Research Article

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Evolution and Production of a Clinical-Grade Adenovirus Expressing the Transgene Tissue Inhibitor of Metalloproteinase-3 (TIMP-3) Through Genome Engineering

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Abstract

Background: First-generation human adenoviral serotype 5 (HAdV5)-based vectors remain one of the most widely utilised gene transfer vectors for both experimental gene transfer and translational development for vaccine delivery and clinical gene therapy. We previously performed non-Good Manufacturing Practice (GMP), laboratory-grade production of a first-generation, replication-deficient vector expressing Tissue Inhibitor of Metalloproteinase-3 (TIMP-3) as a gene therapy approach to prevent vein graft disease following coronary artery bypass grafting. We have shown in pre-clinical in vitro, ex vivo and in vivo studies that adenoviral gene transfer of TIMP-3 reduces vascular smooth muscle cell (VSMC) proliferation, promotes VSMC apoptosis and reduces neointima formation in human saphenous vein ex vivo and in a pig vein graft model in vivo. Our next step was to translate HAdV5-TIMP-3 to the clinic; however, adenoviral-mediated TIMP-3 over-expression can be cytotoxic in multiple cell types. In this study, we describe the vector which led to successful production of a GMP batch of vector for a Phase 1 first-in-human clinical trial.

Methods and Results: Initially, a low passage seed stock of a plaque-purified clone of HAdV5-TIMP-3 produced in either the pAdEASY system or based on the pJM17 system were compared and assessed in GMP production in a standard clinical grade 293 cell batch. Both vector configurations were assessed by PCR, Sanger DNA sequencing, and western blotting and immunofluorescence for TIMP-3 expression following transduction of HeLa cells. Transfer of the vector from the laboratory to standard clinical grade manufacturing protocols led to loss of TIMP-3 expression and the emergence of contaminating HAdV5 variants with different transgene configurations, including reversion of E1 DNA sequence. Loss of TIMP-3 expression, vector mutations and sequence rearrangements were observed in multiple clones from each configuration by vector passage 5-6. Next, three strategies were undertaken to facilitate vector production at clinical grade: (i) use of a less-active cytomegalovirus immediate early promoter (CMVIEP), (ii) reverse orientation of the expression cassette to reduce TIMP-3 expression levels and (iii) incorporation of tetracycline (Tet) operator sequences to silence TIMP-3 expression in 293 Tet Repressor (TetR) helper cell lines. Strategy iii resulted in successful completion of ten stable passages of vector amplification in commercial 293 T-Rex cells as evidenced by stable AdTIMP-3 batches (HAdV5.CMV.TO.TIMP-3) with expected DNA sequence and equivalent expression levels of TIMP-3 relative to the original non-GMP HAdV5-TIMP-3. AdTIMP-3 production over 10 passages in GMP-grade 293TetR cell lines was sufficient to maintain expected genome configuration assessed by PCR and sequencing, low particle: infectious unit ratios and stable TIMP-3 expression by western blotting. Transfer of the pre-master viral seed stock of HAdV5-CMV.TO.TIMP-3 to clinical grade manufacture produced a high titre batch of HAdV5.CMV.TO.TIMP-3 which passed QP (qualified person) release and was approved for use in a first-in-human clinical trial to assess its utility in preventing saphenous vein graft disease.

Conclusions: In summary, transfer of experimental batches of first-generation HAdV5 vectors expressing TIMP-3 into clinical grade manufacture required silencing of TIMP-3 expression during the multi-passage scale-up required. Tetracycline regulation is sufficient to achieve this only where expression is completely silenced and can be achieved in GMP-certified 293TetR cell lines.

Introduction

Gene therapies, especially those utilising adenoviral vectors, have seen a remarkable rise in clinical adoption, and have broadly demonstrated excellent safety and efficacy, particularly in the fields of oncology [1,2] and in vaccine development towards COVID-19 [3-5]. Unfortunately, adenoviral vectors have seen limited adoption towards cardiovascular disease (CVD), despite them remaining the largest cause of death worldwide [6]. Although viral gene therapy for CVD is yet to manifest, several important clinical trials utilising adenoviral vectors have provided promising evidence that CVDs such as angina can be treated using gene therapy [7-10].

First generation human adenoviral serotype 5 (HAdV5) vectors remain one of the most widely utilised gene transfer vectors for both experimental gene transfer and translational development for vaccine delivery and clinical gene therapy. Production of HAdV5 at laboratory scale in the widely used adherent HEK293 cell line is robust and reproducible and widely performed utilising commercial kits, with generation of recombinant HAdV5 vectors typically performed using approaches such as the pAdEASY system [11], although alternative producer cell lines including PER.C6 [12] are also used. Translation of experimental HAdV5 vectors into clinical grade products requires optimisation and development of protocols able to produce vector batches of high quality and at high titres with acceptable particle/ infectious unit ratios, using serum-free suspension, GMP-grade HEK293 cells or alternative GMP-certified producer cell lines and GMP compliant raw materials typically not used in R&D. While this has been achieved for a range of HAdV5 vectors expressing different transgenes, including the COVID-19 spike protein [5], and tumour necrosis factor alpha and interleukin-2 for the treatment of ovarian cancer [1,13], with successful progression to clinic, there have been previous reports that for HAdV5 vectors expressing toxic transgenes, clinical scale up and production have proved extremely challenging [14].

Coronary artery bypass graft (CABG) surgery is an interventional procedure performed to alleviate angina and mitigate the risk of myocardial ischaemia in patients with advanced, multi-vessel atherosclerosis. CABG is commonly-performed, with more than 14,000 surgeries performed annually in the UK [15] and more than 400,000 annually in the USA [16,17]. During CABG surgery, a vessel is removed, most commonly the great saphenous vein, and an autologous transplant performed to bypass the coronary artery occlusion. Clinical studies have demonstrated that 10-20% of CABG surgeries fail in the first 12-18 months post-surgery [18-20], and even 40-50% in 5-10 years post-surgery [21-23]. CABG failure, reflected by recurrent angina or myocardial infarction, may be managed by percutaneous coronary intervention or medical therapy, whereas re-do CABG is rarely undertaken due to the risk of sternotomy damaging the underlying arterial graft [24-28]. In particular, these secondary surgical interventions after a failed first-time CABG present with poor clinical outcomes [29-32] and an increased economic stress to healthcare systems, emphasising the need for novel therapeutic strategies.

CABG failure is a complex, multi-factorial process, however, phenotypic switching of vascular smooth muscle cells (VSMCs) from a quiescent to "synthetic", pro-migratory and proliferative, state is a well-established pathological process leading to CABG failure [33,34]. Synthetic VSMCs secrete matrix-degrading metalloproteinases (MMPs), a family of enzymes important in the progression of vascular disease and neointimal hyperplasia through their ability to degrade extracellular matrix [35-38], facilitating further VSMC migration and proliferation. Migrating, proliferative VSMCs eventually invade the intimal layer of the vasculature, where they occlude the grafted vessel though the progressive thickening

of the intima (neointimal hyperplasia). Left untreated, neointimal hyperplasia reflects an accelerated atherosclerosis-like process which results in a re-occurrence of symptoms in the patient that requires further intervention [39,40].

MMP activity is governed by a family of endogenous inhibitors, known as Tissue Inhibitor of Metalloproteinases (TIMPs). We have previously demonstrated in both an *ex vivo* human saphenous vein model [41] and porcine CABG model [41,42] that overexpression of TIMP-3 via first generation human adenovirus serotype 5, (HAdV5-TIMP-3) results in substantially reduced neointima formation through inhibiting VSMC migration and proliferation, and promoting apoptosis, suggesting that gene transfer of TIMP-3 via adenoviral-mediated delivery could be a promising strategy to prevent saphenous vein graft failure. These promising pre-clinical results prompted us to embark on clinical manufacturing of HAdV5-TIMP-3.

Suppression of toxic transgenes during viral propagation has been reported previously [14,43,44], and various strategies to silence transgene expression during scale-up have been developed. These findings illustrate the challenges encountered when scaling-up production of vectors to larger volumes in preparation for clinical studies, thus providing lessons which could be valuable for researchers wishing to progress to clinical studies utilising viral vectors overexpressing transgenes that are cytotoxic in the producer cell line. Moving from preclinical experiments to clinical studies requires large-scale (typically 50-250 Liter) manufacture and multiple passaging of the vector to produce Master Viral Seed Stocks (MVSS), Drug Substance and Drug Product.

Here, we describe the results of diverse vector design studies and demonstrate that we solved the challenges we encountered and manufactured HAdV5-TIMP-3 to clinical grade and scale for a first-in-human clinical trial (ISRCTN43650325).

Materials and Methods

Cell lines used

MAX Efficiency Stbl2 Competent Cells (Life Technologies, Catalogue #10268-019 Lot #1521023), HeLa cells, HEK293 (Life technologies Catalogue #11631-017 Lot #906940), HEK293-TetR (Trex) (Life technologies, Catalogue #51-0012 Lot #1264787), Procell92.S (GSK plc, London, United Kingdom).

Cell culture

HEK293 and HEK293-TetR cells were cultured in Dulbecco's Modified Eagle Medium (Life technologies, Catalogue #11995 Lot #1459826) supplemented with non-heat inactivated (gamma irradiated) fetal bovine serum (Gibco, Catalogue #10101-145), 200 mM glutamine (Gibco, Catalogue # 25030-024), and blasticidin (Life Technologies, Catalogue #46-1120 (R210-01).

MAX Efficiency Stbl2 Competent Cells were cultured in LB broth or LB agar (Soy peptone, Organotechnie, Catalogue #19649, Yeast extract, BioSpringer, Catalogue #0203/O-PW-L, NaCl, Merck, Catalogue #116224), supplemented with 50 mg/mL kanamycin (Gibco, catalogue # 11815-024).

Procell-92.S

The pre-GMP Procell-92.S research-grade cell bank frozen at passage 37 (p37) was prepared at Okairos Srl (Naples, Italy). The p37 research-grade cell bank originated from the isolation and further propagation of cells from human embryonic kidney (HEK-293) cells. The Procell-92.S master cell banks (MCBs) were prepared in GMP conditions at Advaxia Biologics (formerly Advent) (Pomezia, Italy). The Procell-92.S MCB lot #A.0005 frozen at passage 44 (p44) was prepared by further expansion from the p37 research-grade Procell-92.S cell bank. Due to limited quantities of the p44 MCB, a second Procell-92.S MCB lot #C.0013 frozen at passage 50 (p50) was prepared by further expansion from the p44 Procell-92.S MCB lot #A.0005. Cells were grown in CD293/L-Glutamine growth medium and frozen in DMSO-containing growth medium. No animal-derived raw materials were used in the manufacture.

Preparation of pAdeasy plasmids for transfection

One plaque pure clone of HAdV5-TIMP-3 produced using the pAdEasy system or the pJM17 system [45,46] was provided by University of Glasgow. Clones were confirmed

to be free of replication-competent adenovirus using 96 well plate plaque dilution assay in HeLa cells [47]. Each well was assessed daily for 8 days for the appearance of viral plaques with none observed (data not shown). Additionally, infection of seed stocks into HeLa cells with daily assessment for 14 days revealed no observation of emerging bacteria, fungi, or yeast infection (data not shown). Mycoplasma was assayed using Minerva Biolabs VenorGeM PCR kit with Promega GoTaq and was observed to be negative (data not shown). The other variants (pAdEasy.CMVTIMP-3REV, pAdEasy.SHCMVTIMP-3 and pAdEasy.CMVTOTIMP-3) were prepared at Batavia Biosciences (Leiden, The Netherlands). The plasmids were introduced by transformation into Stbl2 bacterial cells (Life Technologies, #10268-019), by the heat shock principle. The transformed Stbl2 cells were subsequently grown on animal component-free LB agar (Soy peptone, Organotechnie Product #19649, Yeast extract, BioSpringer, Product #203/O-PW-L, NaCl, Merck, Product #116224) containing 50 µg/mL kanamycin (Gibco, Product #11815-024, Lot #1440929). One single colony was picked and subsequently grown up to a 3 mL culture by which the plasmid was checked to have contained its original form by miniprep procedure. After confirmation, this 3 mL culture was subsequently used to perform a streak on animal component-free LB agar containing 50 µg/mL kanamycin. This streak was repeated one more time. After the second streak a maxiprep culture (NucleoBond Xtra Maxi Plus EF Macherey-Nagel, Product #740426.10) was inoculated using animal component-free LB broth containing 50 µg/mL kanamycin. The plasmid was subsequently isolated using an Endotoxin-free maxiprep kit (Macherey-Nagel, # 740424.50). The RNAse present in the kit was not used as it is of animal origin; instead, an RNAse T1 derived from the fungus *Aspergillus oryzae* was used (Roche, Product #10109193001). Sequences of the obtained plasmid DNAs were confirmed by restriction enzyme analyses (*Bgl*II, *Pac*I *Xba*I, *Not*I, *Nco*I). Before transfection, the pAdeasy plasmids were digested with *Pac*I restriction enzyme and subsequently cleaned by chaotropic salt treatment. After digestion by *Pac*I two fragments are expected for each vector: ~31 kb and 4.5 or 2.9 kb depending on the origin of recombination in the bacteria used to generate the full adenoviral genome. If the recombination occurred between left arms of the adenoviral sequence, 2.9kb should be visible. The presence of the fragment ~ 4.5 kb evidences the recombination at origin of replication.

Isolation, propagation, purification and titration of adenoviral vectors

The HAdV5.CMVTO.TIMP-3 vector was generated by the transfection procedure. In brief, the obtained plasmids were linearized using BSA-free *Pac*I restriction endonuclease and subsequently cleaned by chaotropic salt treatment (Nucleospin Extract II kit, Macherey-Nagel) and incubated for 20 minutes at 65°C. A total of 4 µg linearized plasmid was used in the DNA mixture prepared in DMEM (Gibco, Product #11995-065). Lipofectamine 2000 (Invitrogen, #11668-019) was added to the DNA mixture at a DNA:Lipofectamine 2000 ratio of 1 µg:10 µL and incubated at room temperature for 30-40 minutes. HEK293 or HEK293-TetR cells seeded in a T25 flask one day prior to transfection, were washed with DMEM and the transfection mixture was incubated on the cells for 4 hours at 37°C and 10% CO_2. Then, the transfection mixture was removed, and culture medium was added. After incubation at 37°C and 10% CO_2 for 2 days, the transfected cells were passaged to a T75 flask and incubated until the development of cytopathic effect (CPE). As CPE development did not progress to full CPE, 3 mL of cells from the T75 flask were passaged to a new T75. Four days after the passage, full CPE was obtained and the total crude material was harvested, stored at -20°C and subsequently used for reinfection of HEK293/HEK293-TetR cells seeded in a T75 flask one day prior to the reinfection. By the reinfection, full CPE was obtained at day 2 past infection and the total crude material was harvested and stored at -20°C. This material was used to perform the first plaque purification. In total 10 plaques were picked and propagated on HEK293/HEK293-TetR cells seeded in a 24-well plate format.

Plaque purification and stability testing

For each vector, 3 plaques were selected for sequential passages in T25 flasks up to passage 10. After sequential passage 6 and 10 the 3 selected plaques per vector were analysed by transgene PCR for stability

determination and for TIMP-3 expression by western blot analysis. The HAdV5.CMVTO.TIMP-3 vector was purified from the cell pellet by a three-step freeze/thaw cycle and a two-step CsCl centrifugation procedure. The host cell DNA was degraded by Benzonase treatment. The obtained viral aliquots were snap frozen on liquid nitrogen and stored at -80°C.

Viral particle and infectious particle concentration determination

The viral particle (vp) concentration (vp/mL) of the purified HAdV5.CMVTO.TIMP-3 preMVS was determined by OD260 measurement in the presence of 1% SDS. The obtained titre was $1.36x10^{12}$ vp/mL, resulting in a total $2.1x10^{13}$ vp. The infectious particles concentration (TCID50/mL) was determined in duplicate by TCID50 assay on different days using 911 cells. The obtained titre was 10.13 Log10 TCID50/mL (351-088) and 10.48 Log10 TCID50/mL (351-106), which are within the 0.5 Log variance of the assay. The average TCID50 titre is therefore 10.31 Log10 TCID50/mL.

Transgene PCR and sequence analysis

The transgene region integrity was determined by PCR using the proofreading polymerase Phusion (NEB; cat# M0530S) and the transgene region primers TG-F and TG-R (Table 1); located just before the CMV promoter and after the polyA respectively, ensuring entire coverage of the transgene sequence. The input plasmid pAdEasy.CMVTO.TIMP-3 was used as a positive control. The transgene PCR product of expected size was confirmed by sequence analysis.

Vector identity PCR

The vector identity was determined by PCR using the Taq polymerase (Invitrogen; cat# 10342-020), the E3 region primers (E3-F and E3-R) and E4 region primers (E4-F and E4-R) (for primer sequences see appendix A). As positive control the input plasmid pAdEasy.CMVTO.TIMP-3 was used.

DNA extraction

DNA extraction was performed using a Qiagen QiAamp DNA Mini Kit, following the manufacturer's instructions; DNA was eluted in a final volume of 50 µL.

PCR conditions

PCR was performed using Promega GoTaq Polymerase following manufacturer's instructions, using a DNA Engine Tetrad PTC-225 Thermal Cycler. 10 µL PCR was run on 1% TBE gel. For TIMP-3 and pShuttle homologous primers, Promega 100 bp ladder was used to determine product size. For HAdV5-specific primers, Promega 1kb ladder was used to determine product size.

PCR conditions

1. 95°C, 2 minutes
2. 95°C, 1 minute
3. 53°C, 1 minute
4. 72°C, 2 minutes
5. Steps 2-4 repeated for 40 cycles
6. 72°C, 5 minutes
7. 4°C

DNA sequencing analysis

DNA sequencing analysis was outsourced to the University of Glasgow's Polyomics service.

Restriction digest

The genomic structure of P4 and P10 purified viruses was checked by three different restriction patterns (*Sph*I, *Apa*LI/*Bsr*GI, *Sna*BI/*Not*I). HAdV5easyTIMP-3 plasmid was included as control.

SDS-PAGE and western immunoblotting

HEK293 or HEK293-TetR cells were transfected with *Pac*I-digested Ad5.CMV.TIMP-3, HAdV5.CMVTO.TIMP-3, Ad5.SHCMV.TIMP-3, or Ad5.CMV.TIMP-3REV as described to produce viral vectors. Cells were washed 3x with PBS, and 50 µL Laemmli 2x running buffer/well was added, and samples stored at -20°C. 50 µL PBS was added to the cell lysates, and 25 µL sample separated on a 12% polyacrylamide gel (Novex) by SDS-PAGE in MOPS running buffer (Life Technologies) at 170 volts for 70 minutes. Gels were transferred using PVDF membranes (GE Healthcare) pre-soaked in methanol at 300 mA (250 volts) for 2 hours. Membranes were blocked overnight at room temperature in non-fat dry milk (Biorad) made up in PBST on a rolling mixer. TIMP-3 was detected using anti-TIMP-3 rabbit polyclonal primary antibody at 1:500 (cat#AB6000, EMD Millipore) overnight at room temperature and Goat anti-rabbit IgG-HRP (cat# 170-6515, Biorad) secondary antibody at 1:5000

dilution for 2 hours. Blots were washed 3x with PBST, 10 minutes per wash, before being incubated for 5 minutes in Pierce ECL2 solution (Thermo Fisher Scientific #80196). Bands were visualised using a ChemiDoc imaging system (Biorad). Magic Mark (Life Technologies #LC5602) pre-stained marker was used as a marker. As a negative control, lysate obtained from untransduced A549 cells was used.

Purity determination

The purity of the HAdV5.CMVTO.TIMP-3 preMVS was determined by electrophoresis. SDS-PAGE was performed under denaturing conditions, which results in disintegration of the adenoviral particle and separation of the individual proteins that constitute the HAdV5.CMVTO.TIMP-3 vector. Four different amounts ($1x10^9$ vp, $2x10^9$ vp, $3x10^9$ vp and $4x10^9$ vp) were loaded on the gel together with four concentrations of BSA (0.5 µg, 0.25 µg, 0.1 µg and 0.05 µg). The size of the observed protein bands was related to the Novex Sharp Pre-stained Marker migration pattern. The apparent sizes were used to putatively assign the bands on the gel to specific proteins. After instant blue staining (Expedeon; cat# 194-ISB1L), the gel was scanned, and the intensity of the bands were determined by the Gel Pro 6.0 software. BSA was used as an internal marker of known concentration.

High-performance size exclusion chromatography

High performance size exclusion chromatography (HP-SEC) was performed to assess viral aggregation from Ad5.CMVTO. TIMP-3 virus using a Dionex HPLC UltiMate 3000 System and the below protocol.

HP-SEC buffer: 10 mM Sodium Phosphate, 50 mM NaCl, pH 7.00, Milli-Q water 0.45 µm filtered, Column storage solution: 20% Ethanol, Methanol 10 % for HPLC seal wash, Tris - KCl buffer for System Suitability control: 50mM Tris-HCl, 100 mM KCl.

1. System suitability control

Suitability control mix: Thyroglobulin bovine (THG), MW 669 kDa, Bovine serum albumin (BSA), MW 69 kDa, Tris - KCl buffer.

90 µL of Milli-Q water were added into an HPLC vial, and 30 µl of the suitability control mix added and mixed by pipetting up and down.

2. Preparation of samples

Samples and reference standards were homogenized before use by gently pipetting up and down several times. For Test Samples, ~5 x 10^{10} vp were injected. Thw injection volume was calculated based on the vp concentration of the sample as measured by optical density, with injection volumes between 30 and 100 µL most commonly used. The required volume for the injection plus an extra volume of 15 µL were transferred into a HPLC vial. For the reference sample, two injections of 100 µL were required, so at least 230 µL were filled in one HPLC vial. One or two HPLC vials were filled with 1000 µL of filtered Milli-Q water.

3. HPLC system preparation for sample analyses

The inlet of channel A was inserted into the HPSEC buffer, the inlets of channel B and C into Milli-Q water and the inlet of channel D to the column storage solution. The seal wash line was inserted in seal wash solution (10% Methanol solution), and the guard and analytical HP-SEC columns installed. The columns were secured inside the HPLC column compartment. All lines were purged with the corresponding solutions; to equilibrate, the HPSEC buffer was pumped through the column for 30 minutes using 0.8 mL/min flow and pressure monitored throughout.

4. HP-SEC sample set

One Milli-Q water injection of 100 µL was first performed. To confirm the system suitability, 50 µL of the system suitability control was injected before the first and after the last reference standard in the sequence. The reference standard was analysed before and after the Test Samples. The Test Samples were injected in duplicate in each run.

5. Running the HPSEC sample set

The HPLC vials were placed at the corresponding positions in the auto-sampler, and the sequence run started; when finished the analytical column and the guard column were flushed, and finally the system cleaned.

6. Data processing and evaluation

Peaks between 0.1 and 15 minutes of sample chromatogram were integrated (15.5 min is the formulation buffer peak). The %

difference between retention times (RT) or peak areas was calculated using the following formula: % difference = (x1 – x2/Mean of x1 + x2) x 100; Where x1 and x2 are either RT or peaks area.

7. System suitability control

A mixture of two reference standards was implemented as a system suitability control: the high and low molecular weight globular proteins' thyroglobulin bovine (669 kDa) and Bovine serum albumin (69 kDa). As column performance verification, we checked that the thyroglobulin bovine peak came earlier than the BSA peak, and the difference in elution time between the two standards was at least 1 min. We ensured that the peak of the reference sample comes earlier than the thyroglobulin bovine peak (an average molecular weight of Adenovirus is 1.5 MDa).

8. Test samples

Both chromatograms of Bulk Drug Substance (purified formulated virus) and reference material were overlayed. Finally, we ensured that the elution times of monomer peak are similar to the reference (max 10% discrepancy). For duplicate injections, the chromatograms were comparable to each other when overlaid.

TIMP-3 Immunofluorescence Staining

HeLa cells were plated onto sterile glass coverslips in 24-well plates at $4x10^4$ cells/well. 24 hours later, cells were transfected with HAdV5-TIMP-3 or RAd35 (LacZ expressing recombinant adenoviral vector [48] at either 5, 50, or 250 pfu/mL for 3 hours. The media was then changed and left for 48 hours. Cells were then fixed in 4% PFA, washed 3x with 1x PBS, permeabilised with 0.3% Triton X100 in PBS, and blocked with 5% normal donkey serum, 1% BSA for 1 hour at room temperature. Cells were incubated with anti-TIMP-3 antibody (cat#AB6000, EMD Millipore) at 1:500 dilution (or 1:1500 dilutions for rabbit IgG control) and incubated for 1 hour at room temperature. Cells were then washed 3x with 1x PBS and incubated with goat anti-rabbit Alexa 488 secondary antibody. Cells were mounted using ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, Massachusetts, USA).

Hexon immunostaining

A 24 well plate of Procell 92.-S cells (GSK, London, UK) were infected with serial dilutions of HAdV5-TIMP-3 purified virus. 48 hours later, the cells were fixed, incubated with an anti Hexon primary antibody and the signal was detected by a secondary antibody HRP conjugate.

Results

Use of standard viral production methods results in a loss of TIMP-3 expression and the emergence of HAdV5 structural variants

The programme was initiated with the manufacturer Finnvector to produce HAdV5-TIMP-3. Although the programme started well in small scale optimisation with transfer of vector production to GMP master cell banks of HEK293 cells owned by Finnvector, it quickly became clear that HAdV5-TIMP-3 production through multiple passages was not compatible with the vector maintaining transgene expression through the multiple rounds of vector expansion/passaging.

During test production of toxicological batch grade HAdV5-TIMP-3 (mimicking GMP production) it was observed that sequential amplification of HAdV5-TIMP-3 through these standard "clinical grade adenovirus production" processes led to loss of the transgene-expressing viral vector with concomitant emergence of "contaminant" vectors with a different genome configuration (Figure 1). We performed PCR and gel electrophoresis on viral lysates from individual viral clones to confirm the presence of both TIMP-3 and the pShuttle vector (used as part of the AdEasy system) (TIMP-3 634bp product; top left panel, pShuttle specific primers 799bp product; top middle panel) and for absence of contaminating E1 band (2.4kb product contaminated, 2.034kB band for TIMP-3, 3.998kB band for wtHAdV5; top right panel). We noted that while bands of the expected size were observed from isolated clones at earlier passages, further rounds of passaging resulted in either a complete loss of PCR product (for example, clone 11 showed bands of expected size at passage 2, but this was absent at passage 3), or the emergence of bands of unexpected sizes (Figure 1A). We then performed DNA sequencing on individual viral clones which revealed that genomic rearrangements such as frameshift mutations emerged through subsequent passages (Figure 1B).

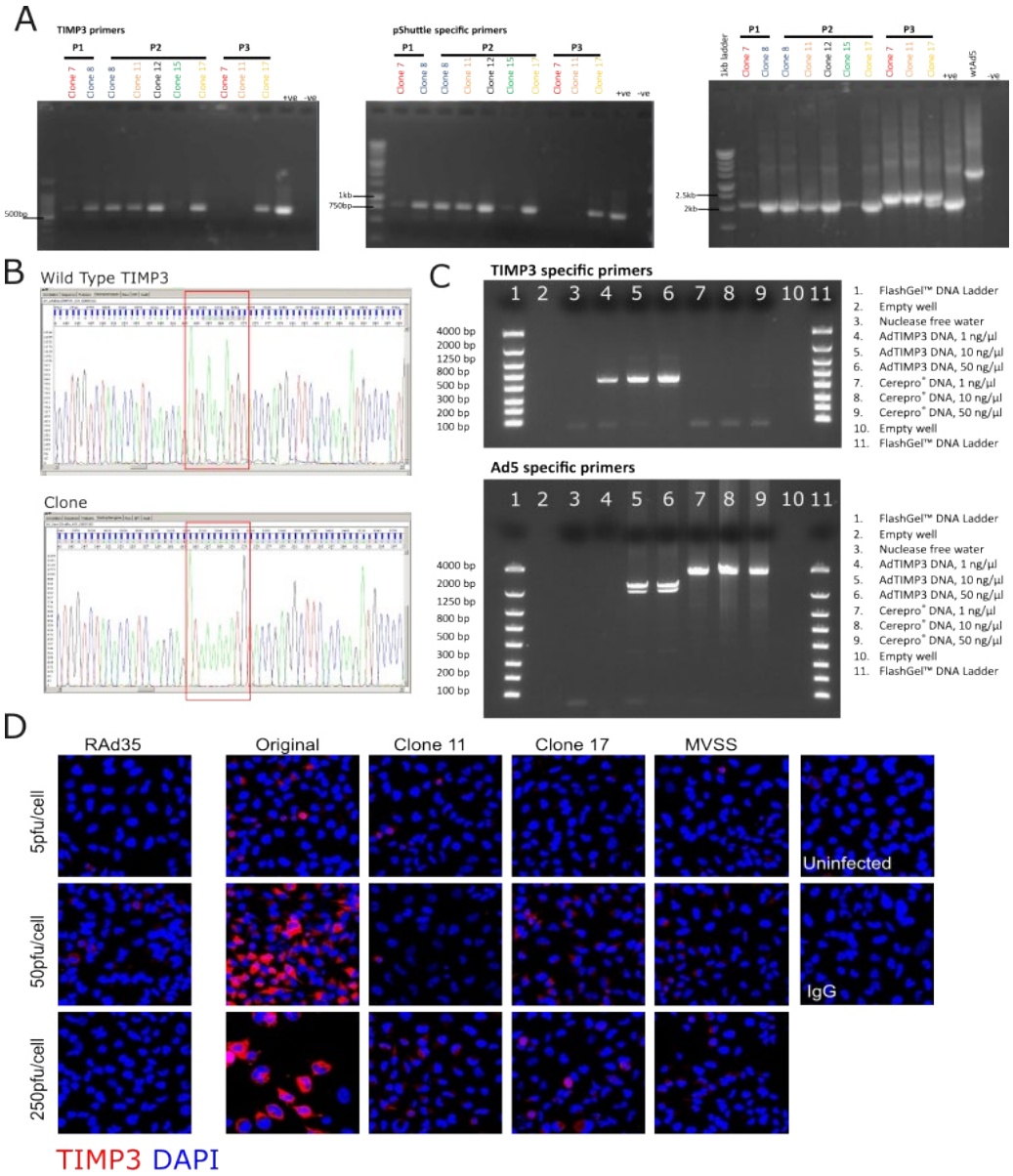


Figure 1: HAdV5.TIMP-3 Clone Screening Demonstrates Viral Vector Instability by Traditional Scaling Methods.

A. PCR results using TIMP-3-specific primers (634bp product; top left panel), pShuttle specific primers (799bp product; top middle panel) and for absence of contaminating E1 band (2.4kb product contaminated, 2.034kB band for TIMP-3, 3.998kB band for wtHAdV5; top right panel). Positive control is the original virus. "P" refers to passage number. "+ve" refers to an original plaque from the University of Glasgow HAdV5.TIMP-3; "-ve" refers to water. **B**. DNA sequence electropherograms illustrating the presence of frameshift mutations corresponding to the HAdV5 E1 sequence. **C**. PCR with TIMP-3-specific and HAdV5-specific primers was carried out using HAdV5-TIMP-3 DNA or Cerepro DNA after transfection as indicated. **D**. Immunofluorescent imaging of TIMP-3 (red) and DAPI (blue) of indicated clone after transfection with either HAdV5-TIMP-3 or RAd35 as negative control at 5, 50 or 250pfu/cell, as indicated.

Subsequently, we undertook PCR with TIMP-3-specific and HAdV5-specific primers using HAdV5-TIMP-3 DNA and the control adenoviral gene therapy vector Cerepro (sitimagene ceradenovec) [49] as template. A correct size fragment (634bp) was detected from HAdV5.TIMP-3 template DNA using 1, 10 and 50 ng of template but was not amplified from negative control (Cerepro) (Figure 1C, upper). A correct size fragment (3528 bp) was amplified from Cerepro using HAdV5 specific primers. However, HAdV5 specific primers amplified two fragments from the HAdV5. TIMP-3 DNA, corresponding to intact TIMP-3 virus (2023bp) and mutated virus containing partial E1 sequence (2400 bp) (Figure 1C lower). These were not detectable in the originally supplied seed stock of HAdV5-TIMP-3.

We then performed immunofluorescence staining for TIMP-3 in HeLa cells transduced at either 5, 50 or 250 pfu/cell with HAdV5-TIMP-3 or RAd35 [50] as control to assess TIMP-3 overexpression from individual clones (Figure 1D). Our original HAdV5-TIMP-3 stock demonstrated dose-dependent TIMP-3 expression, indicative of consistent TIMP-3 over-expression. In comparison, individual clones from subsequent passages produced using the standard manufacturing protocols demonstrated an almost complete loss of TIMP-3 expression. Collectively these results demonstrated that standard viral production protocols were not suitable for the scaling of a standard configuration first generation HAdV5 vector expressing TIMP-3 constitutively from the CMVIEP.

Silencing of TIMP-3 over-expression using tetracycline repression is required for sustained HAdV5-TIMP-3 stability during manufacturing

We then worked with Batavia Biosciences (Leiden, The Netherlands) to assess different strategies for their impact on HAdV5-TIMP-3 stability. We undertook three approaches to create three new plasmids with the aim of ensuring correct genomic configurations and TIMP-3 over-expression over 10 passages. These strategies included the incorporation of two TET operator sequences into the Ad5TIMP-3 expression cassette to block transgene expression during production [51] (pAdEasy.CMVTetOTIMP-3) (Supplementary Figure 1), reversal of the expression cassette in the E1 region to reduce transgene expression (pAdEasy.CMVTIMP-3REV) and the use of a lower activity (SHCMV) promoter [14,52] to drive TIMP-3 transcription (pAdEasy.SHCMVTIMP-3). We additionally utilised the original pAdEasy.CMV.TIMP-3 plasmid alongside the new vectors in these assays (Supplementary Figure 2). After plasmid production, the sequences of the obtained plasmid DNAs were confirmed by restriction enzyme analysis and DNA sequencing. Both the restriction enzyme analysis and sequence analysis confirmed correct configuration of all pAdeasy TIMP-3 plasmid variants (Supplementary Figure 3A, 3B). The full restriction digest fragment sizes are given in Supplementary Table 1. *Pac*I-digested and cleaned plasmids were used to generate the viral vector on the HEK293 cell line (Ad5.CMV.TIMP-3, Ad5.SHCMV.TIMP-3 and Ad5.CMV.TIMP-3rev) or TREx HEK293 cell line (Ad5.CMVTO.TIMP-3) by transfection using a DNA:lipofectamine ratio of 1:10 (Supplementary Figure 3C). Each vector required multiple passages, reinfections or a combination of these to obtain full cytopathic effect (CPE). When full CPE was obtained, the vectors were subjected to one round of plaque purification. In total, 10 viral plaques were picked for each vector and subjected to two rounds of propagation. The material obtained by the second propagation was analysed by transgene PCR analysis, identity PCR analysis (the E3 and E4 regions) and transgene expression analysis by western blot. The transgene and identity PCRs showed the expected bands for all plaques analysed (Figure 2A. 2B, Supplementary Figure 4A, 4B, 4D, 4E, 4G, 4H). The western blot analysis for the original configuration Ad5.CMV.TIMP-3 and the lower activity SHCMV promoter Ad5.SHCMV.TIMP-3 demonstrated a total loss of TIMP-3 expression (Data not shown), while the western blot analysis for the reversed configuration Ad5.CMV.TIMP-3REV and the Tet operator TIMP-3 Ad5.CMVTO. TIMP-3 did show TIMP-3 expression (Figure 2C, Supplementary Figure 4F). To confirm the lack of TIMP-3 expression with Ad5. CMV.TIMP-3 and Ad5.SHCMV.TIMP-3, we repeated the western blots and additionally included a plaque from both the original University of Glasgow Ad5.CMV.TIMP-3, and from Ad5.CMVTO.TIMP-3. However, upon repeat western blotting, TIMP-3 expression could once again not be detected from Ad5.CMV.TIMP-3 and Ad5.SHCMV.TIMP-3, despite being detected for the original Ad5.CMV.TIMP-3 and Ad5.CMVTO.TIMP-3 (Supplementary Figure 4C, 4I).

Despite the loss of TIMP-3 expression for Ad5.CMV.TIMP-3 and Ad5.SHCMV.TIMP-3, we chose to continue to assess stability for these constructs, alongside Ad5.CMV.TIMP-3REV and Ad5.CMVTO.TIMP-3. For each vector, three plaques were selected for sequential passages in T25 flasks up to passage 10. After sequential passage 6 and 10, the 3 selected plaques per vector were analysed by transgene PCR for stability determination. All plaques analysed, including the original Ad5.CMV.TIMP-3 clone showed the expected transgene PCR band at both passage 6 and 10, indicating that sequential passaging show no signs of deletions for all vector variants prepared (Supplementary Figure 5).

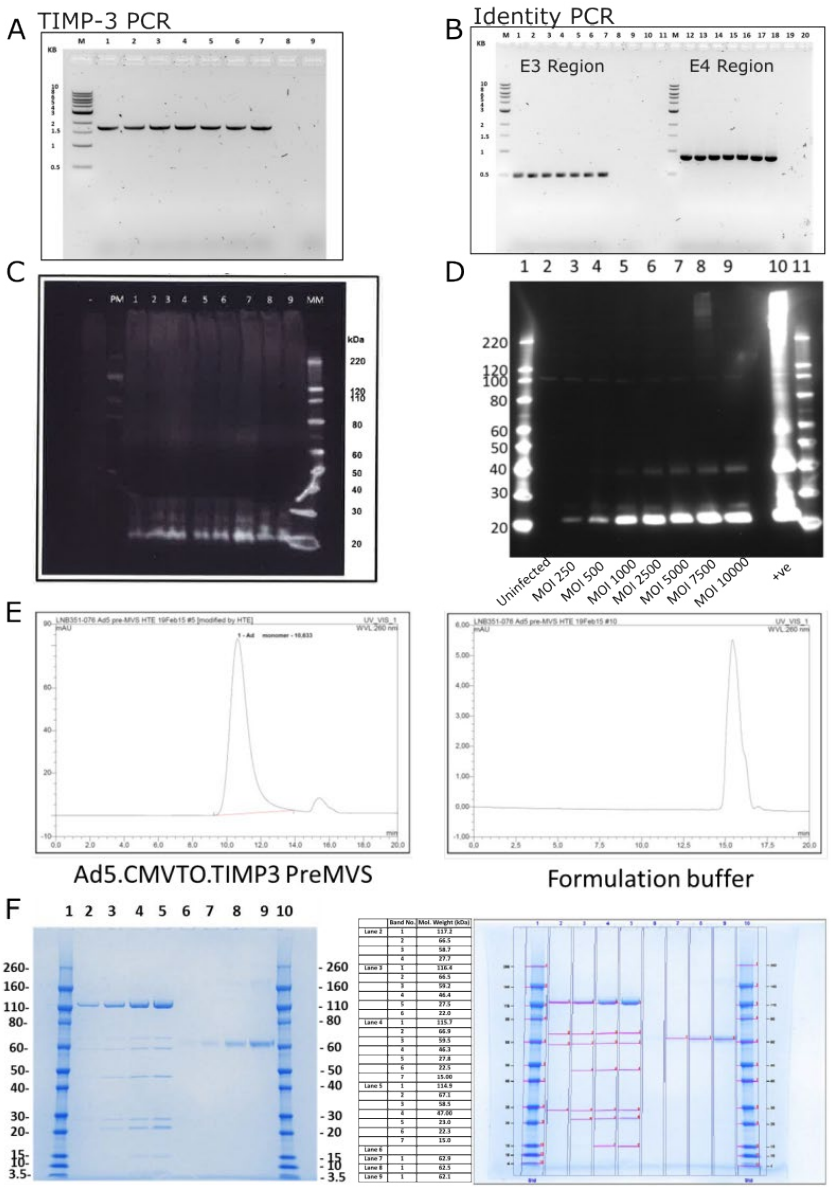


Figure 2: Silencing TIMP-3 Overexpression Using Tetracycline Repression Leads to HAdV5-TIMP-3 Genomic Stability in Pre-MVSS Stocks.

A. TIMP-3 integrity was measured by PCR using primers TG-F & TG-R after 3 rounds of viral passage from HAdV5.CMVTO.TIMP-3 transduction. Lanes 1-6 refer to 3 individual plaques of HAdV5.CMVTO.TIMP-3, with either 100µl (1-3) or 200 µl (4-6) infected into cells. Lane 7 refers to plasmid pAdEasy.CMVTO.TIMP3 positive control, lane 8 is empty, and lane 9 is cell lysate from uninfected cells. **B**. PCR for the E3 (primers E3-F & E3-R) and E4 region (primers E4-F & E4-R) of HAdV5.CMTO.TIMP-3 was performed to assess transgene integrity after 3 rounds of viral passage from HAdV5.CMVTO.TIMP-3 transduction. Lanes 1-6 and 12-17 refer to 3 individual plaques of HAdV5.CMVTO.TIMP-3, with either 100µl (1-3/12-14) or 200 µl (4-6) infected into cells. Lane 7 refers to plasmid pAdEasy.CMVTO.TIMP3 positive control, lane 8 is empty, and lane 9 is cell lysate from uninfected cells. **C.** TIMP-3 expression by western blot analysis; expected size 24 kD; MM: Magic marker (numbers indicate the amount of kDa), PM: Pre-stained marker (Life Technologies); -: negative control; numbers on top indicate the plaque numbers tested. **D.** TIMP-3 expression by western blot analysis (expected size 24 kD). Lane 1 and 11: Magic marker (numbers indicate the amount of kD); Lane 10: positive control; Lane 2: uninfected A549 cells. Lane 3: preMVS at MOI 250, Lane 4: preMVS at MOI 500, Lane 5: preMVS at MOI 1000, Lane 6: preMVS at MOI 2500, Lane 7: preMVS at MOI 5000, Lane 8: preMVS at MOI 7500 and Lane 9: preMVS at MOI 10000. **D**. HPLC size exclusion chromatography was performed to determine viral aggregation; control: Formulation buffer (background). For the sample: peak 1 is the virus peak; second peak is the formulation buffer. **E**. Purity determination by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Table indicates the protein band pattern detected. Lane 1 and 10: Novex Sharp Pre-stained (Invitrogen, numbers indicate the amount of kD); Lane 2: $1x10^9$ vp; Lane 3: $2x10^9$ vp; Lane 4: $3x10^9$ vp; Lane 5: $4x10^9$ vp; Lane 6: 0.05 µg BSA; Lane 7: 0.1 µg BSA; Lane 8: 0.25 µg BSA; Lane 9: 0.5 µg BSA. Hexon: ~116kD, penton base: ~67kD, pIIIa (minor capsid protein): ~59kD, pV (minor core protein): ~47kD, pVI (minor capsid protein): ~28kD pVII (core protein): ~22kD, pVIII and pIX (minor capsid proteins): ~15kD

The sequence analysis of the three plaques of Ad5.CMV.TIMP-3 and the three plaques of Ad5.SHCMV.TIMP-3 showed the same deletion of a "G" nucleotide in the TIMP-3 gene at position 52, causing a frameshift mutation within the TIMP-3 gene. We assumed that this frameshift mutation was present at viral passage 2 and explains the lack of TIMP-3 expression observed in the western blot analysis. For Ad5.CMV.TIMP-3REV plaque #7, an approximate 116 bp deletion was found, starting just before the start codon of TIMP-3. The sequences of plaques #4 and #5 were found to be correct, although not all parts of the CMV-TG-polyA region were covered with clear sequence reads. In contrast, in all three plaques of Ad5.CMVTO.TIMP-3 the sequence was correct. Based on these results, we decided to continue only with Ad5.CMVTO. TIMP-3 plaque #1 to prepare the pre-MVS.

TET repression using the non-GMP T-REx™ System (Thermo-Fisher), in which TIMP-3 expression is silenced in the 293 T-Rex cells (HAdV5-CMV.TO.TIMP-3), resulted in stable maintenance of TIMP-3 over expression across 10 rounds of passage (Figure 2D). We performed western blotting to assess TIMP-3 over-expression at the protein level using cell lysates from A549 cells transduced at increasing multiplicity of infections (250, 500, 1000, 2500, 5000, 7500, 10000 vp/cell) from our pre-master viral seed stock (pre-MVS) lot. We additionally used A549 cell lysate transduced with concentrated early-passage HAdV5-TIMP-3 as a positive control, and untransduced A549 cells as negative control. TIMP-3 expression was detected at the expected size of 24kDa at increasing levels relative to increasing multiplicity of infection from cells transduced with HAdV5-CMV.TO.TIMP-3, demonstrating consistent transgene expression from the pre-MVS stock (Figure 2D). We also noted the presence of extra bands at 27 kD and 40 kD corresponding to glycosylated forms of TIMP-3.

We next assessed whether HAdV5-CMV. TO.TIMP-3 forms aggregates using high performance size exclusion chromatography (HP-SEC). We injected $5.44x10^{10}$ viral particles at a final volume of 40 µL into the column and used formulation buffer as control. The virus produced a single peak with a retention time of around 10 minutes, whereas formulation buffer showed a background peak with a longer retention time (Figure 2E). We also noted a small tail peak from the HAdV5-CMV.TO.TIMP-3, most likely owing to slightly smaller virus particles. Collectively, these data suggest that HAdV5-CMV.TO.TIMP-3 does not form viral protein aggregates.

The purity of HAdV5-CMV.TO.TIMP-3 pre-MVS was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions, which results in separation of the individual proteins that constitute the HAdV5-CMV. TO.TIMP-3 vector (Figure 2F). Four different viral particle amounts ($1x10^9$ vp, $2x10^9$ vp, $3x10^9$ vp, $4x10^9$ vp) (lanes 2-5) were loaded on the gel together with four different concentrations of BSA (0.05 µg, 0.1 µg, 0.25 µg, 0.5 µg BSA) (lanes 6-9). The protein band pattern observed for the HAdV5-CMV. TO.TIMP-3 was as expected for human adenovirus serotype 5, as the visible bands are comparable with known adenoviral-related proteins (~116 kD: Hexon; ~67 kD: Penton base; ~59 kD: pIIIa (minor capsid protein); ~47 kD: pV (minor core protein); ~28 kD: pVI (minor capsid protein); ~22 kD: pVII (core protein), ~15 kD pVIII and pIX (minor capsid proteins). No unexpected or unknown bands were detected.

Collectively, these results demonstrate that production of HAdV5-TIMP-3 using Tet operator sequences to repress TIMP-3 expression during viral production in helper cell lines results in high titre vector production with the correct genomic configuration without loss of transgene expression, which is suitable to produce a master viral stock in a GMP-grade Tet repressor cell line.

The GMP-Grade Procell-92.S Tet repressor cell line can successfully produce HAdV5.CMVTO. TIMP-3 to clinical grade

We sought to identify a GMP-grade 293-TET repressor helper cell line for adenoviral vector production for clinical trials and identified GSK's Procell-92.S Tet repressor cell line. Through GSK's service company, Reithera, we initiated assessment of the suitability of Procell-92.S for HAdV5-TIMP-3 production via small scale preparations produced over 10 sequential passages (to mimic GMP production). Reithera transfected 10 µg of linearised pAdEasy.CMVTO.TIMP-3 DNA into Procell-92.S Tet repressor cells and remade the preMVS stock (data not shown). The vector was then amplified up to

passage 4 and purified. Adenoviral vectors were purified from cells by caesium chloride gradient and viral particle concentration measured. We observed equivalent virus particle titre at each passage, indicating that viral production using the Procell-92.S Tet repressor cell line does not result in viral instability (Figure 3A). Similar results were observed when cells were further amplified to passage 10 (Figure 3B). Finally, after passage 10, we purified total virus and measured viral production, which confirmed that Procell-8/92.S cells produce HAdV5-TIMP-3 to high titres suitable for transfer to GMP production of clinical trial batches (Figure 3C).

A

Passages	Cells	MOI	Productivity Vp/ml	Productivity Vp/cell
P1	Procell92.S MCB (adapted to grow in adherence) T-25 flask	NA	4,35E+10	7,25E+04
P2	Procell 92.S MCB 50 mL 5 e5 cells/mL	100 vp/cell	1,78E+10	3,57E+04
P3	Procell 92.S MCB 50 mL 5 e5 cells/mL	100 vp/cell	1,16E+10	2,32E+04
P4	Procell 92.S MCB 300 mL 5 e5 cells/mL	100 vp/cell	3,96E+10	7,2E+04
P4 Purified virus			vp/ml	Total vp
			4,80E+11	3,84E+12

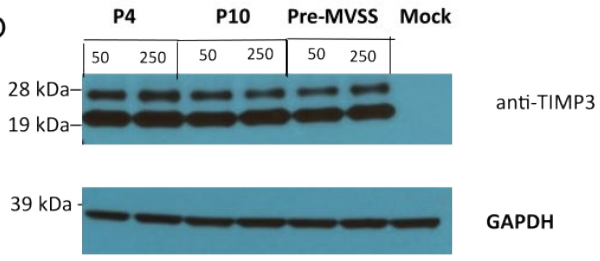
B

Passages	MOI Vp/cell	Volumetric productivity Vp/ml	Cell-specific productivity Vp/cell
P5	100	1,52E+10	3,05E+04
P6	100	1,88E+10	3,77E+04
P7	100	2,06E+10	4,12E+04
P8	100	1,86E+10	3,71E+04
P9	100	1,40E+10	2,80E+04

C

	Vp/ml	Vp tot
Bulk Harvest	6,80E+09	1,02E+12
Purified Virus	2,81E+11	5,63E+11

D



E

Ad5 TIMP3	Vp/mL	IFU/ml	Ratio Vp/IFU
P4 purified virus	4,80E+11	9,25E+09	52
P10 purified virus	2,81E+11	5,81E+09	48

Figure 3: HAdV5.CMVTO.TIMP-3 Production and Stability in GMP-Grade Procell-92.S Tet Repressor Cells.

A. Genetic stability was assessed through serial passages of HAdV5.CMVTO.TIMP-3 infected Procell-92.S Tet Repressor cells. The virus was purified by caesium chloride (CsCl) density gradient at passage 4 and passage 10 and viral particle concentration evaluated. **B**. Cells from passage 5-passage 9 were analysed for genetic stability of HAdV5.CMVTO.TIMP-3. At each passage the virus was purified by CsCl density gradient and viral particle concentration evaluated. **C**. The final passage 10 pre-MVSS HAdV5.CMVTO.TIMP-3 was analysed for genetic stability. The virus was purified by CsCl density gradient and viral particle concentration evaluated. **D**. Western immunoblot for TIMP-3 after either P4, P10 or pre-MVSS HAdV5.CMVTO.TIMP-3 transduction at the indicated multiplicity of infection to A549 cells. The expression level of TIMP-3 from P4, P10 and of the initial seed (pre-MVSS) virus are comparable. **E**. The infectivity of P4 and P10 purified viruses was evaluated in adherent Procell-92.S cells by Hexon Immunostaining and quantified.

To assess TIMP-3 overexpression, we transduced A549 cells at a multiplicity of infection of either 50 or 250 with virus purified from passage 4 and passage 10 cells, or with the pre-MVSS HAdV5-TIMP-3 and left for 3 days. We then assessed TIMP-3 protein expression by western blot. At each passage of HAdV5-TIMP-3 production (up to passage 10), TIMP-3 production was equivalent to the pre-MVSS HAdV5-TIMP-3, with two clear bands visible in all conditions except mock-treated cells, corresponding to the non-glycosylated and glycosylated forms of TIMP-3 (Figure 3D). We then performed hexon immunostaining to assess viral infectivity. The ratio between total virus particles and IFU measured by hexon immunostaining was the same for P4 and P10 vectors (Figure 3E). Finally, purified vectors from passage 4 and passage 10 were analysed by restriction digestion and direct sequencing of the TIMP-3 expression cassette; this confirmed the correct adenoviral and transgene sequence was maintained (data not shown).

Production of clinical grade HAdV5-CMV.TO.TIMP-3 to enable a First-In-Human clinical trial

With the problem of clinical-grade production now solved, we have now synthesised sufficient amounts of HAdV5-CMV.TO.TIMP-3 in order to begin a first-in-human clinical trial Figure 4. We manufactured HAdV5-CMV.TO.TIMP-3 with Advaxia Biologics (formerly Advent) (Pomezia, Italy), who was granted permission from GSK to use their Procell-92.S Tet repressor cell line. Advaxia were successfully able to produce HAdV5-CMV.TO.TIMP-3 to high titres and at sufficient volume to complete a phase I clinical trial. A summary of the release criteria for HAdV5-CMV.TO.TIMP-3 as set out by the European Pharmacopoeia (European Directorate for the Quality of Medicines & HealthCare) is given in Table 1.

Discussion

First-generation, E1/E3-deleted adenoviral vectors expressing a wide variety of transgenes are produced using well-established, reproducible protocols in laboratories worldwide for the development of novel therapies. We have demonstrated that adenoviral-mediated overexpression of TIMP-3 in saphenous vein is effective at reducing neointimal hyperplasia in *ex vivo* human saphenous vein organ culture models [41], and in two short- and long-

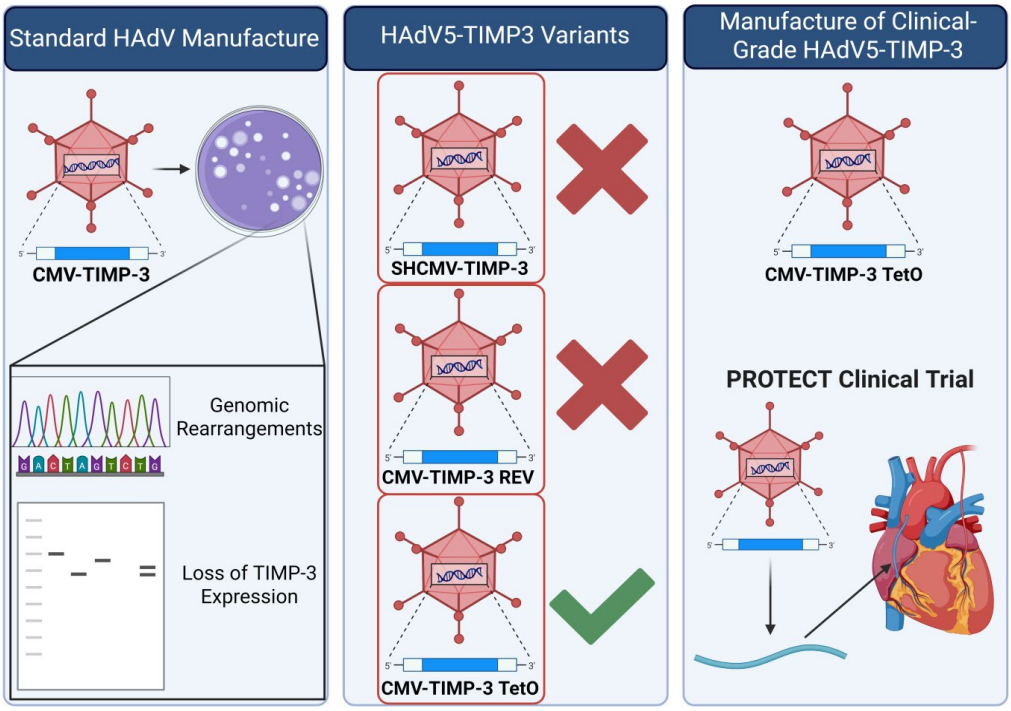


Figure 4. Successful Manufacture of a Clinical-Grade Adenovirus Expressing TIMP-3.
Summary of the data presented in this study. Standard adenoviral manufacturing methods are not suitable to produce HAdV5-TIMP-3, with sequence variants arising, accompanied by loss of transgene expression. Only complete silencing of TIMP-3 expression using tetracycline-mediated repression in GMP-compliant cell lines was suitable to produce a clinical batch of HAdV5-TIMP-3 for using in the PROTECT clinical trial.

Table 1. HAdV5.CMVTO.TIMP-3 Drug Product Specification

The Ad5.CMVTO.TIMP-3 drug product is controlled using the specification shown. qPCR: quantitative polymerase reaction; IFU: infectious units; LAL: limulus amoebocyte lysate; CFU: colony forming units.

Test (Method)	Acceptance Criterion	Batch Number	
		19-RD00006-PR	J.0008
Bioburden [1] (Ph. Eur. 2.6.12)	≤ 1 CFU/10 mL	N.D.	0 CFU/10 mL
Endotoxin (LAL method) (Ph. Eur. 2.6.14)	≤ 10 EU/mL	0.1 EU/mL	0.635 EU/mL
Visual inspection (Ph. Eur. 2.9.20)	Clear to slightly opaque solution essentially free from visible particles	N.D.	Clear solution essentially free from visible particles
Particulate Contamination (Ph. Eur. 2.9.19 Method I – Light obscuration particle count test)	≥ 10 µm: ≤ 6000 particles/vial ≥ 25 µm: ≤ 600 particles/vial	N.D	≥ 10 µm: 3 particles/vial ≥ 25 µm: 1 particles/vial
Transgene expression (Western Blot)	Expresses	Expresses	Expresses
Ratio vector particle concentration by qPCR genome quantitation / Infectious virus concentration by IFU titration (Calculation)	≤ 300 vp/ifu	15 vp/ifu	23 vp/ifu
Vector particle concentration (vp/mL) (qPCR genome quantitation)	Report results (vp/mL)	3.7×10^{11} vp/mL	5.2×10^{11} vp/mL
Infectious virus concentration (IFU titration)	≥ 1.3×10^{10} ifu/mL	2.4×10^{10} ifu/mL	2.3×10^{10} ifu/mL
Sterility test by direct inoculation (Ph. Eur. 2.6.1)	Absence of growth	Absence of growth	Absence of growth
Endotoxin (LAL method) [1] (Ph. Eur. 2.6.14)	≤ 10 EU/mL	N.D.	0.755 EU/mL
Container Closure Integrity Test (Dye ingress test)	No dye ingress	N.D.	No dye ingress
Extractable volume (Ph. Eur. 2.9.17)	≥ 0.5 mL	N.D.	0.60 mL
pH (Ph. Eur. 2.2.3)	6.9 – 7.9	7.3	7.3
Osmolality (Ph. Eur. 2.2.35)	> 265 mOsMol/Kg	417 mOsMol/Kg	424 mOsMol/Kg

Notes:

[1] Tested on Step 1 of the DP manufacturing process, as described in P.3.3 Description of Manufacturing Process and Process Controls.

CFU: colony forming unit; N.D: not done; LAL: limulus amoebocyte lysate; EU: endotoxin units; qPCR: quantitative polymerase reaction.

term studies in a porcine arterial bypass model [41,42]. These therapeutic effects are primarily incurred through increased apoptosis of VSMCs [53], and thus reduced neointima formation. The results also demonstrate that over-expression of TIMP-3 is cytotoxic in producer cells, to the extent that standard procedures for viral production are not possible. We are now in a position to translate these preclinical results into a phase I clinical trial [ISRCTN43650325] but to do so, required manufacture scale-up to produce clinical-grade HAdV5-TIMP-3. Here, we have outlined the challenges we have faced during this process, illustrating that traditional adenoviral manufacturing steps are unsuitable to produce HAdV5-TIMP-3. Further, we exemplify a variety of approaches we undertook to solve this problem and delineate that only complete silencing of TIMP-3 expression using tetracycline-mediated repression in an appropriate Tet

cell line results in the production of HAdV5-TIMP-3 to suitable levels. Through this method, we have successfully manufactured HAdV5-TIMP-3 at high titres to clinical grade, enabling us to begin our first-in-human clinical trial (public Clinical Study Registration: ISRCTN43650325) [54], "A randomised Placebo-contROlled Trial of AdTIMP-3 to prEvent Coronary artery bypass grafT failure" (PROTECT) at the NHS Golden Jubilee Hospital, Glasgow, United Kingdom. In this phase I prospective, randomised, open-label, dose-ranging, phase 1 trial of HAdV5.CMVTO.TIMP-3 to prevent CABG failure, HAdV5.CMVTO.TIMP-3 will be delivered *ex vivo* to the saphenous vein for individual patients following harvesting from the leg and immediately before surgical grafting in the heart. The sample size is 12 participants including three sequential groups of four participants, with each group receiving a sequentially higher dose of HAdV5.CMVTO.TIMP-3 (cohort 1: 2.5 x 10^9 ifu/mL, cohort 2: 7.5 x 10^9 ifu/mL, cohort 3: 2.5 x 10^{10} ifu/mL) with progression in each group overseen by the Safety Review Committee. The randomisation procedure involves allocation of the study therapy or matched placebo at random (1:1) to the saphenous vein graft intended for the left or right coronary artery. Therefore, each participant will receive active therapy. The primary objective of this study is to determine a tolerable dose of HAdV5.CMVTO.TIMP-3 which can be used in future, phase II/III studies by evaluating safety and dose-limiting toxicity, overseen by a Safety Review Committee convened by the trial sponsor (NHS Greater Glasgow and Clyde Health Board). Additionally, we will assess the degree of saphenous vein graft occlusion and narrowing (lumen loss), if any, by 12-months post-surgery by use of multidetector CT angiography. Finally, we will collect data on patient reported outcome measures (PROMS) including the EQ5D-5L, Seattle Angina, and DASI questionnaires, and possible adverse reactions and major adverse cardiovascular events (MACE) - including death, re-hospitalisation for cardiovascular events including for recurrent myocardial infarction or heart failure and repeat revascularisation of a native coronary artery or graft. These results will inform whether HAdV5-CMV.TO.TIMP-3 is effective as a novel gene therapy for CABG failure. Clinical follow-up of individual participants will continue for 5-years.

More broadly, the data presented here illustrate some of the challenges that can occur when there is a need to express cytotoxic transgenes from adenoviral vectors, and highlights the lack of a standardised, "one size-fits all" approach for solving these challenges. Rather, specific problems that arise will depend on the specific transgene being expressed, the degree of over-expression required, and its precise functions and degree of toxicity to cells. Individual research groups may require a suite of suitable cell lines, techniques, and collaborations that offer bespoke, adaptable solutions to solve manufacturing challenges, some of which have been delineated in this work.

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Author Contributions

S.A. Nicklin, A.H. Baker, A. Lemckert, M. Havenga, S. Colloca, C. Berry, and S. Carmichael designed the project and experiments. V. Ammendola, R. Dakin, K. White, N. Britton performed experiments and analysed data. S.D. Brown wrote the initial manuscript, with further edits suggested by S. A. Nicklin and A. H. Baker. All authors except A. Lemckert (deceased) reviewed and approved the final manuscript.

Authors will consider reasonable requests for the datasets in this manuscript, however due to third-party restrictions the authors cannot guarantee access to the data.

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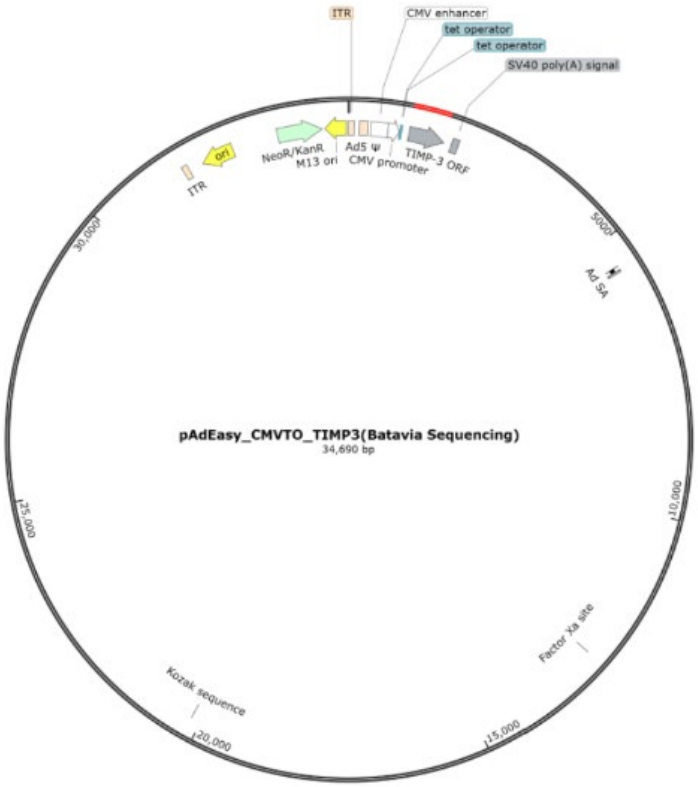
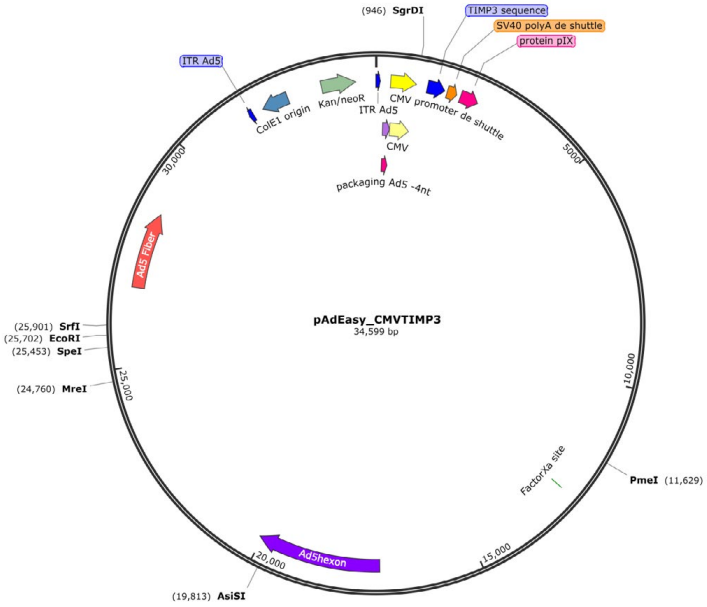
Supplementary Figures



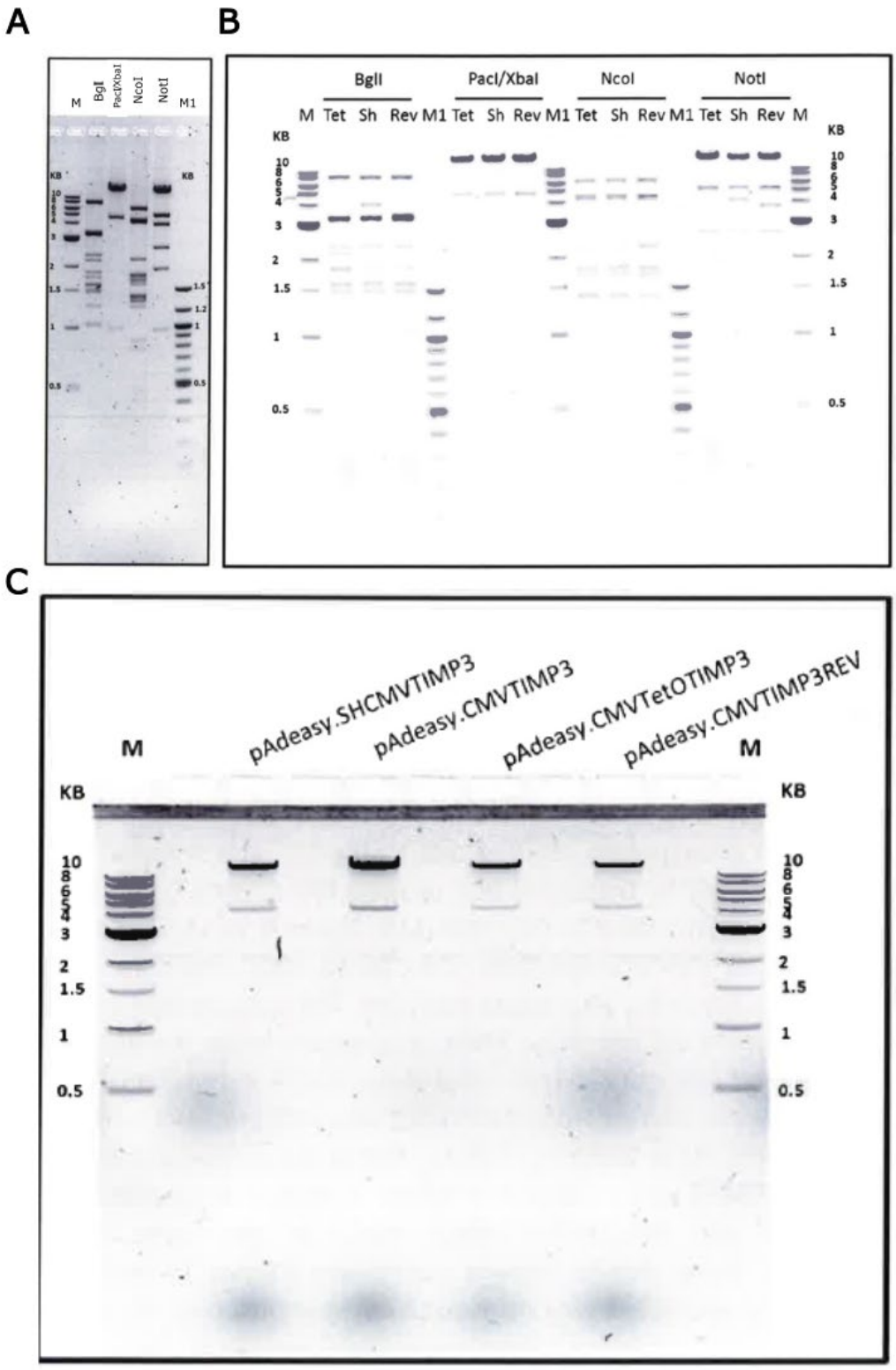
Supplementary Figure 1: Detailed Genetic Map of pAdEasy.CMVTO.TIMP3 Plasmid.

Tet: tetracycline, ORF: open reading frame, CMV: cytomegalovirus, CMVTO: cytomegalovirus tetracycline operator, SV40: simian virus 40, ori: origin of replication.



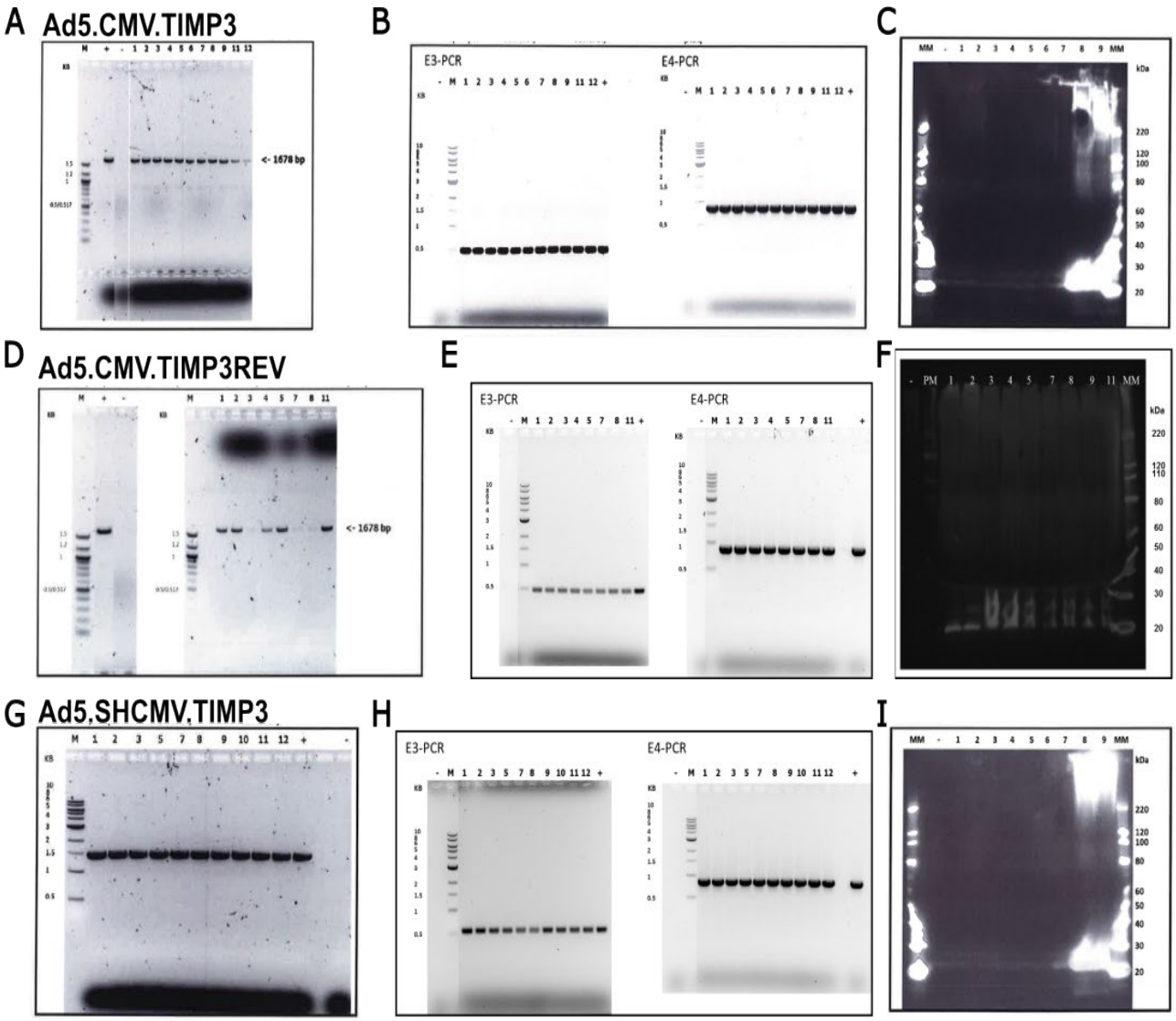
Supplementary Figure 2: Detailed Genetic Map of pAdEasy.CMVTIMP3 Plasmid.

Tet: tetracycline, ORF: open reading frame, CMV: cytomegalovirus, SV40: simian virus 40, ori: origin of replication.



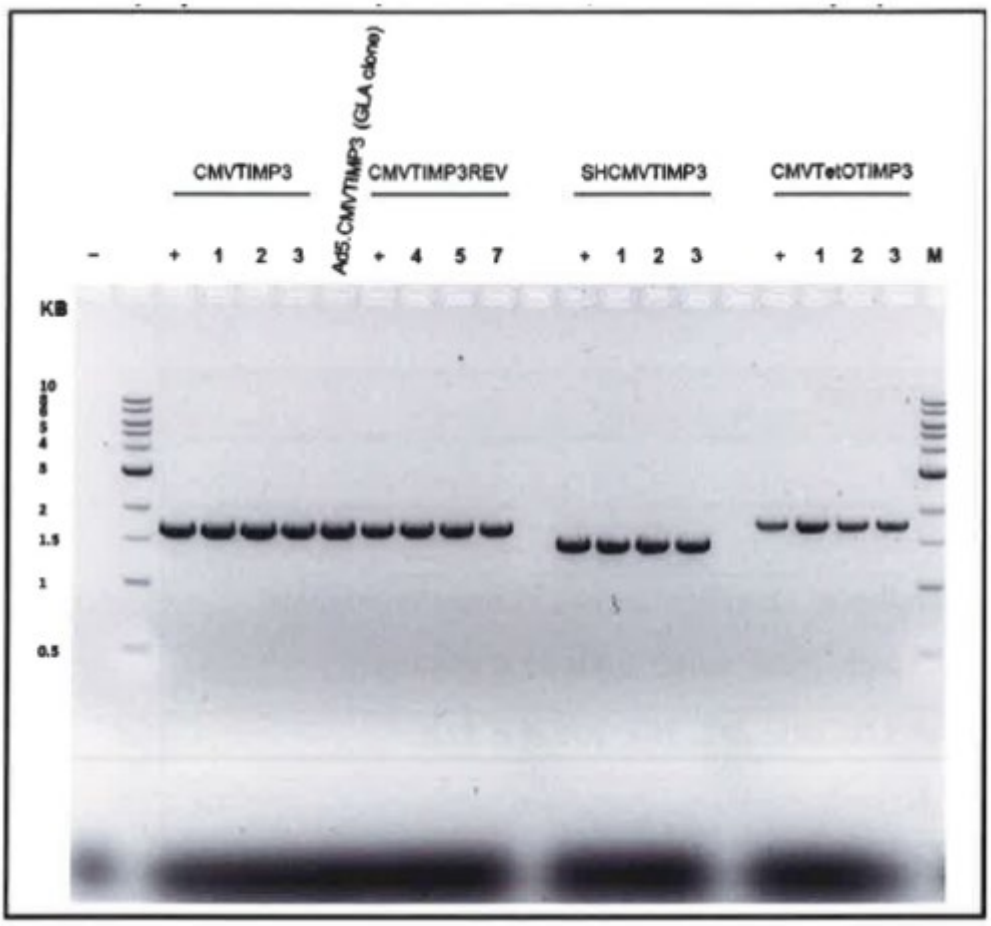
Supplementary Figure 3: Restriction Digests of pAdEasy.CMV.TIMP-3 Plasmids.

A. Restriction digest as indicated from pAdEasy.CMV.TIMP-3 plasmid. M= 1kb ladder, M1= 100bp ladder. **B**. Restriction digest as indicated from either pAdEasy.CMVTetOTIMP-3 (Tet), pAdEasy.SHCMVTIMP-3 (Sh) or pAdEasy.CMVTIMP-3REV (Rev). M= 1kb ladder, M1= 100bp ladder. **C.** *Pac*I restriction digest of pAdEasy TIMP-3 vectors as indicated prior to transfection. Bands are observed at ~31kb and ~4.5kb.



Supplementary Figure 4: Stability Testing of HAdV5.CMV.TIMP-3Variants.

A, D, G. Transgene PCR analysis of indicated HAdv5.CMV.TIMP-3 variants. (A) HAdV5.CMV.TIMP-3, expected band size 1678bp, (D) HAdv5.CMV.TIMP-3REV, expected band size 1678bp, (G) HAdV5.SHCMV.TIMP-3, expected band size 1462bp. Numbers indicate plaque numbers, + is plasmid control, - is water. **B, E, H**. Identity PCR analysis of indicated HAdv5.CMV.TIMP-3 variants. (B) HAdV5.CMV.TIMP-3, expected band size E3 475bp, E4 853bp, (E) HAdv5.CMV.TIMP-3REV expected band size E3 475bp, E4 853bp, (H) HAdV5.SHCMV.TIMP-3 expected band size E3 475bp, E4 853bp. Numbers indicate plaque numbers, + is plasmid control, - is water. **C, F, I.** Western blot analysis of TIMP-3 expression from indicated HAdv5.CMV.TIMP-3 variants. (C) HAdV5.CMV.TIMP-3, expected band size 24kD. Numbers 1-3 refer to plaque numbers 1-3 from viral passage 2, numbers 4-6 refer to plaque numbers 1-3 from viral passage 3, number 7 refers to original University of Glasgow HAdV5.CMV.TIMP-3 at viral passage 2, number 8 and 9 refer to HAdV5.CMVTO.TIMP-3 plaque number 1 at viral passage 2 and 3, respectively. MM refers to Magic Marker, - refers to uninfected HeLa cells. (F) HAdv5.CMV.TIMP-3REV, expected band size 24kD. Numbers indicate plaque numbers, PM refers to pre-stained marker, MM refers to Magic Marker, - refers to uninfected HeLa cells. (I) HAdV5.SHCMV.TIMP-3, expected band size 24kD. Numbers 1-3 refer to plaque numbers 1-3 from viral passage 2, numbers 4-6 refer to plaque numbers 1-3 from viral passage 3, number 7 refers to original University of Glasgow HAdV5.CMV.TIMP-3 at viral passage 2, number 8 and 9 refer to HAdV5.CMVTO.TIMP-3 plaque number 1 at viral passage 2 and 3, respectively. MM refers to Magic Marker, - refers to uninfected HeLa cells.



Supplementary Figure 5. Transgene PCR of HAdV5.CMV.TIMP-3 Variants at Viral Passage 10.

TIMP-3 PCR analysis from viral plaque isolated at passage 10 from HAdV5.CMV.TIMP-3 variants as indicated. M refers to marker, - refers to water control, + refers to plasmid control. Numbers indicate plaque numbers at viral passage 10.