Introduction

Vascular endothelial growth factor A (VEGF-A) and its receptors play important roles in neovascularization. VEGFR1 (also known as Flt-1) is a most potent receptor among others in terms of binding affinity.

Flt23K receptor (FLT domains 2 and 3 tagged with KDEL) is able to bind and sequester VEGF-A and can be used to suppress neovascularization.

The delivery and long-term incorporation of the Flt23K gene is a major hurdle. Incorporation of the Flt23K gene into the genome by transpositional insertions, leads to long term reduction of neovascularization in response to an injury without the need for repeated intraocular injections.

The PiggyBac (PB) transposon is a mobile genetic element that efficiently transposes the gene of interest into host chromosomes via a "cut and paste" mechanism.

During transposition, the PB transposase recognizes transposon-specific inverted terminal repeat sequences (ITRs) and efficiently moves the contents from the original sites and integrates them into TTAA chromosomal sites.

Methods

To achieve genomic integration of Flt23K gene, we utilized transposon based helper-independent piggybac plasmid (pGGENIE). Flt23K and DsRed Express 2 cDNA were cloned in pGGENIE. The plasmid lacking the transposase (pBT gene) was used as a control. To examine genomic integration and expression of transgenes in vitro, we transfected the plasmids into HeLa cells and cultured up to 10 passages. Fluorescence microscopy was done to check the transfection efficiency with DS Red fluorescence. Also, Flt23K expression and activity was examined by western blot with Anti-VEGFR1 antibody and anti-VEGF antibody. The same blot was normalized with anti-GAPDH antibody. Genomic integration was analyzed using a PCR based genome walking approach. For in vivo study we used a laser induced neovascular model. Plasmids with 10% neuroproteor were injected into the vitreous of C57BL/6J mice. Choroidal neovascularization (CNV) was induced by laser photocoagulation one month after plasmid injection. CNV was stained with Isolectin GS-IB4 one week after laser injury and CNV volume was calculated with the confocal microscope software.

Results

Figure 1. Schematic representation of transposon based helper-independent piggybac plasmid (pGGENIE). CAG promoter drives IRES mediated co-expression of Flt2-3K and DsRed Ex-2. The transgene cassette is delimited by 3’ and 5’ ITRS.

Figure 2. HeLa sustained DsRed expression, 10 passages after transfection of piggybac.

Figure 3. PCR based genome walking in HeLa cells: Four prominent bands were amplified in piggybac transfected HeLa cells (lane b). No prominent amplification in control HeLa cells (lane c). Sequencing of amplified PCR products indicated random integration into the genome.

Figure 4. Over expression of Piggybac-FLT23K reduced VEGF-A protein expression in HeLa cells. This result indicated Flt23K not only sequesters VEGF-A inside cells but also reduces the protein expression.

Figure 5. ELISA for VEGF-A from HeLa culture medium: Flt2-3K suppress hypoxia induced VEGF-A expression.

Figure 6. Retina flat mount after two months of piggybac plasmid injection (intravitral). (a) DsRed spot on retina surface. (b) Enlarged spots (60X)

Figure 7. Confocal images of Isolectin GS-IB4 stained tissue within laser scars show CNV membranes. CNV size was reduced in Piggybac+Flt2-3K injected eyes.

Figure 8. CNV volume was significantly reduced in Piggybac+Flt2-3K injected eyes.

Conclusion

Our results indicate that nonviral gene therapeutic approach based on Flt23K expression in an in vitro cell model was significantly pronounced and showed a distinctly reducing effect in choroidal neovascularization in a C56BL/6J Mouse model. Our approach can be extrapolated as a novel way to treat pathological neovascularization by long term expression of Flt23K.