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Targeting Mueller Cell-derived VEGF With Short Hairpin RNA

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Introduction

Retinal vascular diseases associated with hypoxic, avascular retina include retinopathy of prematurity (ROP), retinal vein occlusion, and diabetic retinopathy. Increased vascular endothelial growth factor (VEGF) signaling is common to these and can lead to aberrant angiogenesis. However, VEGF also has important neurotrophic effects and is required for photoreceptor survival[1]. Therefore, determining where VEGF is produced and the function of specific splice variants is important to understand pathological mechanisms.

Using the Penn 50/10 rat model of oxygen induced retinopathy (OIR)[2], we have previously shown that $VEGF_{164}$ is the predominant retinal splice variant, and is upregulated at postnatal days (P) 12, 14, and 18 compared to pups raised in room air[3] (Fig. 1 A). Similar findings were identified for VEGF protein[4] (Fig. 1 B). VEGF expression was detected in the outer nuclear layer by *in situ* hybridization, corresponding to CRALBP- labeled Mueller cells, (Fig. 1C).

Purpose

1) To determine the silencing effects of different VEGFA and $VEGF_{164}$-specific shRNA sequences on VEGF expression.

2) To package sequences that display high silencing activity into lentivectors designed to target Mueller cells *in vivo*[5].

Methods

1) Creation of $VEGF_{120}$ and $VEGF_{164}$ HEK reporter cell lines: HEK cells were transfected with lentiviral vectors containing either rat $VEGF_{120}$ or $VEGF_{164}$ cDNA followed by a green fluorescent protein (GFP) reporter driven by a cytomegalovirus (CMV) promoter, and a second CMV promoter driving production of viral packaging elements.

2) Transfection of HEK reporter cells with VEGFA and $VEGF_{164}$ - specific shRNA: Four commercially available VEGFA shRNAs, each packaged into a lentiviral transfer vector, were used to transduce HEK rat $VEGF_{120}$ and $VEGF_{164}$ reporter cell lines. Real time PCR was then used to determine percent silencing (Figure 2).

shRNA target sequences (two each targeting VEGFA and $VEGF_{164}$) were cloned into expression plasmids containing a red fluorescent reporter gene driven by the CMV promoter. Both HEK VEGF reporter cell lines were transfected with each of the silencing plasmids. Flow cytometry was then used to determine percent silencing (Figure 3)

3) Real Time PCR: 48 hours post transduction of HEK VEGFA and $VEGF_{164}$ reporter cell lines with each of the four lentivectors containing the commercially available VEGFA shRNAs, silencing of VEGFA mRNA was determined by real time PCR. Silencing was expressed as a percentage of VEGF message found in untransduced reporter cells (Figure 2).

4) Fluorescence-activated cell sorting (FACS): 48 hours post transfection FACS analysis was used to determine percent silencing of GFP as an indirect measure of VEGFA and $VEGF_{164}$ silencing. We gated on red cells and measured mean fluorescent intensity (MFI) of GFP. Silencing was calculated as the difference in GFP MFI between untransfected and transfected cells and expressed as a percentage of untransfected cells (Figure 3).

Results

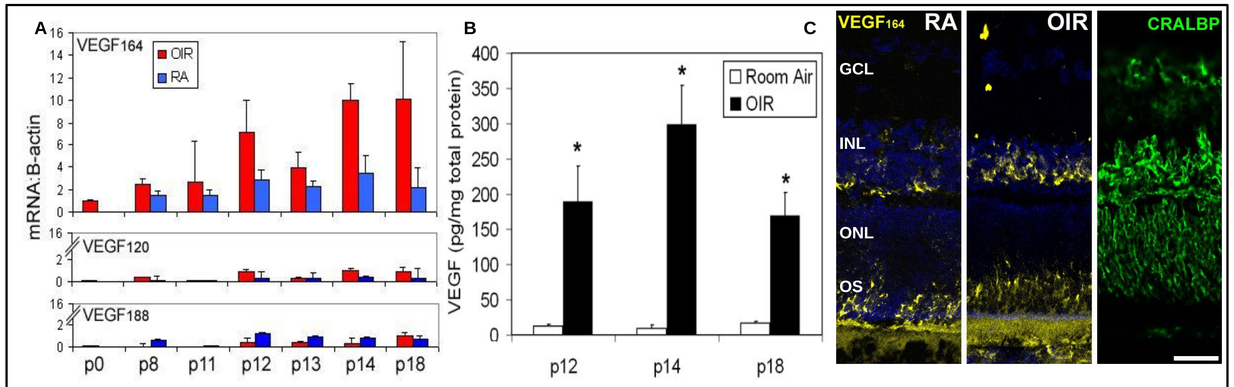


Figure 1. VEGF expression in the rat 50 10 OIR model. A) Real-time PCR values of VEGF splice variant expression in the 50/10 OIR model. The predominant splice variant, $VEGF_{164}$, has significantly increased expression compared to room air (RA; ANOVA, $p<0.001$). Each time point had at least 5 retinas from different pups taken from at least 2 litters (previously published[2]). B) VEGF concentration was increased in the 50/10 OIR model compared to RA at all time points (overall ANOVA $p<0.001$; post-hoc Student's t-tests * $p<0.001$). In 50/10 OIR model, VEGF concentration at P14 was greater than at P12 or P18 (ANOVA $p<0.001$; posthoc Student's t-tests, * $p<0.001$; n=6 for each time point). C) Fluorescence micrograph of retinal cryosections from the 50/10 OIR model (center) and RA control (left) at P14. $VEGF_{164}$ message is labeled by *in situ* hybridization and shows $VEGF_{164}$ expression localized to the outer nuclear layer (ONL), where Muller cells were identified by positive cellular retinaldehyde-binding protein (*CRALBP*) immunostaining (right). Ganglion cell layer (GCL); inner nuclear layer (INL); ONL; outer segments (OS); scale bar = 50um.

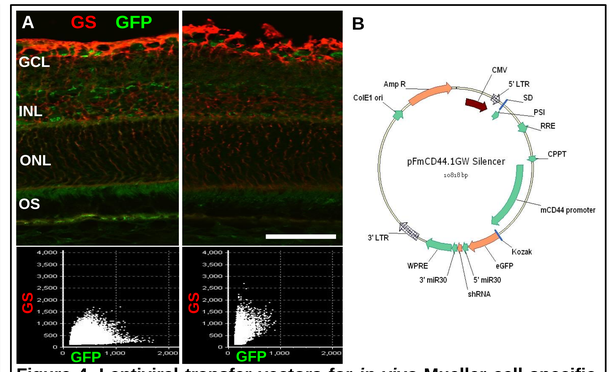


Figure 4. Lentiviral transfer vectors for *in vivo* Mueller cell specific targeting of VEGFA and $VEGF_{164}$-specific shRNAs. A) Fluorescence micrographs (upper panels) of rat retinal cryosections at P14 after subretinal injection (left) with empty pFMCD44.1GW Mueller cell specific lentivector containing a GFP reporter at P7 and uninjected, fellow eye (right). Sections were immunostained for glutamine synthetase (GS in red) to label Mueller cells and GFP (green); scale bar = 50um. Scatter plots (lower panels) confirm colabeling of GS and GFP in injected eye. B) Map of Mueller cell specific lentivirus expression plasmid containing a GFP reporter gene, which was used to verify Mueller cell specific expression in A.

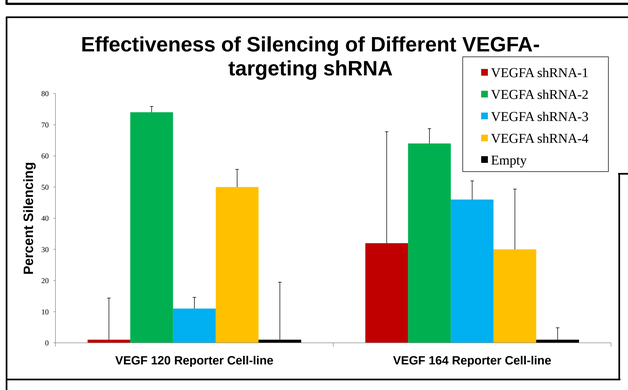
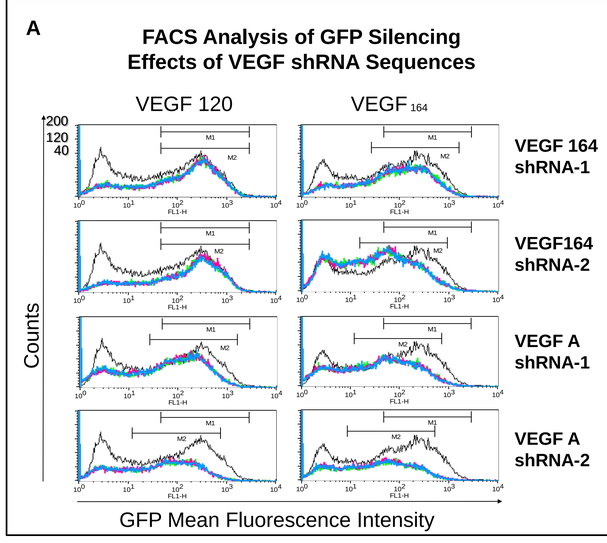


Figure 2. Silencing of VEGFA mRNA by different VEGFA shRNAs. Forty eight hours post transduction with shRNA lentivectors, real time PCR results show that VEGFA shRNA-2 (green bars) displays the most silencing effect on both $VEGF_{120}$ (~75%) and $VEGF_{164}$ mRNA (~65%), indicating the sequence is likely to efficiently silence VEGFA *in vivo*.



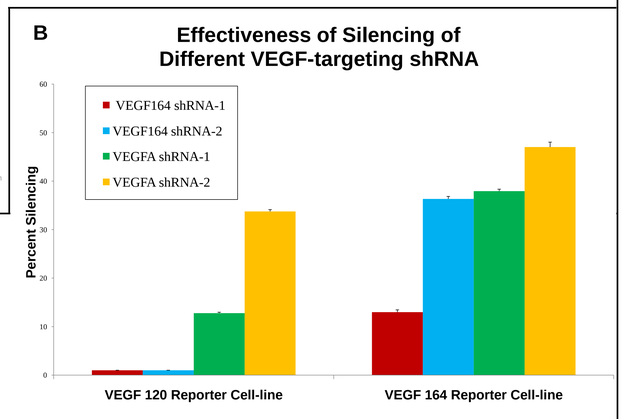


Figure 3. Silencing of $VEGF_{120}$ and $VEGF_{164}$ protein by different VEGFA and $VEGF_{164}$ shRNAs. A) FACS analysis of $VEGF_{120}$ and $VEGF_{164}$ reporter cell GFP expression forty eight hours post transfection with each shRNA plasmid. The greater the shift from M1 to M2 (ΔM) correlates directly with a larger reduction in GFP expression, and indirectly with a higher silencing of reporter cell VEGF. B) Histogram depicting ΔM as a percentage of untransfected reporter cells. $VEGF_{164}$ shRNA-2 (blue bars) displays minimal silencing of $VEGF_{120}$ and relatively high silencing of $VEGF_{164}$, indicating it has selective silencing of the $VEGF_{164}$ splice variant. Conversely, VEGFA shRNA-2 (yellow bars) displays relatively high silencing of both $VEGF_{120}$ and $VEGF_{164}$, indicating it has non-selective silencing for VEGFA splice variants.

Conclusions & Future Directions

- Selective silencing of VEGF splice variants or VEGFA is possible using shRNAs on HEK rat VEGF reporter cell lines.
- Silencing specific VEGF splice variants may prove useful when inhibiting VEGF, which has physiologic and pathologic effects.
- The shRNA sequences that generated the most robust silencing of VEGFA (all splice variants) and the most selective silencing of $VEGF_{164}$ will be used to study the effects of Mueller cell-specific VEGF silencing on the rat 50/10 OIR model.

References

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Funding

NIH R01EY017011

NIH R01EY015130

March of Dimes 6-FY08-590

Financial Disclosures: None

SUPPORTED BY

Research to Prevent Blindness

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