.⁸⁰ Immunostaining revealed that the α -PC-PEG aptamer significantly reduced α -SMA-positive (α -SMA $^{+}$) areas ($17.35 \pm 1.97 \times 10^3$ μ m ^{2}) compared with the

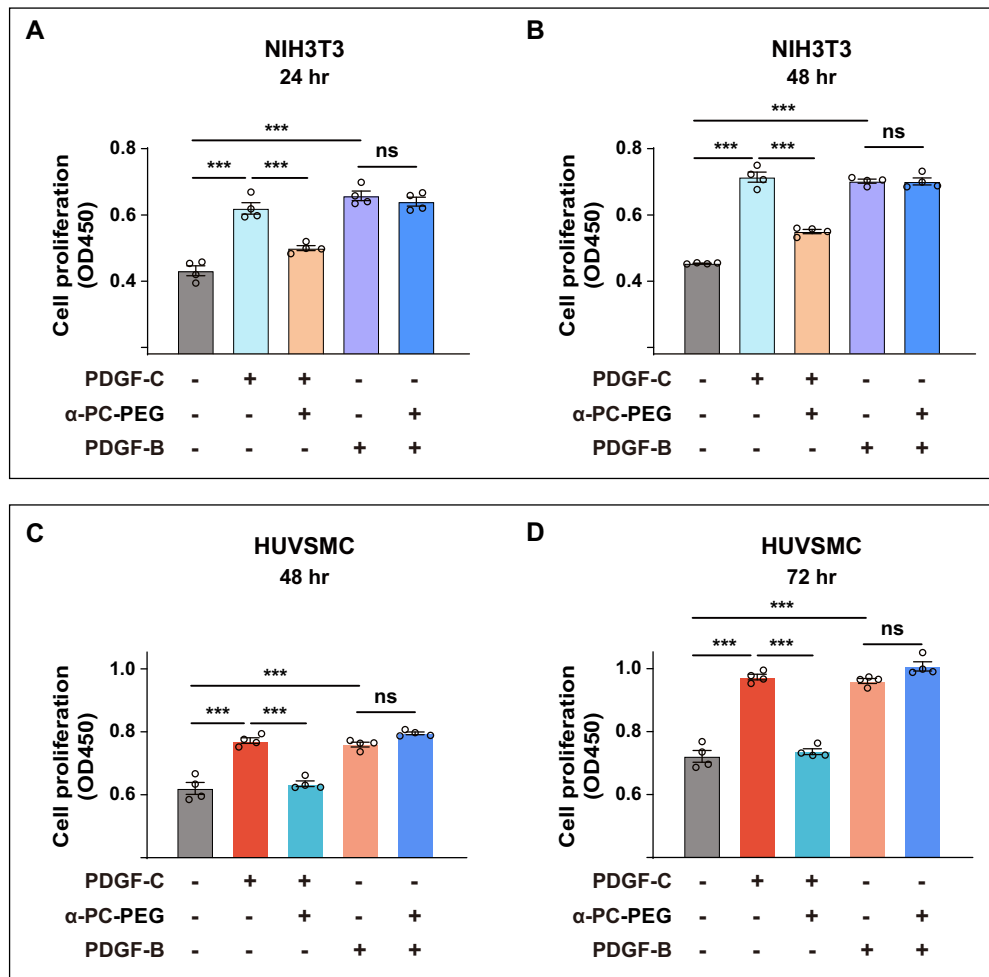


FIGURE 4. The α -PC-PEG aptamer inhibits PDGF-C-induced proliferation of both fibroblasts and vascular smooth muscle cells. (A, B) A CCK-8 assay demonstrating that the α -PC-PEG aptamer inhibited PDGF-C-induced, but not PDGF-B-induced, proliferation in NIH/3T3 cells at 24 hours (A) and 48 hours (B). Adjusted $P < 0.001$ for both PDGF-C versus BSA, and PDGF-C versus PDGF-C + α -PC-PEG aptamer at both time points. Adjusted $P < 0.001$ for PDGF-B versus BSA at both indicated time points, $P = 0.85$ and $P = 1.0$ for PDGF-B versus PDGF-B + α -PC-PEG aptamer at 24 and 48 hours, respectively. (C, D) A CCK-8 assay showing that the α -PC-PEG aptamer inhibited PDGF-C-induced, but not PDGF-B-induced, proliferation of HUVMSCs at 48 hours (C) and 72 hours (D). Adjusted $P < 0.001$ for both PDGF-C versus BSA and PDGF-C versus PDGF-C + α -PC-PEG aptamer at both time points. Adjusted $P < 0.001$ for PDGF-B versus BSA at both time points, $P = 0.14$ and $P = 0.08$ for PDGF-B versus PDGF-B + α -PC-PEG aptamer at 48 hours and 72 hours, respectively ($n = 4$). Data are represented as mean $\pm$ SEM. ** $P < 0.01$, * $P < 0.001$ (one-way ANOVA with Sidak's post hoc test).**

control group ($94.34 \pm 7.81 \times 10^3 \mu\text{m}^2$) (Figs. 8B, 8C). Similarly, the COL1-positive (COL1⁺) areas were also substantially reduced in the mice treated with the α -PC-PEG aptamer ($9.79 \pm 0.73 \times 10^3 \mu\text{m}^2$ vs. $24.52 \pm 1.19 \times 10^3 \mu\text{m}^2$ in control mice), suggesting decreased extracellular matrix deposition⁸¹ (Figs. 8B, 8C). These data thus demonstrate that the α -PC-PEG aptamer effectively suppresses subretinal fibrosis in the CNV model.

DISCUSSION

In this study, we identified and characterized a novel anti-PDGF-C DNA aptamer (α -PC), which specifically binds to PDGF-C, blocks its downstream signaling, and inhibits PDGF-C-induced proliferation and migration of both fibroblasts and vascular smooth muscle cells. In vivo, the α -PC-PEG aptamer effectively inhibited laser-induced CNV and subretinal fibrosis in mice. Strikingly, in anti-VEGF-refractory aged mice, the α -PC-PEG aptamer markedly

reduced CNV, but anti-VEGF treatment failed to do so. These findings highlight a potential therapeutic value of the α -PC aptamer in the treatment of neovascular diseases and suggest a possibility of targeting PDGF-C to overcome the limitations of anti-VEGF therapy.

Mounting evidence has suggested that PDGF-C may be a promising target for the treatment of neovascular diseases. Clinical studies revealed significant upregulation of PDGF-C in the retinas of AMD patients and other neovascular pathologies.^{31,32} Consistently, our qRT-PCR results also demonstrated that, in a laser-induced CNV model, PDGF-C expression markedly increased, whereas the other PDGF family members (PDGF-A, PDGF-B, and PDGF-D) showed no significant upregulation. PDGF-C has been reported to be highly expressed in endothelial tip cells and contributes to endothelial motility and choroidal capillary sprouting, but this is not the case for PDGF-B.^{22,37} Moreover, sequence and structural analysis by Reigstad et al.³⁶ revealed that PDGF-C shares greater homology to VEGF-A than to PDGF-B,

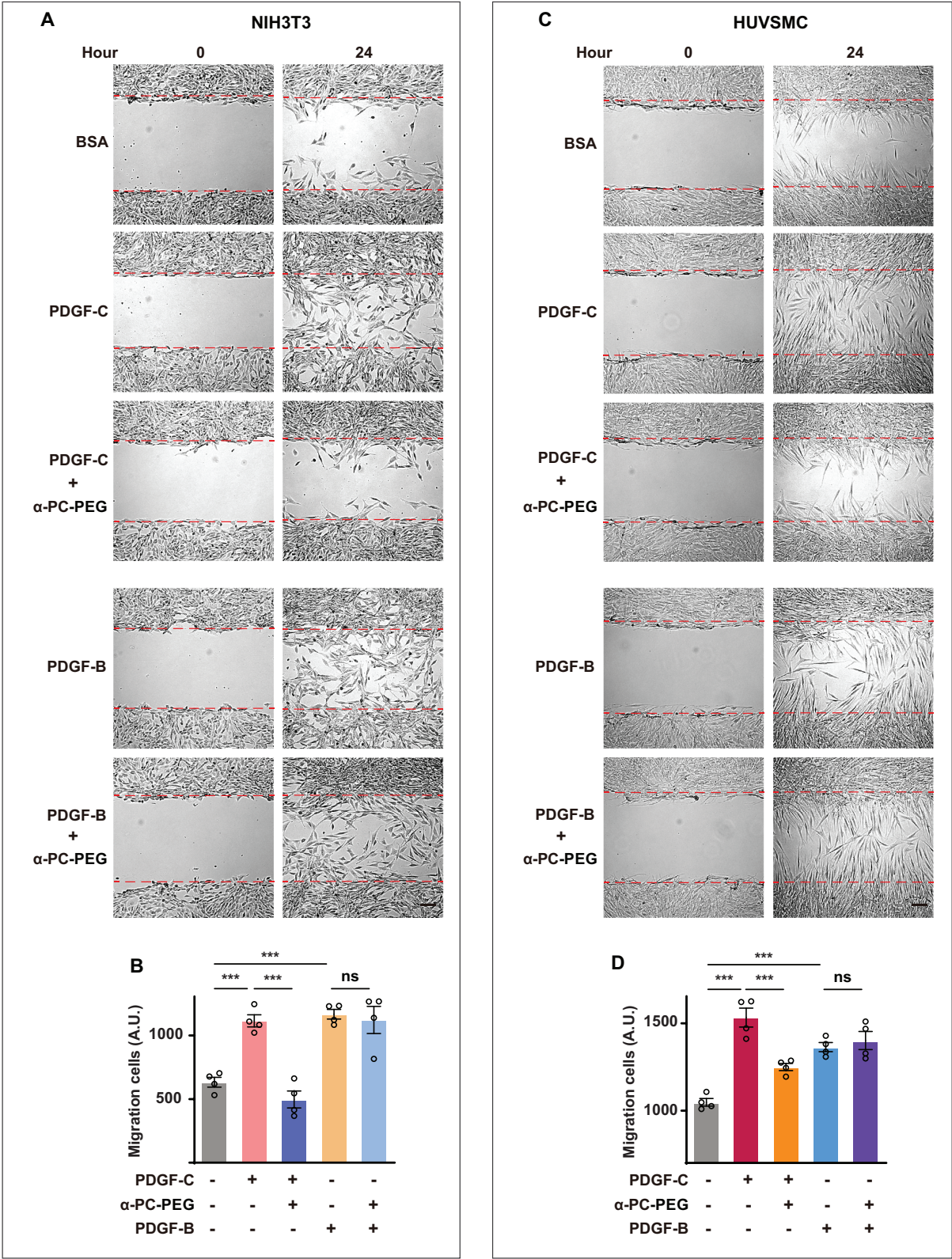


FIGURE 5. The α -PC-PEG aptamer inhibits PDGF-C-induced cell migration. (A, B) Representative phase contrast images from a wound healing assay (A) and corresponding quantifications (B) show that the α -PC-PEG aptamer inhibited PDGF-C-induced, but not PDGF-B-induced, migration of NIH/3T3 cells. Adjusted $P = 0.0004$ for PDGF-C versus BSA, $P < 0.001$ for PDGF-C versus PDGF-C + α -PC-PEG aptamer, $P = 0.001$ for PDGF-B versus BSA, and $P = 0.98$ for PDGF-B versus PDGF-B + α -PC-PEG aptamer. (C, D) Representative phase contrast images from a wound healing assay (C) and corresponding quantifications (D) show that the α -PC-PEG aptamer inhibited PDGF-C-induced, but not PDGF-B-induced, migration of HUVMCs. Adjusted $P < 0.001$ for PDGF-C versus BSA, $P = 0.0004$ for PDGF-C versus PDGF-C + α -PC-PEG aptamer, $P = 0.001$ for PDGF-B versus BSA, and $P = 0.94$ for PDGF-B versus PDGF-B + α -PC-PEG aptamer ($n = 4$). Data are represented as mean $\pm$ SEM. *** $P < 0.001$ (one-way ANOVA with Sidak's post hoc test).

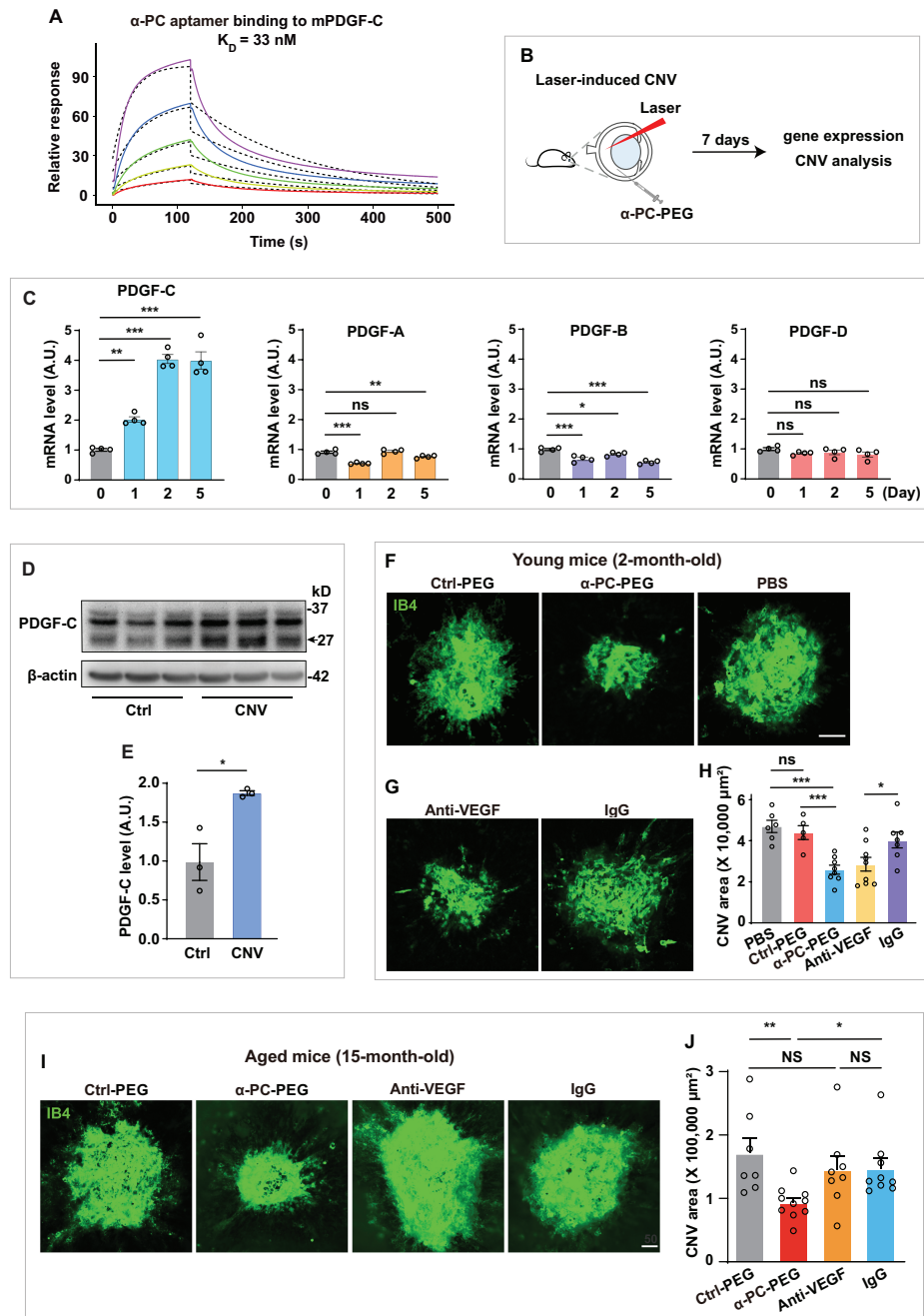


FIGURE 6. The α -PC-PEG aptamer represses choroidal neovascularization in both young mice and anti-VEGF refractory aged mice. (A) SPR sensorgrams showing the binding kinetics of the α -PC aptamer to mPDGF-C. Lines from bottom to top represent increasing concentrations of recombinant mPDGF-C (12.5, 25, 50, 100, and 200 nM). (B) Schematic diagram illustrating the experimental procedure for the laser-induced CNV model. (C) qRT-PCR results showing the mRNA levels of PDGF family members in the retina following laser treatment, indicating that only PDGF-C was consistently and significantly upregulated from 1 to 5 days post-laser injury ($n = 4$). One-way ANOVA with Dunnett's post hoc test was used to compare each post-laser time point (day 1, day 2, day 5) to the control group (day 0, no CNV) yielded the following adjusted P values: for PDGF-C, $P = 0.0034$ (day 1), $P < 0.001$ (day 2), and $P < 0.001$ (day 5); for PDGF-A, $P < 0.001$ (day 1), $P = 0.87$ (day 2), and $P = 0.007$ (day 5); for PDGF-B, $P < 0.001$ (day 1), $P = 0.035$ (day 2), and $P < 0.001$ (day 5). For PDGF-D, the expression was not significantly altered (adjusted $P = 0.30$ at day 1, $P = 0.47$ at day 2, and $P = 0.15$ at day 5). (D, E) Western blots (D) with corresponding quantification (E) show increased PDGF-C protein levels in the retina following laser-induced injury ($n = 3$). $P = 0.02$ for laser injury versus control (Student's t -test). (F, G) Representative fluorescence images of choroidal wholemounts stained with IB4 (green) from 2-month-old young mice treated with α -PC-PEG aptamer, anti-VEGF, or control treatments following laser-induced CNV. (H) Quantitative analysis of CNV lesion size in F and G shows that the α -PC-PEG aptamer suppressed CNV. One-way ANOVA with Sidak's post hoc test yielded adjusted $P = 0.0009$ for α -PC-PEG aptamer versus Ctrl aptamer, $P = 0.001$ for α -PC-PEG aptamer versus PBS, and $P = 0.036$ for anti-VEGF versus control IgG. (I) Representative fluorescence images of choroidal wholemounts stained with IB4 (green) from 15-month-old aged mice treated with α -PC-PEG aptamer, Ctrl aptamer, anti-VEGF, or control IgG. (J) Quantification of CNV lesion areas in I, showing that the α -PC-PEG aptamer, but not anti-VEGF, reduced CNV. Statistical analysis was conducted using Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons. Adjusted $P = 0.0063$ for α -PC-PEG aptamer versus Ctrl aptamer and $P > 0.99$ for anti-VEGF versus control IgG ($n = 5$ –10 for F to J). Scale bar: 50 μm . Data are represented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

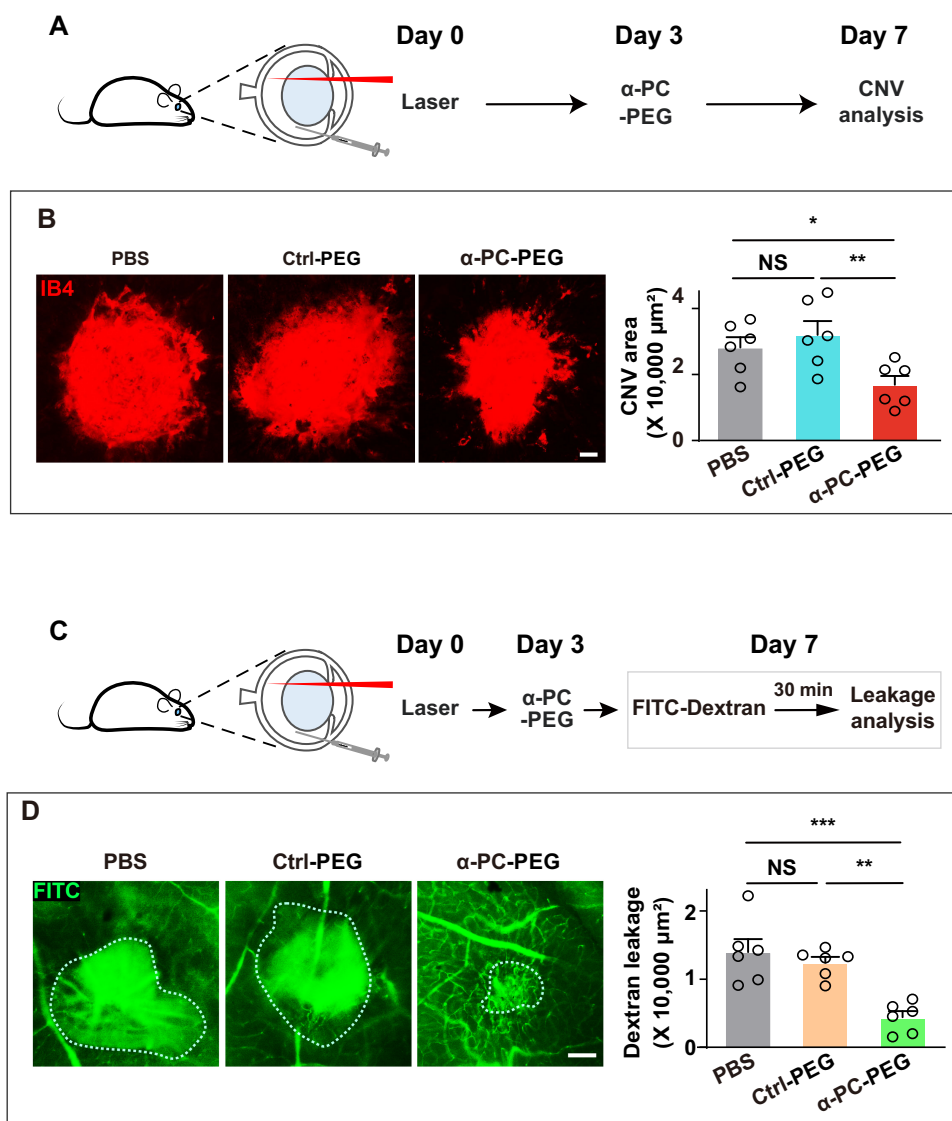


FIGURE 7. The α -PC-PEG aptamer reduces established CNV and attenuates vascular leakage. (A) Schematic diagram illustrating the experimental procedure. (B) Representative fluorescence images and quantitative analysis of choroidal wholemounts stained with IB4 (red) from mice treated with PBS, Ctrl aptamer, or α -PC-PEG aptamer. One-way ANOVA with uncorrected Fisher's least significant difference post hoc test yielded $P = 0.033$ for α -PC-PEG versus PBS, $P = 0.0065$ for α -PC-PEG versus Ctrl aptamer, and $P = 0.43$ for Ctrl aptamer versus PBS. (C) Schematic diagram of the vascular leakage assay. (D) Representative images and quantifications showing FITC-dextran extravasation (green) in choroidal flatmounts from mice treated with PBS, Ctrl aptamer, or α -PC-PEG aptamer. One-way ANOVA with Sidak's post hoc test yielded adjusted $P = 0.0004$ for α -PC-PEG versus PBS, $P = 0.0019$ for α -PC-PEG versus Ctrl aptamer, and $P = 0.81$ for Ctrl aptamer versus PBS. For all panels, $n = 6$ per group. Scale bar: 20 μm in B and 100 μm in D. Data are represented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

suggesting that PDGF-C might be functionally more akin to VEGF-A. This is consistent with the fact that, in the PDGFR-binding domain, PDGF-C has a low sequence homology (9% identity and 36% similarity) to PDGF-B.⁸² These lines of evidence thus highlight important roles of PDGF-C in CNV pathogenesis and further support PDGF-C as a promising therapeutic target for wAMD.

In this work, we found a remarkable effect of the α -PC-PEG aptamer in suppressing CNV in a mouse model. Indeed, neutralizing PDGF-C can both inhibit neovessel growth and destabilize pathological vascular structures.³³ Our data further demonstrated that the α -PC aptamer specifically blocked the binding of PDGF-C to PDGFR α and suppressed PDGF-C-mediated signal transduction and

consequent cell proliferation and migration. PDGF-C acts on multiple PDGFR α -expressing cell types, including endothelial cells, vascular mural cells (pericytes and smooth muscle cells), fibroblasts, and inflammatory cells, thereby promoting multiple aspects of pathological angiogenesis, such as pericyte and smooth muscle cell recruitment, endothelial motility, fibroblast activation, and inflammatory responses, all of which exacerbate neovascularization.^{29,83–85} Moreover, under certain conditions, fibroblasts may adopt endothelial-like features and directly contribute to or mimic pathological angiogenesis.^{86,87} Therefore, the α -PC-PEG aptamer can target multiple cellular aspects critical to neovascularization and has a highly attractive therapeutic value for the treatment of neovascular diseases.

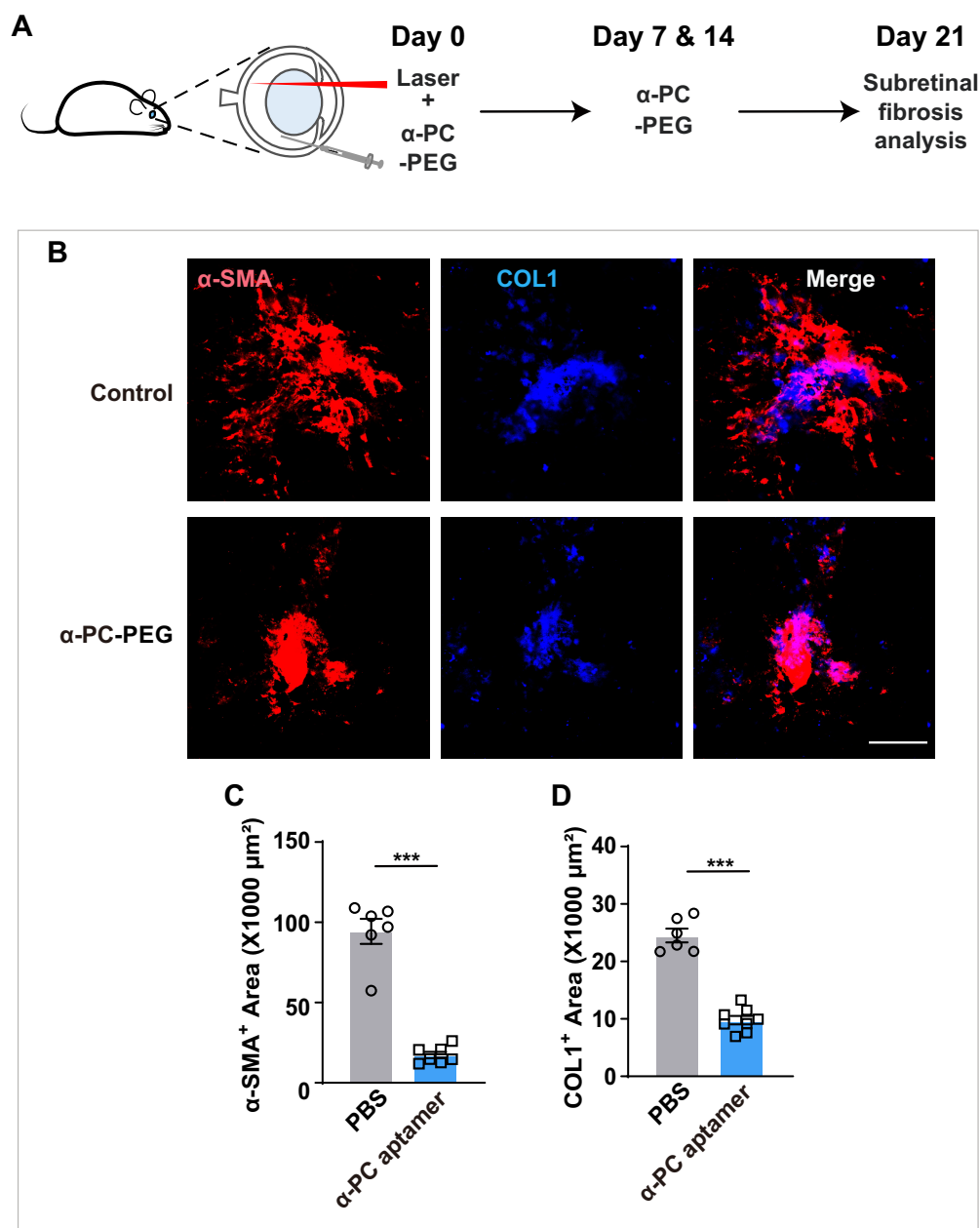


FIGURE 8. The α -PC-PEG aptamer suppresses subretinal fibrosis in CNV. (A) Experimental design of the anti-fibrosis study in 2-month-old mice with laser-induced CNV and fibrosis and the timeline of α -PC-PEG aptamer treatment. (B–D) Representative fluorescence images of choroidal wholemounts stained with α -SMA or COL1 from mice treated with or without the α -PC-PEG aptamer for 3 weeks after laser injury, showing that the α -PC-PEG aptamer reduced subretinal fibrosis. Student's *t*-test showed $P < 0.001$ for α -PC-PEG aptamer versus Control for both α -SMA (B, C) and COL1 (B, D) ($n = 6$ –8). Scale bar: 50 μ m. Data are represented as mean $\pm$ SEM. *** $P < 0.001$.

Although anti-VEGF therapy has revolutionized the treatment of neovascular diseases, a subset of wAMD patients remains refractory to it, underscoring a major unmet clinical need. It has been shown that in aged mice anti-VEGF agents fail to suppress pathological angiogenesis,^{59,88} likely due to increased inflammation and oxidative stress,^{59,89,90} which can activate other compensatory pro-angiogenic pathways. We have previously shown that PDGF-C is a potent inducer of inflammation by activating the ETV1/CXCR4 signaling axis.³⁰ Other groups have also reported that PDGF-C is a potent pro-inflammatory cytokine that induces chemokine expression and leuko-

cyte recruitment to exacerbate inflammation.^{29,91,92} Moreover, oxidative stress can act as both an upstream activator and downstream mediator of the signaling activities of PDGFR α ,^{93,94} which is the primary receptor for PDGF-C. Therefore, inhibiting PDGF-C may alleviate both inflammation and oxidative stress, apart from suppressing its pro-angiogenic effect. Indeed, several studies have implicated PDGF-C as a key compensatory factor responsible for resistance to anti-VEGF therapy in ocular and tumor neovascularization.^{34,63} Combined inhibition of PDGF-C and VEGF has previously been shown to suppress pathological angiogenesis more efficiently than monotherapy in preclinical

CNV models.⁶³ In this work, we showed that the α -PC-PEG aptamer effectively reduced CNV in anti-VEGF-refractory aged mice. Crucially, biochemical analyses showed that the aptamer is highly selective for PDGF-C and does not affect VEGF-A-induced VEGFR2 activation, supporting its ability to overcome anti-VEGF resistance, and could be a highly promising strategy for mono-, combination, or sequential therapies for the treatment of neovascular diseases, particularly in patients with poor responses to anti-VEGF-A therapies.

Subretinal fibrosis is a key complication and major cause of irreversible vision loss in wAMD.⁹⁵ Although VEGF blockade reduces vascular leakage and neovascularization, its inability to prevent or inhibit the development of fibrotic scars underscores an urgent need for new or complementary therapeutic strategies. PDGF-C/PDGFR signaling has been implicated in fibrotic remodeling across multiple organs, including kidney, lung, liver and heart,⁹⁶ as well as gastrointestinal tissues, where targeting PDGF-C was shown to be effective in reducing fibrosis.³⁰ Consistently, we found that the α -PC-PEG aptamer markedly reduced subretinal fibrosis in a laser-induced CNV mouse model. Indeed, the α -PC-PEG aptamer blocked the effect of PDGF-C on profibrotic cells, specifically, vascular smooth muscle cells and fibroblasts, both of which contribute to fibrosis by producing extracellular matrix proteins.^{97,98} Thus, PDGF-C targeting may simultaneously suppress neovascularization and fibrotic scarring, thereby addressing two central drivers of vision loss in wAMD.

Clinically approved aptamers to date have been mostly RNA based—for example, avacincaptad pegol (IZERVAY)⁹⁹ and pegaptanib (Macugen).⁴⁶ However, DNA aptamers offer significant advantages, such as intrinsic chemical stability, lower production cost, and amenability to chemical modification. These benefits have attracted increasing interest in the research and applications of DNA aptamers.^{100,101} In this study, we developed a PEG-modified DNA aptamer targeting PDGF-C to leverage the stability and manufacturing benefits. The α -PC aptamer exhibits a very high affinity for human PDGF-C ($K_D \sim 20$ pM), which surpasses those of many reported aptamers and is comparable to therapeutic antibodies,^{47,102} highlighting its promising therapeutic potential for PDGF-C targeting.

In summary, in this work, we identified a novel DNA aptamer against PDGF-C (α -PC aptamer) that specifically binds to PDGF-C and blocks PDGF-C/PDGFR α signaling and inhibits both choroidal neovascularization and subretinal fibrosis in mice. Of particular importance, the α -PC aptamer proved effective in inhibiting CNV in anti-VEGF-refractory aged mice, demonstrating promising therapeutic potential of the α -PC aptamer in the treatment of neovascular diseases, particularly in neovascular diseases that are resistant to anti-VEGF therapy.

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