A safety study of high concentration and high frequency intravitreal injection of conbercept in rabbits

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The novel anti-VEGF drug conbercept has been used in the treatment of several retinal neovascular diseases. Owning to the alteration of the structure, the newest drug is capable of combining more molecular targets and present higher affinity to the angiogenesis promoting factors. However, it is unknown whether it will cause any unwanted effects like other anti-VEGF agents. We studied the short-term safety of high concentration and high frequency intravitreal injection of conbercept in rabbits. Intraocular pressure, fundus-photography, ERGs were applied. Retinal morphology, the amount of apoptotic cells and protein levels of IL-6, IL-8 and TNF-α in the aqueous humor were determined. Retinal proteomics was detected using tandem mass tags (TMTs) quantitative mass spectrometry. The difference of IOP, ERGs, protein levels of inflammatory factors among rabbits received conbercept and PBS was not significant (P > 0.05). Fundus photographs and retinal morphology of animals in the conbercept-injected groups mimic those observed in the PBS-injected groups. No TUNEL-positive cell was seen in the retinal ganglion cell layer in the conbercept-injected groups. Proteomics did not show significant changes of inflammation or apoptosis associated proteins in the conbercept-injected eyes. We conclude that intravitreal injection of high concentration and high frequency conbercept is well tolerated at least in a short-term in rabbits.

In the past decade, intravitreal injections of anti-vascular endothelial growth factor (VEGF) agents have been successfully used in the treatment of several retinal neovascular diseases which were incurable not long ago. Ranibizumab and bevacizumab were the two most extensively used anti-VEGF drugs. Recently, aflibercept and conbercept, two new anti-VEGF agents have been developed. Owning to the alteration of the drug structure, the novel anti-VEGF drugs are capable of combining more molecular targets and present higher affinity to the angiogenesis promoting factors1,2. Clinically, the new drugs make it possible to prolong the interval between multiple injections3, are effective in some patients non-responsive to ranibizumab and bevacizumab3,4, and even work in some severe patients with increased dosage. With a similar structure and effect to aflibercept, the newest anti-VEGF drug conbercept is a recombinant fusion protein composed of the second Ig domain of VEGFR1 and the third and fourth Ig domain of VEGFR2 to the constant region (Fc) of human IgG1. It is designed as a receptor decoy with high affinity for all VEGF isoforms and PIGF1,5,6.

Although clinical trials have proved anti-VEGF drugs exhibit satisfactory safety in many neovascular retinal diseases, patients undergo intravitreal injections still suffer from some unwanted adverse events, even if the occurrence is low. Moreover, long-term repeated injections increase the risk of the small-probability ocular side effects7-9. In addition to the adverse events that caused directly by the injection procedure, some side effects may be associated with the drugs themselves. These drug-related side effects include endophthalmitis6, increase of intraocular pressure2, retinal toxicity, and decrease of retinal function7,10. It has been reported that bevacizumab may lead to an increase of intraocular and systemic concentrations of IL-6 and IL-8 in patients11,12 and a loss...
of retinal ganglion cells (RGCs) in rats. Significant reduction of electroretinogram (ERG) a-wave and b-wave amplitudes in isolated bovine retinas after application of aflibercept has been reported, suggesting the drug might also affect retinal function.

The improved anti-VEGF effect of aflibercept and conbercept may be attributed to expansion of their targets and increase of affinity. In addition to blocking VEGF-A receptor as ranibizumab and bevacizumab do, both aflibercept and conbercept combine VEGF-B and placental growth factor (PIGF), which promote neovascularization and permeability of the blood vessel. However, VEGF-B and PIGF are involved in normal physiological functions. There are increasing concerns with regard to whether blocking these targets may cause any unwanted side effects. In addition, PIGF exerts a protective effect on retinal neuronal cells but it is still unknown whether it is an indispensable protective factor in the retina. Thus, one can never overemphasize the importance of the safety issue of the new agents, especially the safety concerns may be exaggerated in a diseased retina.

We studied whether high dosage and high frequency intraocular application of conbercept would cause unwanted ocular adverse effects. The affinity, pharmacokinetic and systemic tolerability of intravitreal injection of conbercept in rabbit has been studied. By using the same model, we evaluated the safety of intraocular administration of conbercept via morphological, functional and biological assessments. We also studied the retinal proteomics in the conbercept treated eyes.

Results

Intraocular pressure. The averaged IOPs (Fig. 1) after a single injection of PBS, or 0.2, 0.5, 2.0 mg of conbercept were 14.4 ± 1.3, 14.7 ± 2.2, 14.2 ± 1.9 and 15.1 ± 1.4 mmHg at day 4; 15.7 ± 1.8, 16.2 ± 2.4, 15.2 ± 1.3 and 15.8 ± 2.3 mmHg at day 7; 16.0 ± 2.0, 15.6 ± 1.8, 16.2 ± 1.9 and 16.9 ± 2.2 mmHg at day 14. The IOPs of the six-injection F and G groups were 15.3 ± 2.4 and 15.9 ± 1.9 mmHg on the 7th day after the final weekly-injection. There was no significant difference (p > 0.05, n = 6) in IOP after the single injection of 0.2, 0.5, 2.0 mg and the six weekly injections of 0.2 mg conbercept compared to the PBS groups.

Figure 1. The intraocular pressure (IOP) in rabbits after a single injection of 50 μL PBS, 0.2, 0.5 or 2.0 mg of conbercept and 6 multiple injections of 50 μL of PBS or 0.2 mg of conbercept. There was no significance in IOP among the conbercept-injected groups and the PBS-injected groups (p > 0.05). The results are mean ± SD (n = 6).
Fundus examination. After a single intravitreal injection of different doses of conbercept, no signs of abnormalities or inflammation was seen in the fundus (Fig. 2d–l) when compared to the PBS-injected groups (Fig. 2a,b and c). The vitreous was clear and the vascular pattern appeared normal. No vascular narrowing, dilatation or tortuosity, retinal detachment, hemorrhage or optic nerve head changes were seen. Similar results were found in the six-weekly conbercept-injected group (Fig. 2m) and the PBS-injected group (Fig. 2n).

ERG. The representative ERG responses (Fig. 3A) of the conbercept- and PBS-injected rabbits and the amplitude-intensity profiles (Fig. 3B) were exhibited. The ERG waveforms and the amplitudes of the averaged a- and b-wave recorded under various stimuli in the conbercept-injected groups mimicked those in the PBS-injected groups. There was no significant differences ($p > 0.05, n = 5$) between all the pairs in any of the recordings. The ERG showed no evidence of retinal functional damage after application of conbercept.

Histology. No apparent structural changes or sign of toxicity were observed in the conbercept-injected groups and the appearance of the retina were similar to the PBS control groups (Fig. 4). No sign of retinal degeneration,

Figure 2. Fundus photographs taken on the 4th, 7th, 14th day after a single injection of 50 μL PBS (a–c) or 0.2, 0.5, or 2.0 mg of conbercept (d–l) and on the 7th day after the 6th multiple injections of 50 μL PBS (m) or 0.2 mg of conbercept (n). The results observed in the conbercept-injected groups mimic those in the PBS-injected groups.
disorganization, thinning, cell loss, or hypocellularity was observed in these groups. The difference in the number of cells in the GCL in all the groups was not significant ($p > 0.05, n = 3$). There was no significant difference in the thickness of the inner nuclear layer (INL) among all the groups ($p > 0.05, n = 3$).

**TUNEL.** TUNEL-positive cells were not seen in the GCL of any animals injected with different doses and frequencies of conbercept and PBS (Fig. 5). No significant difference in apoptotic cell was observed among the...
Figure 4. Retinal histology on the 4th, 7th, 14th day after a single injection of 50 μL PBS or 0.2, 0.5, 2.0 mg of conbercept and on the 7th day after the 6th multiple injections of 50 μL PBS or 0.2 mg of conbercept. There was significant difference in the cell number in the ganglion cell layer (GCL) and the thickness of inner nuclear layer (INL) (p > 0.05). The results were mean ± SEM (n = 3). Scale bar: 50 μm.
conbercept injected and the PBS-injected groups. In the NMDA-injected group which was the positive control, positive cells were seen in the GCL.

ELISA. Aqueous humor was collected at day 4, 7, 14 after a single intravitreal injection of different doses of conbercept or 50 μL of PBS and at day 7 after the 6th weekly-injection of 50 μL PBS or 0.2 mg conbercept. The differences of the IL-6, IL-8 and TNF-α protein expression among the conbercept groups and the PBS groups was not significant (p > 0.05, n = 4) (Fig. 6). The concentrations of the cytokines in the LPS-injected group which served as a positive control were higher than those of the other groups (p < 0.001, n = 3–4).

Proteomics analysis. Using the TMT quantitative mass spectrometry, a total of 6042 proteins were quantified from the rabbit retina on the 4th, 7th and the 14th day after intravitreal injection of 0.5 or 2.0 mg conbercept. Compared with the untreated control eyes, 250 proteins presented changes greater than 2 folds at either dosages or time point in the conbercept-injected eyes. Among them as shown in Tables 1, 2 and 3, 50 proteins were up-regulated, 57 were down-regulated and the rest 143 proteins showed inconsistent changes at different times or dosages.

While most of the protein presented a fold change less than 3 folds, VEGF receptor 1 (VEGFR1) was the only protein that showed more than a 10-fold increase. In the 0.5 mg-injected group, the protein level of VEGFR1 was 3.85 times, 3.32 times and 2.57 times higher than the untreated group at the 4th, 7th and 14th day respectively. In the 2.0 mg-injected group it was 18.35 times, 3.46 times and 6.73 times higher. The complement C3 alpha chain isoform X1 partial 1 and fibrinogen alpha chain were the second highest increased proteins. It reached a 5-fold increase at day 7 in the 0.2 mg injected rabbits. The most decreased protein was cyclic GMP-AMP synthase, which decreased to 0.11 fold at day 14 in the 0.5 mg group.

We especially examined the changes of growth factors and proteins associated with inflammation and apoptosis. Neither of those proteins presented a fold change greater than 2. VEGF-B, PIGF and interleukins including interleukin-1 and interleukin-17 were not identified.

To better understand the functions of the differentially expressed proteins, we performed KEGG pathway analysis. The top 20 significant enriched pathway terms are shown in Fig. 7.
Discussion
We investigated the safety of intraocular injection of high concentration and high frequency of a novel anti-VEGF agent conbercept in rabbit. No drug-related side effects were detected with IOP, fundus, retinal morphology and function assessments. No apoptotic cells were found in the retinal GCL in the conbercept injected eyes. We did not observe alternations of the major pro-inflammatory cytokines. Considering the high concentration and high frequency intravitreal injection did not cause unwanted side effects, we concluded that conbercept is well tolerated in rabbit in a short term observation.

Most of the transient elevation of IOP after intravitreal injection is believed to be caused by a sudden increase of vitreous volume and can recover within a few minutes to a few hours. However, it was reported that a limited number of AMD patients developed sustained elevation of IOP after single or repeated intravitreal injection of anti-VEGF drugs, which lasted from several weeks to even 1 or 2 years. The increase of IOP at long-term might be attributed to the drug itself. Nevertheless, we did not observed elevation of IOP among all the groups in our study.

Although overproduction of VEGF is deleterious, adequate concentrations of VEGF may be important for the eye to maintain normal functions including vascular development and neurons survival. PIGF, a member of the VEGF family, also exerts a role in the protection of neuron in the retina. It was showed that potent inhibitors of all VEGF-A isoforms significantly diminished the protective effects of ischemic preconditioning on neurons. In addition, VEGF-A120 plays a supporting role in the survival of normal RGCs. It was also reported that multiple injections of high doses of up to 5.0 mg bevacizumab in rabbits would induce photoreceptors apoptosis at 1 week after injections. In a murine model, systemic administration of a viral vector expressing soluble VEGF receptor-1 led to a significant decline in ERG responses. Since conbercept is a multi-target VEGF blocker and binds all isoforms of VEGF-A, VEGF-B, and PIGF with high affinity, it arouses a concern whether it will lead to unwanted retinal neuron death and dysfunction. The number of GCL cells and the thickness of INL were not changed and no TUNEL-positive cells were seen in the RGC layer of the conbercept-injected groups. Functionally, the ERG waveforms were normal and the amplitudes of the a- and b-waves were similar to the PBS injected control group. We did not observed retinal and choroidal structural abnormalities under light microscope. Thus, our data are in agreement with other studies that inhibition of VEGF or prolonged blockade of ocular VEGF receptors with conbercept would not cause morphological and functional damage to the retina.

Despite rarely happens, intraocular inflammation is a serious concern after intravitreal anti-VEGF treatment. We detected the protein expressions of IL-6, IL-8 and TNF-α in the aqueous humor of conbercept injected eyes. The levels of these inflammatory cytokines are similar to those in the PBS injected groups. Even though it is reasonable that the human fusion protein might cause inflammatory responses in rabbits, however, we did not observe such phenomenon.

Figure 6. Protein levels of IL-6, IL-8 and TNF-α in the aqueous humor. Samples were collected on the 4th, 7th, 14th day after single injection of 50 μL PBS or 0.2 mg, 0.5 mg, 2.0 mg of conbercept and on the 7th day after the 6th multiple injections of 50 μL PBS or 0.2 mg of conbercept as measured by ELISA. Positive control was collected 24 hours after injection of 100 μg of LPS. No statistically significant difference was found among the conbercept-injected groups and the PBS-injected groups. The protein levels of the three inflammation cytokines in the LPS-injected groups were significantly higher than all the other groups (p < 0.001). The results are mean ± SEM (n = 3~4). #Undetectable.
| Accession number | Gene symbol | Protein name | Fold change |
|------------------|-------------|--------------|-------------|
| 65584547         | FLT1        | vascular endothelial growth factor receptor 1 | 3.85        |
| 65583776         | MKRN2       | probable E3 ubiquitin-protein ligase makorin-2 isofrom X5 | 2.10        |
| 65585155         | NFKBIZ,  | NF-kappa B inhibitor zeta isoform X2 | 4.04        |
| 291405684        | YPEL2       | protein yippee-like 2 | 1.04        |
| 655826988        |            | LOW QUALITY PROTEIN: 39S ribosomal protein L20, mitochondrial-like | 3.61        |
| 655841362        | KLHDC3      | kelch domain-containing protein 3 | 1.20        |
| 655879793        | DLGAP3      | LOW QUALITY PROTEIN: disks large-associated protein 3 | 1.14        |
| 655878481        | RC3H2       | reuin-2 isoform X4 | 1.97        |
| 655846130        | BCAN        | brevac core protein | 3.23        |
| 655834754        | RRM15B      | putative RNA-binding protein 15B | 1.60        |
| 291389567        | ZFCH3H      | zinc finger C3H1 domain-containing protein | 2.41        |
| 655600655        | VPS13A      | vacuolar protein sorting-associated protein 13A | 1.20        |
| 315360765        | EPT1, SEL1  | *ethanolaminephosphotransferase 1 | 1.20        |
| 655901171        |            | maestro heat-like repeat-containing protein family member 1 | 2.26        |
| 655837568        |            | RNA-binding protein 48-like | 1.43        |
| 655862221        | DNAJB12     | LOW QUALITY PROTEIN: dnaJ homolog subfamily B member 12 | 1.62        |
| 129270090        | CRYAA       | *alpha-crystallin A chain | 1.88        |
| 65578703         | CMTM4       | CKL-like MARVEL transmembrane domain-containing protein 4 | 1.14        |
| 655834907        | BAP1        | ubiquitin carboxyl-terminal hydrolase BAP1 | 1.26        |
| 65582768        | CRYBB1      | beta-crystallin B1 | 1.93        |
| 65582947         | GATM        | LOW QUALITY PROTEIN: glycine amidinotransferase, mitochondrial | 1.20        |
| 291416535        | UVSSA       | UV-stimulated scaffold protein A, partial | 1.22        |
| 655883513        |            | LOW QUALITY PROTEIN: carboxyl reductase [NADPH] 1-like | 2.08        |
| 655896223        | FAM118A     | protein FAM118A | 1.79        |
| 655850611        |            | retinal-specific ATP-binding cassette transporter-like | 1.73        |
| 655739906        | BAZ2A       | LOW QUALITY PROTEIN: bromodomain adjacent to zinc finger domain protein 2A | 2.10        |
| 126723630        | LGALS3      | *galexct-3 | 1.35        |
| 291387457        | SRAI        | steroid receptor RNA activator 1 | 1.10        |
| 655846332        | GBA         | glucosylceramidase isoform X2 | 2.26        |
| 655857145        | BMPR1B      | bone morphogenetic protein receptor type-1B isoform X2 | 2.35        |
| 655872717        | ABCA5       | ATP-binding cassette sub-family A member 5 isoform X2 | 2.16        |
| 655752526        | CDK17       | cyclin-dependent kinase 17 | 2.17        |
| 655771130        | SAMD4B      | protein Smad homolog 2 | 1.20        |
| 65585844         | PCDH10      | protocadherin-10 isoform X2 | 1.50        |
| 655842878        | CEP57L1     | centrosomal protein CEP57L1 isoform X5 | 1.21        |
| 655605232        | SORBS2      | sorbin and SH3 domain-containing protein 2 isoform X9 | 1.67        |
| 655841271        | TREML2      | trem-like transcript 2 protein | 9.55        |
| 291406631        |            | ATPase inhibitor, mitochondrial-like | 1.71        |
| 65572731         | LETMD1      | LETM1 domain-containing protein 1 isoform X2 | 2.53        |
| 291406313        | MAPT        | microtubule-associated protein tau isoform X11 | 5.27        |

Continued
Significantly up-regulated proteins after intravitreal injection of conbercept. *Means the proteins are not predicted proteins.

| Accession number | Gene symbol | Protein name | 4–0.5 mg | 4–2.0 mg | 7–0.5 mg | 7–2.0 mg | 14–0.5 mg | 14–2.0 mg |
|------------------|-------------|--------------|----------|----------|----------|----------|----------|----------|
| 29139405         | MRT04       | mRNA turnover protein 4 homolog | 1.36     | 1.20     | 2.30     | 1.45     | 1.22     | 1.59     |
| 655890274        | OGG1        | N-glycosylase/DNA lyase | 1.86     | 1.57     | 1.92     | 1.38     | 1.38     | 2.89     |
| 291410889        | PGR6        | prostaglandin E(2) 9-reductase | 1.24     | 2.20     | 1.84     | 1.37     | 1.11     | 1.88     |
| 655835184        | FXK         | FX-domain-containing protein kinase-like protein isoform X4 | 1.13     | 1.67     | 2.08     | 1.27     | 2.33     | 1.10     |
| 655601008        | APOA1       | apolipoprotein A-1 isoform X1 | 1.11     | 2.85     | 1.09     | 1.24     | 1.34     | 1.31     |
| 655864723        | ARPP19      | cAMP-regulated phosphoprotein 19 | 1.14     | 1.40     | 1.22     | 1.20     | 1.22     | 2.06     |
| 655875742        | PCSK1N      | proSAAS | 1.24     | 1.76     | 1.75     | 1.17     | 1.83     | 2.05     |
| 291405564        | ASC2        | acid-sensing ion channel 2 | 1.59     | 1.68     | 2.17     | 1.15     | 1.14     | 1.02     |
| 147903853        | ATP2A1, ATP2A3, SERCA1a | sarcoplasmic/endoplasmic reticulum calcium ATPase 1 | 2.09     | 1.08     | 1.25     | 1.14     | 1.11     | 1.28     |
| 291398822        | PARS2       | probable proline-tRNA ligase, mitochondrial | 1.75     | 1.58     | 2.15     | 1.11     | 1.40     | 1.06     |

We used 10-plex TMT-labeled proteomic quantification to analysis the variation of proteins in the retina of conbercept injected eyes. This technique has been demonstrated to be a powerful method to reach very large coverage of the proteome and to discover differentially expressed proteins (DEPs). Although the proteomic was based on a database of rabbit protein, it could help us to understand the protein alternation after intraocular administration of conbercept. To the best of our knowledge, this is the first study to explore DEPs in the retina after intraocular administration of an anti-VEGF drug.

Among 6042 proteins quantified, we identified 250 proteins (~4%) altered by more than 2.0-fold or less than 0.5-fold with greater than 95.0% probability at least at one time point or dosage in the eyes applied with 0.5 or 2.0 mg conbercept at day 4, 7 or 14. The only protein that reached more than 10-fold increases was VEGFR1. It was not surprise since conbercept is a recombinant fusion protein contains several ligand binding domains including VEGFR1. Increase of VEGFR1 should be a consequence of increase of exogenous conbercept. On the other hand, the data proved the efficacy of the assay.

Proteomic analysis did not show significant changes of cell death or inflammation associated proteins in the conbercept-injected eyes. No increase of cytokine, chemokine or neuroinflammation related proteins was observed. Compared with the untreated group, conbercept did not cause substantial changes of the expressions of growth factors. Although the complement C3 alpha chain isoform X1 partial 1 was detected, no any other related proteins were found. In the complement and coagulation cascade pathway, the levels of three fibrinogens were higher than the control but these proteins are also related with platelet activation. Thus, alternation of the complement C3 alpha chain isoform X1 partial 1 maybe associated with platelet activation, rather than inflammation. Platelet activation was detected as the most enriched pathway after conbercept injection. Fibrinogen alpha chain, fibrinogen beta chain and fibrinogen gamma chain isoform X2 increased at day 7 after injection. It has been shown that application of ranibizumab and bevacizumab may contribute to a risk of systemic thromboembolic events in elderly patients. Up-regulation of three fibrinogens after conbercept injection might raise a concern whether the proteins could be a potential risk factor. Research revealing the fibrinogen concentration in circulation would be helpful.

Mitochondrial adenosine triphosphate synthase (ATPase inhibitor) was up-regulated at all three time points. Bevacizumab was reported to show mild mitochondrial toxicity at clinically doses. Five mitochondrial proteins were altered significantly with four up-regulated involved in steroid biosynthesis, lipoic acid metabolism, pyridine metabolism, glycerophospholipid metabolism and one down-regulated in amino acid metabolism.

In the protein processing in endoplasmic reticulum, alpha-crystallin A chain was approximately 2-fold at day 4 and 1.5-fold increased at day 7 and 14 after injected with both dosages of conbercept. Alpha-crystalline is of physiological and nutritional importance in the maintenance of health of the retina.

The Graves disease carrier protein (GDC) was down-regulated after conbercept injection at all three time points and decreased more than 5 folds in the 2.0 mg group at day 7. GDC is recognized in patients with active Graves disease (GD).

In the riboflavin (Vitamin B2) pathway, the expression of riboflavin (RF) kinase in the conbercept-injected groups decreased at all three time points. ATP:riboflavin kinase catalyzes the synthesis of cofactor flavin mononucleotide (FMN) by transforming riboflavin and ATP into FMN and ADP. RF is of physiological and nutritional importance in the maintenance of health of the retina.

There are inevitable limitations for this study. First, we injected recombinant human fusion protein into the rabbit eyes. The data was obtained form a rabbit database and the protein information could not be completely used to predict the outcome in human. Second, the cutoff we set for protein changes is 2 folds. Thus we can’t exclude the possibility that a protein presents a fold change less than the threshold will not exert functional changes. However, since such investigation can’t be duplicated in human subjects, the first retinal proteomic study...
| Accession number | Gene symbol | Protein name                                      | Fold change |
|------------------|-------------|--------------------------------------------------|-------------|
| 655828813        | RAPH1       | ras-associated and pleckstrin homology domains-containing protein 1 | −0.77 −0.44 −0.88 −0.90 −0.74 −0.96 |
| 291416104        | RG58        | regulator of G-protein signaling 8               | −0.87 −0.83 −0.80 −0.89 −0.84 −0.39 |
| 291406868        | PIK3R1P1    | phosphoinositide-3-kinase-interacting protein 1  | −0.20 −0.40 −0.81 −0.89 −0.95 −0.23 |
| 655603509        | LRRC4C/LRRC4| leucine-rich repeat-containing protein 4 C        | −0.93 −0.38 −0.98 −0.88 −0.97 −0.52 |
| 291384766        | MPPED2      | metallophosphoesterase MPPED2                    | −0.58 −0.25 −0.42 −0.88 −0.93 −0.46 |
| 291382897        | TEX10       | testis-expressed sequence 10 protein             | −0.51 −0.67 −0.84 −0.88 −0.86 −0.27 |
| 655886298        | ZNF629      | zinc finger protein 629                          | −0.73 −0.49 −0.95 −0.84 −0.80 −0.94 |
| 291403838        | MAPK5       | mitogen-activated protein kinase kinase kinase 5 | −0.70 −0.44 −0.90 −0.82 −0.89 −0.80 |
| 655876070        | SPIN3       | spinulin-3                                       | −0.50 −0.15 −0.63 −0.82 −0.91 −0.98 |
| 655896069        | MED15       | mediator of RNA polymerase II transcription subunit 15 isoform X3 | −0.36 −0.46 −0.54 −0.82 −0.71 −0.63 |
| 655605182        | SNX25       | sorting nexin 25                                 | −0.12 −0.38 −0.63 −0.77 −0.83 −0.24 |
| 655633227        | DMXL1       | LOW QUALITY PROTEIN: dmX-like protein 1         | −0.68 −0.42 −0.82 −0.77 −0.58 −0.68 |
| 291402173        | Sipa1L2     | signal-induced proliferation-associated 1-like protein 2 | −0.98 −0.41 −0.88 −0.76 −0.99 −0.84 |
| 291408938        | MED4        | mediator of RNA polymerase II transcription subunit 4 | −0.54 −0.40 −0.66 −0.74 −0.38 −0.55 |
| 655859905        | UBE2G1      | ubiquitin-conjugating enzyme E2 G1              | −0.51 −0.36 −0.86 −0.74 −0.75 −0.54 |
| 655896886        | DNAJC16     | dnal homolog subfamily C member 16 isoform X2    | −0.71 −0.67 −0.48 −0.71 −0.77 −0.51 |
| 655837812        | DMX2L       | Friend virus susceptibility protein 1-like       | −0.61 −0.72 −0.48 −0.66 −0.49 −0.67 |
| 655891444        | PGS1        | LOW QUALITY PROTEIN: CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidylinositoltransferase, mitochondrial | −0.44 −0.35 −0.73 −0.62 −0.61 −0.62 |
| 655842073        | MB21D1      | cyclic GMP-AMP synthase                          | −0.63 −0.57 −0.11 −0.61 −0.45 −0.70 |
| 655600047        | DDX58       | probable ATP-dependent RNA helicase DDX58 isoform X2 | −0.47 −0.91 −0.43 −0.60 −0.66 −0.80 |
| 655901439        | ras-related protein Rab-9A-like                  | −0.48 −0.63 −0.87 −0.59 −0.92 −0.96 |
| 655875606        | Cdk16       | cyclin-dependent kinase 16 isoform X7            | −0.34 −0.55 −0.83 −0.57 −0.22 −0.70 |
| 655847855        | COL11A1     | collagen alpha-1(XI) chain isoform X1            | −0.60 −0.55 −0.50 −0.56 −0.82 −0.46 |
| 655844088        | RMND1       | required for mitotic nuclear division protein 1 homolog isoform X2 | −0.32 −0.41 −0.71 −0.54 −0.64 −0.35 |
| 655902770        | interferon-induced GTP-binding protein Mx1       | −0.53 −0.96 −0.47 −0.53 −0.57 −0.85 |
| 655828284        | STAT1       | signal transducer and activator of transcription 1-alpha/beta | −0.51 −0.89 −0.50 −0.53 −0.60 −0.92 |
| 291384499        | RRP8        | ribosomal RNA-processing protein 8               | −0.74 −0.53 −0.87 −0.51 −0.83 −0.38 |
| 655600642        | RFK         | ribollavin kinase                                | −0.46 −0.77 −0.56 −0.50 −0.49 −0.50 |
| 655871198        | KRT15       | keratin, type I cytoskeletal 15                  | −0.59 −0.92 −0.44 −0.49 −0.88 −0.88 |
| 655877487        | ARMCX2      | armadillo repeat-containing X-linked protein 2   | −0.97 −0.68 −0.91 −0.49 −0.81 −0.34 |
| 655895404        | DDX55       | ATP-dependent RNA helicase DDX55 isoform X2      | −0.81 −0.46 −0.61 −0.49 −0.76 −0.73 |
| 291409274        | 1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial | −0.91 −0.74 −0.67 −0.44 −0.75 −0.64 |
| 291402773        | ITGA11      | integrin alpha-11                                | −0.57 −0.74 −0.38 −0.34 −0.67 −0.64 |
| 291389217        | KRT1        | keratin, type II cytoskeletal 1                  | −0.67 −0.78 −0.40 −0.44 −0.82 −0.78 |
| 298919207        | RLA-A3      | *MHC class I antigen-like precursor              | −0.32 −0.55 −0.39 −0.40 −0.46 −0.77 |
| 655883687        | cytosolic carnitine palmitoyltransferase 1        | −0.26 −0.43 −0.59 −0.40 −0.71 −0.30 |
| 284055498        | AFF4, RA_m002_JrmFRA6Br | *AFF4/FMR2 family member 4                | −0.63 −0.36 −0.52 −0.40 −0.37 −0.41 |
| 291389201        | KRT2        | keratin, type II cytoskeletal 6A-like            | −0.45 −0.45 −0.38 −0.38 −0.70 −0.83 |
| 291394553        | OSBP3       | oxysterol-binding protein-related protein 3      | −0.44 −0.49 −0.23 −0.37 −0.41 −0.61 |
| 655812471        | serine/arginine repetitive matrix protein 5-like | −0.42 −0.55 −0.51 −0.33 −0.62 −0.47 |
| 655730595        | KRT72       | keratin, type II cytoskeletal 72                 | −0.58 −0.71 −0.49 −0.33 −0.82 −0.74 |
| 655897183        | CLASRP      | CLK4-associated serine/arginine rich protein isoform X2 | −0.48 −0.62 −0.51 −0.32 −0.38 −0.26 |

Continued
Table 2. Significantly down-regulated proteins after intravitreal injection of conbercept. *Means the proteins are not predicted proteins.  † Means down-regulation.

| Accession number | Gene symbol | Protein name | Fold change |
|------------------|-------------|--------------|-------------|
|                  |             |              | 
|                  |             |              | Day 4–0.5 mg | Day 4–2.0 mg | Day 7–0.5 mg | Day 7–2.0 mg | Day 14–0.5 mg | Day 14–2.0 mg |
| 655603057        | PRKIR       | 52kDa repressor of the inhibitor of the protein kinase | −0.93 | −0.17 | −0.80 | −0.32 | −0.73 | −0.39 |
| 655840152        | C12H6orf47  | uncharacterized protein C6orf47 homolog | −0.31 | −0.32 | −0.28 | −0.31 | −0.20 | −0.32 |
| 291389221        | KRT3, CK-3, K3 | keratin, type II cytoskeletal 3 | −0.53 | −0.74 | −0.35 | −0.31 | −0.87 | −0.55 |
| 291406083        | keratin, type I cytoskeletal 14 | −0.44 | −0.39 | −0.33 | −0.30 | −0.60 | −0.70 |
| 655730654        | KRT2        | LOW QUALITY PROTEIN: keratin, type II cytoskeletal 2 epidermal | −0.60 | −0.91 | −0.32 | −0.29 | −0.80 | −0.60 |
| 126722900        | *lipophilin AL precursor | −0.29 | −0.25 | −0.41 | −0.29 | −0.26 | −0.38 |
| 655891028        | LOW QUALITY PROTEIN: D-dopachrome decarboxylase-like | −0.59 | −0.24 | −0.90 | −0.27 | −0.26 | −0.38 |
| 291393010        | LACC1       | laccase domain-containing protein 1 | −0.33 | −0.38 | −0.11 | −0.26 | −0.26 | −0.36 |
| 126722998        | *lipophilin CL2 precursor | −0.21 | −0.26 | −0.37 | −0.25 | −0.29 | −0.50 |
| 291389193        | KRT85       | keratin, type II cuticular Hb5 | −0.23 | −0.22 | −0.22 | −0.22 | −0.48 | −0.22 |
| 291404267        | SLC25A16    | graves disease carrier protein | −0.25 | −0.32 | −0.66 | −0.19 | −0.54 | −0.60 |
| 655872869        | KRT34       | LOW QUALITY PROTEIN: keratin, type I cuticular Ha4 | −0.22 | −0.20 | −0.17 | −0.18 | −0.32 | −0.19 |
| 655879141        | UBL3        | ubiquitin-like protein 3 | −0.18 | −0.26 | −0.60 | −0.15 | −0.44 | −0.63 |
| 291407186        | MAP7D2      | MAP7 domain-containing protein 2 isoform X7 | −0.17 | −0.48 | −0.75 | −0.13 | −0.11 | −0.49 |
| 291389187        | keratin, type II cuticular Hb6 | −0.21 | −0.14 | −0.18 | −0.13 | −0.27 | −0.20 |

in anti-VEGF injected eye still provide important information with regard to the molecular changes in the retina. Based on these data, it is practical to confirm whether the proteins are actually altered and to explore their significances. In addition, it is also possible to decide whether supplement treatments are necessary. For example, if it is confirmed that riboflavin kinase activity is lower in anti-VEGF injected eyes and consequently causes unwanted effect, it might be necessary to supply the patients with flavinmononucleotide.

We concluded that intravitreal injection of high concentration and high frequency of conbercept is well tolerated at least in a short-term in rabbit. Our study offers a comprehensive and intuitionistic overlook on the alteration of protein expression in the retina injected with conbercept. The data provided important information for the future clinical study and for designing therapeutic protocols.

Methods
Animals. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University, Chongqing, China. The animals were fed with standard laboratory food and water in an air-conditioned room with a 12-hour light-dark cycle.

One hundred and eleven pigmented Chinchilla rabbits, weighing 2 to 3 kg, were used. Only the right eye of each animal was injected and the left eye was untreated. Rabbits were randomized into nine groups. Group A (n = 3) did not receive any injection and was labeled as the blank. Group B (n = 27) received intravitreal injection of 50 μL of PBS and was labeled as control group. Groups C (n = 18), and D, E (n = 27 each) received intravitreal doses of 0.2, 0.5 and 2.0 mg/eye of conbercept on Day 0 respectively. Groups F and G (n = 6 each) received six weekly injections of 0.2 mg/eye of conbercept respectively. Groups H and I (n = 3 each) received 400 nmoles N-Methyl-D-Aspartate (NMDA) and 100 μg lipopolysaccharide (LPS) respectively and were labeled as the positive control groups for TUNEL and ELISA essays.

The vitreous volume of a rabbit is approximately 1.5 mL and that of a human is about 5 mL. As the dose of 0.5 mg or less conbercept is frequently used in humans, the doses of 0.2, 0.5 and 2.0 mg for conbercept in rabbits are about 1.3, 3.3, and 13.3 times of that in human.

Intravitreal injection. Intravitreal injection was performed in sterile conditions. Rabbits were anesthetized with injection of 3% phenobarbital sodium solution (30 mg/Kg) through the ear vein. After corneal surface anesthesia with oxybuprocaine hydrochloride eye drop (Santen Pharmaceutical Co., Ltd, Osaka, Japan), a 27-gauge needle attached to a 1 mL syringe was introduced into the vitreous cavity 3.5 mm posterior to the superotemporal limbus. The needle tip was directed towards the center of the vitreous under direct visualization. The conbercept solutions or PBS (50 μL) was slowly administered into the vitreous. To prevent reflux, the needle was held in place for 30 seconds before withdrawal. At the end of the procedure, lincomycin hydrochloride eye drops were applied.

Intraocular pressure. At day 4, 7, 14 after the single intravitreal injection of conbercept (0.2 mg, 0.5 mg and 2.0 mg) or PBS, and at day 7 after the 6th weekly-injection of conbercept (0.2 mg) or PBS, IOP was measured using a Schiotz tonometer (66 Vision Tech., Suzhou, China). Rabbits were anaesthetized with intraperitoneal injection
| Accession number | Gene symbol | Protein name | Fold change |
|------------------|-------------|--------------|-------------|
| 655874492        | TPCN1       | two pore calcium channel protein 1 | Day 4: 1.68, Day 14: 0.95 |
| 655846261        | RIT1        | GTP binding protein Rit1 | Day 4: 1.20, Day 14: 1.19 |
| 655759055        | RPS19BP1    | active regulator of SIRT1 | Day 4: 2.08, Day 14: 2.10 |
| 655868318        | NSMCE4A     | LOW QUALITY PROTEIN: non-structural maintenance of chromosomes element 4 homolog A | Day 4: 1.76, Day 14: 1.78 |
| 655892261        | SP2         | LOW QUALITY PROTEIN: transcription factor Sp2 | Day 4: −0.91, Day 14: −0.94 |
| 291387997        | EPDR1       | epidermal retinol dehydrogenase | Day 4: 1.09, Day 14: 1.10 |
| 65584071         | HEATR3      | HEAT repeat-containing protein 3 | Day 4: 2.18, Day 14: 2.14 |
| 655879209        | PIBF1       | proline-rich protein 14 | Day 4: 1.16, Day 14: 1.17 |
| 65588452         | THAP1       | THAP domain-containing protein 1 isoform X2 | Day 4: 1.13, Day 14: 1.15 |
| 655854110        | KALRN       | kalirin isoform X20 | Day 4: 4.29, Day 14: 4.30 |
| 655828138        | FKBP7       | peptidyl-prolyl cis-trans isomerase FKBP7 | Day 4: −0.33, Day 14: −0.34 |
| 291409542        | INTS5       | integrator complex subunit 5 | Day 4: −0.81, Day 14: −0.86 |
| 291409404        | KCNJ13, KIR7.1 | inward rectifier potassium channel 13 isoform X1 | Day 4: 1.32, Day 14: 1.33 |
| 655604456        | STIM2       | stromal interaction molecule 2 isoform X2 | Day 4: −0.29, Day 14: −0.30 |
| 291409135        | NR2F2       | COUP transcription factor 2 isoform X3 | Day 4: 1.20, Day 14: 1.21 |
| 655870532        | MRPS23      | 28S ribosomal protein S23, mitochondrial isoform X2 | Day 4: 1.20, Day 14: 1.21 |
| 655886914        | TRIM4       | tripartite motif-containing protein 4 isoform X2 | Day 4: 1.04, Day 14: 1.05 |
| 291393060        | HNRPAL1     | heterogeneous nuclear ribonucleoprotein A1-like | Day 4: −0.49, Day 14: −0.50 |
| 291407324        | LANCL3      | lanC-like protein 3 | Day 4: −0.66, Day 14: −0.69 |
| 655605545        | STON1       | stonin-1 isoform X2 | Day 4: 1.14, Day 14: 1.15 |
| 65583182         | MOBP        | myelin-associated oligodendrocyte basic protein | Day 4: 1.30, Day 14: 1.31 |
| 655603503        | CHH11orf74  | uncharacterized protein C11orf74 homolog | Day 4: −0.49, Day 14: −0.50 |
| 655836760        | ZNF532      | zinc finger protein 532 isoform X3 | Day 4: 2.50, Day 14: 2.55 |
| 291403541        | RBM23       | probable RNA-binding protein 23 | Day 4: 1.02, Day 14: 1.03 |
| 655879659        | OSCP1       | protein OSCP1 isoform X4 | Day 4: 1.29, Day 14: 1.30 |
| 291383827        | TAGLN       | transgelin | Day 4: 1.45, Day 14: 1.46 |
| 291401266        | C15H4orf32  | uncharacterized protein C1orf32 homolog | Day 4: −0.48, Day 14: −0.50 |
| 291394485        | TIMM21      | mitochondrial import inner membrane translocase subunit Tim21 | Day 4: −0.87, Day 14: −0.89 |
| 655899294        | C3          | complement C3 alpha chain isoform X1, partial | Day 4: 1.20, Day 14: 1.22 |
| 284005533        | MAP1A       | *microtubule-associated protein 1A | Day 4: 1.28, Day 14: 1.30 |
| 655601080        | PRDM10      | PR domain zinc finger protein 10 isoform X2 | Day 4: 1.21, Day 14: 1.23 |
| 655903015        | ARHGGEF1    | LOW QUALITY PROTEIN: rho guanine nucleotide exchange factor 1 | Day 4: −0.52, Day 14: −0.54 |
| 655859423        | RGS7        | regulator of G-protein signaling 7 isoform X4 | Day 4: −0.95, Day 14: −0.97 |
| 655878265        | ZBTB43      | zinc finger and BTB domain-containing protein 43 | Day 4: 1.03, Day 14: 1.04 |
| 291383390        | CHH9orf41   | UFOP586 protein C9orf41 homolog | Day 4: 1.06, Day 14: 1.08 |
| 655832408        | PIBF1       | progesterone-induced-blocking factor 1 | Day 4: −0.32, Day 14: −0.33 |
| 291401111        | FGA         | fibrinogen alpha chain | Day 4: 1.41, Day 14: 1.43 |
| 291401007        | TIA1        | T-lymphoma invasion and metastasis-inducing protein 1 | Day 4: −0.86, Day 14: −0.88 |

Continued
| Accession number | Gene symbol | Protein name                                                                 | Fold change |
|------------------|-------------|------------------------------------------------------------------------------|-------------|
| 291406107        | NKIRAS2     | cleftless homolog 2 inhibitor-interacting Ras-like protein 2                  | −0.47       |
| 291401109        | FG8         | fibrinogen beta chain                                                        | 1.28        |
| 655601664        | ALG9        | alpha-1,2-mannosyltransferase ALG9 isoform X2                                | −0.76       |
| 291395292        | PLCXD3      | PI-PLC-X domain-containing protein 3                                          | −0.92       |
| 291402327        | FBXO28      | F-box only protein 28                                                         | 1.06        |
| 291402543        | DSTYK       | dual serine/threonine and tyrosine protein kinase isoform X2                  | −0.88       |
| 655897677        | KDELR1      | ER luminal protein retaining receptor 1                                       | 1.28        |
| 126723746        | ALB         | *serum albumin precursor                                                      | −0.91       |
| 291413693        | MRPL41      | 39S ribosomal protein 141, mitochondrial                                      | −0.78       |
| 655902607        | phosphofurin acidic cluster sorting protein 2                                | −0.71       |
| 655883487        | PTPRE       | LOW QUALITY PROTEIN: receptor-type tyrosine-protein phosphatase epilon        | −0.49       |
| 655856160        | FGG         | fibrinogen gamma chain isoform X2                                             | 1.15        |
| 655862391        | TRIP4       | activating signal co-integrator 1 isoform X3                                 | −0.54       |
| 655603017        | LOW QUALITY PROTEIN: transmembrane protease serine 13-like                   | 1.20        |
| 655608615        | PSME4       | proteasome activator complex subunit 4                                        | 1.63        |
| 655602331        | LIPT2       | putative lipoyltransferase 2, mitochondrial                                   | −0.79       |
| 655805767        | RRN3        | RNA polymerase I-specific transcription initiation factor RRN3 isoform X2     | 1.16        |
| 157781959        | TPM2        | *tropomyosin 2 (beta)                                                         | 3.88        |
| 655807516        | LOW QUALITY PROTEIN: THUMP domain-containing protein 1-like                  | −0.97       |
| 291380041        | PAK7        | serine/threonine-protein kinase PAK 7                                         | −0.74       |
| 291406137        | NAGLU       | alpha-N-acetylgalactosaminidase                                                | 1.59        |
| 655858943        | USP6NL      | LOW QUALITY PROTEIN: USP6 N-terminal-like protein                             | −0.88       |
| 655601434        | FXYD6       | FXYD domain-containing ion transport regulator 6 isoform X4                   | 1.31        |
| 126723638        | PAPSS2      | *bifunctional 3′-phosphoadenosine 5′-phosphosulfate synthase 2               | −0.38       |
| 655897422        | uncharacterized protein C16orf74 homolog                                    | −0.50       |
| 655801810        | RBF0X1      | RNA binding protein fox-1 homolog 1                                          | −0.62       |
| 655879231        | RFC3        | replication factor C subunit 3 isoform X2                                     | −0.97       |
| 655827806        | LRP2        | low-density lipoprotein receptor-related protein 2                           | 2.13        |
| 126723185        | ADCY10, SAC | *adenylate cyclase type 10                                                   | 1.20        |
| 291394610        | NOD1        | nucleotide-binding oligomerization domain-containing protein 1               | 1.20        |
| 655897113        | ERCC1       | DNA excision repair protein ERCC-1                                            | 1.20        |
| 291408033        | TCEAL5      | transcription elongation factor A protein-like 5                              | 1.10        |
| 291395159        | FAM134B     | protein FAM134B                                                              | −0.90       |
| 291403287        | ACTC1       | actin, alpha cardiac muscle                                                   | 3.87        |

Continued
| Accession number | Gene symbol | Protein name                                                                 | Fold change                   |
|------------------|-------------|------------------------------------------------------------------------------|-------------------------------|
| 291393596        | ELP6        | elongator complex protein 6 isoform X2                                       | 1.25                          |
| 291410032        | CBR3        | carboxyl reductase [NADPH]                                                   | −0.62                         |
| 130493079         | TNNI2, Tnn1 | α-troponin 1, fast skeletal muscle                                           | 2.18                          |
| 655605874         | KCNIP3      | calasin isoform X3                                                           | 1.04                          |
| 291400645         | QRTTD1      | queuine tRNA-ribosyltransferase subunit QRTTD1                               | −0.56                         |
| 291412004         | #N/A        | mitogen-activated protein kinase 8 isoform X1                                | 1.28                          |
| 655895776         | SYVN1       | LOW QUALITY PROTEIN: E3 ubiquitin-protein ligase synoviolin                  | 1.38                          |
| 655663205         | ZNF346      | zinc finger protein 346 isoform X2                                          | 1.96                          |
| 65566440          | PBLD        | phenazine biosynthesis-like domain-containing protein isoform X2             | −0.80                         |
| 156119398         | MYL1        | *myosin light chain 1/3, skeletal muscle isoform                             | 2.84                          |
| 291403770         | GEMIN2      | gem-associated protein 2 isoform X1                                          | 1.25                          |
| 291404856         | TRUB1       | probable tRNA pseudouridine synthase                                          | 1.21                          |
| 655853659         | HRG         | histidine-rich glycoprotein                                                   | −0.90                         |
| 655602610         | APBB1       | amyloid beta A4 precursor protein-binding family B member 1 isoform X6       | −0.88                         |
| 655861319         | GLRX2       | glutaredoxin 2 isoform X2                                                     | −0.34                         |
| 291392203         | SMARCAL1    | SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 | 1.85                          |
| 655868632         | myosin-2    |                                                                               | 2.36                          |
| 291404152         | FUT11       | alpha-(1,3)-fucosyltransferase 11                                            | 1.03                          |
| 291416252         | EXOSC4      | exosome complex component RRP4                                                | −0.83                         |
| 291402469         | HSD11B1     | corticosteroid 11-beta-dehydrogenase isozyme 1                               | −0.48                         |
| 655838173         | GPR98       | G-protein coupled receptor 98                                                | 1.19                          |
| 655599632         | STX17       | syntaxin-17                                                                  | −0.28                         |
| 655848676         | FUBP1       | far upstream element-binding protein 1 isoform X14                           | −0.82                         |
| 655874201         | ANAPC5      | anaphase-promoting complex subunit 5 isoform X3                              | −0.71                         |
| 655841323         | GLTSCR1L    | GLTSCR1-like protein isoform X2                                               | −0.99                         |
| 655889301         | PPL         | periplakin                                                                    | −0.85                         |
| 291406958         | PPTC7       | protein phosphatase PTC7 homolog                                             | −0.46                         |
| 655758942         | MYBPC1      | myosin-binding protein C, slow-type, partial                                  | 1.43                          |
| 291393095         | COMMD6      | COMM domain-containing protein 6 isoform X2                                  | −0.42                         |
| 291402968         | PGB         | GPI mannose transferase 3                                                     | −0.90                         |
| 655835568         | DALRD3      | DALR anticonod-binding domain-containing protein 3                            | −0.87                         |
| 291346000         | SMPDL3A     | acid sphingomyelinase-like phosphodiesterase 3                               | −0.76                         |
| 655892424         | AHRGFE10L   | rho guanine nucleotide exchange factor 10-like protein                       | 3.28                          |
| 655902632         | TNNI1       | troponin T, slow skeletal muscle                                             | 2.50                          |
| 655897791         | SH3BPSL     | SH3 domain-binding protein 5-like isoform X2                                 | −0.70                         |
| 655706784         | SLA2        | src-like-adapter 2                                                           | −0.47                         |

Continued
| Accession number | Gene symbol | Protein name                                      | Fold change |
|------------------|-------------|--------------------------------------------------|-------------|
|                  |             | Day 4–0.5 mg | Day 4–2.0 mg | Day 7–0.5 mg | Day 7–2.0 mg | Day 14–0.5 mg | Day 14–2.0 mg |
| 655902451        | LAMA5       | LOW QUALITY PROTEIN: laminin subunit alpha-5     | −0.81       | 1.12        | −0.96        | −0.63        | −0.65        | −0.27        |
| 291405504        | TMEM199     | transmembrane protein 199                        | 1.09        | 1.53        | −0.91        | −0.63        | −0.94        | 2.42         |
| 655603581        | CRY2        | cryptochrome-2                                   | 1.13        | −0.36       | 1.01         | −0.63        | −0.83        | 1.31         |
| 655729065        | KRT7        | LOW QUALITY PROTEIN: keratin, type II cytoskeletal 7 | −0.73       | 1.43        | −0.35        | −0.63        | −0.74        | −0.92        |
| 655841393        | CUL9        | LOW QUALITY PROTEIN: cullin-9                    | 1.35        | −0.79       | −0.52        | −0.63        | 1.53         | −0.48        |
| 655894938        | PPP62       | serine/threonine-protein phosphatase 6 regulatory subunit 2 | −0.42       | −0.55       | −0.71        | −0.63        | 1.06         | 1.86         |
| 291387257        | CSNK1G3     | casein kinase I isoform gamma-3 isoform X8       | −0.91       | −0.87       | −0.89        | −0.62        | −0.85        | 4.01         |
| 291397177        | TFB1M       | dimethyladenosine transferase 1, mitochondrial   | 2.06        | 1.53        | 2.55         | −0.60        | 1.74         | 1.32         |
| 291403160        | ZNF106, ZFP106 | zinc finger protein 106                           | 1.01        | −0.52       | 1.07         | −0.55        | 1.09         | −0.45        |
| 655867504        | MRPL43      | LOW QUALITY PROTEIN: 39S ribosomal protein L43, mitochondrial | 1.19        | 1.13        | −0.96        | −0.53        | 1.03         | −0.32        |
| 655765030        | MB          | LOW QUALITY PROTEIN: myoglobin                   | −0.91       | −0.82       | −0.81        | −0.49        | 1.11         | −0.81        |
| 655601432        | #N/A        | FXYD-domain-containing ion transport regulator 6 isoform X3 | −0.56       | −0.38       | 1.05         | −0.46        | −0.93        | −0.44        |
| 291389209        | keratin, type II cytoskeletal 5 isoform X1       | −0.63       | 1.11        | −0.45        | −0.46        | −0.73        | −0.76        |
| 655795749        | DHODH       | dihydroorotate dehydrogenase (quinone), mitochondrial | −0.75       | −0.90       | 1.20         | −0.46        | −0.85        | −0.91        |
| 291410767        | histone H2A type 1-H                             | 1.02        | 1.13        | −0.50        | −0.46        | −0.83        | 1.33         |
| 655837047        | GPNMB       | transmembrane glycoprotein NMB                   | −0.58       | 1.19        | −0.39        | −0.46        | −0.76        | −0.66        |
| 655864222        | MYLPF       | myosin regulatory light chain 2, skeletal muscle isoform type 2 | 1.48        | −0.33       | −0.46        | −0.45        | −0.66        | −0.68        |
| 291387979        | XK4         | XK-related protein 4                             | −0.57       | −0.74       | −0.87        | −0.45        | 1.04         | 1.54         |
| 655771366        | NCCRP1      | F-box only protein 50 isoform X2                 | 1.59        | −0.22       | −0.46        | −0.44        | −0.39        | −0.67        |
| 655828240        | OSGEL1      | probabile tRNA N6-adenosine threonylcarbamoyltransferase, mitochondrial isoform X2 | 1.01        | −0.73       | −0.94        | −0.44        | −0.38        | −0.63        |
| 655871287        | KRT10       | keratin, type I cytoskeletal 10 isoform X2       | −0.60       | 1.25        | −0.33        | −0.43        | −0.92        | −0.71        |
| 126723437        | ENO3, ENO1  | *beta-enolase*                                   | −0.68       | −0.66       | 1.07         | −0.43        | −0.45        | 1.68         |
| 655835901        | #N/A        | band 4.1-like protein 3 isoform X19              | −0.77       | 1.21        | 1.46         | −0.41        | −0.95        | 1.45         |
| 655831942        | LATS2       | serine/threonine-protein kinase LATS2 isoform X2 | 1.11        | −0.62       | 1.07         | −0.41        | −0.81        | 1.07         |
| 655842035        | OGFR1L      | LOW QUALITY PROTEIN: opioid growth factor receptor-like protein 1 | −0.29       | 1.02        | −0.81        | −0.38        | −0.77        | −0.50        |
| 655840529        | C12H5orf136 | uncharacterized protein C6orf136 homolog isoform X2 | 1.06        | −0.52       | −0.81        | −0.38        | 1.09         | 1.44         |
| 291389971        | keratin, type I cytoskeletal 18                  | −0.60       | 1.23        | −0.28        | −0.37        | −0.64        | −0.72         |
| 291387122        | SMC6        | structural maintenance of chromosomes protein 6 | 1.03        | −0.60       | −0.31        | −0.36        | −0.31        | −0.52        |
| 291403457        | hsc70-interacting protein                       | −0.45       | −0.12       | 1.20         | −0.34        | 1.68         | −0.10        |
| 655839030        | EMB         | embigin                                          | 1.13        | −0.36       | 1.19         | −0.30        | −0.76        | −0.54        |
| 655877350        | RAB9B       | ras-related protein Rab-9B                      | 1.23        | 1.79        | 1.67         | −0.28        | 1.99         | −0.41        |
| 655901361        | IGS15       | ubiquitin-like protein IGS15                     | −0.49       | −0.94       | −0.45        | −0.28        | −0.66        | 1.19         |
| 655897078        | CD3EAP      | DNA-directed RNA polymerase I subunit RPA34      | 1.90        | −0.82       | 2.44         | −0.25        | 1.99         | 1.54         |
| 655868318        | NSMCE4A     | LOW QUALITY PROTEIN: non-structural maintenance of chromosomes element 4 homolog A | 1.76        | 1.38        | 1.86         | 2.44         | 1.99         | −0.67        |

Table 3. Significantly altered proteins with inconsistent changes after intravitreal injection of conbercept. *Means the proteins are not predicted proteins. ~Means down-regulation.
of 1 mL/kg pentobarbital sodium. An average of five consecutive readings by the same observer was applied for analysis.

**Fundus Photography.** The pupils were dilated with tropicamide eye drops (Shenyang Xingqi Pharmaceutical Co. Ltd, Shenyang, China) 30 minutes prior to imaging. The fundus photography of the rabbit eye was performed using a digital fundus camera system under anesthesia.

**Electroretinogram.** Electroretinogram (RetiMINER System, AiErXi Medical Equipment Co., Ltd., Chongqing, China) was recorded at day 4, 7, 14 after the single intravitreal injection and at day 7 after the 6th weekly intravitreal injection. After dark adaptation, rabbits were anesthetized with pentobarbital sodium. Pupils were dilated and Burian-Allen corneal bipolar electrodes (Hansen Laboratory, Iowa City, Iowa) were applied as the corneal electrodes. The ground electrode was placed subcutaneously on the back. Dark- and light-adapted ERGs were recorded followed a previous procedure32.

**Histological Evaluation.** Animals were sacrificed with an injection of overdose sodium pentobarbital under deep anesthesia. Eyeballs were enucleated and half of the eyecup was fixed with 4% paraformaldehyde for 24 hours at room temperature33. Tissues were embedded in paraffin and 4-μm sections were cut through the optic disc and stained with hematoxylin and eosin (HE). The images of each section were acquired. The number of cells in the GCL was counted in a region of 800 to 1500 μm from the center of the optic nerve head on both sides. The thickness of inner nuclear layer (INL) was measured in three areas at a distance of 500 to 1000 μm from the edge of optic disc. Four sections of each eye were measured, and data were averaged for each eye. All measurements and analysis were performed in a masked manner.

**TUNEL.** The terminal dUTP-mediated nick-end labeling (TUNEL) was performed to detect the apoptosis cells in the retina33. Sections were mounted with fluorescein-FRAGEL media. Staining was performed according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Samples were permeabilized in 100μL of 20μg/mL proteinase K for 20 minutes, equilibrated with 100μL of 1% TDT buffer for 30 minutes at room temperature and labeled with 60μL TDT labeling reaction mixture for 1 hour at 37 °C. Sections were photographed and the TUNEL-positive cells were counted between 1000 to 1500μm from the center of the optic disc on both sides in the GCL and INL.

**ELISA.** The concentrations of IL-6 (RayBiotech, Norcross, GA), IL-8 (R&D Systems, Minneapolis, Minnesota, CA) and TNF-α (RayBiotech) in 100μL aqueous humor were determined according to the manufacturers' protocols33. The absorbance at 450 nm wavelength was measured using a multifunction microplate reader (Molecular Devices).

**Protein Extraction.** Retinas were isolated from the eyeball and frozen in liquid nitrogen and lysed using protein extraction buffer (8 M urea, 0.1% SDS) containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA) on ice for 30 min and then centrifuged at 16,000 × g for 15 minutes at 4 °C. The supernatant was collected and protein concentration was determined by BCA assay kits (Pierce, Rockford, IL, USA).

**TMT labeling and fractionation of labeled peptide.** Tandem mass tag (TMT) labeling was performed according to the manufacturer's instructions (Pierce). Proteins were precipitated by pre-chilled (−20 °C) acetone. After resuspension, proteins were digested overnight at 37 °C by using 2.5μg of trypsin. One tube of TMT10 Label Reagent was added to each 100μg sample and the reaction was carried out at room temperature for 1 hour. After
labeling, ten tissue samples were combined for one measurement. For fractionation of the labeled peptides, samples were first lyophilized and reconstituted. A total of 40 fractions were collected which were concatenated to 20 fractions, vacuum dried and stored at −80 °C until further analysis.

**LC-MS/MS Analysis.** The LC-MS/MS analysis was carried out by Capitalbio Technology with a Q Exactive Mass Spectrometer (Thermo Scientific, San Jose, CA). Mass spectrometry analysis was performed in a data dependent manner with full scans (300–1,800 m/z) acquired using an Orbitrap mass analyzer at a mass resolution of 70,000 at 400 m/z in Q Exactive. Twenty most intense precursor ions from a survey scan were selected for MS/MS from each duty cycle and detected at a mass resolution of 35,000 at m/z of 400 in Orbitrap analyzer. All the tandem mass spectra were produced by higher-energy collision dissociation (HCD) method. Dynamic exclusion was set for 20 seconds.

**Data analysis.** Proteome Discoverer software (Ver. 1.4, Thermo Scientific) was used to perform database searching against the Oryctolagus cuniculus database (46551 proteins) using the Sequest algorithms. Following settings were applied: precursor mass tolerance of 15 ppm, fragment mass tolerance of 20 mmu. Only high confident peptides with a global FDR < 1% based on a target-decoy approach were included in the results. In the TMT quantitation workflow the most confident centroid method was used with an integration window of 20 ppm. For protein quantitation, only unique peptides were used to quantify proteins.

Enriched pathways were analyzed in a command-line program KOBAS 2.0. We used the whole genome as the default background distribution to identify the significantly enriched pathways statistically in a set of sequences. For each pathway that occurs in the set of genes, we counted the total number of genes in the set that were involved in the pathway. We then calculated the p value using a hypergeometric distribution. If a whole genome has N total genes, among which M are involved in the pathway under investigation, and the set of genes has n total genes, among which m are involved in the same pathway, the p value for the pathway is calculated as follows:

Because a large number of KEGG pathways are considered, multiple hypotheses tests are performed. To reduce the Type-1 errors (i.e. false positive discoveries), we performed an FDR correction with a default cutoff of 0.05.

**Statistical Analysis.** The IOP results were presented as mean ± SD and the other data were mean ± SEM. Statistical analysis was performed using the GraphPad Prism (GraphPad Prism 5, Inc., San Diego, CA, USA). The results were analyzed by one-way ANOVA followed by Bonferroni correction for multiple comparisons. p less than 0.05 was considered statistically significant.

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**Acknowledgements**

This study is supported by Kanghong Biotechnology Co. Ltd, Chengdu, and in part by the National Natural Science Foundation of China grants (81271033, 81470621), Chongqing Science and Technology Commission (2014pt-sy10002) and National Key Clinical Specialties Construction Program of China. The authors alone are responsible for the content and writing of the paper.

**Author Contributions**

J.W., B.L., X.K. and Q.W. conceived the idea and designed the experiments. J.W., C.L., L.T. and Y.Q. performed all the experiments. J.W. and B.L. analyzed data and wrote the manuscript. B.L. reviewed and revised the manuscript.

**Additional Information**

**Competing Interests:** The authors declare that they have no competing financial interests.

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