Developing a gene therapy for the genetic blood clotting disorder: Von Willebrand Disease

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Layman's summary

Von Willebrand Disease (VWD) is the most common blood clotting disease, effecting around 1% of the population (1). VWD is caused by mutations within the Von Willebrand Factor (VWF) gene. VWF is a protein involved in blood clotting and provides an interaction between the blood clotting factors and the site of injury. Insufficient VWF production can lead to symptoms ranging from mild bleeding from minor wounds to severe life-threatening bleeding episodes depending on the amount of VWF produced. Current treatment methods involve regular injections of plasma concentrates which contain the VWF protein to stop the bleeding. An alternative would be to produce a gene therapy which introduces a working gene into cells to compensate for the malfunctioning gene and produce long term functional proteins for blood clotting. This project will develop viruses which will carry a functional gene into cells to produce functional VWF proteins. The VWF gene will need to be split between three viruses to allow them to be carry the gene successfully into cells due to the genes overall large size. After development of these viruses, they will be tested on cells, firstly, to determine successful functional VWF protein production and will then be injected into VWD disease mouse models which mimic symptoms of the disease. If successful, the time taken to stop bleeding in the gene therapy treated VWD mouse should be comparable with a healthy mouse.

Background

Von Willebrand Disease (VWD) is a monogenic blood clotting disease, caused by mutations within the Von Willebrand Factor (VWF) gene (figure 1). Mutations within the VWF gene can cause a range of phenotypes from: prolonged bleeding from minor wounds, due to a partial deficiency in VWF production as seen in type 1 VWD patients, to severe bleeding episodes as seen in type VWD 3 patients, who produce no functional VWF. For these type 3 VWD patients, treatment involves regular injections of plasma concentrations containing the VWF protein as a preventative measure, which, even with stringent screening processes, contains a risk of blood borne infection (2).

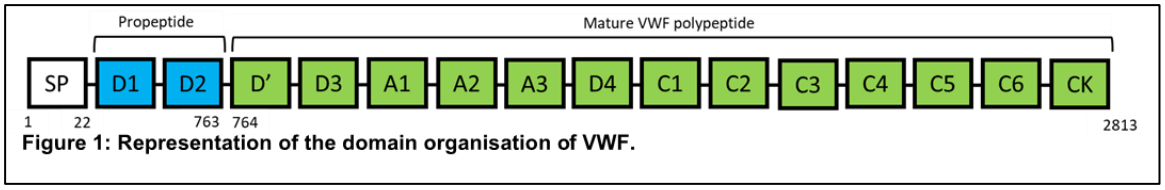

Figure 1: Representation of the domain organisation of VWF.

VWF is essential in haemostasis binding to fibrillar collagen and extracellular matrix proteins when the subendothelial matrix becomes exposed after vascular injury occurs. Once anchored, VWF tethers to platelet GPIbα receptors via its A1 domain, causing platelet arrest at the site of injury and the subsequent activation of the platelets (1). Aggregation of platelets can then occur, and the formation of thrombi can begin to arrest bleeding. So, with no functional protein produced type 3 VWD patients cannot produce this interaction between platelets and the site of vascular injury, leading to excessive bleeding as thrombi cannot be formed.

VWFs haemostatic properties are increased when it exists in a multimer state. Multimerization occurs within the *trans*-Golgi, after the propeptide (domains D1 and D2 – as seen in figure 1) is cleaved from the mature VWF polypeptide by furin. The propeptide then acts as a disulphide isomerase catalysing disulphide bond formation between the D3 domains of the mature VWF polypeptides producing multimers. It has been demonstrated that VWF function decreases proportionally to its size, demonstrating the importance of the production of high molecular weight multimers (HMWM) (3).

Current treatment methods have undesirable features (repeat injections) and risks, one alternative to overcome this would be to create a gene therapy for VWD. VWD is both an ideal and challenging candidate for gene therapy. VWD is a monogenic disorder so the reintroduction of a functional gene should correct the defective phenotype. However, the coding region for VWF exceeds 8.4 kilobases and approaches the carrying capacity of most viral vectors used in gene therapy. The ideal candidate as a viral vector for a gene therapy is the adeno-associated virus (AAV). Compared to WT AAV, recombinant AAV (rAAV) does not undergo integration into the host DNA and remains episomal to the nucleus. The risk of random integration events remains as low as 0.1%, which is the lowest of all viral gene therapy vectors (4), making AAV the safest viral vector. It also has the ability to transduce mitotic and post-mitotic cells, produces a low immune response and long-term expression. However, the biggest limitation of AAV is its limited packaging capacity, with a maximum capacity of 5 kilobases. So, a gene vector needs to be designed to fit this capacity.

Other unsuccessful attempts have been made to create a gene therapy for VWD: for example, a lentiviral vector was shown to produce a complete correction of the bleeding VWD phenotype, however, this was only seen within 1/3rd of mice (5). This lentivirus can also integrate randomly into the host genome, making it a less ideal candidate for gene therapy. In previous experiments performed within this laboratory a truncated VWF vector was produced with the D4 to C6 domains removed to overcome the packaging limitations of AAV. This truncated vector (rAAV-VWF$_{\Delta D4C6}$) was co-transfected with the propeptide present on a separate construct. As demonstrated by Wise *et al.,* the propeptide needs to be present but not attached to the mature VWF polypeptide to produce multimers (6). This methodology produced a significant increase in the VWF protein (figure 2A) compared to control media, however, VWF multimerization was not seen to occur when compared to control reference plasma (figure 2B). It is hypothesised that the unsuccessful multimer formation was due to the truncation of the mature VWF polypeptide which affected its ability to interact with the propeptide and form HMWM.

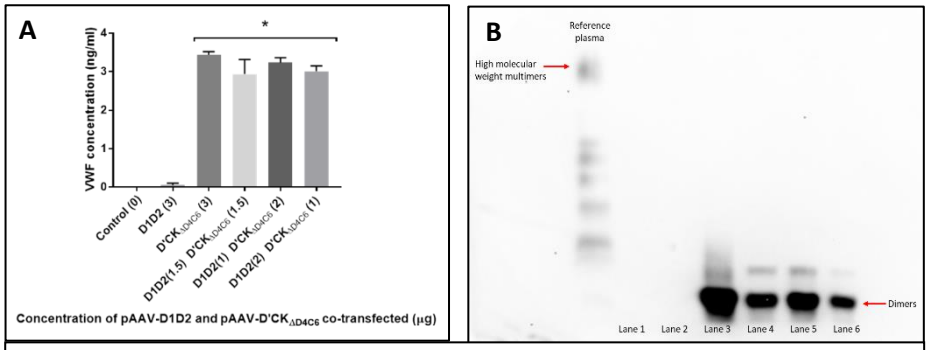

Figure 2: (A) Co-transduction of rAAV-D1D2 and rAAV-VWF$_{\Delta D4C6}$ into HEK293T cells (B) Multimer gel assay of co-transfected rAAV-D1D2 and rAAV-VWF$_{\Delta D4C6}$ cell lysate. Lane 1: control with no added plasmid, Lane 2: 3µg of rAAV-D'CK$_{\Delta D4C6}$, Lane 4: rAAV-D1D2 (1.5µg) and rAAV-D'CK$_{\Delta D4C6}$ (1.5µg), Lane 5: rAAV-D1D2 (1µg) and rAAV-D'CKΔ$_{D4C}$6 (2µg), rAAV-D1D2 (2µg) and rAAV-D'CK$_{\Delta D4C6}$ (1µg).

The design of the vectors within this research project will follow on from this work using the propeptide construct that was produced for these previous experiments. The truncation of the VWF gene will be removed and a hybrid dual vector will be produced splitting the full length mature VWF polypeptide between two vectors. This will overcome the packaging limitation of AAV and should produce a full length vector *in vitro*. This will be possible as the vector wil be designed to recombine using either trans-splicing where inverted terminal repeat (ITR) sequences within AAV recombine readily intracellularly to form circular concatemers (7). The vector will also contain an overlapping region which will allow homologous recombination to take place between the two vectors, this will occur via the overlapping AP gene fragment which is highly recombinant (8). Once the vectors have been recombined, the overlapping sequences and ITRs will be spliced out during transcription and produce the full length mRNA. This technique has been shown to produce a 80% transduction efficiency compared to a single vector and has successfully transduced 50% of heart cells when designed to treat dystrophic cardiomyopathy(9). The propeptide, on a separate plasmid, should then interact with the full length mature polypeptide to produce HMWM.

The hypothesis is that when the three rAAV-VWF constructs are co-transduced into *in vitro* and *in vivo* models the 5' and 3' rAAV-VWF vectors will recombine to form a full length mature VWF polypeptide via trans-splicing or homologous recombination. The rAAV-D1D2 vector will produce the propeptide protein which will act as a disulphide isomerase on the full length mature protein to produce HMWM. Within the type 3 VWD mouse model this will restore HMWM VWF protein production, which will be sufficient to arrest bleeding.

Scientific methodology

Aim 1: Successful development and design of rAAV-VWF constructs to fit within AAV vector packaging constraints: The rAAV-D1D2 vector was previously created within this laboratory (figure 3). The 5' and 3' VWF-AAV constructs will be produced using the NEBuilder HiFi assembly method. Forward and reverse primers will be designed to amplify each specific element from their respective plasmids (as described in red, figure 3) using polymerase chain reaction (PCR), which will isolate each specific vector fragment, including the split within the VWF gene. The primers will also introduce an overlapping sequence which will be used for the final assembly of the vectors. PCR reactions will be separated by agarose gel electrophoresis to isolate each element and excise bands of the correct size, which will be purified using *E.coli* transformation. The backbone pAAV-GFP plasmid will be replicated via transformation into *E.coli* cultures and Midi-prepped for purification. The backbone will then be digested with an appropriate restriction enzyme to open the backbone ready for insertion. A NEBuilder HiFi Assembly Kit will be used to assemble the overlapping sequences for each specific element producing the assembled constructs. The correct assembly will be confirmed by sequencing the vectors after further purification of the constructs. An identical vector will be produced containing a split GFP

gene instead of the VWF gene. This will then allow confirmation of trans-splicing or homologous recombination between the two vectors, as HEK293 cells will fluoresce green under a fluorescence microscope if the two vectors have successfully spliced together after co-transfection.

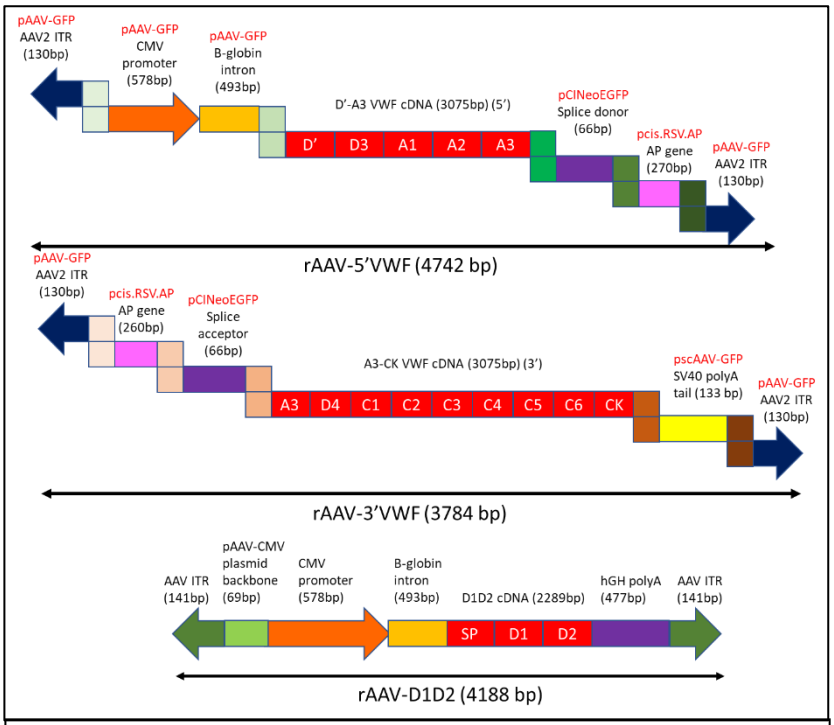

Figure 3: Vector design of the three separate constructs: rAAV-5'VWF, rAAV-3'VWF and rAAV-D1D2. The coloured blocks represent the overlapping regions used within the production stage. The red text indicates the plasmid each fragment is isolated from.

Aim 2: Successful production, packaging and purification of the three rAAV-VWF constructs: The rAAV-VWF plasmids will be separately transfected into stable HEK293 cells to produce the virus. The HEK293 cell line will be used as it is highly transfectable. Due to the limited packaging capacity of AAV, essential replication genes have been removed and will be co-transfected on separate plasmids during the virus production stage. Each plasmid will be co-transfected with a pRC2 plasmid (containing the AAV *rep* and *cap* genes which are essential for viral packaging) and a phelper plasmid (containing helper genes from the adenovirus which mediate AAV replication) in a ratio of 1:1:1 using the standard transient triple transfection technique. This will produce an AAV2 construct which will be used for *in vitro* experiments as they transfect HEK293 cells more efficiently. After liver promoter optimisation, AAV8 constructs will be produced as they have a superior transduction efficiency in hepatocytes. This will be achieved using the same technique with the plasmids pAAV8.2 and pAdΔF6. This transfection will occur in an opti-MEM media (a reduced media) which will allow the transfection of the three plasmids into the HEK293 cells. This media will be replaced after 4 hours to MEM (supplemented with amino acids, penstrep and foetal calf serum) to allow virus replication without killing the cells. After incubation at 37°C for 48 hours, the cells will be harvested using EDTA and the rAAV-VWF constructs will be extracted from the cells using a freeze-thaw technique.

This crude AAV lysate will then need to be purified to increase its transduction efficiency. This can be achieved using the optimised CsCl gradient purification protocol as designed by Ayuso *et al.* (10). Purification and packaging of the produced AAV vectors will be assessed using a Coomassie protein stain (GelCode Blue safe protein) after running the AAV purified vectors on a standard SDS-PAGE gel. This will allow the separation by size of the proteins present in the lysate and if successfully purified and packaged there should only be three clear bands present at 63 kDa, 73 kDa and 87 kDa.

Aim 3: *In vitro* assessment of rAAV-VWF constructs to establish successful and functional VWF production: The rAAV-VWF constructs will then be co-transduced into HEK293 cells to assess their ability to create functional VWF *in vitro.* Different concentrations of the three plasmids will be co-transduced to assess the optimised ratio for transduction. 2ml of HEK293 cells will be split into 6 wells plates and grown for 48 hours at 37°C in MEM (supplemented with amino acids, penstrep and foetal calf serum). After 48 hours the HEK293 cells will be co-transduced with varying ratios of the three rAAV-VWF vectors and incubated for a further 48 hours at 37°C. 1ml of media will be harvested and the cells lysed with lysis buffer (50 mM Tri-HCl, 150mM NaCl, 1 mM EDTA, 1% v/v Tritron X), centrifuged at 13000 rpm for 7 minutes and the pellet discarded.

An ELISA will then be performed to quantitatively evaluate the production of VWF at each co-transfection ratio. An ELISA will be performed using standard techniques with a dilution of 1:1000 polyclonal rabbit anti-VWF (Dako, Cat no. A0082) and detected using 1:1000 Anti-VWF- horseradish peroxidase (HRP) (Dako, Cat No. P0226), and will be quantified using the SIGMAFAST OPD tablets producing a colour change which can measured via the absorbance using a plate reader set to 492 nm. ANOVA tests will be performed between the different transduction ratios to determine the optimum.

A multimer assay will be used to determine if HMWM are produced by the HEK293 cells by qualitatively assessing the distribution of VWF multimers depending on their size, as seen in figure 2B. A multimer gel will

be prepared (2.24g Tris, 0.75 glycine, 1.2% SeaKem HGT agarose and 0.1% SDS dissolved in deionised water at pH9) and run on a non-reducing SDS-PAGE in multimer gel running buffer (100 mM tris, 150 mM glycine and 0.1% SDS) with samples diluted 1 in 10 in multimer buffer (10 mM Tris-HCl and 5mM EDTA at pH 8 and 3% SDS, 10% glycerol and 300µg/ml Bromophenol Blue). Samples are run at 60 V for 20 minutes and then 35V for 4 hours. The multimer gel is then equilibrated in 10mM dithiothreitol for 10 minutes and blotted by standard techniques onto a nitrocellulose membrane. The membrane is blocked using skimmed dried milk powder, stained with Anti-VWF-HRP, visualised using Immobilon Western HRP substrate and imaged using the iBright Imaging system.

The co-investigator Prof. Amaia Cadiñanos-Garai will screen and optimise the enhancer and promoter combinations before the *in vivo* application. Furthering her research, tests on the optimised vectors' protein production will also be assessed within this laboratory, repeating the earlier experiments as described above (ELISA and multimer assays) to confirm there is no damaging effect of the new enhancer/promoter combination on the actual protein production.

Aim 4: Pilot *in vivo* assessment of rAAV-VWF constructs within VWD disease mouse models to determine any corrections in VWD phenotypes: VWF knockout mice ($VWF^{-/-}$) with a C57BL/6J background as well as wild type (WT) C57Bl/6J mice (The Jackson Laboratory, USA) will be used for the *in vivo* experiments. These mice have the neomycin cassette inserted into intron 5 of VWF, disrupting VWF production. 5 mice will be used for each group as this is a pilot study to assess the viability and capability of the rAAV-VWF constructs. Four groups will be used: Control ($VWF^{-/-}$, no injection), Sham ($VWF^{-/-}$, injection of saline), Treatment ($VWF^{-/-}$, injection of rAAV-VWF), WT C57Bl/6J (positive control). These mice models are essential and comparisons will be made between the different groups to understand the effects of the rAAV-VWF constructs on VWD phenotypes.

The rAAV-VWF vectors will be administered by hydrodynamic gene transfer which is efficient in the *in vivo* transfection of liver cells and causes no long-term damage to the mice. This technique produces increased dynamic force which causes increased permeabilization of the plasma membranes of parenchyma hepatocytes allowing the DNA to enter the cells (11). This will be performed as previously described by De Meyer *et al.* (12) on 4 week old mice, using a 27-gauge needle to inject a dose of the vector diluted in 0.9% saline via the tail vein, after the mouse has been anaesthetised with isoflurane.

Bleeding times will be assessed within the mice using a tail clip assay to determine if there has been a correction in the bleeding phenotype associated with VWD. Using the protocol stated by Wang *et al.* (5), tails will be cut 1cm from the terminal tip and immersed in warmed PBS. The bleeding time will be recorded as the time taken for complete cessation of blood flow. If mice do not stop bleeding in 8 minutes they will cauterised to prevent further blood loss. A comparison will then be performed between $VWF^{-/-}$ and WT mice. This assay will be completed before rAAV-VWF administration, 3 weeks, 8 weeks and 12 weeks post injection.

Mouse blood will be collected pre-injection to determine no VWF production and then 3 days, 1 week, 2 weeks, 4 weeks, 8 weeks, 10 weeks and 12 weeks post injection. The mice will be anaesthetised using isoflurane and blood samples will be taken via retro-orbital puncture. This blood will be used for ELISAs (which will quantify VWF production) and multimer assays (confirm HMWM formation) as described above. The VWF concentrations and HMWM production of the positive control (normal VWF), control (no VWF) and treatment groups will be compared to quantify and evaluate VWF production *in vivo.*

After 12 weeks, the mice liver will be harvested for immunohistochemistry assessment to determine the localisation of VWF expression to the liver cells. Using a protocol developed by Portier *et al.* (13), the liver will be fixed with paraformaldehyde, blocked and stained with a polyclonal rabbit anti-human VWF antibody. After washing, it will be stained with fluorescein isothiocyanate labelled swine anti-rabbit Ig antibody to allow visualisation of VWF. Slides will also be mounted using Prolong Gold antifade with DAPI to determine the nucleus within each cell and will be visualised using a Scanning Confocal Microscope.

Contribution of the Co-investigator: Screening of three hepatospecific promoters for VWF vector Professor Amaia Cadiñanos-Garai. With a PhD and post-doc stay in the field of gene therapy and regulation of gene expression, I am currently working in the development of an adeno-associated virus (AAV) for the treatment of Wilson's Disease (14). With my experience in vector development, I will assist Professor Kozlowski´s project in the generation of a hybrid dual AAV virus for the treatment of VWD. My specific role will be to guide the screening of different enhancer-promoter combinations that will direct liver specificity of the therapeutic vector.

VWF is primarily expressed in endothelial cells; however, the high resistance these cells show to transfection drives the possibility of targeting VWF expression in an alternative way. The liver, due to its high protein expression profile, has been widely targeted by a variety of gene therapy vectors. Alongside, several literature sources report that VWF is also expressed, and in high level, in the liver (12), suggesting that targeting hepatocytes and inducing VWF expression and secretion in them could be a promising approach.

During the first stages of the project, CMV promoter will be used, mainly due to its high expression inducing profile. CMV is a widely known promoter, which is commonly used at initial stages of vector development. However, inactivation events, organ inspecificity and low liver expression levels in *in vivo* models counter-indicate its use. With this in mind, once the actual construct is optimized we aim to elect an alternative promoter that induces protein expression to levels comparable to those induced by CMV in a hepato-specific manner. To this purpose, the efficiency of three chimeric constructs will be screened; Ealb-AAT, EII-AAT and α-antitrypsin (AAT) (5). They all contain α-antitrypsin promoter, which regulates the expression of a serum proteinase inhibitor, synthetized in the liver and widely used in liver directed gene therapies (14). Two of the promoters contain specific enhancer regions which will further induce protein expression and liver specificity. EII is the human hepatitis B virus enhancer, known to drive the liver specific expression. Evenly, albumin enhancer (Ealb) is also known to induce hepato-specificity. With these two enhancers and the virgin promoter, we aim to design a highly specific, efficient and translationally possible construct. Firstly, using specific restriction enzymes, the chimeric promoter combinations will be cloned into our vector. After sequence confirmation, huh7 human liver cell line transfection will be performed and levels of VWF's compared between transfected and non-transfected cells by western-blot using specific anti-VWF antibodies. In parallel, non-hepatic cells will be transfected to ensure vectors hepato-specificity. Transfection conditions will be optimized, and cell viability tested using trypan blue. If constructs show hepato-specificity and ability to induce VWF expression, viral particles will be produced and huh7 cells infected. Protein expression levels from the *in vitro* model will be compared to the pilot *in vivo* study and based on these data a specific construct chosen. Guaranteeing a highly efficient promoter and high expression levels of our protein, will significantly influence the translational value of this vector, as it will enable the use of less vector units, which will reduce the potential side effects of introducing an exogenous molecule into the organism.

Programme of work

The estimated time of completion of this grant is 24 months. Milestones correspond to each aim within the scientific methodology section. The shaded sections represent the time in which each milestone should be completed in.

Milestone	Period of time (months)					
	4	8	12	16	20	24
Vector development	■					
Virus development and purification		■				
In vitro testing and optimisation			■	■		
In vivo testing					■	■

Costing

		Cost (for 24 months)
Staff	1 post-doc research associate	£111,408
Equipment	None – all supplied within the current laboratory	£0
Consumables	Laboratory consumables - £15,000 per year (cell biology)	£60,000
	- £15,000 per year (molecular biology)	
	Animal study costs - £5000 for required number of mice	£5900
	- £600 for cage and upkeep (14 weeks for 20 mice)	
	- £300 anaesthetic and other incurred mouse costs	
Overhead	Staff cost x 1.3	£144,830.40
Total		**£322,138.40**

Justification of resources

A post-doc research associate will be hired for the 24-month period, with experience in the molecular and cellular techniques used as well as animal handling experience. This will allow the project to be completed within the time frame, limiting time occurred costs. The haematology laboratory has previous experience working on molecular and cell biology concepts and therefore already contains the large equipment required e.g. PCR MultiGene Optimax Thermal Cycler and Scanning confocal microscope etc. therefore incurring zero cost. Laboratory consumables will incur a significant cost as many different techniques will be used, as discussed within the scientific methodology section. The different cell lines used, antibodies, kits for vector development, restriction endonucleases etc. will need to be purchased over the two years. Due to only a pilot

study being run for the *in vivo* experiments, animal costs have been limited due to the small sample size. However, the cost of the $VWF^{-/-}$ mice are expensive due to the company prices for ordering this specific strain of mouse. Also, due to the increased number of times the mice need to be anaesthetised (every time blood is taken, and the bleeding times are assessed), costs have to be considered for renting the procedure room as well as buying the anaesthetic.

Timeliness and novelty:

The recent success of other AAV gene therapies for the blood clotting disorders Haemophilia A and B demonstrate the timeliness of this project. Haemophilia A and B AAV gene therapy human trials are currently underway and have produced significant improvements to the patients within the trials (15,16). Following this success, the development of further AAV therapies is certain. So, the production of an AAV VWD gene therapy, is the obvious next step as VWD is more common within the population then Haemophilia. The novelty of this research is shown within the development of the AAV gene vector. Even though dual hybrid AAV vectors have been used in other gene therapy construct design, a successful triple plasmid co-transduction with hybrid dual vector production is a novel technique. This will also be novel within VWD gene therapy research as only one previous experiment has used AAV vectors and the project was abandoned due to poor results (5).

Impact

If this research is successful, the technique developed can be applied by other academics to develop further gene therapies for genetic disorders which are currently unsuccessful due to their larger gene size. This will also be beneficial for patients of monogenic disorders as further innovation within the gene therapy sector should occur and new gene therapies should be developed for previously untreatable conditions. The development of a gene therapy for VWD will produce a positive social and economic impact as patients will only require a single administration of the gene therapy vector occurring a one-off cost and decreasing time in hospital or doctor surgery's. This will increase the quality of life for type 3 VWD patients as the risk of severe bleeding episodes will be decreased and their time within hospital environments will be reduced. Running patient engagement programs to update type 3 VWD patients on the progress being made on the gene therapy experiments will provide interaction and education to the public and increase optimism to the VWD community about future treatment options.

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