The University of Utah
Moran Eye Center

Knockdown of Müller cell specific VEGF reduces retinal neovascularization in a rat model of retinopathy of prematurity

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Purpose

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

Introduction

Retinopathy of prematurity (ROP) remains a leading cause of childhood blindness and is increasing in frequency in developing countries. The pathophysiology of ROP can be described by two stages : 1) first, delay in physiological vascular development causing avascular retina and 2) later abnormal neovascularization that grows into the vitreous.

VEGF is one of the most studied factors leading to IVNV. 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 new intravitreal neovascularization (IVNV) with total retinal detachment, even one year after treatment[2].

Using a relevant model of ROP, we found that inhibition of VEGF with a neutralizing intravitreal antibody not only led to recurrent IVNV and reduced postnatal growth, but also upregulation of angiogenic factors, including erythropoietin.[3] Targeting the cell type that overexpresses VEGF may lead to safer treatments for ROP.

Methods

1. **Lentivirus with shRNA to VEGFA targeting Müller cells:** As previously described[4], two shRNAs to VEGFA or luciferase were created and tested for specificity to VEGFA knockdown using reporter cell lines in HEK293 cells. The shRNA having the greater knockdown was then embedded within a miR30 microRNA and cloned into the lentivector pFMCD44 with a GFP tag that was shown to target Müller cells. Sensitivity and specificity of the lentivector was confirmed in vitro and in vivo and was presented previously.
2. **Animal model:** As reported[5], within 4 hours of birth, pups and their dams were placed into an Oxycycler (Biopsherix, NY), which cycled oxygen between 50% and 10% every 24 hours for 14 days. Litter numbers were 12 and 14 pups for each experiment to assure consistency in outcomes. Two hours preceding the end of the 50% oxygen cycle at postnatal day 8 (p8), 1μl of lentivector containing VEGFA-shRNA or Luc-shRNA and was given to pups by subretinal injection. All pups were harvested at p18.
3. **Retinal flat mount and protein:** For each pup, one eye was prepared for retinal flat mounts and the other for protein for VEGF (ELISA) or in situ hybridization. After enucleation, eyecups without cornea, lens and vitreous were fixed in 4% paraformaldehyde (PFA) for one hour on ice, and Isolectin B4 stained retinal flat mounts were prepared. Retinal images were analyzed[6] for avascular/total retinal area (AVA%) and IVNV% using ImageJ.
4. **In Situ Hybridization:** 10 um sections from fresh frozen uninjected eyes were processed for *in situ hybridization* for $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ splice variants using the Fluorescence In Situ Hybridization (FISH) kit following the manufacturer's instructions.

Acknowledgements

R01 R01EY015130 MEH, R01EY017011MEH
Financial Disclosures: None

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Results

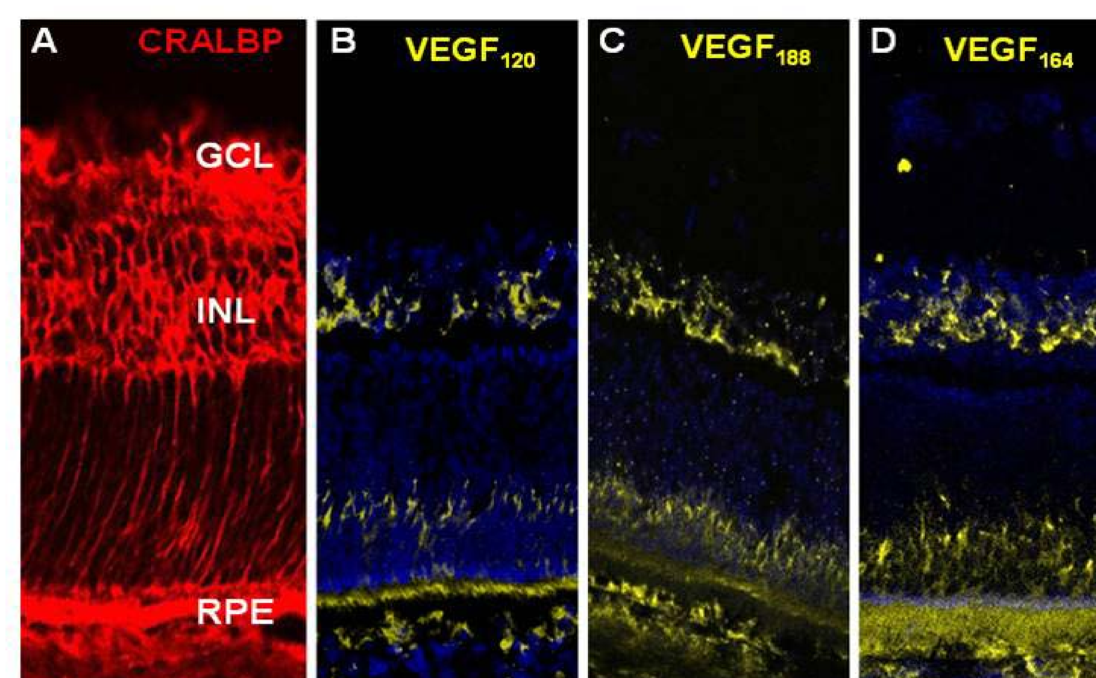
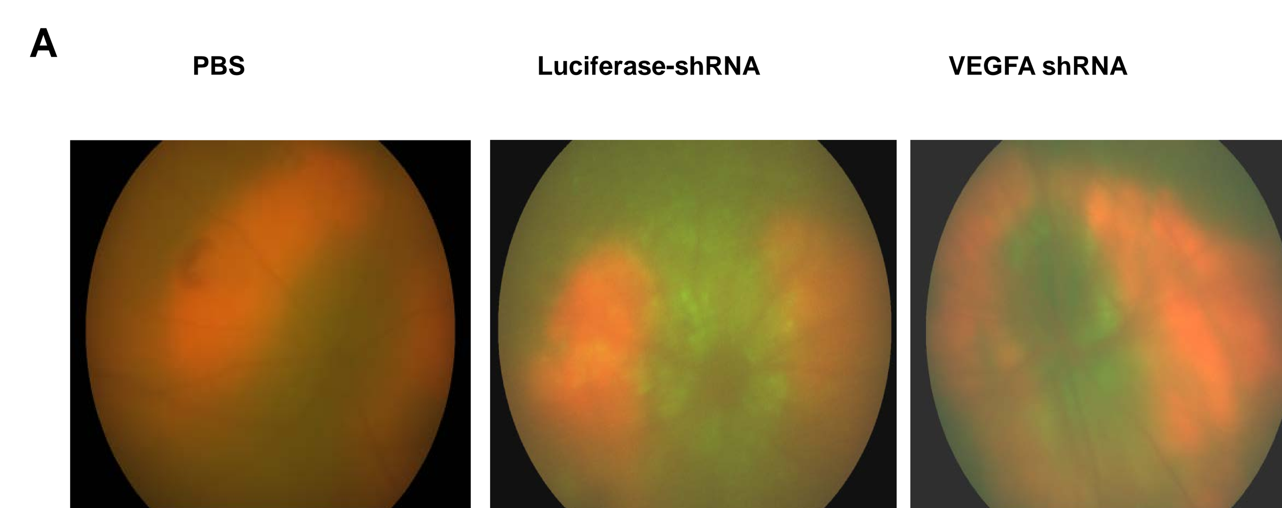


Figure 1. Messenger RNA (mRNA) of VEGF splice variants localized in inner nuclear layer corresponding to CRALBP-labeled Müller cells in 50/10 OIR model. Fluorescence in situ hybridization of VEGF splice variants (B-D) localized to retinal layers corresponding to CRALBP labeled Müller cells (A).



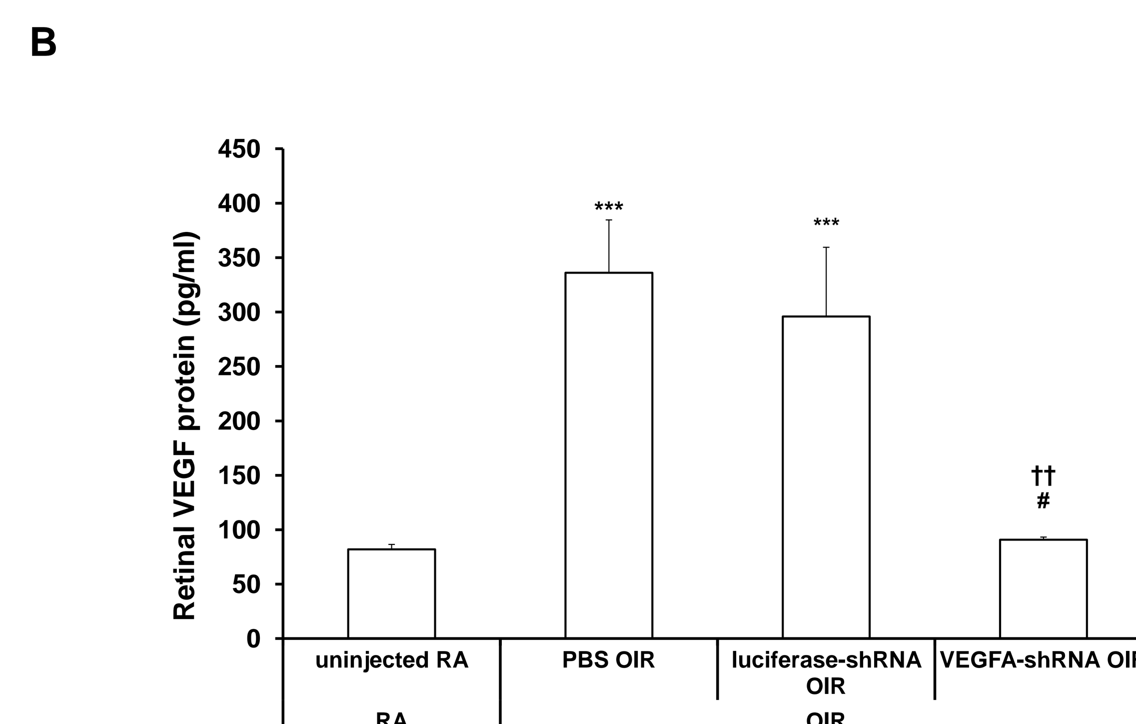


Figure 2. In vivo analysis of lentivector-delivered shRNA transduction in retina of pups raised in 50/10 OIR model at p18. (A) Micron III images show GFP expression in retinas of pups with lentivirus injection; (B) ELISA of retinal VEGF protein at p18. All data are means ±S.E.M. ***p< 0.001 *vs.* RA; #p<0.05 *vs.* PBS inj; ††p<0.01 *vs.* luciferase-shRNA.

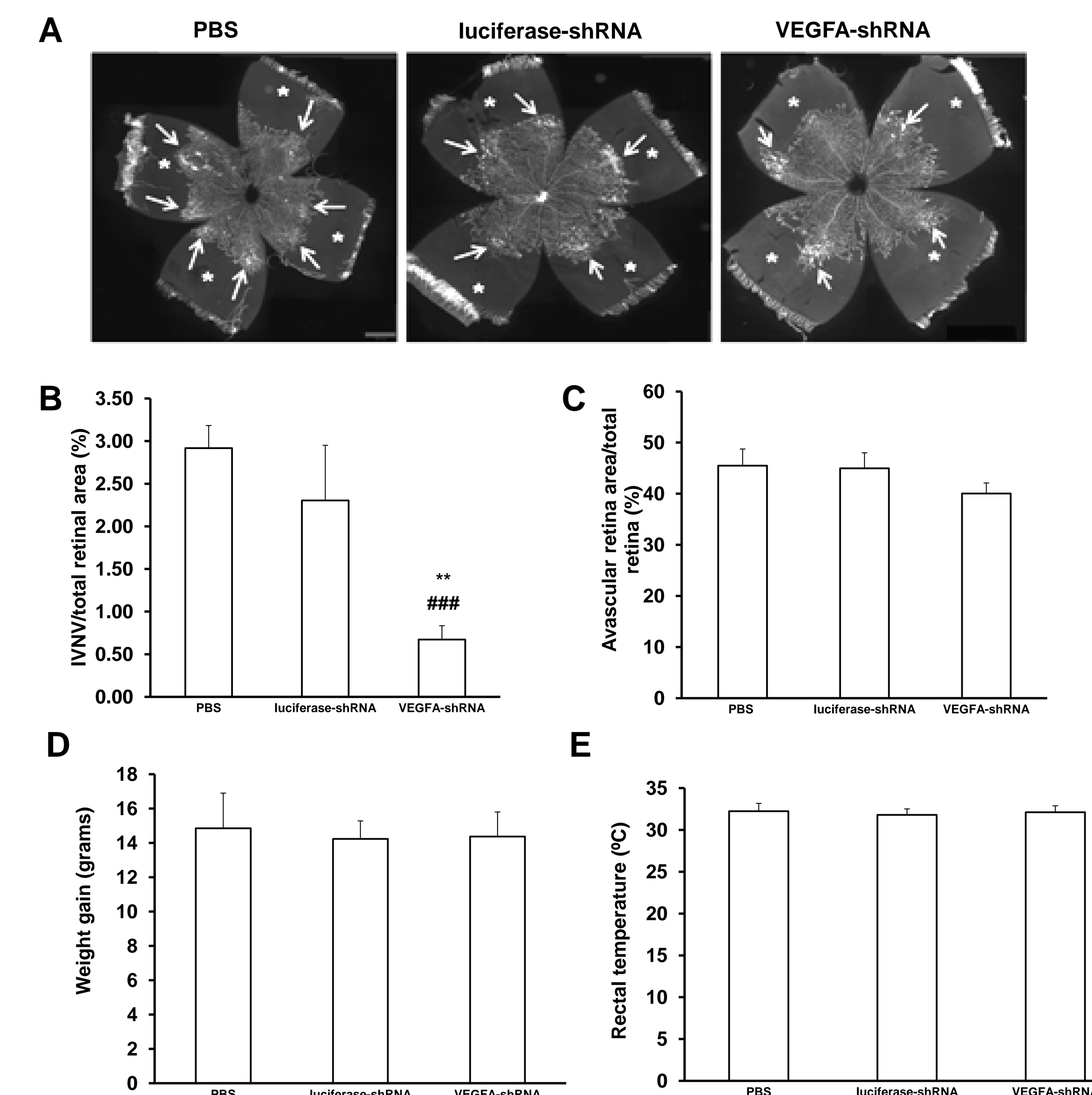


Figure 3. Lentivirus-derived VEGFA-shRNA reduces intravitreous neovascularization (IVNV) without interfering with physiological retinal vascular development and body weight, pup temperature in 50/10 OIR model. (A) Images of retinal flatmounts at p18 following subretinal injections in each group (PBS, control-luciferase, VEGA shRNA); Quantification of IVNV(B) and Avascular area (AVA) (C) in each group. (D) Weight gain of pups from postnatal day 7 (p7) to p18; (B) Rectal temperature at p18. All data are means ±S.E.M. **p< 0.01 *vs.* PBS inj; ###p<0.001 *vs.* luciferase-shRNA inj.

Conclusions

1. In the rat 50/10 OIR model, knockdown of Müller cell-derived VEGFA significantly reduced IVNV compared to control Luc-shRNA or PBS injection.
2. VEGFA-shRNA was able to reduce retinal VEGF protein levels to that of p18 room air raised pups.
3. There is no effect on body weights and rectal temperature with lentivirus subretinal injection.

References

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