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. 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), we have previously shown that VEGF-A
t is the predominant retinal splice variant, and is upregulated at postnatal days (P) 12, 14, and 18 compared to pups raised in room air (Fig. 1 A). Similar findings were identified for VEGF protein (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
t specific shRNA sequences on VEGF expression.
2) To package sequences that display high silencing activity into lentivectors designed to target Mueller cells in vivo.

Methods

1) Creation of VEGF
t and VEGF
t HEK reporter cell lines: HEK cells were transfected with lentiviral vectors containing either rat VEGF
t or VEGF
t 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
t specific shRNA: Four commercially available VEGF shRNAs, each packaged into a lentiviral transfer vector, were used to transduce HEK rat VEGF
t and VEGF
t reporter cell lines. Real-time PCR was then used to determine percent silencing (Figure 2).

3) Real time PCR: 48 hours post-transduction, real time PCR was used to determine percent silencing (Figure 3).

4) Fluorescence-activated cell sorting (FACS): 48 hours post-transduction, FACS analysis was used to determine percent silencing of GFP as an indirect measure of VEGF and VEGF
t 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
t, 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). 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-test *p<0.001). In 50/10 OIR model, VEGF concentration at P14 was greater than P7 (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
t expression is labeled by in situ hybridization and shows VEGF
t expression localized to the outer nuclear layer (ONL), where Muller cells were identified by positive cell nuclear retinohyaline binding protein (CRALBP) immunostaining (right). Ganglion cell layer (GCC); inner nuclear layer (INL); ONL, outer segments (OS); scale bar = 500m.

Figure 2. Silencing of VEGFA mRNA by different VEGFA shRNAs. Forty eight hours post-transduction with shRNA vectors, real-time PCR results show that VEGF shRNA-2 (green bars) displays the most silencing effect on both VEGFA (~75%) and VEGF
t, mRNA (~50%), indicating the sequence is likely to efficiently silence VEGFA in vivo.

Figure 3. Silencing of VEGF	n and VEGF	n protein by different VEGFA and VEGF	n shRNAs. A) FACS analysis of VEGF	n and VEGF	n reporter cell GFP expression forty eight hours post-transfection with each shRNA plasmid. The greatest shift from M1 to M2 (~60%) correlates directly with a larger reduction in GFP expression, and indirectly with a higher silencing of reporter cell VEGF. B) Histogram depicting percentage of untransfected reporter cells. VEGF	n shRNA-2 (blue bars) displays minimal silencing of VEGF	n and relatively high silencing of VEGF	n. Conversely, VEGF shRNA-2 (yellow bars) displays relatively high silencing of both VEGF	n and VEGF	n, indicating it has non-selective silencing for VEGFA splice variants.

Conclusions & Future Directions

• Selective silencing of VEGF splice variants or VEGF 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 VEGF (all splice variants) and the most selective silencing of VEGF	n will be studied to the effects of Mueller cell-specific VEGF silencing on the retinal 50/10 OIR model.

References


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