Protective effects of a novel drug RC28-E blocking both VEGF and FGF2 on early diabetic rat retina

Qian-Hui Yang¹, Yan Zhang¹, Jing Jiang², Mian-Mian Wu¹, Qian Han¹, Qi-Yu Bo¹, Guang-Wei Yu¹, Yu-Sha Ru¹, Xun Liu¹, Min Huang³, Ling Wang³, Xiao-Min Zhang¹, Jian-Min Fang⁴, Xiao-Rong Li¹

¹Tianjin Medical University Eye Hospital, Tianjin Medical University Eye Institute, College of Optometry and Ophthalmology, Tianjin Medical University, Tianjin 300384, China
²School of Pharmacy, Binzhou Medical University, Yantai 264003, Shandong Province, China
³Remegen, Ltd., Yantai 264006, Shandong Province, China
⁴School of Life Sciences and Technology, Tongji University, Shanghai 200092, China

Co-first authors: Qian-Hui Yang, Yan Zhang, Jing Jiang and Mian-Mian Wu

Correspondence to: Jian-Min Fang. School of Life Sciences and Technology, Tongji University, Shanghai 200092, China. jfang@tongji.edu.cn; Xiao-Rong Li. Tianjin Medical University Eye Hospital, Tianjin Medical University Eye Institute, College of Optometry and Ophthalmology, Tianjin Medical University, Tianjin 300384, China. xli@tmu.edu.cn

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Abstract

- **AIM:** To investigate protective effects of a novel recombinant decoy receptor drug RC28-E on retinal damage in early diabetic rats.
- **METHODS:** The streptozotocin (STZ)-induced diabetic rats were randomly divided into 6 groups: diabetes mellitus (DM) group (saline, 3 μL/eye); RC28-E at low (0.33 μg/μL, 3 μL), medium (1 μg/μL, 3 μL), and high (3 μg/μL, 3 μL) dose groups; vascular endothelial growth factor (VEGF) Trap group (1 μg/μL, 3 μL); fibroblast growth factor (FGF) Trap group (1 μg/μL, 3 μL). Normal control group was included. At week 1 and 4 following diabetic induction, the rats were intravitreally injected with the corresponding solutions. At week 6 following the induction, apoptosis in retinal vessels was detected by TUNEL staining. Glial fibrillary acidic protein (GFAP) expression was examined by immunofluorescence. Blood-retinal barrier (BRB) breakdown was assessed by Evans blue assay. Ultrastructural changes in choroidal and retinal vessels were analyzed by transmission electron microscopy (TEM). Content of VEGF and FGF proteins in retina was measured by enzyme linked immunosorbent assay (ELISA). The retinal expression of intercellular cell adhesion molecule-1 (ICAM-1), tumor necrosis factor-α (TNF-α), VEGF and FGF genes was examined by quantitative polymerase chain reaction (qPCR).
- **RESULTS:** TUNEL staining showed that the aberrantly increased apoptotic cells death in diabetic retinal vascular network was significantly reduced by treatments of medium and high dose RC28-E, VEGF Trap, and FGF Trap (all P<0.05), the effects of medium and high dose RC28-E or FGF Trap were greater than VEGF Trap (P<0.01). GFAP staining suggested that reactive gliosis was substantially inhibited in all RC28-E and VEGF Trap groups, but the inhibition in FGF Trap group was not as prominent. Evans blue assay demonstrated that only high dose RC28-E could significantly reduce vascular leakage in early diabetic retina (P<0.01). TEM revealed that the ultrastructures in choroidal and retinal vessels were damaged in early diabetic retina, which was ameliorated to differential extents by each drug. The expression of VEGF and FGF2 proteins was significantly upregulated in early diabetic retina, and normalized by RC28-E at all dosages and by the corresponding Traps. The upregulation of ICAM-1 and TNF-α in diabetic retina was substantially suppressed by RC28-E and positive control drugs.
- **CONCLUSION:** Dual blockade of VEGF and FGF2 by RC28-E generates remarkable protective effects, including anti-apoptosis, anti-gliosis, anti-leakage, and improving ultrastructures and proinflammatory microenvironment, in early diabetic retina, thereby supporting further development of RC28-E into a novel and effective drug to diabetic retinopathy (DR).
- **KEYWORDS:** diabetic retinopathy; vascular endothelial growth factor; fibroblast growth factor 2; recombinant decoy receptor; retinal damage; diabetes

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RC28-E protects early diabetic rat retina

INTRODUCTION

Diabetes mellitus (DM) threatens the public health with the rising prevalence around the globe. Diabetic retinopathy (DR), one of the severe microvascular complications of DM[1], is the leading cause of blindness in the working age population[2]. Thus far, the majority of therapeutic modalities to DR have focused on symptom relief. For example, laser therapy prevents dramatic deterioration in visual function, yet hardly improves visual acuity and generates damage in neuroretina[3]. Glucocorticoid drugs can alleviate diabetic macular edema caused by breakdown of blood-retinal barrier (BRB) and retinal vessel leakage, however they may cause adverse effects such as cataract, elevated intraocular pressure[4-5] and endophthalmitis. In addition, surgeries like vitrectomy are traumatic, and their efficacies on restoring visual functions are not satisfactory[6]. Therefore, it is necessary to develop a novel, effective, and safe therapeutic modality based on the pathogenesis of DR.

Up to now, vascular endothelial growth factor (VEGF) is considered as an important proinflammatory, proangiogenic, and vascular permeability factor in the pathogenesis and development of DR[7], and its protein levels were elevated in the vitreous fluid of the diabetic patients[8]. Emerging ophthalmic drugs thus target this pathogenic factor. For instance, ranibizumab is an Fab fragment of a humanized monoclonal antibody to VEGF[9], and conbercept is a recombinant decoy receptor that fuses human IgG1 Fc with the Fab fragment containing domain 2 of VEGF receptor 1 (VEGFR1) and domains 3 and 4 of VEGFR2[10]. However, the main problems incurred by the anti-VEGF drugs include more than 50% non-response rate and rapid drug resistance[6,11], indicating that the factors besides VEGF might also be responsible for DR pathogenesis.

Previous studies have suggested that fibroblast growth factor 2 (FGF2) expression is upregulated both in the retinas of diabetic animals[12-14] and in the vitreous samples of diabetic patients[15-16]. At the cellular and molecular level, FGF2 acts as a potent mitogen for vascular endothelial cells[17], and exerts a synergistic effect on biological activities of VEGF[18]. Furthermore, numerous studies have implicated that simultaneous targeting on VEGF and FGF2 may be more efficacious in treating angiogenesis-related diseases[19-20]. Based on this rationale, a novel recombinant decoy receptor drug, designated as RC28-E (Remegen, Ltd., Yantai Biopharmaceutical company, China), that can bind both VEGF and FGF2 with high affinity and specificity. The Fab fragment of RC28-E contains domain 2 of VEGFR1, domain 3 of VEGFR2, as well as domains 2 and 3 of FGFRI[21]. The antiangiogenic effects of RC28-E have been confirmed both in vascular endothelial cells and in xenograft tumor model[21]. Therefore, it is hypothesized that RC28-E may generate protective effects on early diabetic retina by dual blockade of VEGF and FGF2. To test this hypothesis, RC28-E was intravitreally injected into streptozotocin (STZ)-induced early diabetic rats, 6wk following the diabetic induction, the apoptosis of retinal vessels, the activation of retinal glial cells, the BRB breakdown, the ultrastructural changes in retinal and choroidal vessels, and the expression of proinflammatory factors were examined. In the meanwhile, VEGF Trap, a decoy receptor of VEGF that has already been used in the clinics[22] and FGF Trap, a decoy receptor of FGF2 containing domains 2 and 3 of FGFRI in its Fab fragment[23], were included as positive control drugs. This study provides experimental bases for further development of RC28-E into a novel drug to DR.

MATERIALS AND METHODS

Animals Male Sprague-Dawley (SD) rats (age 8-10wk, body weight 220-250 g) were purchased from Experimental Animal Center in the Chinese Academy of Military Medical Sciences. Rats were housed in specific pathogen free animal facility of Tianjin Medical University, and fed ad libitum with food and drink. The ambient temperature was 25°C±1°C, the relative humidity was 40%±5%, and the illumination was under 12-h light: 12-h dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University (permission number: SYXK2009-0001), and in accord with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Diabetic Induction The rats were injected with 2% STZ dissolved in sodium citrate buffer (pH 4.5; Amresco Chemical Co., Solon, OH, USA) at 45 mg/kg through vena caudalis to induce diabetes. At 72h following the injection, the rats with the blood glucose levels higher than 16.7 mmol/L were considered diabetic and included in the subsequent experiments (n=240); whereas the normal controls (n=40) were intravenously injected with an equal volume of sodium citrate buffer.

Animal Grouping and Intravitreal Injections The diabetic rats were randomly divided into 6 groups (n=40/group): the DM group (saline, 3 μL/eye); VEGF Trap group (VEGF Trap-Eye, Regeneron, Tarrytown, NY, and Bayer HealthCare, Berlin, Germany, 1 μg/μL ×3 μL/eye); FGF Trap group (FGF Trap, Remegen Co., Ltd., Yantai, China, 1 μg/μL ×3 μL/eye); low dose RC28-E group (RC28-E, Remegen Co., Ltd., Yantai, China, 0.33 μg/μL ×3 μL/eye); medium dose RC28-E group (1 μg/μL ×3 μL/eye); and high dose RC28-E group (3 μg/μL ×3 μL/eye). The normal control group (saline, 3 μL/eye) was also included. All the animals were intravitreally injected in the right eye with the corresponding solution with a microsyringe equipped with a 33 Gauge needle as previously described[24-25]. The intravitreal injections were performed at week 1 and 4 following the intravenous injection.

Physiological Parameters of Streptozotocin-induced Diabetic Rats The body weight of all the experimental
animals were recorded on the weekly basis. The blood glucose levels of all the rats were measured at day 3 and week 6 after the intravenous injection of STZ or sodium citrate buffer. At week 4 following the induction of diabetes, the averaged daily water consumption of the diabetic rats and normal counterparts was monitored for 2wk.

**Trypsin Digestion of Retina Followed by TUNEL Staining**

At week 6 after the intravenous injection, the rats were deeply anesthetized, the eyeballs were fixed in 4% paraformaldehyde (PFA) at 4°C for 72h. The intact retinas were isolated under a dissection microscope and gently shaken overnight in deionized water at room temperature (RT). The retinas were then transferred to 1% Triton-X 100 (Sigma-Aldrich, St. Louis, MO, USA) and shaken for 2h at 37°C, after which they were subjected to 2-3h digestion at 37°C with 3% crude trypsin (Amresco Chemical Co., Solon, OH, USA). Subsequently, the retina was mounted on the slide with photoreceptor side upwards. It was extensively flushed with drop-wise deionized water from a curved needle mounted on a 1-mL syringe till the parenchyma was substantially exfoliated, revealing a translucent retinal vascular network. The vascular network was monitored for 2wk.

The vascular network was subjected to a 2-3h digestion at 37°C with 3% crude trypsin (Amresco Chemical Co., Solon, OH, USA). Subsequently, the retina was mounted on the slide with photoreceptor side upwards. It was extensively flushed with drop-wise deionized water from a curved needle mounted on a 1-mL syringe till the parenchyma was substantially exfoliated, revealing a translucent retinal vascular network. The vascular network was monitored for 2wk.

**Immunofluorescence of Glial Fibrillary Acidic Protein Staining**

The rats were anesthetized and perfused through left ventricle with cold phosphate buffered solution (PBS) and 1% PFA. The eyeballs were fixed in 4% PFA for 1h at RT, washed with PBS, dehydrated in 10% sucrose for 1h and 20% sucrose for 2h, cryo-embedded in OCT. (Sakura Finetek, Torrance, CA, USA), and stored at -80°C. The comparable part of the frozen eyeballs was cryo-sectioned (5 μm in thickness). The sections were incubated with a primary rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP) (1:1000, Abcam, Cambridge, MA, USA) at 4°C overnight. After extensive washes, the slides were stained next day with 4’,6-diamidino-2-phenylindole (DAPI)-containing ProLong Gold Antifade reagent (Life Technologies, Beijing, China) and photographed under a fluorescence microscope (BX51; Olympus Optical Co. Ltd., Tokyo, Japan) as previously described[25].

**Evans Blue Assay**

The BRB breakdown was examined by Evans blue assay at week 6 after the diabetic induction as described elsewhere[24]. Briefly, the rats were injected with Evans blue solution (Biotopped Co., Ltd., Beijing, China) through tail vein. At 2h after the injection, blood (1 mL) was collected from inner canthus and centrifuged. The supematant was diluted (1:100) with methanamide (Amresco Chemical Co., Solon, OH, USA). The absorbance at 620 nm, with the correction at 740 nm was measured by an Infinite 200 PRO Multimode Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). The concentration of Evans blue in the blood was calculated. Then the rats were thoroughly perfused with warm sodium citrate buffer. The retinas were isolated and dry weight recorded. The Evans blue dye leaked into the retina parenchyma was extracted with methanamide. The portion with low molecular weight was enriched by ultrafiltration. The absorbance of the filtrate at 620 nm with correction at 740 nm was measured as described above. The permeability of Evans blue in retina was calculated according to the following formula: Permeability (μL/g·h) = [Evans blue content in retina (μg)/ retina dry weight (g)] / [time-averaged Evans blue concentration in blood (μg/μL) × circulating time (h)]

**Transmission Electron Microscopy**

The eyeballs, with anterior segments removed, were fixed in a pre-cooled 2.5% glutaraldehyde solution (pH 7.4) followed by 1% osmium tetroxide solution. Then the samples were dehydrated in graded ethanol solutions, treated with propylene oxide, embedded in Epon812, and subjected to ultra-thin sections (about 50 nm). The sections were subsequently stained with uranium acetate and lead citrate, and photographed by a Hitachi-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

**Enzyme-linked Immunosorbent Assay**

At week 6 following the intravenous injection, total proteins of the rat retinas were extracted using a Tissue Protein Extraction Kit (CWbiotech, Beijing, China), and the total protein concentration was determined using a BCA Protein Assay Kit (CWbiotech, Beijing, China). The content of VEGF and FGF2 protein in the retina samples was determined by enzyme linked immunosorbent assays (ELISAs) according to the manufacturer’s protocols (Rat VEGF Quantikine ELISA Kit, cat. RRVOO; Mouse/Rat FGF basic Quantikine ELISA Kit, cat. MFB00; R&D systems, Minneapolis, MN, USA), the protein concentration (pg/mL) of VEGF and FGF2 was normalized to the total protein concentration (mg/mL).

**RNA Extraction, Reverse Transcription and Quantitative Polymerase Chain Reaction**

At week 6 after the diabetic induction, total RNA from rat retinas was extracted using a TRIzol Plus RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA), the concentration and purity of total RNA were determined with a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Of 1 μg total RNA was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The quantitative polymerase chain reaction (qPCR) reaction mixture contained 3 μL of cDNA template, forward and reverse primers of target genes (Table 1),
and 5 μL SYBR Green FastStart 2X Master Mix (Roche, Branford, CT, USA). The qPCR was performed as previously described\cite{24-25}. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene. The data were analyzed by $2^{-\Delta\Delta C_{t}}$ method.

**Statistical Analyses** The statistical analyses were performed using SPSS 13.0 software (SPSS Inc., San Diego, CA, USA). The normality of quantitative data was confirmed by Shapiro-Wilk test, and the data were expressed as mean±SD. The homogeneity of variance was verified by Levene test. Then the differences in physiological parameters between DM and normal rats were analyzed by Two-way ANOVA followed by Tukey post hoc; other quantitative data were analyzed by one-way ANOVA followed by Tukey post hoc. A $P$ value less than 0.05 was considered significant.

**RESULTS**

**Physiological Parameters** During the first 2 wk after the diabetic induction, the body weight of diabetic rats showed a slight decrease, and then increased slowly till week 4 following the induction, and remained stable thereafter. By contrast, the body weight of normal rats exhibited a close to linear increment, and was significantly higher than that of the diabetic rats at each time point (all $P<0.01$; Table 2).

<table>
<thead>
<tr>
<th>Groups</th>
<th>D 0</th>
<th>D 3</th>
<th>W 1</th>
<th>W 2</th>
<th>W 3</th>
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<td>26.87</td>
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</table>

D: Day; W: Week; DM: Diabetes mellitus. $^aP<0.01$ vs DM.

Whereas the blood glucose levels of the normal controls at the 2 time points were 8.04±0.95 mmol/L and 6.60±0.74 mmol/L, respectively (Table 3). As for water consumption, the averaged volume of drinking water of diabetic rats was 3-4 fold that of the normal control group (all $P<0.001$, DM vs normal; Table 4).

**The Apoptotic Cell Death in Retinal Vascular Network** The retinal vascular networks were outlined by DAPI counterstaining of cell nuclei (Figure 1). No apoptotic cell death was detected in the normal group; whereas the number of apoptotic cells was significantly increased in the retinal vascular network of DM group as compared to the normal controls ($P<0.01$; Figure 1). The number of apoptotic cells in low dose RC28-E group was decreased, but was not significantly different from that in the DM group ($P=0.110$; Figure 1). The numbers of apoptotic cells in medium and high dose RC28-E groups, VEGF Trap, and FGF Trap groups were significantly lower than that in the DM group ($P<0.01$, medium dose RC28-E vs DM; $P=0.001$, high dose RC28-E vs DM; $P<0.01$, VEGF Trap vs DM; $P<0.05$, FGF Trap vs DM; Figure 1). The numbers of apoptotic cells in the medium and high doses RC28-E groups and FGF Trap

<table>
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<td>25.97±4.98</td>
<td>32.05±2.65</td>
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<tr>
<td>Normal</td>
<td>8.04±0.95$^a$</td>
<td>6.60±0.74$^a$</td>
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D: Day; W: Week; DM: diabetes mellitus. $^aP<0.01$ vs DM.

**Table 1 qPCR primers**

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<th>Gene</th>
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<td>R:5’-CTCCTGTTGAAATGGCAGGACAT-3’</td>
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<td>R:5’-GGGCTCTGAAAACCATGAACT-3’</td>
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</table>

ICAM-1: Intercellular cell adhesion molecule-1; TNF-α: Tumor necrosis factor-α; VEGF: Vascular endothelial cell growth factor; FGF2: Fibroblast growth factor 2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.
group were significantly lower than that of VEGF Trap group \((P<0.01, \text{ medium dose } \text{RC28-E} \text{ vs } \text{VEGF Trap}; P<0.01, \text{ high dose } \text{RC28-E} \text{ vs } \text{VEGF Trap}; P<0.05, \text{ FGF Trap vs VEGF Trap})\); Figure 1). The results suggest that all the drugs, including RC28-E, VEGF Trap and FGF Trap have an anti-apoptotic effect in early diabetic retinas, and the medium and high dose RC28-E as well as FGF Trap exert better protection from neurodegeneration than low dose RC28-E and VEGF Trap.

**Expression and Distribution of Retinal Glial Cell Marker Glial Fibrillary Acidic Protein** In the normal retinas, GFAP was mainly localized to the outer nuclear layer (ONL), with scattered filaments and spot-like staining extending into the outer plexiform layer (Figure 2). In contrast, the GFAP staining in the retinas of DM group was predominantly located to the inner nuclear layer, inner plexiform layer, and ganglion cell layer (GCL), and the staining intensity was substantially augmented; moreover, such staining revealed the morphological changes of retinal glial cells, with pseudopodia extending from GCL to ONL, indicating the reactive gliosis in early diabetic retinas (Figure 2). In all the RC28-E treatment groups and VEGF Trap group, only a little GFAP staining was observed in the GCL. In the FGF Trap-treated retinas, GFAP staining was observed across the retinal layers, but with much less intensity than that in the retinas of DM group (Figure 2). These results suggest that RC28-E at all dosages and VEGF Trap may have a greater inhibitory effect on reactive gliosis than FGF Trap in early diabetic retinas.

**Blood-retinal Barrier Breakdown and Retinal Vessel Leakage** The retinal vascular leakage, as detected by the amount of Evans blue dye in retinal parenchyma, in the DM group was significantly higher than that of the normal control group \((P<0.001; \text{ Figure 3})\). Furthermore, treatment of the early diabetic retinas with high dose RC28-E significantly reduced the vascular leakage to the normal level \((P<0.001, \text{ DM vs high dose } \text{RC28-E}; P=0.137, \text{ high dose } \text{RC28-E} \text{ vs normal})\); Figure 3). However, there was no significant difference in the retinal vascular leakage between medium dose RC28-E, low dose EC28-E, VEGF Trap, and FGF Trap groups and DM group \((P>0.05; \text{ Figure 3})\).

**Ultrastructural Changes in Retinal Microvessels** In the normal group, the basement membranes of choroidal and retinal capillaries were intact, the lumens unobstructed; the

<table>
<thead>
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<th>D 2</th>
<th>D 3</th>
<th>D 4</th>
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<tr>
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<td>627.50</td>
<td>730.00</td>
<td>792.50</td>
<td>785.00</td>
<td>882.50</td>
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<td>781.25</td>
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<tr>
<td>Average</td>
<td>206.67 (^a)</td>
<td>193.33 (^b)</td>
<td>233.33 (^b)</td>
<td>180.00 (^b)</td>
<td>200.00 (^b)</td>
<td>193.33 (^b)</td>
<td>166.67 (^b)</td>
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<td>140.00 (^b)</td>
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D: Day; DM: Diabetes mellitus; \(^{ab}<0.001\) vs DM.

Figure 1 The apoptotic cells death in retinal vascular network of each experimental group A: The representative images of TUNEL staining of retinal vascular network in each group and the positive and negative controls of TUNEL staining. Arrows indicate the TUNEL-positive cells. Scale bar=20 μm. B: The apoptotic cells in each experimental group were quantified. \(^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001.\)
nuclei and collagen fibers in the ONL were neatly arranged (Figure 4). While in the DM group, the stratified basement membranes were observed in choroidal and retinal capillaries. The endothelial cells were swelled and protruded towards the lumen, the blood cells were congested, both of which lead to the stenosis and deformation of capillary lumens (Figure 4). Among the RC28-E treatment groups, the most obvious changes lied in the capillary ultrastructures. As the dosage increased, the lumens of choroidal and retinal microvessels became gradually smooth and the basement membrane stratification progressively improved, with the RC28-E at high dose generating the optimal protective effects (Figure 4). In the FGF Trap group, the basement membranes of choroidal microvessels were stratified; the endothelial cells in the retinal vessels approximating the inner limiting membrane protruded toward the lumen, hereby a few vascular lumens were almost occluded (Figure 4). The choroidal capillary basement membranes in the VEGF Trap group were stratified, yet the lumens of both choroidal and retinal vessels were still open (Figure 4).

Expression of Vascular Endothelial Growth Factor and Fibroblast Growth Factor 2 Proteins in the Retinas The expression of VEGF protein in the retina homogenate of DM group was significantly upregulated as compared with that of normal group (P<0.01, normal vs DM; Figure 5A), however, this upregulation was significantly reduced to the normal level by the intervention of RC28-E at all dosages (P=0.05, RC28-E low dose vs DM; P=0.179, RC28-E low dose vs normal; P=0.01, RC28-E medium dose vs DM; P=0.435, RC28-E medium dose vs normal; P=0.05, RC28-E high dose vs DM; P=0.489, RC28-E high dose vs normal; Figure 5A). As expected, VEGF Trap, but not FGF Trap, significantly reduced the VEGF protein content in the diabetic retina homogenate (P<0.05, VEGF Trap vs DM; P=0.156, FGF Trap vs DM; Figure 5A). On the other hand, the FGF2 protein level in the early diabetic retinas was increased as compared to that in the normal controls, and the difference was at the verge of statistical significance (P=0.060, normal vs DM; Figure 5B). Additionally, low and medium dose RC28-E significantly reduced the FGF2 protein levels (both P<0.05; Figure 5B).
whereas high dose RC28-E resulted in a trendy decrease ($P=0.067$), in comparison to that in the DM group. FGF Trap, but not VEGF Trap, significantly reduced the free FGF2 protein content in the diabetic retinal homogenate ($P<0.05$, FGF Trap vs DM; $P=0.161$, VEGF Trap vs DM; Figure 5B). These results suggest that RC28-E at all dosages can reduce the concentrations of its free target proteins, i.e. VEGF and FGF2, to the normal levels in early diabetic retinas; whereas the single target drugs can only act on their corresponding targets.

Expression of Proinflammatory Factors in the Retinas The expression of the proinflammatory factor intercellular cell adhesion molecule-1 (ICAM-1) and tumor necrosis factor-α (TNF-α) exhibited a similar trend. Both were dramatically upregulated in early diabetic retinas (Both $P<0.05$, normal vs DM; Figure 6A and 6B), which was then substantially decreased by RC28-E at all dosages (All $P<0.05$, RC28-E at all dosages vs DM; Figure 6A and 6B). VEGF Trap and FGF Trap also inhibited the upregulated expression of both proinflammatory factors, however to a less extent than RC28-E.
did (Figure 6A and 6B). Moreover, no significant difference was found in VEGF transcript levels among the experimental groups, except a trend increase in the DM group (Figure 6C), this result was consistent with our recent study and presumably due to the selection of VEGF reference sequence for qPCR primer design [24]. In contrast, FGF2 mRNA abundance was significantly augmented in the DM group (P<0.05, normal vs DM; Figure 6D), which was tapered by RC28-E in a dose-dependent manner, although there was no statistical significance among the experimental groups. FGF Trap intervention lowered the FGF2 transcript level, whereas VEGF Trap did not, in early diabetic retinas (Figure 6D).

**DISCUSSION**

The Fab fragment of RC28-E contains extracellular domain 2 of VEGFR1 and extracellular domain 3 of VEGFR2, the structure that is identical to that in VEGF Trap. In addition, the RC28-E Fab incorporates extracellular domains 2 and 3 of FGFR1. The Fab of RC28-E is fused to the Fc portion of human IgG1. Therefore, RC28-E can bind both VEGF and FGF2 with high affinity without eliciting the downstream signaling pathways of either factor [21]. In the current study, we administered the innovative drug RC28-E to STZ-induced diabetic rats, and reported the remarkable protection of early diabetic retina from apoptotic cell death in vascular network, aberrant activation of glial cell, BRB breakdown and vessel leakage, impairment in the ultrastructures of microvessels and neuroretina, and exacerbation of proinflammatory microenvironment.

Intravenous injection of STZ into the SD rats triggered selective death of islet β cells in pancreas, causing hyperglycemia due to the absolute deficiency of insulin secretion (Table 3). The rats also exhibited weight loss (Table 2), polydipsia (Table 4), polyuria, hyperphagia, and other typical metabolic symptoms of DM [27]. The STZ-induced type 1 diabetic rat model does not experience self-remission or recovery over time as a result of the STZ-induced permanent destruction of islet β cells.

Intravitreal injections of RC28-E, VEGF Trap, and FGF Trap had no significant effect on systemic symptoms and metabolic parameters in diabetic rats (Tables 2-4), indicating that these decoy receptors to VEGF, FGF2, or both exert their effects through local, instead of systemic, mechanisms.

At the tissue level in the retina of early diabetic rats, intravitreal injections RC28-E at medium and high doses significantly decreased the number of apoptotic cells in the vasculature (Figure 1), and the protective effects were similar to FGF Trap and better than VEGF Trap (Figure 1). On the other hand, the GFAP staining intensity, an indicator of glial cell reactive activation [28], was substantially subdued by RC28-E at all doses and VEGF Trap; whereas FGF Trap exerted an apparently less inhibition on reactive gliosis under early diabetic condition (Figure 2). These results suggest that single target Traps behave differentially on pathological phenotypes in early diabetic retina, and dual blockade with RC28-E at sufficient concentration may generate the optimal effects. Indeed, the transmission electron microscopy (TEM) results revealed dose-dependent changes in the ultrastructures of outer and inner BRB, as well as that of outer neuroretina, with RC28-E at high dose providing the best protection (Figure 4).

However, the results of Evans blue assay were unexpected. Although the dye leakage in retina was boosted 1.5 fold under early diabetic condition, all the drug treatments, except that of RC28-E at high dose, did not alleviate the diabetes-induced retinal vessel leakage (Figure 3). This could be due to the inappropriate timing for intravitreal injections, in view of a recent parallel study testing the anti-leakage function of a naturally-occurring ocular peptide, α-melanocyte-
interleukin 1β and nuclear factor-κB genes, including ICAM-1 and TNF-α through activation of the pathological phenotypes in the STZ-induced diabetic rats during the stage of nonproliferative DR, to which most of VEGF Trap and FGF Trap only normalized the protein levels similar to those in the normal controls (Figure 5). Whereas proteins in the groups treated with RC28-E at all doses were in the early diabetic retinas (Figure 5), and the levels of both significantly reduce the free VEGF and FGF2 protein levels the high affinity and specificity to both VEGF and FGF2, did rat model of diabetes. At the molecular level, RC28-E, with be the insufficient concentrations of the single target Traps to VEGF signaling with either EC28-E at all doses or VEGF Trap the protective effects of this novel drug in these cells.

In conclusion, RC28-E, a novel recombinant decoy receptor drug blocking the activities of both VEGF and FGF2, reduces apoptotic cell death in retinal vasculature, inhibits reactive cell death, and identifies the signaling pathway mediating the protective effects of this novel drug in these cells. These results support further development of RC28-E into a novel and effective drug to DR.

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