**Article**

# Targeted Delivery of FLT-Morpholino Using Cyclic RGD Peptide

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**Purpose:** We previously showed that intravitreal injection of the sFLT morpholino-oligomer (FLT-MO) suppresses laser-induced choroidal neovascularization (CNV) in mice by decreasing the membrane bound form of Flt-1 while increasing the soluble form of Flt-1 via alternative splicing shift. In this study, we examined whether cyclic RGD peptide (cRGD) can promote morpholino-oligomer accumulation in CNV following tail vein injection, and whether systemic cRGD conjugated FLT-MO (cRGD-FLT-MO) suppresses CNV growth.

**Methods:** cRGD conjugated fluorescent morpholino-oligomer (cRGD-F-MO) was injected via tail vein into mice with previous retinal laser photocoagulation and examined for cRGD-F-MO accumulation in CNV. To examine whether cRGD-FLT-MO suppresses CNV growth, mice were tail-vein injected with cRGD-FLT-MO, cRGD conjugated standard morpholino-oligomer (cRGD-STD-MO), or Dulbecco's Phosphate-Buffered Saline (DPBS) 1 and 4 days postlaser photocoagulation. Seven days postlaser photocoagulation, eyes were harvested and laser CNV was stained with isolectin GS-IB4, allowing quantification of CNV size by confocal microscopy.

**Results:** cRGD-F-MO accumulation in CNV commenced immediately after tail vein injection and could be observed even 1 day after injection. cRGD-FLT-MO tail vein injection significantly suppressed CNV size ($2.7 \times 10^5 \pm 0.3 \times 10^5$ $\mu m^3$, $P < 0.05$ by Student's $t$-test) compared with controls (DPBS: $5.1 \times 10^5 \pm 0.6 \times 10^5$ $\mu m^3$ and cRGD-STD-MO: $5.5 \times 10^5 \pm 0.8 \times 10^5$ $\mu m^3$).

**Conclusions:** cRGD peptide facilitates morpholino-oligomer accumulation in CNV following systemic delivery. cRGD-FLT-MO suppressed CNV growth after tail-vein injection, demonstrating the potential utility of cRGD peptide for morpholino-oligomer delivery to CNV.

**Translational Relevance:** Current therapy for neovascular age-related macular degeneration involves intravitreal injection of anti-vascular endothelial growth factor drugs. Our results indicate that CNV can be treated systemically, thus eliminating risks and hazards associated with intravitreal injection.

## Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible visual blindness over the age of 50 in the western world.[1–5] Currently, neovascular AMD is treated with anti-vascular endothelial growth factor (VEGF) therapies, which include anti-VEGF antibodies (Avastin®, Lucentis®, Genetech, South San Francisco, CA) or recombinant protein (Eylea®, Regeneron, Tarrytown, NY). Anti-VEGF therapy not only decreases choroidal neovascularization (CNV) size, but improves visual acuity in approximately one third of treated patients.[6,7] Although various genetic and environmental factors have been identified as associated with neovascular AMD,[8,9] the success of anti-VEGF therapy indicates that the primary pathological pathways of AMD

require VEGF for neovascular development and sustenance. However, repeated and long-term intravitreal injections may cause endophthalmitis, retinal detachment, intraocular pressure elevation, ocular hemorrhage, and systematic complications,[10] although current improved sterile technique and protocol reduce the risk of endophthalmitis greatly.[11] Therefore, the ability to locally target VEGF activity at the site of CNV through venous injection would greatly reduce the patient's risks and burdens.

Previously, we showed that splicing shift of VEGF receptors (FLT1 or KDR) by morpholino oligomers suppressed the growth of laser-induced CNV in intravitreally injected mice.[12,13] Both morpholino oligomers can shift membrane bound form of receptors (mbFLT1 or mbKDR) to soluble form receptors (sFLT1 or sKDR). The membrane bound form of VEGF receptors can promote neovascularization through autophosphorylation of intracellular domains upon VEGF stimulation. On the other hand, soluble form VEGF receptors, which naturally exist via alternative splicing and polyadenylation, inhibit neovascularization by sequestering the ligand and preventing VEGF receptor activation. Thus, the morpholino-based strategy can inhibit angiogenesis by increasing VEGF antagonist (sFLT or sKDR) and decreasing the relevant signaling receptor (mbFLT or mbKDR). Successfully targeted morpholino oligomer delivery has the potential to open a new therapeutic field. To target CNV, we utilized cyclic RGD peptide (cRGD). The RGD (arginine–glycine–asparagine) motif is known as a minimal motif of integrin binding. Among the various types of integrins, integrin $\alpha_v/\beta_3$ preferentially binds to RGD motifs and is highly expressed in tumors and neovascular lesions, which include CNV.[14–17] In this study, we examined the targeting efficiency of morpholino oligomer to laser CNV via tail vein delivery in mouse, and whether cRGD-conjugated FLT-MO (cRGD-FLT-MO) could inhibit laser-induced CNV growth.

## Methods

### cRGD Conjugation to Morpholino Oligomer

Morpholino oligomers were synthesized as 5′ primary amine and 3′fluorescein modified oligomers (Gene Tools, Philomath, OR). Morpholino oligomer sequences are: sFLT_MO (mouse): 5′-CTTTTTGCCG CAGTGCTCACCTCTA-3′; STD_MO: 5′-CCTCTTACCTCAGTTACAATTTATA-3′.[12] Cyclic RGD with cysteine [cyclo (Arg-Gly-Asp-d-Phe-Cys)]

was ordered from Peptides International, Inc. (Louisville, KY).[18,19] A SIAB (N-succinimidyl[4-iodoacetyl]aminobenzoate; Thermo Fisher Scientific, Waltham, MA) reaction was used to conjugate the morpholino oligomer and cyclic RGD. Initially, each morpholino oligomer was dissolved in phosphate buffered saline (PBS) (pH = 8) to 2 mM. SIAB was dissolved in dimethylformamide to 100 mM. The same volume of each solution was mixed (1:50 molar ratio), then incubated at room temperature for 60 minutes. After equilibrating a desalting column with borate buffer (50 mM sodium borate, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.5), cyclic RGD was added at a 1:12 molar ratio (morpholino oligomer:cRGD) and the reaction was carried out overnight. The conjugation was confirmed after removing unconjugated cRGD through a desalting column (Fig. 1A), and high performance liquid chromatography (HPLC) was used for further purification of the product (Fig. 1B).

### Mouse Aortic Endothelial Cell Culture

Primary mouse aortic endothelial cells (MAECs) were obtained from Cell Biologics (Chicago, IL) and maintained following the manufacturer's protocols. One day after plating MAEC in six-well plates, cRGD morpholino oligomer or control morpholino oligomer (no cRGD) were added at the indicated concentration. After additional 2 days of incubation, the medium was changed and fluorescence was observed by EVOS FL AUTO (Thermo Fisher Scientific).

### Quantitative Real-Time PCR and Enzyme-Linked Immunosorbent Assay (ELISA)

After MAEC culture with morpholino oligomer, total RNA was purified with an RNeasy mini kit (Qiagen, Germantown, MD), followed by DNaseI treatment (Sigma-Aldrich, St. Louis, MO). cDNA was prepared with an RT Omniscript kit (Qiagen) using oligo-dT. Real-time polymerase chain reaction (PCR) was carried out with SYBR green PCR (Qiagen) using previously described primers and PCR reaction conditions.[12,13] Real-time data were analyzed with the ΔΔCt method. sFLT protein quantification from the conditioned culture medium and mouse serum was performed by Mouse sVEGF R1/Flt-1 ELISA (R&D Systems, Inc., Minneapolis, MN) following the manufacture's instruction.

### Animals

Eight- to 12-week-old C57/BL6J mice were purchased from The Jackson Laboratory (Bar Harbor,
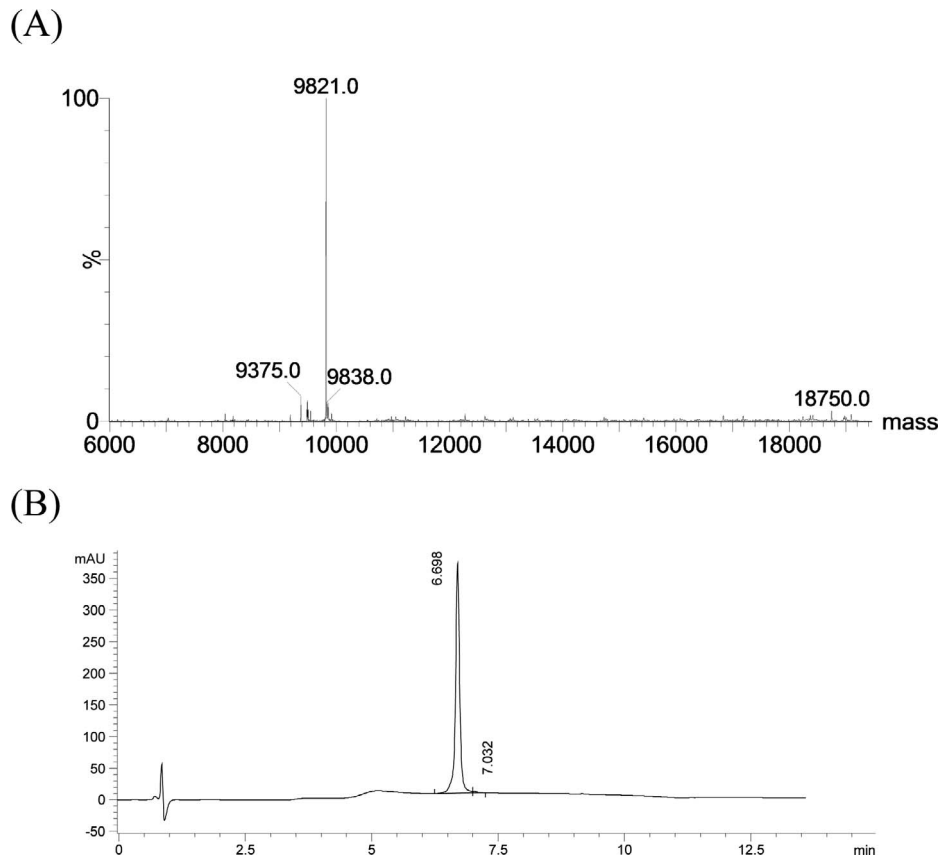
**Figure 1.** cRGD conjugation to morpholino oligomer. (A) The conjugation between morpholino oligomer and cRGD peptide through SIAB reaction was confirmed by Mass Spectrometry. A peak (9821 Da), which is very close to the expected mass (9820.65 Da), was observed. (B) Further HPLC purification of cRGD-morpholino oligomer. We collected the single peak and use it for the further experiments.

ME) and used in this study. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

## Laser Photocoagulation and Tail Vein Injection

Laser photocoagulation in mouse was done as described previously.[20,21] Briefly, after anesthetizing C57/BL6J mice with ketamine/xylazine, both eyes received laser photocoagulation (532 nm, 120 mV, 100 ms, 100 μm). To assess CNV volume, 40 nmol (80 μL) of control or experimental morpholino oligomers were administrated via tail vein 1 and 4 days postlaser injury. Seven days following laser injury, the eyes were harvested and analyzed for CNV volume. For examination of morpholino oligomer accumulation in CNV, cRGD-F-MO (40 nmol) was administered via tail vein injection 7 days after laser injury. The fluorescence of cRGD-F-MO in laser CNV was analyzed using Spectralis (Heidelberg Engineering,

Franklin, MA) using fluorescein angiography/infra-red (FA/IR) mode.

## CNV Volume Measurement in Choroid Flatmount with Confocal Microscope

CNV volume measurement was described previously.[20,21] Briefly, after fixing the eyes with 4% paraformaldehyde in PBS, choroidal specimens were stained with Alexa Fluor® 647 conjugated isolectin GS-IB4 (Thermo Fisher Scientific). After mounting the choroid as a flatmount, stained specimens were scanned and CNV volume was measured with a confocal microscope (Olympus, Waltham, MA).

## Results

### cRGD-FLT-MO Can Be Delivered via Tail Vein

To deliver morpholino oligomer via tail vein injection, we utilized cRGD [cyclo (Arg-Gly-Asp-d-
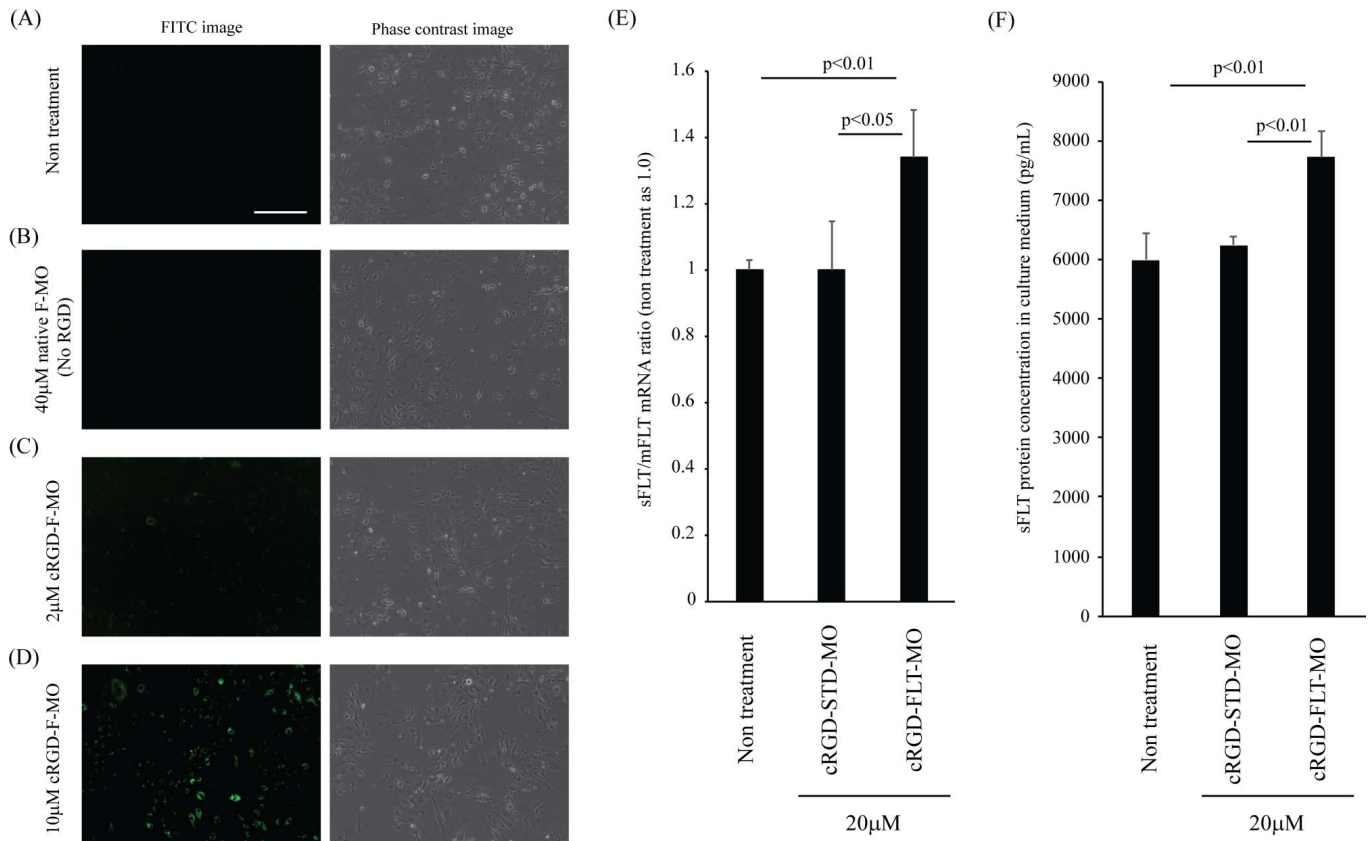
**Figure 2.** cRGD facilitates morpholino oligomer uptake in vitro in MAECs. (A) Control, no treatment; (B) 40 μM morpholino oligomer without cRGD conjugation; (C) 2 μM cRGD conjugated morpholino oligomer; and (D) 10 μM cRGD conjugated morpholino oligomer. Each morpholino oligomer is conjugated with fluorescein. *Scale bar* is 250 μm. (E) sFLT/mFLT mRNA ratio by real-time PCR. cRGD-FLT-MO treatment showed 1.4-fold increase of sFLT/mFLT ratio compared with the controls ($n = 4$). (F) sFLT protein concentration in the conditioned culture medium were examined by ELISA. cRGD-FLT-MO increased sFLT protein 1.28-fold compared with the controls ($n = 3$). The error bars are standard deviation. *P*-value was calculated with Student's *t*-test.

Phe-Cys), M.W. 578.65 Da]. The conjugation between cRGD and morpholino oligomer (5′fluorescein) via SIAB was confirmed by mass spectroscopy (Fig. 1A). The molecular mass of our (control) standard morpholino oligomer with 5′fluorescein and 3′NH$_2$ is 9083 Da. The estimated mass and observed mass after cRGD conjugation via SIAB are 9820.65 and 9821 Da. Therefore, morpholino oligomers were effectively conjugated with cRGD peptide using our conditions. After further HPLC purification (Fig. 1B), the yield was estimated at ∼80% using 260-nm optical density.

## cRGD Peptide Promotes FLT-MO Uptake and Function in Endothelial Cells In Vitro

To examine whether cRGD conjugation enhances the uptake of morpholino oligomers in MAEC cultures, we added cRGD conjugated fluorescence morpholino oligomer (cRGD-F-MO) or native fluo-

rescence morpholino oligomer (F-MO) to MAEC. After 2 days incubation, the fluorescence images were obtained (Figs. 2A–D). While fluorescence was not observed in untreated and 40-μM native F-MO treated cells (Figs. 2A, 2B), we found that 2-μM cRGD-F-MO was sufficient to detect fluorescent signal in cultured MAEC (Fig. 2C), with higher concentrations of cRGD-F-MO yielding higher numbers of positive cells (Fig. 2D). Thus, cRGD conjugation efficiently introduces morpholino oligomer to endothelial cells.

Next, we examined cRGD conjugated FLT morpholino oligomer (cRGD-FLT-MO) function in vitro. Two days after adding cRGD-FLT-MO or cRGD conjugated standard morpholino oligomer (cRGD-STD-MO) to MAEC, sFLT/mFLT mRNA ratio was determined by real-time PCR (Fig. 2E). We found that 20-μM cRGD-FLT-MO significantly increased the sFLT/mFLT mRNA ratio by 1.4-fold
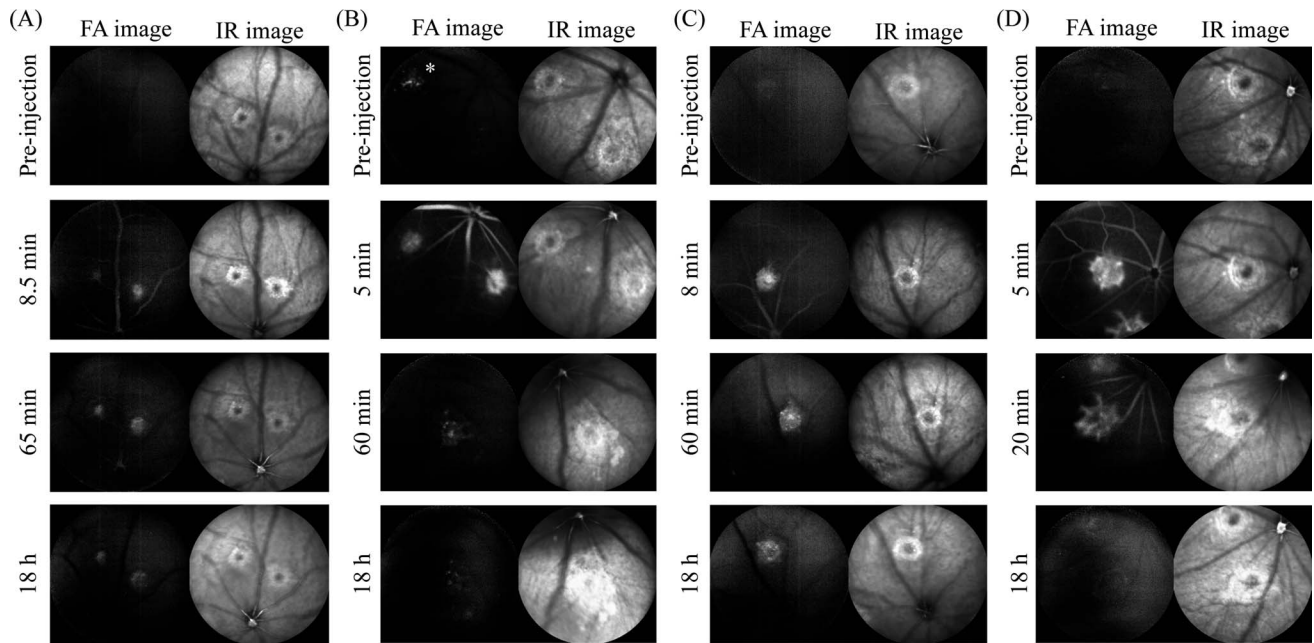
**Figure 3.** cRGD peptide conjugation enhances morpholino oligomer delivery to laser-induced CNV. (A–D) Four different mouse eyes were observed over time pre- and post-cRGD-F-MO tail vein injection. FA images and infrared (IR) images are shown. *Asterisk* (*) indicated CNV with strong autofluorescence.

compared with the control morpholino. sFLT concentration in the conditioned culture medium was measured by ELISA (Fig. 2F). cRGD-FLT-MO increased sFLT protein expression in culture medium by 1.28-fold compared with the control morpholino.

## cRGD Enhances FLT-MO Accumulation in CNV and Suppresses CNV Size

To examine whether cRGD morpholino oligomer accumulates in areas of laser CNV in vivo, we injected cRGD-F-MO via tail vein 7 days postlaser photocoagulation in mice. CNV fluorescence was observed over time using the fluorescence angiography mode of Heidelberg Spectralis (Figs. 3A–D). Some of the laser-induced CNV lesions showed strong auto-fluorescence (asterisk on Fig. 3B) before F-MO injection and were subsequently not used for this analysis. Fluorescence within CNV and retinal vessels was visualized immediately after the tail vein injection. Although the fluorescence in retinal vessels disappeared 60 minutes post-F-MO injection, the fluorescence from CNV could still be observed. The following day (18 hours), CNV lesions continued to fluoresce (Figs. 3A–C), although some areas of CNV showed only trace levels (Fig. 3D). After this time point, retinal cryosections of each cRGD-F-MO eye were prepared, in which FITC fluorescence could be observed within the laser-induced CNV (Figs. 4A, 4B). CNV from DPBS tail vein injected mouse also exhibited some fluorescence (Fig. 4A), which we perceived to be auto-fluorescence. However, CNVs from cRGD-F-MO injected mice showed increased FITC fluorescence in CNV over background auto-fluorescence (Fig. 4B). Thus, cRGD peptide enhances morpholino oligomer accumulation in mouse laser CNV.

Next, we examined whether cRGD-FLT-MO could suppress CNV growth. We injected 40 nmol (80 μL) of cRGD-STD-MO and cRGD-FLT-MO or the same volume of DPBS via tail vein at 1 day and 4 days postlaser photocoagulation. At 7 days postlaser photocoagulation, we examined CNV by fluorescence angiography. Figure 5A shows representative images of fluorescence angiography. Each CNV was ranked on a graded scale of 1 to 4 with ascending size (Supplemental Figure). We found that the number of CNV lesions scored as grade 1 (small CNV) was significantly higher in cRGD-FLT-MO tail vein injected mice compared to DPBS or cRGD-STD-MO injected mice, respectively ($\chi^2$ test, $P < 0.05$).

We also measured CNV volume by immunostaining. Figure 5B shows representative images of laser CNV with each treatment. We found that cRGD-FLT-MO tail vein injection suppress 50% CNV growth compared with the controls (Fig. 5C).
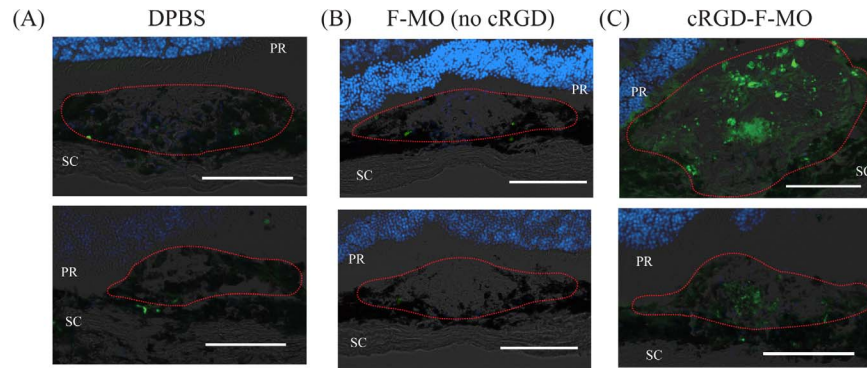
**Figure 4.** cRGD-F-MO accumulation in CNV. One day post-cRGD-F-MO tail vein injection, the CNV fluorescence was observed in cryosection. (A) DPBS, (B) F-MO without cRGD conjugation, and (C) cRGD-F-MO tail vein injection. Two different CNVs in each group are shown. *Scale bar* is 100 μm. The lesion of CNV was delineated by *red dot line*. PR, photoreceptor; SC, sclera.

## cRGD-FLT-MO Did Not Affect Serum sFLT

Finally, we examined whether tail-vein injection of cRGD-FLT-MO alters serum sFLT levels by ELISA (Fig. 6), but did not find a significant difference in serum sFLT between cRGD-FLT-MO injected mice and control mice.

## Discussion

sFLT is known as an endogenous inhibitor of VEGF. The FLT-1 gene alternatively produces sFLT or mbFLT by alternative splicing and polyadenylation. Since mbFLT has tyrosine kinase domains, it is believed to have a proangiogenic affect upon VEGF stimulation. On the other hand, since sFLT lacks tyrosine kinase domains and only binds to VEGF, it is considered to be a decoy receptor and VEGF inhibitor. Previously, we showed that sFLT preserves avascularity of the cornea and retinal photoreceptors/retinal pigment epithelium.[22,23] Moreover, serum sFLT level is correlated to wet AMD prevalence.[24] Thus, sFLT/mbFLT splicing control may be considered an angiogenic switch.

We demonstrated that cRGD conjugated morpholino oligomers penetrate mouse endothelial cells in vitro. However, the change of sFLT/mFLT mRNA ratio was less than that we expected based on previous experiments. Although the reason is unclear, cRGD conjugation may decrease the splice blocking activity of morpholino oligomers. Alternative conjugation using linkers, such as polyethylene glycol with sites of peptidase release, may help recover morpholino oligomer activity in cells. Also, it is possible that a single cRGD peptide conjugation is insufficient for the efficient transit of morpholino oligomers into
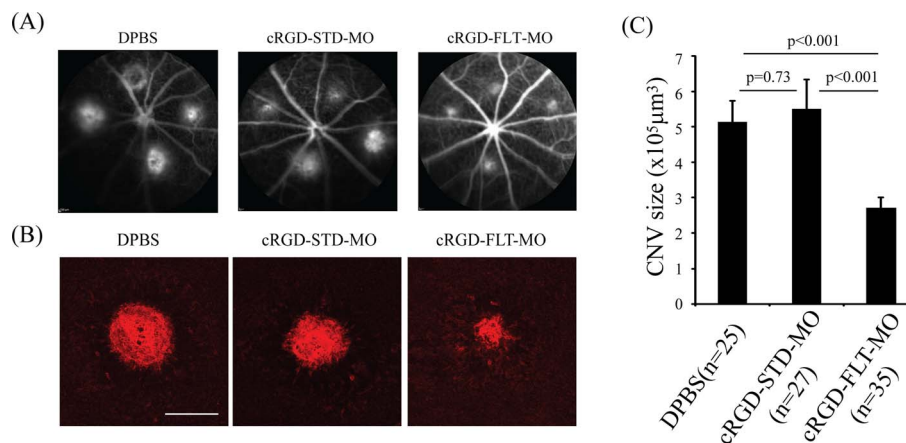


**Figure 5.** cRGD-FLT-MO tail vein injection suppresses laser-induced CNV in mouse. (A) Representative images of fluorescence angiography. (B) Representative CNV images of each treatment by confocal microscope with Alexa Fluor 647 conjugated isolectin GS-IB4. *Scale bar* is 200 μm. (C) The average CNV volume of each group. cRGD-FLT-MO significantly suppressed CNV growth compared with the controls.
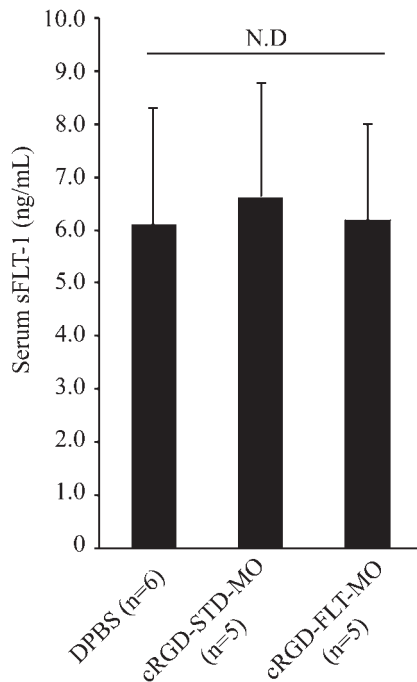
nmol, which corresponds to 23 μg cRGD) did not show inhibition of CNV growth by tail vein injection. This suggests that elevated concentrations of cRGD may be required to effect CNV inhibition through systemic delivery. Since serum sFLT did not show a significant difference between the control and cRGD-FLT-MO treatments, this suggests that systemic delivery of cRGD can effectively target CNV, although by itself the cRGD peptide may not be a suitable anti-angiogenic treatment for CNV.

Although we assessed serum sFLT levels as an off-target, it is possible that cRGD-FLT-MO may cause other off-target effects. For example, the patient with an injury may experience slower wound healing if undergoing treatment with cRGD-FLT-MO. Also, the repeated systematic injection of cRGD-FLT-MO may develop immunologic reactions. These potential effects should be investigated in the future.

In conclusion, we showed that cRGD peptide enhanced morpholino oligomer accumulation in mouse CNV and that cRGD-FLT-MO suppressed CNV growth. Our results indicate it is possible that neovascular AMD can be treated without intravitreal injection by direct morpholino targeting.

## Acknowledgments

cells. Multiple cRGD peptide conjugations using linkers can potentially increase cellular affinity.

cRGD-FLT-MO suppressed laser-induced CNV via tail vein injection when administrated on 1 and 4 days postlaser photocoagulation. Although we confirmed the accumulation of cRGD-F-MO in CNV after tail vein injection, some CNVs lost the fluorescent signal from accumulated cRGD-F-MO to the background level (Fig. 3D). There are several possibilities. One is that the proliferating CNV lesion may lose morpholino oligomers. Alternatively, the high affinity between cRGD peptide and integrins may prevent cRGD-MO release to the cytosol. To overcome this, esterase sensitive peptide may be inserted between cRGD peptide and morpholino oligomer.

We injected cRGD-morpholino 50 times (twice/20 mice, single/10 mice) via tail vein injection. One mouse died immediately after the injection, and although we could not identify the reason, we believe this accidental death might be due to the tail vein injection itself rather than cRGD-morpholino.

Previous studies showed that cyclic RGD inhibited CNV growth with intravitreal injection (100 μg or 200 μg) in rats.[14] However, our cRGD-STD-MO (40

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