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Müller Cell-derived VEGF164 Knockdown Reduces Retinal Neovascularization In A Rat Model Of Retinopathy Of Prematurity

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Purpose

To determine the efficacy and safety from knockdown of Müller cell expressed vascular endothelial growth factor VEGF164 in the 50/10 model of oxygen induced retinopathy (50/10 OIR model).

Introduction

Retinopathy of prematurity (ROP) affects preterm infants and remains a leading cause of childhood blindness. The frequency is increasing in developing countries. The pathophysiology of ROP can be described by two stages: 1) delay in physiological retinal vascular development, and in cases of high oxygen at birth, some vaso-attenuation of vessels causing avascular retina and 2) later abnormal neovascularization that grows into the vitreous as intravitreal neovascularization (IVNV).

Vascular endothelial growth factor (VEGF) is one of the most studied factors leading to IVNV in ROP and its models. We previously reported that VEGF164 and VEGFR2 were increased in a model representative of human ROP whereas other splice variants and VEGFR1 were unaffected in the model. VEGFA protein was found increased in vitreous from preterm infants who had surgery for stage 4 ROP compared to controls[1]. Following a recent clinical trial testing intravitreal delivery of a broad anti-VEGFA antibody in infants with severe ROP, there have been concerning reports of persist avascular (AVA) retina and vasoproliferation sometimes with total retinal detachment, even one year after treatment[2].

Müller cells express VEGFA in retina. Using a relevant model of ROP, we previously reported that knock down of overexpressed Müller cell-derived VEGFA significantly reduced IVNV[3]. However, VEGFA is also vital to Müller cell survival, and Müller cells provide factors important for the survival of other cell types in the retina. VEGF164 is a prevalent isoform of VEGF involved in pathologic angiogenesis in ROP models[4,5,6]. We asked whether inhibition of Müller cell VEGF164 would reduce IVNV without interfering with physiologic retinal vascular development or affect Müller and other retinal cell types in the rat ROP model.

Methods

1. **Lentivirus with shRNA to VEGF164 targeting Müller cells:** VEGFA and control luciferase shRNAs were created as described[7]. Lentivectors with a CD44 promoter and short hairpin RNA (shRNA) were used to knockdown VEGFA (VEGFA.shRNA) or VEGF164 (VEGF164.shRNA). At least two different shRNAs were created and tested for knockdown efficiency in HEK reporter cell lines for VEGFA.shRNA and VEGF164.shRNA. rMC-1 cells were used to test the specificity of VEGFA.shRNA, VEGF164.shRNA and luciferase control (luc.shRNA). Uninfected cells were used as controls for luc.shRNA.
2. **Animal model:** As reported[8], within 4 hours of birth, pups and their dams were placed into an Oxycycler, which cycled oxygen between 50% and 10% every 24 hours for 14 days. At the beginning of the 50% oxygen cycle at postnatal day 8 (p8), 1 µl of lentivector containing luc.shRNA, VEGFA.shRNA or VEGF164.shRNA was given to pups as a subretinal injection. As an additional control, PBS was injected in some eyes. All pups were harvested at p18 or p25. At least 12 pups/litter are required to assure reproducibility of features in the rat ROP model.
3. **Retinal flat mount and protein:** In each pup, one eye was processed as a lectin stained retinal flat mount and the other for VEGF protein (ELISA). Retinal images were analyzed[7] for avascular/total retinal area (AVA%) and IVNV% using ImageJ.
4. **TUNEL assay:** Eyes from groups luc.shRNA, VEGFA.shRNA, VEGF164.shRNA or PBS were fixed in 4% PFA and processed as 12 µm thick cryosections for immunohistochemical analysis of TUNEL staining. TUNEL positive cells co-labeled with TMR red and DAPI were imaged at 20X and counted at 60 micron intervals.
5. **Morphometric analysis:** Thickness of all retinal cell layers and total retinal thickness was evaluated in DAPI-stained cryosections obtained from groups luc.shRNA, VEGFA.shRNA, VEGF164.shRNA or PBS injected eyes.

Acknowledgements

R01 R01EY015130 MEH, R01EY017011MEH
Financial Disclosures: None

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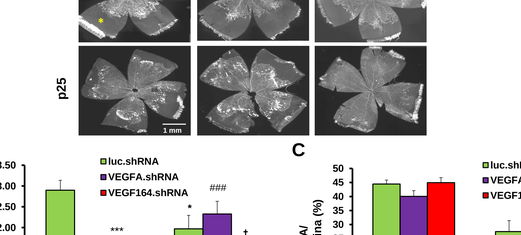
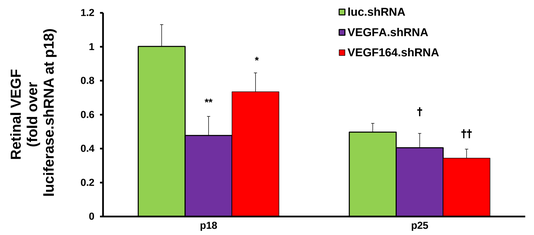
Results

Figure 1. In Vitro Knockdown Efficiency Of VEGF164.shRNA



Viruses containing luciferase.shRNA, VEGFA.shRNA or VEGF164.shRNA-2 were transfected into rMC-1s and real-time PCR was performed to determine the mRNA levels of VEGF120 and VEGF164 . Results were means ± SEM. *p<0.05, ***p<0.001 *versus* uninfected; †p<0.05, ††p<0.005 *versus* luciferase.shRNA (luc.shRNA).

Figure 2. In Vivo Analysis Of ShRNAs Transfected Retina Of Pups Raised In 50/10 OIR Model

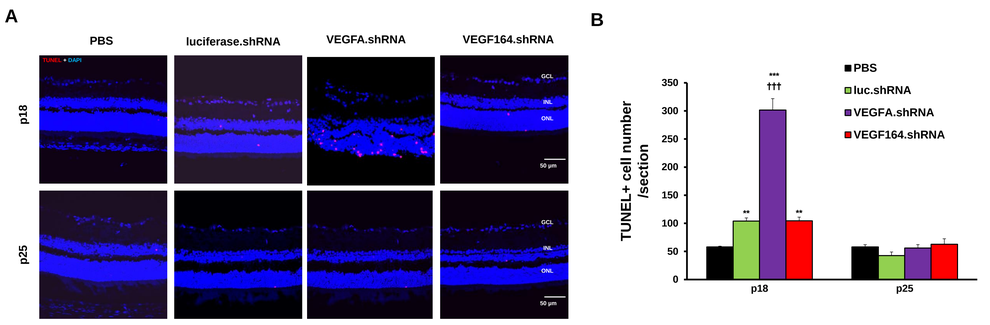


Retinal VEGF levels were measured using ELISA at p18 and p25. Results were means ± SEM. *p<0.05, **p<0.001 *versus* luc.shRNA at p18; †p<0.05, †† p<0.01 *versus* luc.shRNA at p25.

Figure 3. Reduction Of Intravitreal Neovascularization (IVNV) By VEGF164.shRNA Compared To VEGFA.shRNA

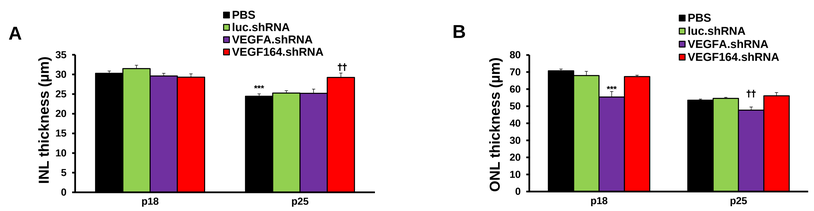
Retinal flat mount images from each group (luc.shRNA, VEGFA.shRNA, VEGF164.shRNA) were analyzed at p18 and p25 (A). % IVNV (B) and % AVA (C) were quantified at p18 and p25. Results were means ± SEM. *p<0.05, ***p<0.001 *versus* luc.shRNA at p18; †p<0.01 *versus* VEGFA.shRNA at p25; ###p<0.001 *versus* VEGFA.shRNA at p18 . Arrow indicates IVNV and star represents AVA.

Figure 4. Safety Of Using VEGF164.shRNA Compared To VEGFA.shRNA



TUNEL-stained images taken at 20X for p18 and p25 in each group (PBS, luciferase.shRNA, VEGFA.shRNA, VEGF164.shRNA) (A). TUNEL positive cells (red) in each group were counted and results were represented in a bar graph format (B). Results were means ± SEM. **p<0.01, ***p<0.001 *versus* PBS at p18; †††p<0.001 *versus* luc.shRNA at p18.

Figure 5. Morphometric Analysis Of Eyes Injected With VEGF164.shRNA And VEGFA.shRNA



Quantification of the thickness of the inner nuclear layer (INL) (µm) (A) (***p<0.001 *versus* PBS at p18; ††p<0.01 *versus* luc.shRNA at p25), and the outer nuclear layer (ONL) (µm) (B) (***p<0.001 *versus* luc.shRNA at p18; ††p<0.001 *versus* luc.shRNA at p25 in DAPI-stained retinal cryosections from p18 and p25 OIR pups treated with luc.shRNA, VEGFA.shRNA and VEGF164.shRNA.Results were means ± SEM.

Conclusions

1. Knockdown of retinal VEGF using shRNAs to Muller cell-derived VEGFA or the splice variant VEGF164 was possible using a lentivector gene therapy strategy.
2. In the rat 50/10 OIR model, knockdown of Müller cell-derived VEGF164 showed long-term inhibition of IVNV compared to VEGFA-shRNA, control Luc-shRNA or PBS injection.
3. VEGF164-shRNA was able to inhibit IVNV more safely than VEGFA.shRNA as determined by effect on outer nuclear layer thickness, but more studies are indicated to determine long-term functions.
4. Future studies are indicated to determine long-term function and structure from inhibition of Muller-cell derived VEGFA or VEGF164.

References

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