Universidad de Oviedo
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Development of Adeno-associated viral (AAV) vectors that specifically express in endothelial cells

A. I. Virtanen Institute for Molecular Sciences
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The research entitled “Development of Adeno-associated viral (AAV) vectors that specifically express in endothelial cells” has been made under my supervision by Ms. Olaya Esparta González graduated in Master in Biotechnology of Environment and Health, at the University of Oviedo in the Molecular Medicine Group laboratory at the A.I. Virtanen Institute at the University of Eastern Finland (Kuopio).

I hereby state that I have read and corrected the present Master Thesis document that I find suitable for its public defence by the student before the designed tribunal. Therefore, I authorize the submission of this Master Thesis to the University of Oviedo, MBEH academic commission.

On Tuesday, 16th July 2019

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1. Abstract

Gene therapy can be a powerful therapeutic option for a multitude of disorders. Importantly, in the last few years gene therapy vectors have been approved for the treatment of specific diseases. In particular, several Adeno-associated viral (AAV) vectors have recently been approved by the FDA for gene therapy. Depending on the required treatment, it can be essential that the vectors are targeted to specific cells or are only expressed in particular cell types. The aim of this study was to develop AAV vectors that specifically express in endothelial cells. Specifically, AAV vectors expressing a putative endothelial-specific enhancer (SE2) derived from a super-enhancer were tested. To begin with AAV transduction in endothelial cells was optimised, different serotypes were tested as well as conditions for the transduction. AAV2 and AAV6 were found to be the most efficient serotypes for endothelial transduction, requiring 1-6 days for maximum expression. In addition, it was noted that Dulbecco's Modified Eagle's Medium (DMEM) enhanced AAV2 transduction. Despite the optimised transduction protocol the AAV vector encoding the putative endothelial-specific enhancer showed very little transduction. The vector was checked by transfection and found to express in endothelial cells. To further investigate the role of this enhancer in the context of the super-enhancer, the endogenous functional activity was also assessed by deleting the different enhancers within the cluster (E1, E2, E3, SE1 and SE2) using the CRISPR-Cas9 technology. The enhancers in the super-enhancer cluster were deleted individually and the expression of the neighbouring genes Nudt7 and Adamts18 was monitored. As a result, it was verified that the enhancers E1 and SE2 have a direct effect on Adamts18, suggesting that the different elements within a super-enhancer do not have individual functions but instead work with one another.

La terapia génica puede ser una poderosa opción terapéutica para tratar distintos trastornos. En los últimos años algunos vectores ya han sido aprobados para su uso en determinadas enfermedades. En concreto, la FDA ha aprobado algunos Adeno-associated viral (AAV) vectors. Dependiendo del tratamiento puede ser importante que los vectores se dirijan a células específicas o se expresen en determinados tipos celulares. El objetivo de este estudio ha sido desarrollar AAV vectors que específicamente expresen en células endoteliales. En concreto se han analizado AAV vectors que expresan el enhancer endotelial SE2 derivado de un super-enhancer. Inicialmente el proceso de transducción de células endoteliales con AAV vectors fue optimizado, analizando diferentes serotipos y condiciones. Los serotipos más prometedores para la transducción de células endoteliales fueron AAV2 y AAV6, necesitando 1-6 días para alcanzar una máxima expresión. Además, se descubrió que el medio Dulbecco's Modified Eagle's Medium (DMEM) aumenta la transducción de AAV2. A pesar de haber optimizado el proceso de transducción, el vector con el enhancer no mostró apenas transducción. Se comprobó también mediante transfección y se descubrió que sí es expresado en células endoteliales. Para investigar más en profundidad el papel de este enhancer, se estudió la actividad funcional endógena de los diferentes enhancers dentro del cluster (E1, E2, E3, SE1 y SE2) mediante deleción con la técnica de CRISPR-Cas9. Los enhancers fueron delecionados individualmente y se analizó la expresión de los genes adyacentes, Nudt7 y Adamts18. Como resultado se observó que E1 y SE2 tienen un efecto directo sobre Adamts18, sugiriendo que los distintos elementos dentro de un super-enhancer actúan conjuntamente.

2. Introduction

2.1. Gene therapy

The World Health Organization has established that ischaemic heart disease is the leading cause of death worldwide (1). The current traditional treatment is not suitable for everyone due to factors such as age or comorbidities (2). Gene therapy stands out offering new solutions that are less invasive, last longer and that can be applied in many fields of medicine (3,4).

Gene therapy is the therapeutic delivery of nucleic acids for the treatment or prevention of disease. Gene therapy was first proposed in 1972 by Friedmann and Roblin (5) but it wasn't until 1990 that the first gene therapy clinical research was approved with limited and temporary clinical benefits; later on the second generation of trials led to positive results but also some detrimental side effects like genotoxicity or immune reactions (3). It was not until recently with increasing knowledge and better vectors that optimistic results have been observed, leading even to some therapeutic drug approvals, such as Glybera for lipoprotein lipase deficiency (6).

In addition to gene delivery, recently different systems for genome modification have also been actively researched and tested. These include zinc-finger nucleases, meganucleases or clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technology (3). Of these, the zinc-finger nucleases was the first to receive approval for clinical trials in 2017 (7).

Most gene therapy clinical trials have focused mainly on cancer (66.6%), followed by monogenic diseases (11.5%), infectious diseases (6.3%) and cardiovascular diseases (6.2%) (4). Up to now they have got good preclinical results although they usually fail clinically. Major limitations in gene therapy are related with poor gene delivery to the desirable site, short transgene expression and immune responses (8). Therefore, the design of new gene delivery vectors is crucial in order to overcome those limitations.

Gene delivery vectors (Figure 1) can be divided into two groups, non-viral physico-chemical methods and recombinant viral approaches. The first category is predominated mainly by the use of naked DNA followed by lipofection. Non-

viral strategies entail less safety risks compared with viral vectors and they have the ability to carry larger amounts of the desirable therapeutic DNA, however, their effects are often transient. Viral vectors are the most popular approach for gene therapy and they are led by adenoviruses (AV) because they have shown high efficiency of transduction, high levels of expression and the possibility of transducing non-dividing cells. AV can carry up to 35kb of DNA, but its main drawback is its transient transgene expression. Other vectors that have gained popularity among the scientific community are lentiviral (LV) vectors and adeno-associated viral (AAV) vectors (9).

LV can carry transgenes of 12-15kb and they are considered useful vectors due to their fast and long transgene expression, since they can integrate in the host genome and potentially provide a life-long cure with a single treatment. Nevertheless, this characteristic also brings some safety concerns since the therapeutic DNA insertion is not in a specific site and its integration site can lead to detrimental effects (10).

AAV vectors have a smaller packaging capacity, around 4.7kb, but they offer a safer therapy because they are non-integrating (3,11). Despite their lack of integration, AAV are able to maintain long enough transgene expression to result in therapeutic effects. There are several natural serotypes with different tissue tropisms, which is very promising for specific tissue targeting (12,13). For these collective reasons, AAV have been gaining in favour and were the first to receive FDA approval for clinical use (14) and as such were used in this study.

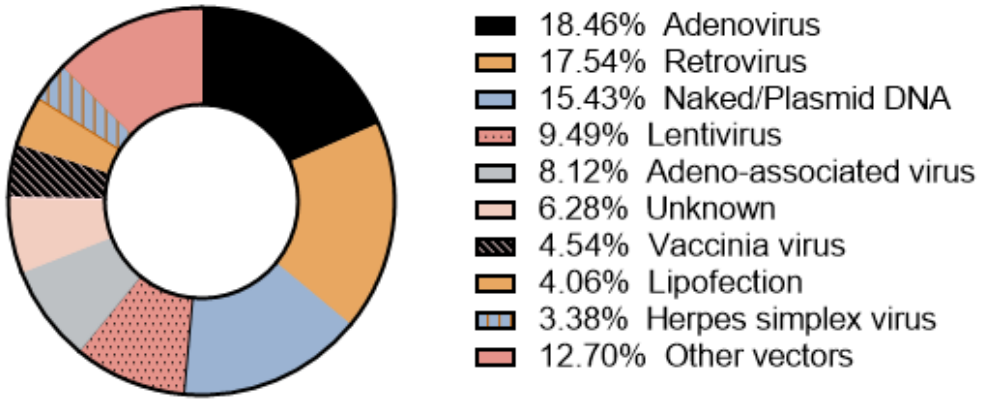


Figure 1. Different vectors used in gene therapy clinical trials worldwide between 1989-2018. Graph made with data from The Journal of Gene Medicine (15).

2.2. AAV vectors

Adeno-associated virus (AAV) is a non-enveloped, non-pathogenic virus that belongs to the *Parvoviridae* family inside the *Dependovirus* genus. It has a small size of 25nm with a single-stranded DNA genome of 4.8kb and it needs co-infection with a helper virus, typically adenovirus or herpesvirus, in order to replicate (16,17). The discovery of this virus was 54 years ago in presence of adenovirus infection and hence its name (18). Initially it was not the centre of attention due to its non-pathogenic nature and therefore there has been a lack of knowledge of this virus until recent years (16).

In its life cycle the virus-host interactions such as cell specificity and antibody recognition are determined by the viral capsid (18). The infection process starts with receptor recognition on the host cells surface such as heparan sulphate proteoglycan or KIAA0319L transmembrane protein (19,20), endocytosis and trafficking to the nucleus (21). The escape of the endosomes before entering in the nucleus occurs with low pH and the entry in the nucleus is not well understood yet but it´s a complex procedure (16,21). Another critical step is inside the nucleus, where the single-stranded transgene is converted into a double-stranded product (17). In the presence of a helper virus it replicates but, in its absence, there is a latency phase where AAV integrates into chromosome 19q13.4. The AAV vectors have the *cis*-active signal and *trans*-active proteins for site-specific integration removed and therefore, they remain episomal (16).

The use of AAV vectors can result in some immunity; an innate response has not been observed (22) but they can trigger cell mediated and humoral response. In cell mediated response the transduced cells are eliminated by cytotoxic T cells although this is not very frequent (23). The humoral immune response is the most common type of immune response to AAV since the majority of patients have been previously exposed to AAV infection and as such have developed neutralising antibodies against the virus (11).

There are several natural serotypes of AAV which offer different tissue tropisms, all of them share genome size and organization and differ in the looped-out domains displayed on the surface. Serotypes from 1-9 have been studied but the most typically used and better understood is AAV2 (12).

When considering a vector for gene therapy some aspects are taken into account; the capacity to target specific cell-types and tissues, the ability to transfer to the nucleus and provide a long-term transgene expression and lack of toxicity (2,16). AAV stands out as a promising vector because it is non-pathogenic, transgenes do not integrate and thus, carries less risk, it has long-term transgene expression (23) and a wide range of serotypes offering the possibility of specific tissue targeting. The only concerns are related with the small packaging capacity, which might be overcome by engineering (16), and pre-existing immunity, which can be overcome by selecting a serotype that has not circulated in the population before (17).

Viral vectors can be further improved by limiting their expression to specific cell types. In AAV, different serotypes can limit the transduction to tissue but not to a specific cell type. One possible means of limiting expression of a viral vector is to insert a regulatory element within the vector that limits the expression of the therapeutic gene to a specific cell type. One such regulatory element could be an enhancer, in particular an enhancer from a super-enhancer cluster. These enhancer clusters are of particular relevance because they have been associated with specific cell type lineages.

2.3. Super Enhancers

An enhancer is a distal short regulatory elements that control some specific genes expression together with a proximal promoter (24). In 1981 the first enhancer was identified in simian vacoulating virus 40 (SV40) genome and it increased the expression of β-globin gene in Hella cells (25).

In 2013, Whyte et al proposed that clusters of enhancers form super-enhancers (26). The definition of a super-enhancer entailed a group of putative enhancers close to each other in the genome, usually no further away than 12.5kb, across a long range of genomic DNA. They bind of a large number cell-type specific master transcription factors such as Oct4, Sox2 and Nanog; they coincide with the presence of high levels of transcriptional coactivator Mediator 1 (Med1); and are characterised by particular histone marks and increased chromatin accessibility as determined by DNase I hypersensitive assay. Importantly super-

enhancer also regulate the expression of genes related with cell identity (24,26–28).

Super-enhancers can regulate cellular identity via cell-type specific signalling routes as well as respond to stimuli such as lineage-determining and cell differentiation signals and repress genes related with cell maintenance (29). However, it is not clear yet if they alone can change the specific cell types or the cell fate (24), many *in vitro* studies has been done regarding the role of super-enhancers but there is still a gap in animal *in vivo* studies (27).

Some genes related to cancer and complex disorders such as auto-immune diseases, diabetes and neurodegenerative diseases are regulated by super-enhancers (30). Aberrant super-enhancers might produce malignancies due to abnormal gene transcription (24). Therefore, one clinical application could be using them as biomarkers for disease diagnostic as well as the risk and progression. In addition, gene therapy could be used to cure these diseases by targeting aberrant super-enhancers and decreasing the detrimental gene expression (24,31). Due to their gene regulation activity, super-enhancers could also mediate the expression of therapeutic genes in gene therapy (24).

In 2013, Hnisz et al defined a list of super-enhancers for 86 human cell types and tissues, including endothelial cells (28). From the list of super-enhancers identified in human umbilical vein endothelial cells (HUVEC), a putative endothelial specific super-enhancer (Figure 2) was selected for further study. This super-enhancer is a cluster of five enhancers (E1, E2, E3, SE1 and SE2) and SE2 has been found to increase transcription of a reporter gene specifically in endothelial cells in the context of lentiviral vectors *in vitro* (Nihay Laham Karam, personal communication). Hence, SE2 is a candidate enhancer that may mediate the expression of a therapeutic gene specifically in endothelial cells.

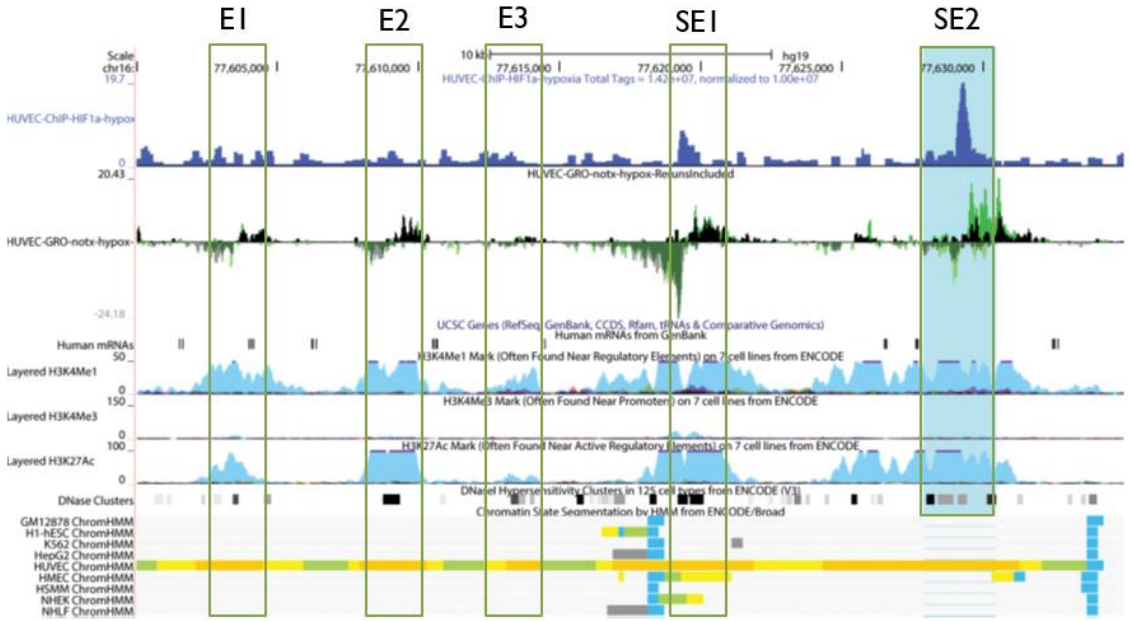


Figure 2. Genome browser view of a putative endothelial specific super-enhancer formed by a cluster of different enhancers (E1, E2, E3, SE1 and SE2).

In addition, the role and hierarchy of the different enhancers within the cluster and how they regulate gene expression has not been understood yet, they could act separately or all together. CRISPR-Cas9 technology can be used to knock-out each individual enhancer and determine their endogenous functional activity. CRISPR stands for clustered regularly interspaced short palindromic repeat and many bacteria and archea have it as an immune mechanism against invading virus (32). In 2012 it was suggested that this natural system could be used for genome editing (33) and since then its application has risen over the years. This editing system works based on 3 main elements, a CRISPR (cr)RNA, a transactivating CRISPR (tracr)RNA and Cas9 nuclease. The crRNA corresponds to the targeting genomic sequence as well as sequnces that enable it to anneal to tracrRNA. The tracrRNA is a structured RNA that binds the Cas9 nuclease, hence the tripartite complex is then targeted to the genome site where the Cas9 makes a double—stranded DNA break (DSB). This powerful genome-modifying system will be instrumental in defining the role of the individual enhancers within the super-enhancer.

3. Objectives

The specific goals of this study were to test the cell-type specificity of the enhancer SE2 *in vitro* in the context of AAV vectors and to determine the endogenous functional activity of the complete super-enhancer which SE2 belongs to.

In order to achieve those aims the project was divided into the following parts with specific objectives:

- The optimization of AAV transduction of endothelial cell, including comparing several serotypes (AAV2, AAV6, AAV8 and AAV9), determining the time course of transduction and the type of media (DMEM, RPMI or Vascular Cell Basal Medium) and supplements (FBS and Heparin Sulphate) used for the transduction.

- The analysis of the transduction efficiency of SE2 in the context of AAV in endothelial cells and comparing it to the negative control FV3.

- The individual deletion of all the enhancers within a super-enhancer cluster (E1, E2, E3, SE1 and SE2) using the CRISPR-Cas9 technology. Cells will be checked for the deletion via PCR of the genomic DNA and deletion positive cells will be tested for gene expression of the neighbouring genes Nudt7 and Adamts18 by RT-qPCR.

4. Materials and methods

4.1. Cell culture

Three human adherent cell lines were used for this study, 293T (human embryonic kidney; ATCC CRL-11268^{TM}), EA.hy926 (endothelial cell line; ATCC CRL-2922^{TM}) and TeloHAEC (aortic endothelial cell line; ATCC CRL-4052^{TM}). They were all obtained from ATCC and cultured according to their instructions.

293T and EA.hy926 were cultured in Dulbecco's Modified Eagle's Medium – high glucose (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 100 units/ml penicillin and 100 µg/ml streptomycin (P/S; complete [c]DMEM). In addition EA.hy926 was cultured in the presence of Hypoxanthine-Aminopectin-Thymidine (HAT) at a final concentration of 0.1 mM, 0.4 µM and 0.016 mM respectively. TeloHAEC was cultured in Vascular Cell Basal Medium (VCBM; dATCC PCS-100-030) supplemented with Vascular Endothelial Cell Growth Kit-VEGF (ATCC PCS-100-041) and P/S (as above, complete [c]VCBM).

All cells were incubated at 37°C and 5% CO_2 in a humidified incubator and were subcultured using trypsin 0.25% (w/v) Trypsin-0.53 mM EDTA (Sigma) to lift the cells after washing with Dulbecco's Phosphate-Buffered Saline (1X) (D-PBS (1X), Thermo Fisher). After incubation at 37°C, trypsin was neutralised with complete media. Cells were counted using CellCountess (Invitrogen) according to the manufacturer instructions.

4.2. Transfection

For plasmid transfections 293T and EA.hy926 were transfected with Lipofectamine 3000 (Invitrogen). 293T cells were seeded in a 6 well plate at 1,500,000 cell/well while EA.hy926 were seeded at 500,000 cell/well in 12 well plates. They were transfected with a pDNA : Lipofectamine 3000 : P300 of 1 µg : 3 µl : 2 µl in OptiMEM. A total of 2.5 µg pDNA/well was added to 293T and 1.25 µg pDNA/well was added to EA.hy926. The transfection complexes were allowed to form at room temperature (RT) for 20 minutes, thereafter they were added to the cells and incubated for 4h, after which the media was changed to fresh complete media. Following the 48h of transfection the cells were harvested by

trypsinization and centrifuged at 800g for 5 minutes at RT. The media was discarded and cell pellets were suspended in 2% formaldehyde in PBS (300 ul for 293T and 100 ul for EA.hy926) and stored at 4°C until flow cytometric analysis.

The plasmids transfected were pLV-GFP (positive control), pAAV-FV3-GFP and pAAV-SE2-GFP (cloned by Nihay Laham Karam and Tuisku Suoranta), all containing the enhanced green fluorescent protein (GFP) as a reporter gene. The positive control plasmid has a strong promoter before the reporter gene in order to induce high level of expression and therefore strong fluorescent signal. Both pAAV plasmids (Figure 3) have a minimal promoter with little transcription ability, pAAV-SE2-GFP has in addition an enhancer element (SE2) preceding the promoter while pAAV-FV3-GFP has a control DNA stuffer (FV3) approximately the same size as SE2.

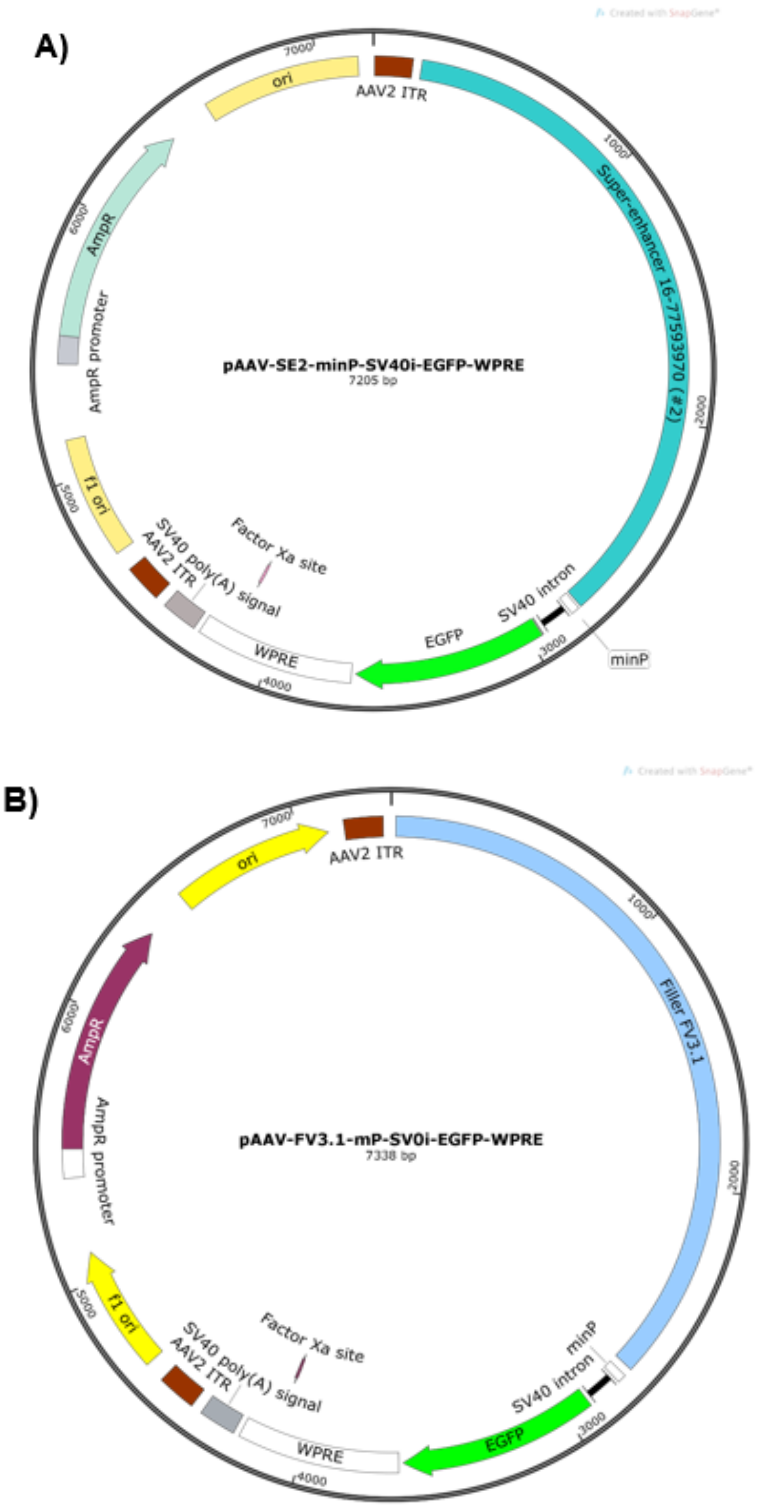


Figure 3. pAAV plasmids for transfecting 293T, TeloHAEC and EA.hy926 cells. A) pAAV-SE2-GFP containing super-enhancer element SE2. B) pAAV-FV3-GFP containing control DNA stuffer FV3. Enhanced green fluorescent protein (EGFP) Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE). Minimal promoter (minP). Bacterial origin of replication (ori). Inverted terminal repeats (ITR). Ampicillin resistance (AmpR).

4.3. Viral Transduction

The cell lines TeloHAEC and EA.hy926 were seeded in 12 well plates at 40.000-50.000 cells/well the day before transduction with AAV particles. On the day of the transduction, the AAV vectors were diluted in culture media, the medium was removed from the cells and 0.5 ml of diluted AAV was added to the cells at 10^4-10^5 vector genomes (vg)/cell. Cells were incubated at 37°C for 5h. After that time the media was aspirated and 1ml/well of the cells own complete media was added. After several days (from 1 to 7 depending on the experiment) the cells were harvested (as above) and they were analysed using flow cytometry. In some experiments, transduced cells were harvested for genomic DNA extraction (kit from Machery-Nagel) and later qPCR analysis. In this case, the cell pellets were stored at -20°C until the genomic DNA extraction.

To optimise transduction efficiency of AAV in endothelial cells, different AAV serotype (2, 6, 8 and 9) preparations of AAV-GFP were tested, as well as the use of different transduction media including RPMI (Roswell Park Memorial Institute), DMEM, cDMEM, VCBM or cVCBM. To determine the effect of FBS (10% in DMEM and 2% in VCBM) and heparin sulphate (0.75 Units/ml) on AAV transduction, media in the absence or presence of these was used for the time of transduction. To determine the optimal collection time after the transduction, a time course was performed, where transduced cells were harvested daily for a week. To investigate the transduction efficiency of our test vectors AAV-FV3 and AAV-SE2, the cells were transduced with either AAV2 or AAV6 serotype preparation of these vectors and the transduced cells were collected after 3 and 2 days, respectively.

4.4. CRISPR-Cas9

293T, EA.hy926 and TeloHAEC cell lines were transfected in triplicates in a 96 well plate with ribonucleoprotein (RNP) complexes in order to delete specifically 5 enhancers (E1, E2, E3, SE1 and SE2) separately using Alt-R® CRISPR-Cas9 System (Integrated DNA Technologies) according to the manufacturer instructions.

Each cell line was transfected with a pair of guide RNAs (gRNAs; Table 1) in order to obtain a complete deletion of the specific enhancers and there were two controls, which were cells transfected only with a single gRNA or tracer RNA (trRNA). The procedure was done by reverse transfection, the complexes were first added into the wells and later the cells were seeded on top in triplicates (60,000-80,000 cell/well for TeloHAEC, 40,000- 80,000 cell/well for 293T and 40,000 cell/well for EA.hy926). After the reverse transfection, cells were incubated at 37°C for 4h, then the old media was removed and fresh complete medium was added. 48 hours later cells were harvested (as above) for flow cytometric analysis and genomic DNA extraction and the rest of the wells were used for clone expansion and selection.

Table 1. CRISPR-Cas9 guide RNAs

Deletion	Matched guides
E1	SE12-1 forward
	CD.Cas9. VSJJ5954.AA
E2	SE12-2 forward
	SE12-2 reverse
E3	CD.Cas9.JBBT6604.AJ
	CD.Cas9.JMFV4355.AA
SE1	CD.Cas9.QHPL0971.AB
	CD.Cas9.MZGV3767.AA
SE2	CD.Cas9.BGJY6208.AA
	CD.Cas9.PFZS7534.AA

4.5. PCR analysis

After the genomic DNA (gDNA) extraction with the kit from Machery-Nagel the concentration of gDNA was measured with NanoDrop 1000 (Thermo Fisher) and a PCR was performed using a Doppio thermal cycler (VWR) in 20 µl reaction volume (20 ng gDNA, 5x Phusion GC Buffer (F-519), 200 µM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer and 0.02 U/µl Phusion Hot Start II DNA Polymerase). All PCR reagents used were from Thermo Fisher. For each enhancer deletion a unique pair of primers was used (Table 2), also the thermic profile was specific for each enhancer deletion (Table 3).

Table 2. Pair of primers for each enhancer deletion

Deletion	Primers for detection	Approximate PCR amplicon size (bp)	
		Wild Type	Deletion
E1	Chr16_SEdel forw	3050	1150
	SE12-1 rev		
E2	SE12-2 forw	2720	218
	SE12-2 rev		
E3	SE12-3 forw	2968	869
	SE12-3 rev		
SE1	SalI-SE1L forw	2294	960
	ClaI -1 rev		
SE2	SE2P560 forw	3620	1092
	SE2L rev		

Table 3. Thermic profiles for each enhancer deletion

E3	SE1	E1, E2 and SE2
98°C for 5 min	98°C for 5 min	98°C for 5 min
35 cycles: 98°C-10s 60°C-10s 72°C-3min	40 cycles: 98°C-10s 68°C-10s 72°C-3min	40 cycles: 98°C-10s 68°C-10s 72°C-4min
Final 72°C for 10 min	Final 72°C for 10 min	Final 72°C for 10 min
Infinite 8°C	Infinite 8°C	Infinite 8°C

The PCR product was run for 1h at 140 volts in an agarose gel (1%) with Midori Green Dye (5%) alongside with 1 Kb Plus DNA Ladder (Thermo Fisher). 4 µl of 6x MassRuler Loading Buffer was added to each sample.

The positive samples for the specific enhancer deletions were expanded for clone selection. Once the colonies were grown they were transferred to a new well in a 48 well plate. These clones were analysed for the deletion by PCR analysis and the positive ones were selected.

4.6. Quantitative PCR

Samples from the CRISPR-Cas9 experiments were treated with TRIzol (Thermo Fisher) for RNA extraction according to manufacturer's instructions. Quantification of the RNA was done with NanoDrop 1000 and samples were kept at -70°C until reverse transcribed into cDNA by reverse transcriptase (RT). Initially, 200 ng random primers and 1 µg RNA sample in final volume of 10 µl diluted in H_2O was incubated at 65°C for 5 minutes and then passed to ice. Then 10 µl of RT mix was added (4µl 5x Buffer RT, 20U Ribolock, 1mM dNTP, 200U ul RT and mH_2O). The RT reactions were incubated at 25°C for 10 min, then 42°C for 1h and 72°C for 10 min. After that qPCR was done in StepOnePlus Real Time PCR System (Applied Biosystems) using primer-probe assays for hActB (Hs.PT.39a.22214847, IDTDNA) as housekeeping gene and the neighbouring genes hNudt7 (Hs.PT.58.39597989, IDTDNA) and hAdamts18 (Hs.PT.58.1326160, IDTDNA). A final volume of 15 µl was made (2x Universal Master Mix, 20x Assay, 2 ul diluted cDNA and H_2O).

5. Results

5.1. AAV Transduction Optimization

5.1.1. AAV Serotypes

In order to optimise the transduction with AAV vectors in endothelial cells several experiments were done comparing the different serotypes, the collection time and the media used.

TeloHAEC and EA.hy926 cells were transduced with AAV-GFP serotypes 2 and 9 using different media (Figure 4). The flow cytometric analysis showed that in TeloHAEC cells, serotype AAV2 gave the lowest fluorescent signal using TeloHAEC's own media [c]VCBM (15.23%) and the highest with DMEM (47.14%). Using RPMI the fluorescent signal was 33.35% and increasing the multiplicity of infection (MOI) to 10^5 in cVCBM increased the transduction efficiency to 36.76%. In EA.hy926 the signal range was between 25.75% and 35.96%, their complete own media obtaining the lowest expression and RPMI the highest expression. Using DMEM alone without FBS the fluorescent signal was 27.08%.

With AAV9 transduction the level of expression was much lower in both cell lines between 0.2 and 1.13% approximately. Only when increasing the MOI to 10^5 in TeloHAEC cells was a significant increase in the level of expression observed, obtaining 8.3% of fluorescent signal.

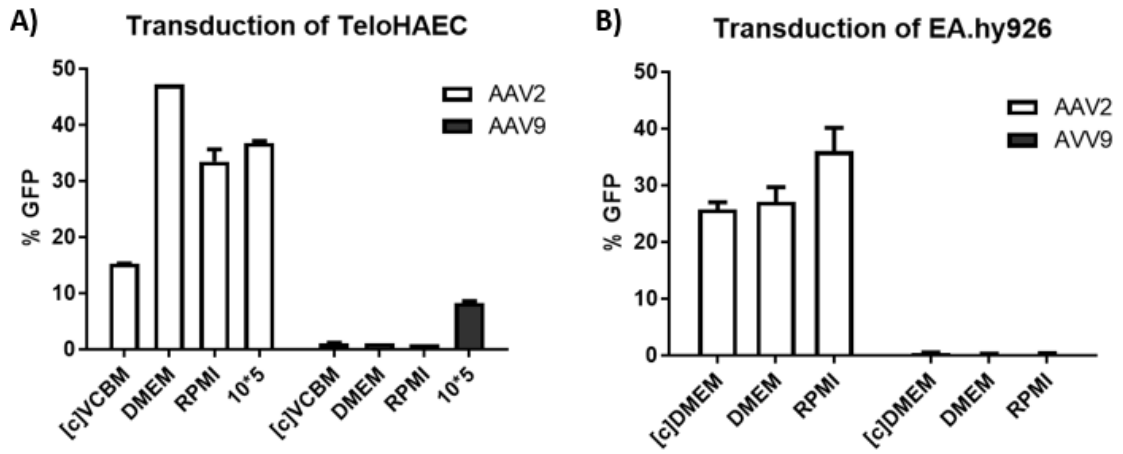


Figure 4. AAV2 and AAV9 transduction efficiency in endothelial cells. TeloHAEC (A) and EA.hy926 (B) cells were transduced with AAV2-GFP and AAV9-GFP (MOI 10^4 and 10^5) using the same cell line complete medium, DMEM or RPMI. Cells were harvested after 3 days and were analysed with flow cytometry.

The next serotype tested was AAV8-GFP which was compared to AAV2-GFP in TeloHAEC cells (Figure 5). Once again, AAV2 presented the best results, reaching a fluorescent signal of 68.53% when using DMEM, followed by 43.41% using RPMI and 31.83% with [c]VCBM. The level of expression of GFP with the transduction of AAV8 was much less than AAV2 ranging between 2.06% to 1%, in different media.

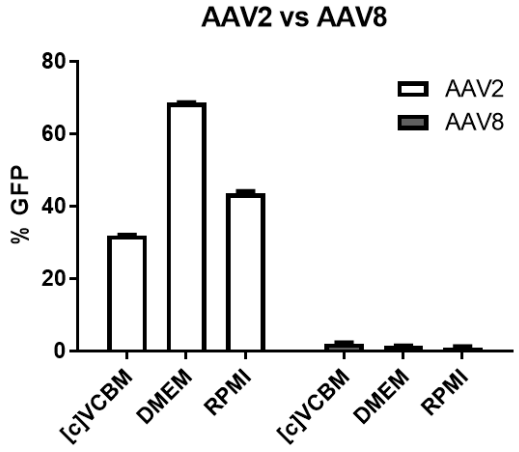


Figure 5. AAV2 and AAV8 transduction efficiency in TeloHAEC. Cells were transduced with AAV2-GFP and AAV8-GFP (MOI 10^4) using [c]VCBM, DMEM and RPMI. Cells were harvested after 3 days and were analysed with flow cytometry.

A comparison of AAV6 and AAV2 transduction efficiency of TeloHAEC is presented in figure 6. AAV2 was more efficient than AAV6 in endothelial cell transduction. For AAV2 the highest fluorescent measurement was 77.29 %, using DMEM. In contrast, the highest transduction efficiency of AAV6 was obtained in presence of [c]VCBM.

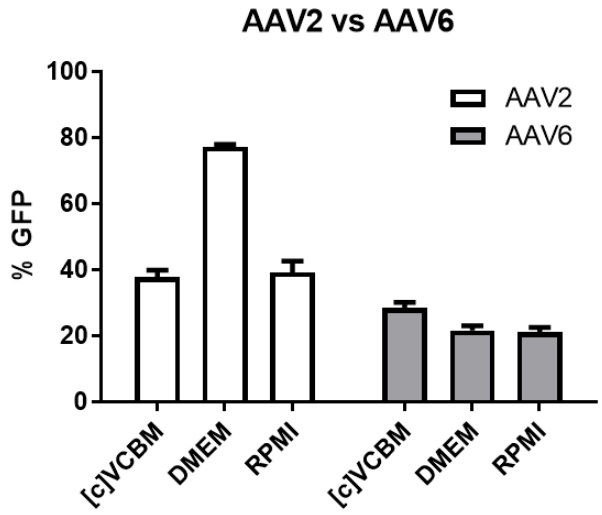


Figure 6. AAV2 and AAV6 transduction efficiency in TeloHAEC. Cells were transduced with AAV2-GFP and AAV6-GFP (MOI 10^4) using [c]VCBM, DMEM and RPMI. Cells were harvested after 3 days and were analysed with flow cytometry.

Comparing all the AAV serotypes used for the transduction of TeloHAEC cells, the best serotype was AAV2 and this was followed by AAV6. Serotypes 8 and 9 do not transduce endothelial cells efficiently and therefore they were not used further in this study.

5.1.2. Time Course

The AAV serotypes were tested following 3 days of transduction, however, different serotypes may have different kinetics of transduction. Therefore, a time course of AAV2 and AAV6 transduction was determined.

AAV2 transduction of endothelial cells increased over time, reaching maximum number of transduced cells after 6 days (figure 7). However, the fluorescence intensity on that day was not the highest, the maximum intensity was reached in earlier time points, between the second and the fourth day after transduction. For

that reason, the optimal time for harvesting cells transduced with AAV2 was the third day after transduction.

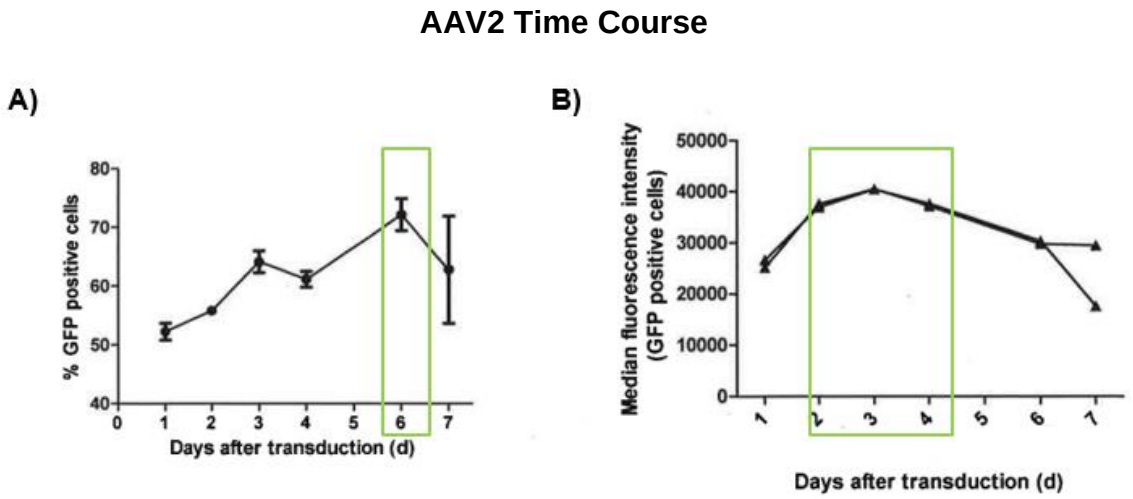


Figure 7. AAV2 time course in TeloHAEC. Cells transduced with AAV2-GFP (MOI 10^4) using DMEM. They were harvested every day for 7 days, except for the fifth day, and were analysed with flow cytometry.

In contrast to AAV2, AAV6 transduction of endothelial cells was quick, and already 1 day after transduction the maximum number of cells were transduced (figure 8) and maintained for the first 3 days. Likewise, the level of GFP expression in the cells as indicated by the median fluorescence intensity was also highest in the early days, especially at day 2, therefore from that moment on all the cells transduced with AAV6 vector were harvested 2 days after transfection.

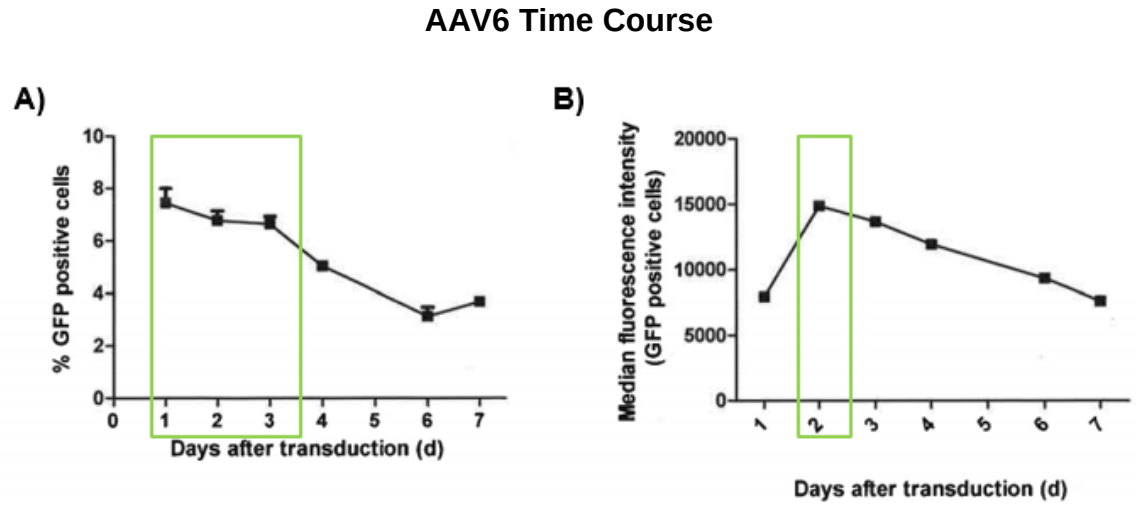


Figure 8. AAV6 time course in TeloHAEC. Cells transduced with AAV6-GFP (MOI 10^4) using [c]VCBM. They were harvested every day for 7 days, except for the fifth day, and were analysed with flow cytometry.

5.1.3. Effect of media supplements

In the previous section, it was clearly shown that AAV transduction can be significantly affected by the transduction media and that various AAV serotypes were differentially affected by the media. In particular, AAV2 transduction was most efficient in DMEM. However, for the remaining serotypes (6, 8 and 9) the best transduction efficiency was obtained using the cell's own complete media, [c]VCBM, followed by DMEM and RPMI. To determine the media components responsible for this, the effects of FBS and Heparan sulphate, a component of [c]VCBM were studied.

In order to understand the difference between DMEM and [c]VCBM in AAV2 in this experiment the effect of FBS in the media was analysed (Figure 9). FBS had little effect on AAV2 transduction in VCBM, however it reduced AAV2 transduction in DMEM. The fluorescent signal using DMEM with and without FBS was 60.53% and 74.8%, respectively.

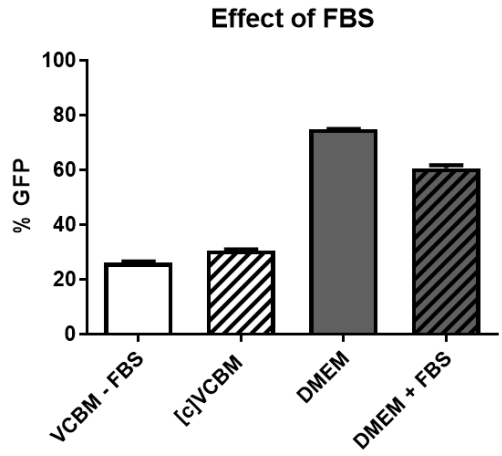


Figure 9. Effect of FBS on GFP expression in TeloHAEC. Cells were transduced with AAV2-GFP (MOI 10^5) using VCBM without FBS, [c]VCBM, DMEM and DMEM with FBS. Cells were harvested after 3 days and were analysed with flow cytometry.

AAV use heparan sulphate proteoglycan as one of their receptors for entry into the cell (21). The VCBM media supplements include heparin sulphate, which is a molecule chemically very similar to it. In [c]VCBM AAV transduction might be negatively affected by the presence of the heparin sulphate, therefore, in this experiment we studied the presence and absence of heparin sulphate (HS) in both VCBM and DMEM (Figure 10).

The results showed that the presence of HS decreased the level of gene expression in both media. The percentage of GFP in their own cell media with or without HS was 22.83% and 29.69%, respectively. The effect of HS on AAV2 transduction was less pronounced in DMEM.

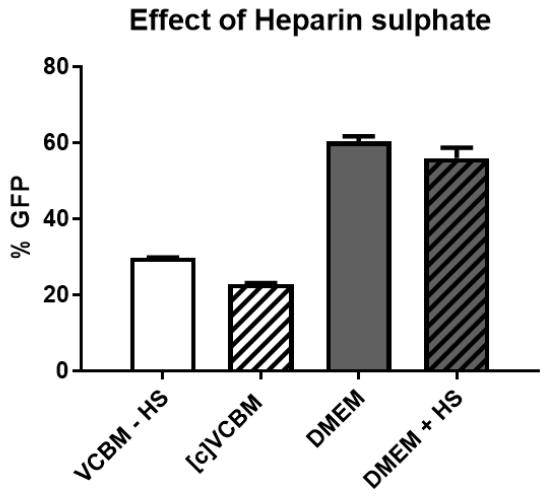


Figure 10. Effect of Heparin sulphate on GFP expression in TeloHAEC. Cells were transduced with AAV2-GFP (MOI 10^5) using VCBM without HS, [c]VCBM, DMEM and DMEM with HS. Cells were harvested after 3 days and were analysed with flow cytometry.

5.2. AAV Transduction of Super-Enhancer

Once the optimization process of the transduction with AAV vectors was done we analysed the transduction efficiency of pAAV-FV3-GFP and pAAV-SE2-GFP (Figure 3) prepared as AAV2 and AAV6 in TeloHAEC and EA.hy926 cell lines.

Unexpectedly, the AAV-FV3-GFP and AAV-SE2-GFP vectors minimally transduced endothelial cells (Figure 11), either as AAV2 or AAV6. In most cases the transduction efficiency was less than 1% and at this level of transduction no effect of SE2 could be seen as compared to the FV3 control.

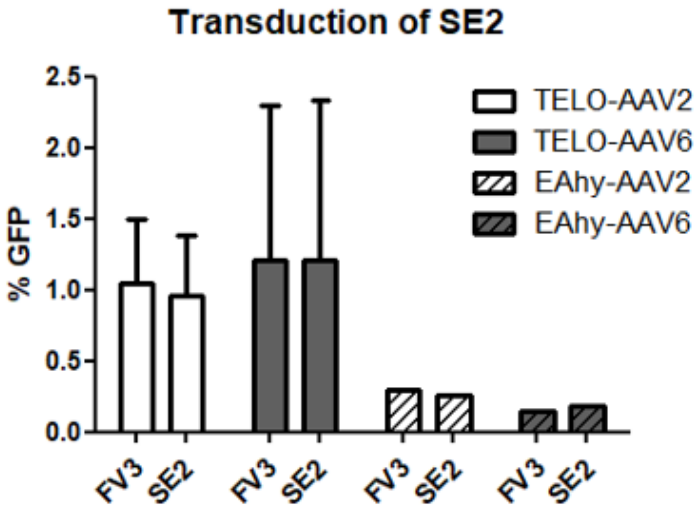


Figure 11. Enhancer SE2 transduction. TeloHAEC and EA.hy926 cells were transduced with AAV2-FV3, AAV2-SE2, AAV6-FV3 and AAV-SE2 (MOI 10^5) using DMEM for AAV2 vectors and their own cell line media for AAV6 vectors. AAV2 cells were harvested after 3 days and AAV6 cells after 2 days and they were analysed with flow cytometry.

In order to check the integrity of the vector plasmids and to confirm the cell-type specificity of the enhancer SE2, pAAV-FV3-GFP and pAAV-SE2-GFP as well as a positive control plasmid (pLV-GFP) were transfected in 293T cells and the endothelial cell line EA.hy926.

After 24 hours of transfection the cells were analysed using a fluorometric microscope to visualize directly the level of GFP expression (Figure 12). The result was that the cells transfected with pLV-GFP expressed much higher levels of fluorescence while the level of GFP expressed by the cells transfected with pAAV-FV3-GFP and pAAV-SE2-GFP was barely detectable.

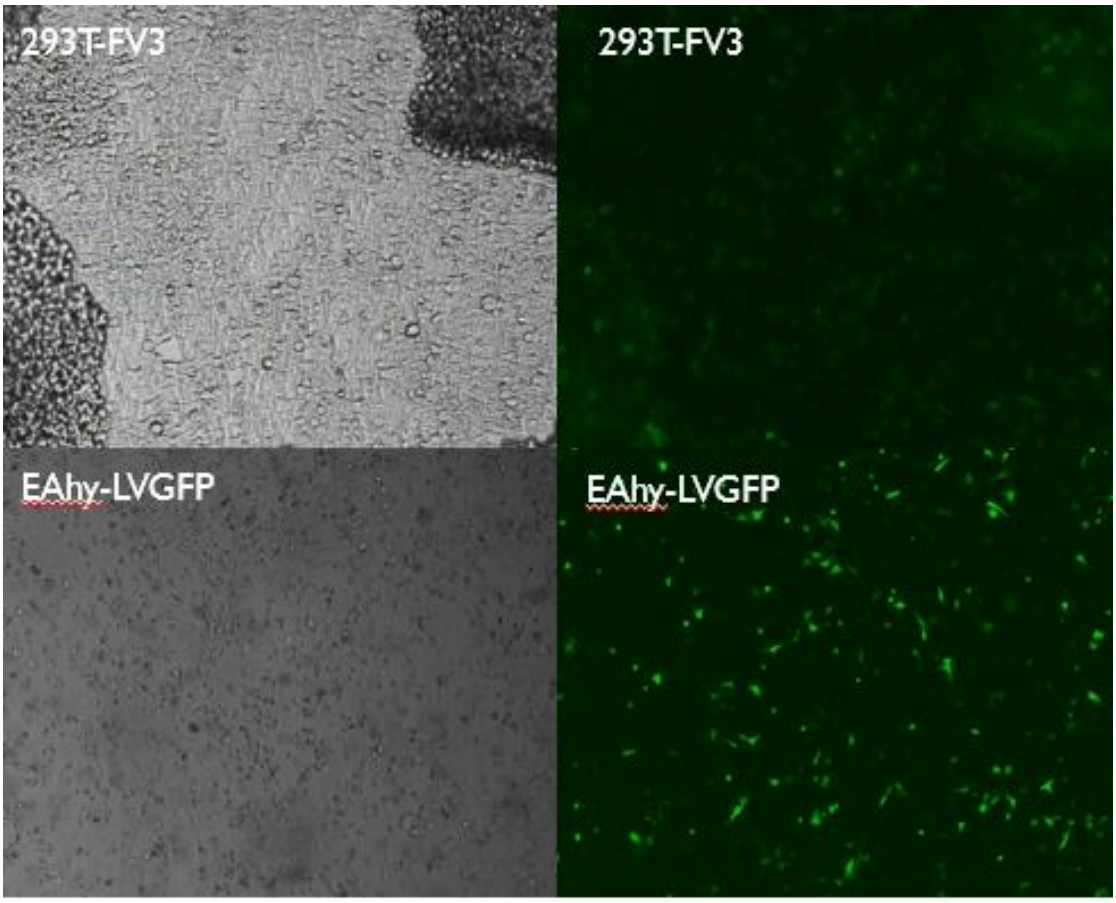


Figure 12. LV and AAV plasmids transfection. Transfection of 293T cells with pAAV-FV3-GFP (up) and EA.hy926 cells with pLV-GFP (down). Images taken with a fluorometric microscope.

After 48 hours of transduction the cells were harvested and analysed by flow cytometry. The comparison between the two cell lines is shown in the figure 13. In 293T cells, whereas the pLV-GFP expressed high levels of GFP in all cells, the pAAV-FV3 and pAAV-SE2 were only expressed in 45.38% and 22.29%, respectively. EA.hy926 cells likewise showed high fluorescent with the positive control plasmid, 60.84%, however, in these cells the pAAV-SE2 was expressed better than the control pAAV-FV3, at GFP positive cells of 14.09% and 8.37% respectively.

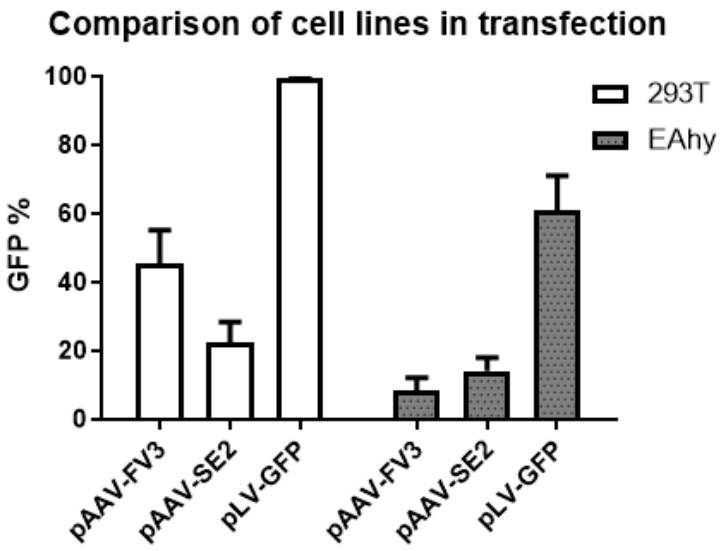


Figure 13. Comparison of cell lines in transfection. 293T and EA.hy926 cells transfected with pAAV-FV3-GFP, pAAV-SE2-GFP and pLV-GFP by Lipofectamine 3000 (Invitrogen). Cells were harvested after 2 days and were analysed with flow cytometry.

5.3. CRISPR-Cas9

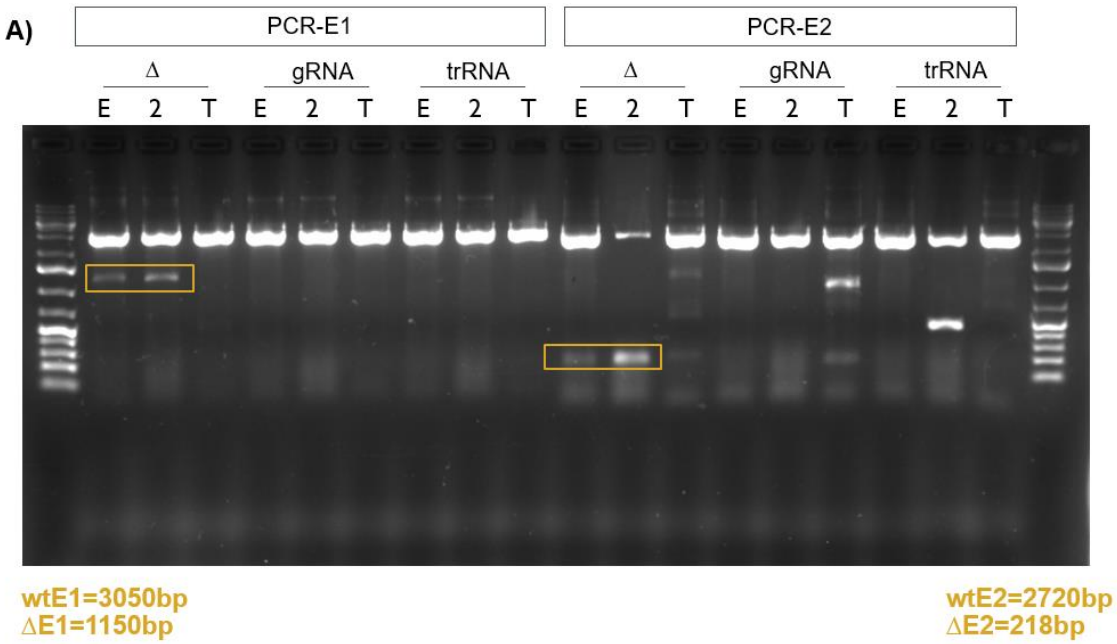
CRISPR-Cas9 technology was used for the specific deletion of the five enhancers (E1, E2, E3, SE1, SE2) within a super-enhancer in cell lines TeloHAEC, EA.hy926 and 293T.

The outcome of four independent deletion experiments is summarised in table 4, where different number of cells were seeded per well. The transfection efficiency was very high (95-100%) in almost all the cell lines except in the first experiment of TeloHAEC, where 60,000 cells/well were seeded and none of them survived. Subsequently, the amount of cells seeded was increased to 80,000 cells/well. The PCR results for TeloHAEC in the next experiments showed that all the five enhancers were successfully deleted, albeit at different levels. EA.hy926 cell line was seeded at 40,000 cells/well and all the five enhancers within the cluster were deleted as well. 293T cells were first seeded at 40,000 cells/well but the survival of the cells was compromised, for that reason in the next experiment the number was also increased to 80,000 cells/well. The PCR analysis showed that 293T cells were positive for the deletion of E1, E2, SE1 and SE2. There were differences between the efficiency of deletion depending on the cell line and the targeted genomic area.

Table 4: Summary of the results from the CRISPR-Cas9 SE deletions

			PCR analysis				
			Deletion				
Experiment	Cell lines	cell/well	E1	E2	E3	SE1	SE2
1	TeloHAEC	60 000	-	-	-	-	-
	EA.hy926	40 000	no	no	yes	no	yes
	293T	40 000	no	no	inconclusive	no	yes
2	TeloHAEC	80 000	no	no	no	no	no
	EA.hy926	40 000	yes	yes	no	yes	inconclusive
	293T	80 000	yes	yes	no	yes	yes
3	TeloHAEC	80 000	no	yes	yes	yes	yes
4	TeloHAEC	80 000	yes	no	no	yes	yes

The deletions were identified by PCR amplifications of the specific genomic regions of the deletions. PCR amplicons were electrophoresed in agarose gels and a sample of these is presented in (Figure 14). In all gDNA samples, except in the deletion of E3 enhancer, the wild type band was present in all the cells (Figure 14A). EA.hy926 and 293T cells presented a clear band of approximately 1150bp in the PCR-E1 and 218bp in the PCR-E2, which correspond with the expected size of the bands with the E1 deletion and E2 deletion respectively (Figure 14A). However, TeloHAEC cells only presented the wild type band in these two PCRs. The E3 deletion appeared to be very efficient, since in pooled cells only the 869bp deletion band was observed (Figure 14B)



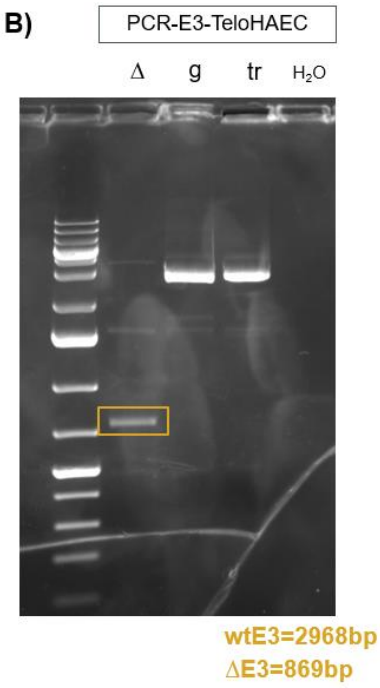


Figure 14. Diagnostic PCR of enhancer deletions. PCR of EA.hy926 (E), 293T(2) and TeloHAEC (T) cell lines transfected with RNP complexes for the deletions (Δ) E1 and E2 (A) and E3 (B) and two controls, guide RNA (g) and tracer RNA (tr) on 1% agarose gel with 1 kb Plus DNA Ladder. Wild type (wt).

The pooled transfected cells were diluted to enable clone selections. Putative clones were expanded, and the presence of the deletion was tested again. Figure 15 demonstrates an example of such an assessment for SE2 deletions in 293T cells. Out of twelve clones from 293T cells, five of them (1, 3, 4, 5 and 7) showed a band with a size of 1092bp which represent the deletion of the SE2 enhancer (Figure 14). In all the clones both the deletion and WT amplicons were present

suggesting either that the cells were not clonal or that the mutation occurred in heterozygous manner.

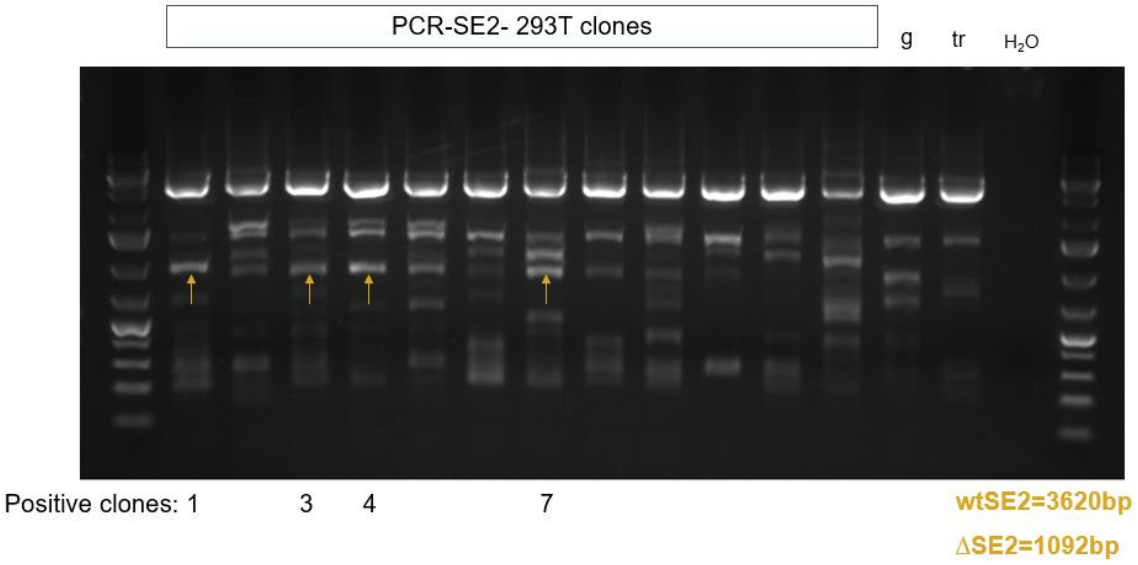


Figure 15. Diagnostic PCR of enhancer deletions. PCR of 293Tclones transfected with RNP complexes for the deletion SE2 and two controls, guide RNA (g) and tracer RNA (tr) on 1% agarose gel with 1 kb Plus DNA Ladder. Wild type (wt). Yellow arrows indicate strongly positive clones.

Deletion positive 293T clones were tested for the effect of the deletion on the neighbouring genes. Using RT-qPCR, the gene expression of the neighbouring genes hAdamts18 and hNudt7 was determined (Figure 16). In all the clones with the deletion of the enhancers E1 (clones 8, 11 and 12) and SE2 (clones 3, 4 and 7) there was a significant decrease in the fold change of hAdamts18. The clone E2-cl9b also showed a significant decrease in the fold change of this gene but not the rest of the clones lacking the E2 enhancer. Some clones such as E1-cl11, E1-cl12, SE1-cl8b and SE2-cl3 also showed a decrease in the fold change of hNudt7 although not very significant.

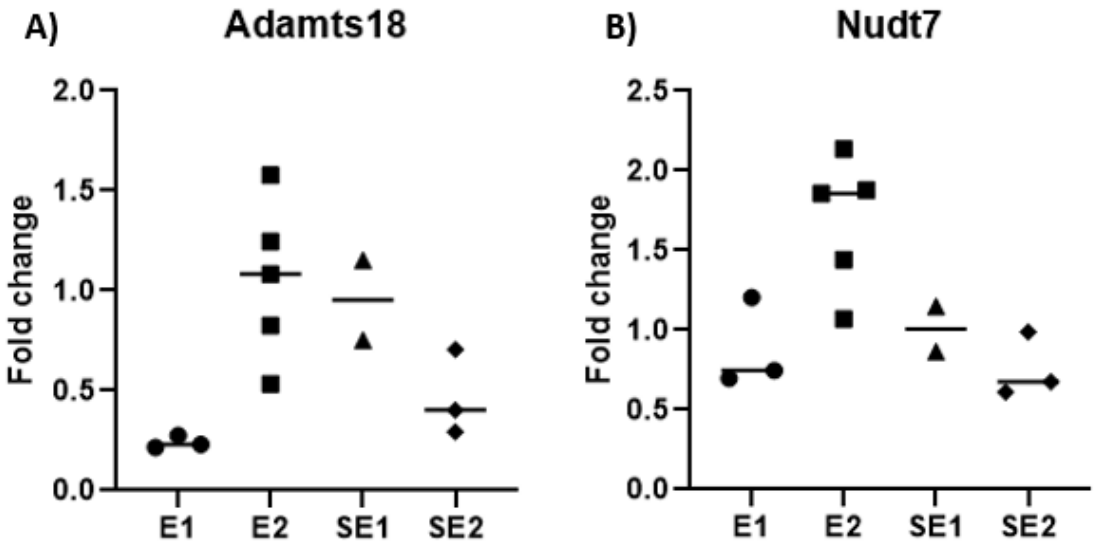


Figure 16: Gene expression in enhancer deletion clones. RT-qPCR results of Adamts18 (A) and Nudt7 (B) genes from 293T positive clones following the deletions of E1, E2, SE1 and SE2.

6. Discussion

The main aim of this study was to investigate the specificity and activity of a putative endothelial specific enhancer in the context of AAV vector. In order to do so, AAV transduction in endothelial cells was first optimised. Four conditions were taken into account; vector serotype, day for cell harvesting after transduction, media and supplements used.

In this study, AAV2 was the best serotype for endothelial cells transduction, followed directly by AAV6. Serotypes AAV8 and AAV9 poorly transduced endothelial cells. These results agree with the literature since it has been described that the tissue tropism of AAV2 is very wide and AAV6 is good transducing human cell lines (34). In contrast, the tissue tropism of AAV8 and AAV9 is mainly for liver and skeletal and cardiac cells as well as cells from pancreas in case of AAV8 and cells from the lungs for AAV9 (13,19). For this reason, the two main serotypes used in the next experiments were AAV2 and AAV6.

Even though AAV2 showed the best transduction rates, it could also be related to the fact that the transduced cells were harvested always three days after the transduction. One possible explanation of why AAV6 serotype led to less GFP expression in the endothelial transduced cells could be related with the optimal day for collection of the cells. AAV2 and AAV6 did have different time course of transduction, interestingly AAV6 was the most rapid serotype and AAV2 the slowest one. Taking into consideration both the number of transduced cells (as indicated by %GFP) and the level of GFP expression (mean fluorescence) the optimal time of transduction of AAV2 and AAV6 was determined to be d3 and d2, respectively. The differences between both serotypes and the day at which they induce higher levels of gene expression might be related with the different composition of their capsids (22), the different receptors which they interact with (19) or the way of endosomal escape or entry in the nucleus of the cells. The internal pathways that these vectors go through are not completely understood yet (18), but it has been suggested in the literature that the capsid uncoating rate plays an important role (23). There are studies with similar results to our data

indicating that AAV2 presents a lag phase compare to AAV6 due to either a less efficient capsid disassembling or a longer time required to pass the nucleus membrane (23). All these factors should be further studied in order to obtain a better understanding of the differences between AAV2 and AAV6.

Surprisingly, in this study we observed a significant effect of media on AAV2 transduction. In particular, DMEM doubled the transduction efficiency of AAV2 compared to the cells own media [c]VCBM. This was not observed for the other serotypes (AAV6, AAV8 and AAV9). For these serotypes the cells own complete media achieved better levels of transduction, while DMEM and RPMI media slightly decreased the level of GFP expression. These results suggested one of 2 scenarios either that something in [c]VCBM inhibited the transduction efficiency of AAV2 or that an ingredient in DMEM supported AAV2 transduction. Subsequently we investigated the effects of both FBS and heparin sulphate, both of which were in [c]VCBM and are feasible culprits, since it has been reported in the literature that serum can affect the transduction efficiency (35) and heparin sulphate is very similar chemically to one of AAV receptors (21).

The presence of FBS did not affect AAV2 transduction in VCBM but decreased AAV2 transduction efficiency in DMEM to a degree. However, this alone did not explain the difference between DMEM and VCBM.

Heparin sulphate is present in [c]VCBM and it could affect the transduction efficiency of AAV2, since it is chemically similar to heparan sulphate proteoglycan, one of the receptors to which this serotype binds (21,22). The results obtained from the transduction of endothelial cells with and without HS in the media confirmed the previous reports in the literature that this compound was inhibiting the transduction efficiency of AAV2 (21). When HS was removed from [c]VCBM, the level of GFP expression increased and the presence of HS in DMEM made the percentage of GFP decrease, although not significantly. Further studies are required to pinpoint the cause of the media-induced difference in AAV2 transduction.

The optimization process was therefore concluded with AAV2 and AAV6 being the best serotypes for transducing endothelial cells, the optimal harvesting days

for the cells were 3 and 2 days after transduction respectively and the ideal media was DMEM for AAV2 and the own cell line media for AAV6.

The second aim of this study was the transduction of endothelial cells with an element (enhancer SE2) of a super-enhancer and its comparison with a negative control (stuffer DNA FV3) with similar size but no biological activity. All in the context of AAV vectors *in vitro.* These vectors failed to transduce endothelial cells. One possible explanation could be that the size of the constructs carrying the enhancer SE2 and the control FV3 was affecting the transduction process, since the optimization experiments were done with vectors carrying a smaller delivery construct. Another possible explanation could be that the vectors were successfully transduced inside the endothelial cell but failed to induce a high level of GFP expression. In future studies, the amount of viral load that enters in the cells should be investigated.

The transfection experiments for the comparison of the enhancer SE2 activity among the different cell lines showed interesting results. Overall the transfection efficiency for EA.hy926 was not as good as for 293T, which was 100%. However, the interesting observation was that the enhancer SE2 drove a higher level of gene expression than the negative control FV3 in the endothelial cells but just the opposite for 293T cells. According to our previous expectations, SE2 activity was specific for endothelial cell types, like EA.hy926. These results could also indicate that the presence of this element might actually inhibit the level of gene expression in 293T cells.

The last objective of this project was to analyse how each enhancer within the super-enhancer cluster affects the gene expression of the neighbouring genes (hAdamts18 and hNudt7). Subsequently, each enhancer was targeted for deletion with CRISPR-Cas9. We successfully induced the deletions of all enhancers, albeit to different levels within a pool of cells, likely reflecting the efficiency of the specific gRNAs. Even following clonal selection, homozygous clones were not obtained, this may either be for technical reasons or biological, in cases where the enhancer is critical for the cell then heterozygotes would have a survival advantage.

Enhancer-deletion positive clones were tested for effects of the enhancer on the expression of its neighbouring genes hAdamts18 and hNudt7. Partial absence of enhancers E1 and SE2 led to a significant and consistent decrease in the gene expression of hAdamts18, which indicates that the endogenous functional activity of enhancer E1 and SE2 is to regulate this gene. Only one out of five E2 clones also showed a notable decrease in the fold change of hAdamts18 but since the rest did not, it is likely that this enhancer is not involved in this gene regulation. The deletion of the enhancer SE1 did not significantly affect the expression of neither hAdamts18 nor hNudt7. Often in genome wide studies, enhancers are linked to their closest neighbour gene, and correlation are drawn from this assumption. In this study we demonstrate, that enhancers belonging to a putative endothelial-specific super-enhancer regulate Adamts18 and not their closer neighbour NudT7, this highlights the need for experimental evidence of gene association to enhancers.

7. Conclusions

In this study we have successfully optimized AAV transduction of endothelial cells, AAV2 and AAV6 were the most efficient serotypes for and Dulbecco's Modified Eagle's Medium (DMEM) enhanced AAV2 transduction. Our aim to achieve cell-type specific expression of AAV continues. The endogenous functional activity of the different elements within a super-enhancer was analysed, which led to the conclusion that the enhancers E1 and SE2 both regulate the expression of the neighbouring gene hAdamts18. Enhancer elements have the potential to drive cell-type expression and therefore should be useful in the context of AAV vectors.

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