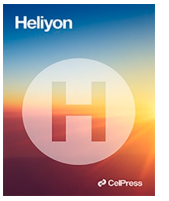
Heliyon 9 (2023) e17067

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Serum-free lentiviral vector production is compatible with medium-resident nuclease activity arising from adherent HEK293T host cells engineered with a nuclease-encoding transgene

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A R T I C L E I N F O

Keywords:
Lentivirus
Gene therapy
Serum
Medium
Nuclease
Transgene
Transient
Transfection
T-cell

A B S T R A C T

At present lentiviral vector production for cell and gene therapy commonly involves transient plasmid transfection of mammalian cells cultivated in serum-containing media and addition of exogenous nuclease to reduce host cell and plasmid DNA impurities. Switching from serum-containing media to chemically-defined, serum free media, and minimising the number of process additions, are both increasingly regarded as necessary steps for simplifying and potentially automating lentiviral vector bioprocessing in future. Here we adapted human embryonic kidney 293T (HEK293T) cells to grow in serum-free media and also modified these cells with transgenes designed to encode a secreted nuclease activity. Stable transfection of HEK293T cells with transgenes encoding the *Staphylococcus aureus* nuclease B (NucB) open reading frame with either its native secretion signal peptide, the murine Igκ chain leader sequence or a novel viral transport fusion protein, all resulted in qualitatively detectable nuclease activity in serum-free media. Serum-free transient transfection of human embryonic kidney HEK293T cells stably harbouring the transgene for NucB with its native secretion signal produced active lentivirus in the presence of medium-resident nuclease activity. This lentivirus material was able to transduce the AGF-T immortal T cell line with a green fluorescent protein reporter payload at a level of 2.05×10^5 TU/mL ($\pm 3.34 \times 10^4$ TU/mL). Sufficient nuclease activity was present in 10 μL of this unconcentrated lentivirus material to degrade 1.5 μg DNA within 2 h at 37 °C, without agitation - conditions compatible with lentivirus production. These observations demonstrate that lentiviral vector production, by transient transfection, is compatible with host cells harbouring a nuclease transgene and evidencing nuclease activity in their surrounding growth media. This work provides a solid basis for future investigations, beyond the scope of this present study, in which commercial and academic groups can apply this approach to therapeutic payloads and potentially omit exogenous nuclease bioprocess additions.

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https://doi.org/10.1016/j.heliyon.2023.e17067
Received 13 February 2023; Received in revised form 30 May 2023; Accepted 6 June 2023
Available online 7 June 2023
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1. Introduction

Lentiviral vectors are a promising tool in therapeutic applications for patients with life-threatening or untreatable diseases [1,2]. Lentiviral delivery systems integrate genetic material into the genome of mitotic and non-mitotic cells, providing long term and stable expression in primary cells such as T cells and dendritic cells [3]. In both basic research and clinical settings lentiviral vectors are predominantly generated by transient transfection of multiple plasmids, each encoding different viral components [4,5]. Viral genes are both modified and distributed across multiple plasmids to reduce the risk of recombination producing replication-competent progeny viruses in the producer cells [6]. Progress is ongoing for construction of safe, high-performance stable transfectant producer cell lines as an alternative to transient transfection [7].

A major concern with current transient transfection vector production platforms is the potential for for residual DNA impurities from the plasmids used in transient transfection, and both DNA and RNA released by dead host cells, to be present in final product [8]. This material could potentially expose patients to virulent genes encoding HIV-1 used for vector packaging, Adenovirus E1 and SV40 T-antigen present in HEK293T producer cells and bacterial genes present in plasmid backbones. Also, lentiviral particles and DNA fragments can both be present in lentiviral process streams as material of highly similar size and negative charge profiles, making it difficult to separate them using filtration and chromatography methods [9]. Therefore, the World Health Organization (WHO) and U.S. Food and Drug Administration (FDA) guidelines recommend that the content and size of residual DNA in the final product dose is limited to 10 ng/dose and no more than 200 base pairs in length [10].

Clinical grade products require minimum amounts of process related-impurities such as host cell proteins, lipids and nucleic acids [10]. Animal-derived materials, such as bovine serum, are a serious concern and add costs to characterisation testing. Serum-free

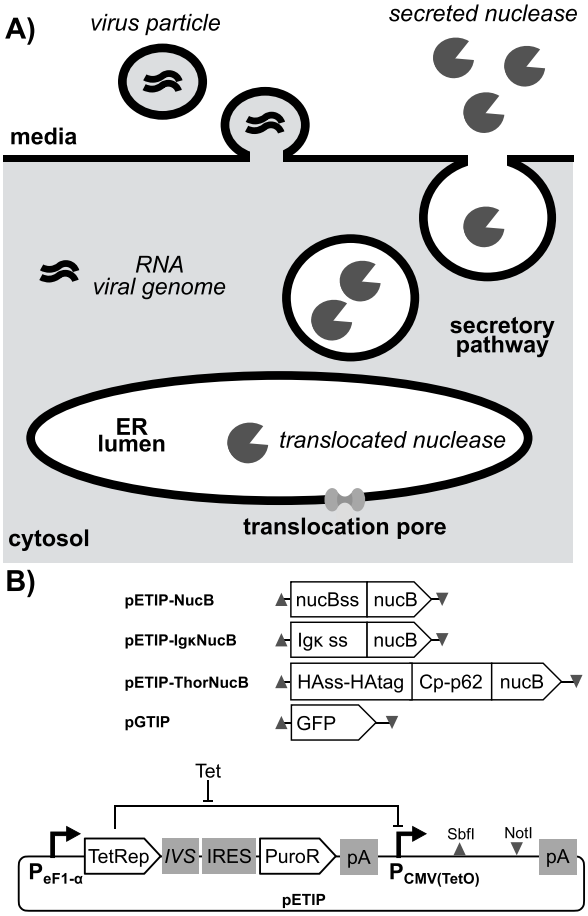


Fig. 1. Design of expression cassettes intended to direct secretion of bacterial nuclease from mammalian cells. A) Schematic overview predicting how viral particle formation, RNA genome packaging and release would be inaccessible to a recombinant nuclease possessing a peptide signal for its translocation to the ER lumen and ultimate transport to the cell exterior via the secretory pathway. **B**) The pETIP plasmids were assembled from fragments encoding NucB with the secretion signal from its native sequence (NucB ss), immunoglobulin-κ (Igκ ss) or the "Thor" domain (HA ss – HA tag – Cp - p62) or d2eGFP. Each fragment was flanked by *SbfI* (upward triangle) and *NotI* (downward triangle) for directional insertion into cognate sites present in the parental plasmid, pETIP. Chimeric intron (IVS), internal ribosome entry site (IRES), polyadenylation signal (pA).

substitute media are recommended to avoid batch-to-batch variation and contamination by potential infectious organisms [11], although this change also removes the benefits from endogenous nuclease properties present in serum [12].

Current production platforms for clinical grade lentiviral vectors reduce residual DNA impurities by treatment with exogenous nucleases, such as Benzonase®, during downstream processing. Treatment with Benzonase® at the recommended concentration of 50 units per millilitre (U/mL) might cost in the order of £50,000, or $90,000, for a 500 L batch of supernatant, making it potentially very costly when scaling up production.

Here we propose an alternative to exogenous nuclease addition in lentiviral vector processing, by modifying the host cell line to secrete nuclease into surrounding growth media. Such an approach has been demonstrated in bacterial production platforms, by introducing transgenes encoding Staphylococcal aureus (*S. aureus*) nuclease B (NucB) to reduce residual DNA and feed viscosity during processing [12–15].

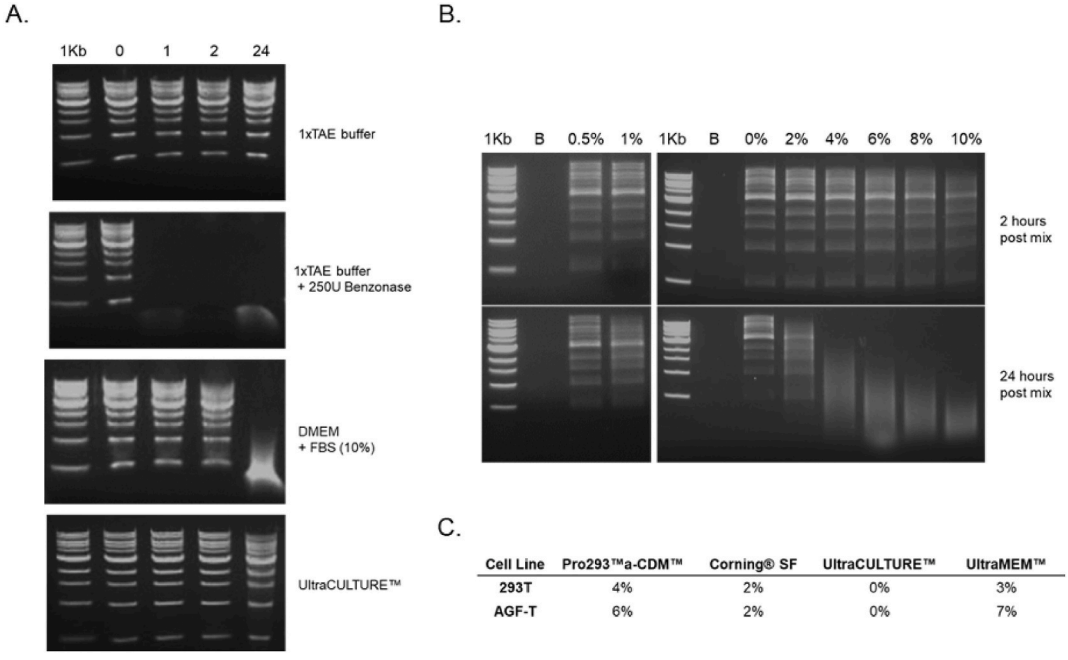
NucB (NCTC 8325) is a Staphylococcal thermo-nuclease, originally characterised due to the high level of heat stability [16]. NucB is a calcium ion dependent, non-specific nuclease that catalyses both DNA and RNA hydrolysis [17]. Interestingly, NucB activity can be enhanced by up to 120% by cell culture media components such as Ca2+, Mg2+, Triton X-100 and Tween 20 [18].

We elected to trial NucB in this study due to its reportedly high levels of activity [19] and the fact it had been shown to be active when expressed in mammalian systems in the past [20]. Mammalian cells have previously been demonstrated to be able to produce bacterial proteins [21,22], despite the fact they can misglycosylate Asn-X-(Ser/Thr) motifs, which are not glycosylation signals in native bacterial proteins, resulting in protein misfolding [22].

Mammalian cells have been shown to be able to translate and secrete bacterial proteins, whether engineered to feature prokaryotic or eukaryotic translocation signal peptides [23,24]. For example, rat Neu7 neural cells were shown to secrete recombinant Proteus vulgaris chondroitinase ABC25. Chinese hamster ovary cells and murine pancreatic 266-6 cells can also produce and secrete the Bacillus subtilis endo 1,4 -β-glucanase enzyme [25]. One reason for this interoperability may be that both eukaryotic and prokaryotic translocation signals tend to predominantly consist of hydrophobic residues [26].

In this work, we describe the construction of a HEK293T cell line adapted to grow in serum-free conditions and harbouring a transgene encoding a *S. aureus* NucB expression cassette. NucB is expressed as a fusion to a secretion signal peptide of either the bacterial *S. aureus* nuclease signal peptide, the mammalian murine Igκ chain leader sequence or a novel fusion protein [27] (Fig. 1), to target the protein for secretion into the surrounding media.

The sole aim of the bench-scale study reported here was to test the hypothesis that engineering a serum-free adapted HEK293T cell line to secrete a detectable level of nuclease activity preserved its ability to produce lentivirus, via transient transfection, at a level



Cell Line	Pro293™a-CDM™	Corning® SF	UltraCULTURE™	UltraMEM™
293T	4%	2%	0%	3%
AGF-T	6%	2%	0%	7%

Fig. 2. Adaptation to serum-free media and detection of nuclease activity in media across a range of serum supplementation levels. 1.5 μg of the same DNA ladder is used in all incubations. **A**). DNA ladder was not incubated with any sample but loaded directly into wells for the lane labelled '1 Kb' in the uppermost gel and the three gel images aligned underneath. DNA ladder was incubated with the sample named at the right of each gel image for the duration in hours indicated above the lanes labelled 0, 1, 2 and 24. **B**) As in panel A, DNA ladder not incubated with sample is present in the lanes labelled '1 Kb' in the upper gel image and the aligned gel image underneath. DNA ladder incubated with 250 U Benzonase® in TAE buffer was loaded in the lanes labelled 'B'. DNA incubated with DMEM supplemented with serum at the indicated v/v percentage was loaded in the lanes labelled according to serum percentage. Duration of incubation in hours is indicated at the right of each gel image. **C**). Cell lines and lowest v/v serum percentage achieved for each serum-free media brand.

comparable with that of the parental cell line. We have purposely omitted from this study any attempt to quantify residual DNA within our modified lentiviral process, as procedures for doing so vary widely in different industrial and clinical settings and residual DNA manifests most readily at larger scales and cultivation intensities than are not typically observed at bench scale. We anticipate that readers engaged in lentiviral vector process development will consider applying the methods reported here and testing their utility for removal of residual DNA from scale production runs with functional payloads.

2. Materials and Methods

2.1. Detection of nuclease activity

Nuclease activity was detected by adding 10 μL of a given sample to 10 μL of a 150 ng/μL solution of 1 kb DNA Ladder, 500bp – 10 kbp, (New England Biolabs), dissolved in nuclease free water (Invitrogen™), at 37 °C for the durations indicated. Reactions were quenched by adding 10 μL of 0.5 M EDTA (Invitrogen™) post incubation and analyzed by 1% w/v agarose gel electrophoresis using tris acetate EDTA (TAE) as gel solvent and buffer and stained with SYBR™ Safe (Invitrogen) according to manufacturer's directions. All agarose gel wells were negative for SYBR™ Safe, as in Fig. 4, however for Figs. 2, 3 and 6 the gel images captured did not have the well in the field of view.

Growth medium samples were prepared by centrifugation of harvested growth media at 1500 rpm for 5 min. Supernatant was then carefully transferred to a new centrifuge tube.

2.2. Plasmid construction and manipulation

Standard molecular biology techniques were used for all plasmid propagation, isolation and analytical procedures. The plasmid pETIP was designed as a parental vector for expression of NucB variants and GFP as a control. The plasmids: pETIP, pGTIP, pETIP-NucB, pETIP-IgκNucB and pETIP-ThorNucB (Fig. 1B), were all assembled using standard molecular biology techniques performed by the commercial service provider, Genscript® (Singapore), with complete sequence data for all expression cassettes available online [28]. The parental pETIP encoded the eF1-α housekeeping promoter upstream of a Tet repressor (TetR) ORF connected to a purmoycin resistance ORF by a synthetic intron and an internal ribosome entry site (IRES), to couple expression of TetR and the puromycin resistance ($Puro^R$) ORF.

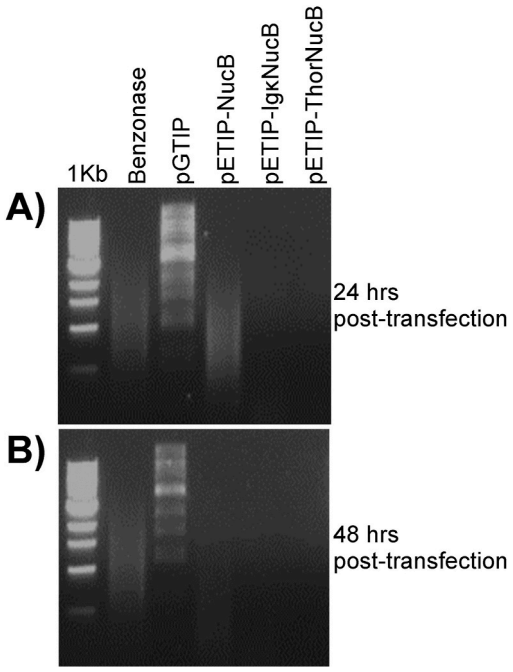


Fig. 3. Detection of nuclease activity in 0.5% v/v serum media from cells transiently transfected with nuclease expression plasmids. Aliquots containing 1.5 μg 1 kB DNA ladder (500bp – 10 kbp) were incubated for 2 h with 4 different samples, or no sample for the lane labelled '1 Kb' in the uppermost gel image **A)** and the aligned gel image **B)** underneath. Ladder incubation was with 250 U Benzonase® in TAE buffer for the lanes labelled 'Benzonase'. Ladder was incubated with growth medium harvested from cells 24 h after they had been transiently transfected, in the presence of 1 μg/mL tetracycline, with either the pETIP-NucB, pETIP-IgκNucB, pETIP-ThorNucB, or pGTIP plasmids, as indicated in the label above the four rightmost lanes of the upper gel image. In the aligned lower gel image, all samples were the same as for the upper gel image except the media samples had been taken 48 h post-transfection.

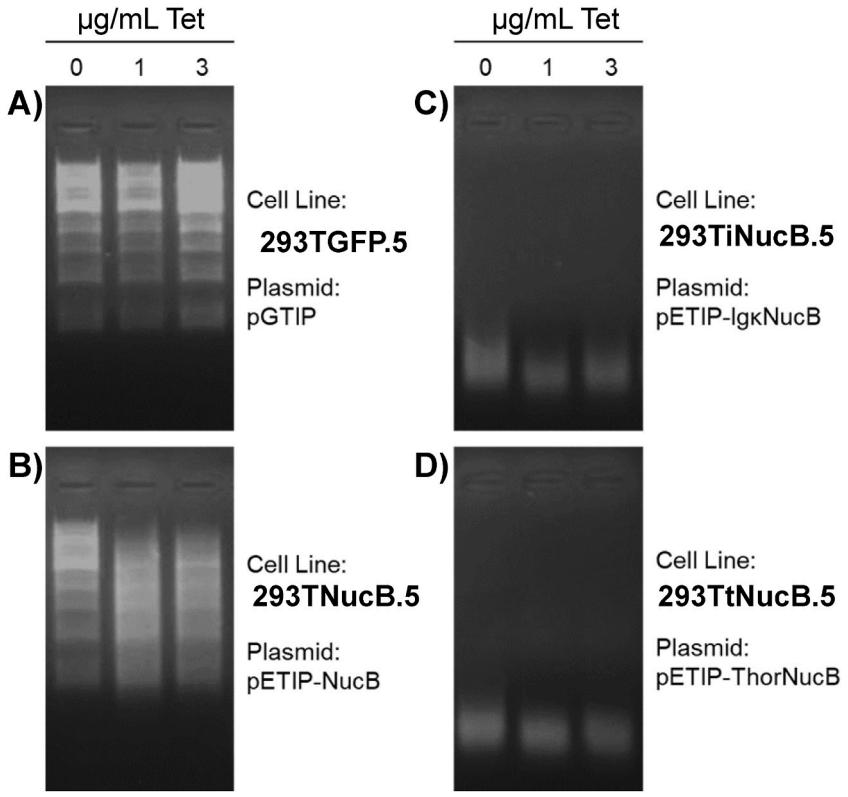


Fig. 4. Detection of nuclease activity in 0.5% v/v FBS media from cells stably transfected with nuclease expression plasmids and incubated with tetracycline. HEK293T cells stably transfected with **A)** pGTIP, **B)** pETIP-NucB, **C)** pETIP-IgκNucB or **D)** pETIP-ThorNucB plasmids were cultivated in the presence of 0, 1 or 3 µg/mL tetracycline for 24 h. For each stably transfected cell line (named on the right of each gel image) a growth medium sample was taken, centrifuged and the supernatant retained. The supernatent sample was incubated with 1.5 µg 1 kB DNA ladder (500bp – 10 kbp) for 2 h then run on a 1% agarose TAE gel. Plasmid and cell line names are given on the right of each gel image and tetracycline concentration present during the 24-h period is indicated above each lane of the uppermost pair of gel images, corresponding also to the lanes in the lower pair of aligned gel images.

A second pETIP expression cassette featured the CMV promoter with tet operator (TetO2) so TetR could repress the CMV promoter, with addition of tetracycline causing derepression. As such, for the family of four assembled plasmids, expression from the second cassette was potentially inducible by addition of tetracycline to growth media. *S. aureus* nuclease (NucB) was encoded with its native transloction signal (NucBss), in a fragment of DNA illiustrated in the uppermost diagram within panel B of Fig. 1 which was used to generate plasmid pETIP-NucB. The NucBss signal was swapped for a mammalian translocation signal (Igκ ss) in the DNA fragment used to generate pETIP-IgκNucB (Fig. 1B, second diagram from top). The NucBss signal was swapped for an engineered translocation domain, termed 'Thor' in the DNA fragment used to generate plasmid pETIP-ThorNucB. The Thor domain consisted of, starting at the amino terminal, an influenza hemagglutinin translocation signal peptide fused directly to an influenza hemagglutinin eptitope tag (HAss-HAtag) followed by a 62-residue region of the amino terminal domain of the Semliki Forest Virus capsid protein (Cp-p62). The final DNA fragment (fourth diagram down from the top of Fig. 1B) encodes green fluorescent protein (GFP) and was used to generate the plasmid pGTIP. All four ORF-encoding fragments were designed to be flanked upstream by an *Sbf*I restriction site (upward pointing triangle) and downstream by a NotI restriction site (downward pointing triangle) for directional insertion into cognate sites present in the parental plasmid, pETIP (lowermost diagram Fig. 1B).

2.3. Mammalian cell cultivation

HEK293T/17 (ATCC® CRL-11268™) and AGF-T Human T (ATCC® CRL-11391™) cell lines were both maintained in DMEM, high glucose, supplemented with 2 mM GlutaMAX™ (Gibco™), 10% vol/vol (v/v) fetal bovine serum (FBS) sourced from Sigma-Aldrich, and penicillin-streptomycin (100 U/mL; Gibco™). Indicated cell lines were adapted grow in UltraCULTURE™ (Lonza), supplemented with 2 mM GlutaMAX™ (Gibco™), 2 mM GlutaMAX™ (Gibco™), and penicillin-streptomycin (100 U/mL; Gibco™) supplemented either with 0.5% v/v Tet System Approved FBS, sourced from Takara Bio, or without FBS supplementation. All cells were incubated at 37 °C with 5% CO_2 and passaged every 3-to-4 days.

2.4. Serum free adaptation of mammalian cell lines

Attempts were made to sequentially adapt and maintain HEK293T and AGF-T cells in Corning® SF media or the following serum-free media brands from Lonza: Pro293™a-CDM™, UltraCULTURE™ and UltraMEM™ (supplemented with 2 mM GlutaMAX™ from Gibco) - all of which were supplemented with penicillin-streptomycin (100 U/mL; Gibco™). Stepwise serum reductions were made by diluting 10% v/v FBS DMEM media with a given serum-free medium brand to reduce overall v/v FBS percentage in 1% increments. When cells were observed to proliferate readily at given FBS percentage, the FBS percentage would then be reduced by a further 1% increment until a percentage was reached at which cells did not readily proliferate (Fig. 2C) or until a media mix with 1% v/v FBS was achieved. At this step, cells were adapted to grow in 0.5% FBS v/v UltraCULTURE™. Cell lines adapted to 0.5% FBS v/v UltraCULTURE™ were then adapted by one further reduction increment to serum-free UltraCULTURE™.

2.5. Transfection of HEK293T cells with nuclease-encoding plasmids

Cells of the HEK293T cell line adapted to grow in 0.5% FBS v/v UltraCULTURE™, termed 293TUC.5, were stably transfected with 10 μg of either pETIP-NucB, pETIP-IgκNucB, pETIP-ThorNucB and pGTIP as control using SuperFect (QIAgen) at a 1:5 DNA:SuperFect mass ratio on a 100 mm dish according to manufacturer's instructions and selected in 100 ng/mL puromycin from 48 h post transfection, along with a plasmid-null negative control. Puromycin-resistant populations arising from this procedure were named as indicated in Fig. 4. Transient transfection with the above cells and plasmids was performed as above except tetracycline was added to 1 μg/mL 24 h post transfection, cell media was characterised 48 h post transfection and puromycin selection was not performed.

2.6. Lentiviral vector production

For a given transient transfection, 2×10^7 cells were seeded into a T175cm [2] flask one day ahead, to achieve a confluence of 80% on the day of transfection start. Branched (average molecular weight 25 kDa, Cat. No. 408727, Sigma Aldrich) polyethylenimine branched (PEI), dissolved in phosphate buffered saline (Gibco™) which had been adjusted to pH 4.5 with HCl, was used as transfection reagent. 1 μL of 10 mM PEI was added to 5 mL of Opti-MEM™ in a 15 mL Falcon tube. In a separate 15 mL Falcon tube, four plasmid solutions were added together and topped up with Opti-MEM™ to a total volume of 5 mL. For this step a sufficient volume of each plasmid solution was added to achieve a mass of 25 μg of the green fluorescent protein (GFP) payload plasmid, pRRLSIN.cPPT. PGK-GFP.WPRE (Addgene #12252), 32.5 μg pMDgp/RRE (Addgene #12251), 25 μg pRSV-Rev (Addgene #12253) and 17.5 μg pMD2.G (Addgene #12259), encoding the VSV-G envelope protetin. The PEI solution and the four-plasmid solution were separately passed through 0.45 μm Millex-HP Syringe Filter units (Millipore, USA) before being combined in a 10 mL transfection solution in a single tube, mixed by gentle inversion and incubated at room temperature for 30 min. Ten minutes prior to the end of this incubation, growth medium was removed from cells and replaced with 10 mL of Opti-MEM™ with 2 mM GlutaMAX™ (Gibco™). At the end of the 30-min incubation the Opti-MEM™ was removed from the cells and replaced with the 10 mL transfection solution. The cells were then incubated at 37 °C with 5% CO_2 for 5 h, after which time the transfection mixture was replaced with 15 mL of growth medium. 48 h post transfection this growth medium was withdrawn from cells and centrifuged at 300 RCF for 5 min to pellet cells and cell debris. The supernatant was removed carefully from pelleted material, passed through a 0.45 μm filter into a 15 mL Falcon tube and stored at 4 °C for 24 h prior to transduction experiments. After this 24-h period all lentiviral supernatant samples were placed at −80 °C for long-term storage.

2.7. Determination of functional lentiviral vector titre

293TUC.5 and 293TUC cells were seeded at 2.5×10^4 cell per well in 48-well plates 24 h prior to transduction, AGF-T UC cells were seeded in the same manner and incubated with lentiviral material on the same day as seeding. Growth media was replaced with lentiviral vector dilutions in fresh growth media. Cells were harvested 72 h after addition of lentiviral material, fixed with 1% PFA (paraformaldehyde; Thermo Scientific™) in PBS and fluorescence measured by flow cytometry, normalised against cells incubated with medium only as negative control. The vector titre in transducing units per mL (TU/mL) was calculated by dividing the total number of positive cells by the volume of vector added in mL. The probability that an individual cell will absorb a given number of viral particles can be modelled using Poisson distribution [29]. Multiple titres were calculated from one representative experiment, where the final viral titre was estimated using the percentage of positive cells and calculated titre to plot a dose dependent curve and calculate for the viral titre value for 5% positive cells. Where a dose dependent curve could not be generated, titre results for positive cell values closest to 5% were used, and negative values were set to 0. In transductions where RetroNectin® (Takara Bio) was used, wells were coated at a concentration of 5 $\mu g/cm^2$ according to manufacturer's instructions.

3. Results

3.1. Design of plasmids encoding secreted nuclease expression

Our concept for engineering a lentiviral host cell with the ability to secrete nuclease was based on our understanding of the mammalian secretory pathway and the topology of lentirvirus assembly. These processes interplay such that viral particle membrane is defined by the plasma membrane and viral particle content is defined by the cytosol (Fig. 1A). We chose to use *S. aureus* NucB (Uniprot:

P00644) as the recombinant nuclease as it has previously been successfully expressed in mammalian cells in its active form as a membrane-bound fusion protein [23]. Although other soluble recombinant bacterial proteins have been successfully expressed and secreted in mammalian cells [30], we sought to de-risk the process of achieving a HEK293T cell capable of introducing nuclease activity into its surrounding growth media by trialling three different secretion signals with the same NucB main enzyme sequence. We designed three NucB variant ORFs (Fig. 1B), utilising the native *S. aureus* nuclease signal peptide (NucB), the mammalian murine Igκ chain leader sequence (IgκNucB), predominantly used in recombinant monoclonal antibody secretion, and a novel viral fusion protein consisting of the influenza virus hemagglutinin signal sequence (HA) and Semliki Forest virus (SFV) capsid protein (ThorNucB), reported to have recorded the fastest secretion time of 15 min post synthesis [27]. We placed the nuclease expression constructs alongside a puromycin resistance gene to enable selection of stable transfectant cell lines.

3.2. Nuclease activity detected in growth media containing 1% v/v serum or higher

The FBS used to supplement cell culture medium typically possesses endogenous nuclease activity [12]. To detect nuclease activity in FBS we incubated 1.5 μg 1 kb DNA ladder (fragments randing from 500 bp to 10 Kbp in size) in a range of serum-free media (Fig. 2A) and DMEM containing FBS (Fig. 2A and B) in concentrations ranging from 0% v/v to 10% v/v, at 37 °C for up to 24 h. This method of detection of nuclease activity has been used extensively by others [12,23] as it gives unambiguous, binary results regarding the presence or absence of nuclease activity plus a potentially useful qualititative indication as to the relative level of nuclease activity present in a given sample. DNA ladder signal remained unchanged in serum-free DMEM, UltraCULTURE™ and DMEM containing 0.5% v/v serum, whereas DMEM containing 1% v/v serum and above showed a time-dependent degradation of DNA ladder. Benzonase® nuclease is a product widely used in industrial lentiviral bioprocessing [31,32] to remove nucleic acid impurities from process streams. As a positive control we incubated 250 units (U) of Benzonase® with DNA ladder. When Benzonase® was incubated with DNA ladder for 1 h or longer, all ladder fragments were degraded to non-visible levels (Fig. 2A and B). Some minor variation was observed, whereby a sub-500 bp fragment band was visible in two incubations (Fig. 2A, second gel image from top, 1-h and 24-h incubations) and zero bands were visible for four incubations (Fig. 2A, second gel image from top, 2-h incubation, and Fig. 2B, all lanes labelled 'B'). As each incubation was performed independently and not always using the same batch of buffer, NEB 1 kb ladder or Benzonase®, we attribute this variation to the status of the reagents.

3.3. Adapting cells to grow in serum-free media

We next attempted to adapt HEK293T and the immortal AGF-T human T cell line to grow in four different brands of serum-free media (Fig. 2C). The intention with this adaptation was to simulate the likely future scenario of an entirely serum-free, distributed manufacturing platform in which universal donor T cells [33] are programmed via lentivirus delivering personalised payloads at bedside. HEK293T and AGF-T cells were successfully adapted to grow in the UltraCULTURE™ brand of serum-free media with zero serum supplementation. For the other three brands of serum-free media, we could only reduce serum to the non-zero levels indicated in Fig. 2C, as the lowest achievable level that still supported cell proliferation. As part of the process of adapting HEK293T and AGF-T cells to serum-free UltraCULTURE™ media, they were also adapted to 0.5% v/v UltraCULTURE™ (see Materials and Methods) as an intermediate step, as this serum level was shown to harbour no nuclease activity, as tested by DNA ladder disappearance (Fig. 2B).

3.4. Nuclease activity detected in 0.5% v/v serum media from cells transiently transfected with nuclease-encoding plasmids

We examined the ability of each of the three nuclease transgenes to bring about detectable nuclease activity in growth medium, after transient transfection of HEK293T cells that had been adapted to grow in 0.5% v/v FBS UltraCULTURE™ (Fig. 3). Tetracycline was added to the media to give final concentration of 1 μg/mL 2 h post transfection, to induce transgene expression, and media samples were taken 24 and 48 h post transfection.

Incubating 1.5 μg 1 kb DNA ladder (500 bp – 10 Kbp) at 37 °C for 2 h in media taken from cells 24 h after transient transfection with the pGTIP plasmid (encoding only GFP expression) showed no evidence of nuclease activity, with the DNA ladder signal largely unchanged from null-treated ladder (Fig. 3 upper gel image). In the same conditions, incubating DNA ladder with 250 U Benzonase® degraded the ladder to a sub-5kb smear. A similar smear to that caused by 250 U Benzonase® was observed when incubating ladder with media taken from cells transiently transfected with the plasmid, pETIP-NucB, encoding NucB with its native secretion signal. Media from transient tranfections with pETIP-IgkNucB and pETIP-ThorNucB degraded the 1.5 μg 1 kb DNA ladder to the extent that no signal was visible. Media taken 48 h after transient tranfection showed broadly the same pattern of ladder disappearance (Fig. 3 lower gel image).

Nuclease activity detected in 0.5% v/v serum media from cells stably transfected with nuclease-encoding plasmids.

We next evaluated the extent to which tetracycline controlled whether detectable nuclease activity was present in the media of polyclonal populations of cells that had been adapted to grow in 0.5% v/v FBS UltraCULTURE™ and stably transfected with each of the three nuclease-encoding plasmids, and the GFP-encoding plasmid. Puromycin-resistant populations arose within 28 days post-transfection and were observed to have no gross increase or decrease in growth performance compared to the parental cell line. Each of these four cell lines was incubated with 0, 1 or 3 μg/mL tetracycline for 24 h and a 10 μL media sample taken and incubated with 1.5 μg 1 kb DNA ladder (500 bp – 10 Kbp) at 37 °C for 2 h (Fig. 4). Stable transfectant cells generated using the GFP-encoding plasmid, pGTIP, yielded media that had no ability to degrade DNA ladder, irrespective of tetracycline incubation. Media from stable transfectant cells arising from the plasmids, pETIP-IgkNucB and pETIP-ThorNucB (encoding IgκNucB and ThorNucB respectively)

degraded all DNA ladder to non-visible levels, whether cells were incubated in the presence or absence of tetracycline. For pETIP-NucB, encoding NucB with its native secretion signal, the equivalent media degraded DNA ladder to a greater extent when tetracycline was present, compared to when tetracycline was absent (Fig. 4B) in the cell line '293TNucB.5'.

3.5. Active lentivirus produced by transient transfection of 293TNucB.5 cells in 0.5% v/v serum media

The nuclease activity we had detected in the growth media of engineered cells (Fig. 4) was consistent with the recombinant nuclease gene being expressed. We were concerned that such expression might negatively impact lentiviral vector production by, for instance, metabolically overburdening cells or by mistrafficked nuclease gaining access to, and degrading, cytosolic nucleic acids, including 'naked' lentiviral genomes that had yet to be packaged. We tested for negative impacts in the engineered 293TNucB.5 cell line, which we had observed to be most tetracycline-responsive. We elected to generate lentiviral vector by transient transfection with the low cost PEI [5,34] reagent in preference to reagents such as Lipofectamine™ (Invitrogen™), FuGene® (Promega) or SuperFect® (Qiagen) which are prohibitively expensive in the context of industrial scale up [5,35]. Transient transfection of 293TNucB.5 cells (Fig. 5), in the absence of tetracycline, with plasmids encoding a VSV G-pseudotyped lentivirus, with GFP payload, yielded a titre of 1.54×10^6 TU/mL ($\pm 3.56 \times 10^5$). The presence of 1 μg/mL tetracycline during transient transfection of 293TNucB.5 cells (Fig. 5), did not markedly impact titre, yielding 1.49×10^6 TU/mL ($\pm 3.5 \times 10^5$). HEK293T cells that had not been engineered with a nuclease-encoding plasmid but had also been adapted to 0.5% v/v FBS UltraCULTURE™, yielded 1.08×10^6 TU/mL ($\pm 2.56 \times 10^5$).

3.6. Nuclease activity detected in serum-free media from a cell line stably transfected with a nuclease-encoding plasmid

Having determined that the presence of nuclease activity in the media did not impede lentivirus production by transient transfection of cells in 0.5% v/v FBS UltraCULTURE™, we next adapted the 293TNucB.5 cell line to growth in only UltraCULTURE™ with zero serum, generating the new cell line, '293TNucB'. We grew 293TNucB cells in zero serum UltraCULTURE™ in the presence and absence of 1 μg/mL tetracycline for 24 h (Fig. 6). Incubating 1.5 μg 1 kb DNA ladder with serum free media from 293TNucB cells grown in no tetracycline resulted in significant degradation of ladder compared to the null experiment where the ladder was mixed only with loading buffer (Fig. 6, compare leftmost and middle lanes). Media from 293TNucB cells that had been incubated with 1 μg/mL tetracycline for 24 h showed further degradation still, with only a smear visible, compared to the media from uninduced cells which left discrete bands of the ladder still visible (Fig. 6, compare rightmost and central lanes).

3.7. Active lentivirus produced by transient transfection of 293TNucB cells in serum-free media

Lentivirus yield from transient transfection of 293TNucB cells was 5.89×10^5 TU/mL ($\pm 1.49 \times 10^5$) when unmodified HEK293Ts adapted to serum-free UltraCULTURE™, termed '293TUC', were used as target cells for titration. When 293TUC cells were used for both lentivirus production and as target cells, the resultant titre was 9.23×10^5 TU/mL ($\pm 2.62 \times 10^5$), a level comparable with that of the 293TNucB cell line (Fig. 7A, leftmost bar pair).

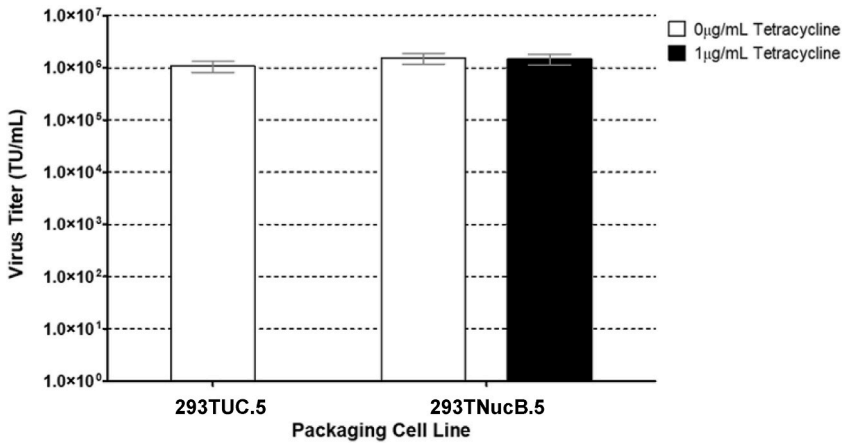


Fig. 5. Lentivirus production in 0.5% v/v FBS media from cells stably transfected with nuclease expression plasmids and incubated with tetracycline. The unmodified HEK293T cell line that had been adapted to grow in 0.5% v/v FBS UltraCULTURE™, referred to as 293TUC.5, and the HEK293T cell line adapted to grow in 0.5% v/v FBS UltraCULTURE™ that had been stably transfected with pETIP-NucB, referred to as 293NucB.5, were each used as host cells for production of lentivirus by transient transfection. 293TUC.5 cells in 0.5% v/v FBS UltraCULTURE™ were used as transduction target cells. 293TUC.5 cells and 293NucB.5 cells yielded lentiviral titres of 1.08×10^6 TU/mL ($\pm 2.56 \times 10^5$) and 1.54×10^6 TU/mL ($\pm 3.56 \times 10^5$) respectively. Transient transfection of 293NucB.5 cells followed by a 5-h incubation with 1 μg/mL tetracycline yielded a titre of 1.49×10^6 TU/mL ($\pm 3.5 \times 10^5$). Titre was calculated using standard curve of known amounts of reference viral material. Errors bars show standard deviation across five tenfold dilutions of lentiviral material arising from a single batch of production by transient transfection.

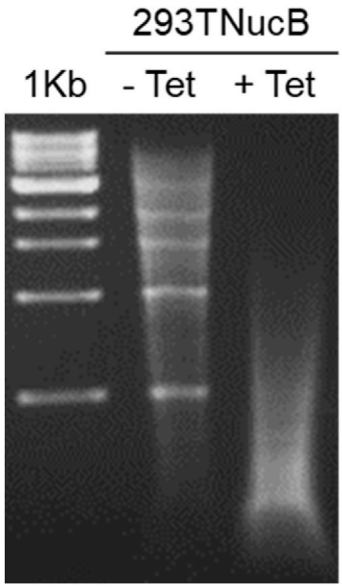


Fig. 6. Detection of nuclease activity in serum-free media from cells stably transfected with a nuclease expression plasmid and incubated with tetracycline. The engineered HEK293T cell line referred to as 293NucB, that had been adapted to grow in serum-free UltraCULTURE™ and stably transfected with the plasmid pETIP-NucB, was incubated in zero or 1 μg/mL tetracycline for 24 h and 10 μL samples of growth media removed and incubated with 1.5 μg 1 kB DNA ladder for 2 h. Reactions were then run on an agarose gel in the lane indicated above the gel image.

We next compared the ability of lentivirus produced from 293TNucB and 293TUC cells to transduce the immortal AGF-T human T cell line, which we had also adapted to growth in serum-free UltraCULTURE™ (Fig. 7A, middle bar pair), termed 'AGF-TUC'. Lentivirus from 293TNucB cells achieved a titre of 1.93×10^5 TU/mL ($\pm 3.14 \times 10^4$), while lentivirus from 293TUC cells achieving the comparable level of 3.31×10^5 TU/mL ($\pm 7.45 \times 10^4$). Including RetroNectin® in the transduction procedure (Fig. 7A, rightmost bar pair) marginally increased transduction of the T cell line by lentivirus from both 293TNucB cells, to 2.05×10^5 TU/mL ($\pm 3.34 \times 10^4$), and 293TUC cells, to 3.40×10^5 TU/mL ($\pm 7.73 \times 10^4$). Nuclease activity was detected, in the same manner as previously, in the unconcentrated lentiviral material derived from 293TNucB cells (Fig. 7B) which was used in all transductions plotted in Fig. 7A. Lentiviral material from unmodified HEK293T cells showed no detectable nuclease acitivity by the method used.

4. Discussion

4.1. *Induction of nuclease transgenes in lentiviral production host cells*

We have previously trialled the use of transgenes encoding Staphylococcal and Serratial nucleases in *Escherichia coli* (*E. coli*) host cells to degrade nucleic acid process stream impurities [36]. Others have also demonstrated that a human DNAse can be used as a process addition to clear DNA impurity from lentiviral process streams [37]. Due its successful prior deployment in *E. coli* process streams, we elected to engineer HEK293T cells, which had been adapted to grow in serum-free media, with transgenes for three variants of Staphylococcal NucB nuclease so they could inducibly effect nuclease activity in their surrounding growth media. These NucB variants featured a secretion signal that was either native to NucB, ported from murine Igκ or consisted of a fusion of secretion signals from influenza HA and SFV capsid. Although expression of all three NucB variants was controlled by the same novel tet-on control unit (Fig. 1), their 'leakiness', with respect to tetracycline induction, varied. Only the native NucB clearly showed less medium-resident nuclease activity when tetracycline was absent (Figs. 4 and 6), while the murine and viral signals resulted in high levels of nuclease activity even in tetracycline-free setups (Fig. 4). This observation suggests the murine and viral signals may have resulted in markedly more active, or more efficiently secreted [26,38–40], NucB enzyme compared to the native signal. Alternatively, the different secretion signal sequence effects may manifest at the mRNA level, via secondary RNA structure. Further characterisation can shed more light on this.

Our *ab initio* rationale for incorporating a novel 'tet-on' expression control unit into the nucleae expression plasmid design (Fig. 1), was to provide the ability to precisely time the initiation of nuclease expression, if it were necessary to do so to preserve the lentiviral titre performance of transiently transfected host cells. Many stable lentiviral producer cell lines feature transgenes in which expression of cytotoxic VSVg envelope is placed under the control of small molecule inducers to maximise the productive lifetime of the cell lines [7,37,41]. In these cases, the burden of demonstrating removal of these small molecules from subsequent process streams is deemed a worthwhile trade-off for the benefits gained from the inducible system [42–44]. In the event, highly detectable levels of media-resident nuclease activity in the presence of tetracycline did not dramaticaly compromise cell growth rates (qualitatively assessed) or lentiviral titre performance compared to unmodified cells (Figs. 6 and 7), suggesting future packaging cell lines with constitutive nuclease

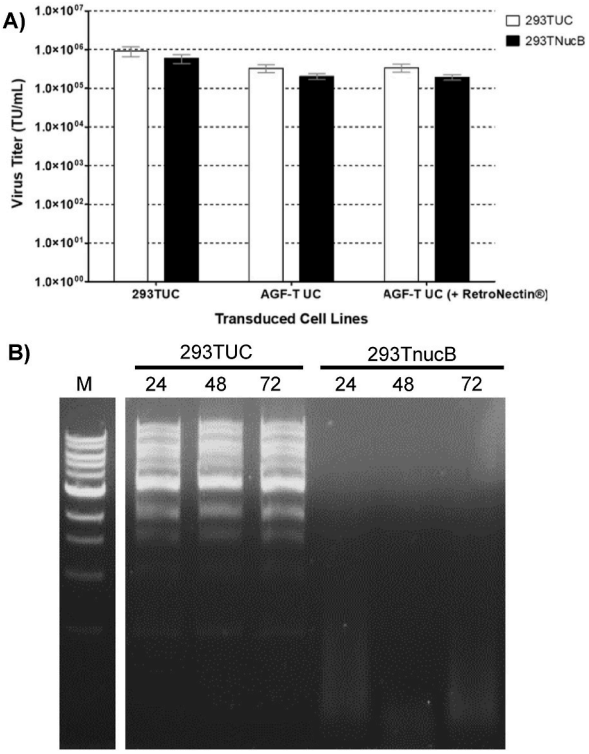


Fig. 7. Lentivirus production in nuclease-active serum-free media from cells stably transfected with a nuclease expression plasmid and incubated with tetracycline. A) The unmodified HEK293T cell line adapted to grow in serum-free UltraCULTURE™, referred to as 293TUC, and 293NucB cells, described in Fig. 6, were each used as host cells for production of lentivirus by transient transfection, with 293NucB cells only supplemented with 1 μg/mL tetracycline 5 h post-transfection. Resultant titres for 293TUC and 293NucB cells were plotted as bar pairs, with transduction targets indicated in the X-axis. When 293TUC cells were used as transduction targets, 293TUC and 293NucB cells yielded titres of 9.23 $\times$ 10^5 TU/mL ($\pm$2.62 $\times$ 10^5) and 5.89 $\times$ 10^5 TU/mL ($\pm$1.49 $\times$ 10^5) respectively. Cells of the immortal AGF-T cell line, adapted to grow in serum-free UltraCULTURE™ and referred to as AGF-T UC, were used as transduction targets. 293TUC and 293NucB cells yielded titres of 3.31 $\times$ 10^5 TU/mL ($\pm$7.45 $\times$ 10^4). and 1.93 $\times$ 10^5 TU/mL ($\pm$3.14 $\times$ 10^4), respectively on AGF-T UC target cells. Respectively, these titres were determined to be 3.40 $\times$ 10^5 TU/mL ($\pm$7.73 $\times$ 10^4) and 2.05 $\times$ 10^5 TU/mL ($\pm$3.34 $\times$ 10^4) when RetroNectin® was present during incubation with AGF-T UC cells. Errors bars show standard deviation across three tenfold dilutions of viral material arising from a single batch of production by transient transfection. **B)** 10 μL samples of lentiviral material from 293TUC and 293NucB cells titred in panel A were taken 24-, 48-, and 72-h post-transfection, incubated with 1.5 μg of 1 kB DNA ladder for 2 h and analyzed by agarose gel in the indicated lanes in the gel image. Comparator DNA ladder, not incubated with media or buffer, was run in the leftmost lane, labelled 'M'. In the panel B gel image, non-relevant intervening lanes were removed by image cropping. Uncropped images available on request.

expression will be compatible with lentivirus production, with no need for a 'tet-on' system or the associated requirement of small molecule bioprocess additions.

4.2. *Serum-free lentiviral bioprocessing*

We measured lentiviral titre performance of unmodified and nuclease-engineered HEK293T cells cultivated in media with low (0.5% v/v) serum (Fig. 5) and no serum (Fig. 7). Data reported in Figs. 5 and 7 were captured from single transient transfection procedures, but with at least three titre measurements, as our aim was to identify only major differences in performance between host cells, such as a tenfold difference in titre or larger. These data are consistent with multiple repeat applications of the HEK293T and nuclease-engineered HEK293T host cells in our hands. Unmodified and nuclease-engineered HEK293T cells both yielded titres of approximately 1 million TU/mL when adapted to growth in media with 0.5% v/v serum (Fig. 5). Adaptation from 0.5% v/v to 0% v/v serum did not reduce titre performance for unmodified HEK293T cells but did result in a reduction from 1.49 $\times$ 10^6 TU/mL ($\pm$3.5 $\times$ 10^5) to 5.89 $\times$ 10^5 TU/mL ($\pm$1.49 $\times$ 10^5) for the nuclease-engineered HEK293T cells (compare Figs. 5 and 7). Further investigation will be needed to determine if this is due to intrinsic factors, such as the stress status of the nuclease-engineered HEK293T cells, or an extrinsic factor such as an influence of medium-resident nuclease activity on the transfection reagents.

With respect to the impact of serum on lentivirus yield perfomance of transient transfection host cells, there are few directly comparable studies in the literature. This is because typically several factors are changed or optimised in parallel when researchers report optimisation of transient transfection for lentivirus production [45]. As a crude comparison, Tirapelle et al. (2022) reported production of approximately 7.5 million TU/mL by adherent HEK293T cells cultivated with media containing 10% v/v serum.

Adaptation to suspension growth in serum-free media reduced this titre performance by two orders of magnitude [46]. Bauler et al. (2020) [3] reported up to ≈50% reduction in titre performance when switching from serum-containing media, adherent cell mode lentivirus production to suspension cell, serum-free media mode. Subsequent increases in titre performance of suspension cell, serum-free media mode production reported by Bauler et al. (2020) [3] required the use of alternative lentiviral vector payload plasmids and intensive optimisation of the transfection procedure.

4.3. Transduction of T cells by lentivirus from nuclease-engineered host cells

Engineered lentiviruses rarely transduce primary and immortalised cells with the same efficiency. For instance, Toscano et al. [47] report higher lentiviral transduction of primary versus immortalised cells, and others have reported the opposite. Here, batches of serum-free lentivirus derived from unmodified and nuclease-engineered HEK293T cells were able to transduce the AGF-T immortal T cell line to a comparable degree. Addition of RetroNectin® did not markedly improve transduction of AGF-T cells (Fig. 7). RetroNectin® is understood to boost lentivirus infectivitity by crosslinking virus particles to VLA-4 and/or VLA-5 integrin receptors on target cells. In this case the non-effect of RetroNectin® may indicate that AFG-T cells do not express sufficient levels VLA-4 and/or VLA-5 integrin ligands for cross linking to have a titre-enhancing effect [42].

In common with all methods where nucleases are a process addition, the approach reported here runs the risk of an immunogenic effect on patients if sufficient recombinant nuclease persists in the product stream. This can be avoided by optimising downstream product purification and impurity removal steps, or by selecting a nuclease proven to be non-immunogenic, either by direct engineering of the enzyme or sourcing an allogenic nuclease. Animal studies could be used to verify if immunogenicity has been removed.

5. Conclusions

This study shows that, in serum-free media, host cells engineered with NucB-based transgenes, that consequently exhibit a media-resident nuclease activity, can be used to make lentivirus via transient transfection. We suggest the serum free viral yield performance of the nuclease-engineered host cell, 293TNucB, was sufficiently close to that of serum-free unmodified HEK293T cells to warrant further investigation. In the first instance, screening clonally-derived populations may identify improved performance compared to the polyclonal populations characterised in this study. Alternative strategies, such as co-transfecting the nuclease-encoding plasmid along with the lentiviral packaging plasmids, would be predicted to achieve similar results. However, such a strategy could reasonably be predicted to come with a 10–30% increase in plasmid production and transfection reagent costs per transient transfection, due to the 10–30% increase in required plasmid mass.

A recent patent application (WO2019175600A1) by Oxford Biomedica (United Kingdom) detailed construction of mammalian cell lines that secrete nuclease and are intended for use in lentivirus production. Unlike the NucB nuclease reported here, nucleases detailed in the Oxford Biomedica 'SecNuc' system underwent mutagenic and codon-usage screening prior to expression in mammalian cells. We suggest this example of commercial implementation of nucleolytic host cells for lentivirus production by transient transfection validates the approach reported here.

We anticipate that future work, beyond the scope of this current study, will apply the cell-engineering strategy reported here to real world clinical processes, where safety and cost reduction is paramount. Bioprocess additions such as Benzonase® are an expensive requirement in current viral vector/vaccine production platforms, to ensure regulators' safety standards are met. If nuclease-engineered host cells are in future shown to sufficiently reduce cellular and plasmid DNA impurities from lentiviral process streams, quantified using reagents such as the Quant-iT™ PicoGreen™ kit (Catalog number: P7589, Thermo Fisher, Waltham, USA), then it may one day be possible to omit costly Benzonase® bioprocess additions. These steps may improve upon current lentiviral bioprocessing and enable the development of new, automated bioprocessing platforms for lentivirus production in the near future.

Author contribution statement

Sadfer Ali: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Milena Rivera: Performed the experiments.

John Ward, Eli Keshavarz-Moore, Chris Mason: Contributed reagents, materials, analysis tools or data.

Darren N. Nesbeth: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

Dr Darren N. Nesbeth was supported by Engineering and Physical Sciences Research Council {EP/G034656/1}

Data availability statement

Data associated with this study has been deposited at Ali S, Trujillo MR; Ward J, Keshavarz-Moore E, Mason C, Nesbeth DN. DNA sequences encoding mammalian expression of Staphylococcal nuclease variants. figshare (2023): Dataset. doi.org/10.6084/m9.figshare.22770212.v1.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are grateful to Didier Trono (University of Geneva, Switzerland) for lentivirus plasmids. We also thank David Darling for early discussions on the concepts developed herein.

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