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Lentiviral vector packaging and producer cell lines yield titers equivalent to the industry-standard four-plasmid process

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Lentiviral vector (LVV)-mediated cell and gene therapies have the potential to cure diseases that currently require lifelong intervention. However, the requirement for plasmid transfection hinders large-scale LVV manufacture. Moreover, large-scale plasmid production, testing, and transfection contribute to operational risk and the high cost associated with this therapeutic modality. Thus, we developed LVV packaging and producer cell lines, which reduce or eliminate the need for plasmid transfection during LVV manufacture. To develop a packaging cell line, lentiviral packaging genes were stably integrated by random integration of linearized plasmid DNA. Then, to develop *EGFP*- and anti-CD19 chimeric antigen receptor-encoding producer cell lines, transfer plasmids were integrated by transposase-mediated integration. Single-cell isolation and testing were performed to isolate the top-performing clonal packaging and producer cell lines. Production of LVVs that encode various cargo genes revealed consistency in the production performance of the packaging and producer cell lines compared to the industry-standard four-plasmid transfection method. By reducing or eliminating the requirement for plasmid transfection, while achieving production performance consistent with the current industry standard, the packaging and producer cell lines developed here can reduce costs and operational risks of LVV manufacture, thus increasing patient access to LVV-mediated cell and gene therapies.

INTRODUCTION

Lentiviral vectors (LVVs) are an attractive cell line engineering option for *ex vivo* cell therapies, particularly chimeric antigen receptor (CAR)-T cell therapy, and have shown promise in the development of *in vivo* gene therapies.[1–4] However, the dependence of LVV production on plasmid transfection is a contributing factor to the high cost of goods associated with this cell therapy modality.[5,6] This is due in part to the operational complexity and risk associated with producing several plasmid batches.[5] In addition, the cost of raw materials associated with plasmid dependence is high: specifically, both the sourcing of large quantities of transfection reagent, and the production and testing of a number of large-scale, Good Manufacturing Practices (GMP)-grade plasmid preparations, are costly.[5,6] Furthermore, the requirement to remove residual plasmid DNA and transfection reagent from LVV preparations contributes to the complexity and thus the cost of downstream processing.[6] Despite these operational and technological drawbacks, the current standard LVV production modality relies on the simultaneous transfection of four plasmids.[7,8] To address these issues, several research groups have attempted to reduce or eliminate plasmid transfection dependence by developing various stable LVV production cell lines, which require production/transfection of only one or no plasmids (reviewed by Ferreira and colleagues[9]).

Stable packaging cell lines have all LVV genetic sequences, except the LVV transfer plasmid stably integrated into the cell genome, thus requiring preparation, testing, and transfection of only one plasmid to produce LVV, as opposed to four for fully transient production. Stable producer cell lines, however, have all LVV-producing elements integrated, thus requiring no plasmid transfection to produce LVV. This is advantageous as scaling up plasmid transfection to

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manufacturing scale is a significant challenge.[5,10,11] In both cases, elimination of the requirement to produce, test, and transfect three or four batches of plasmid DNA reduces the cost, complexity, and operational risk of the manufacturing process.

Although stable LVV production cell lines have the potential to solve several issues, there are technical challenges associated with their development. The first is that, unlike transient systems, stable production cell lines require development before they can be used for a given purpose. For instance, in the case of packaging cell lines, if LVV particles are required to be pseudotyped with a glycoprotein other than what was already encoded by the packaging cell line, then a new cell line would require development. In addition, a new producer cell line requires development each time the cargo gene is changed.

A further technical challenge is that stable LVV production cell lines are often less productive than fully transient systems. When using the four-plasmid transfection method to produce GFP-encoding LVV, one could expect a titer of $\sim 1 \times 10^7$–1×10^8 transducing units (TU)/mL (pre-downstream processing).[12,13] In contrast, when preparing GFP-encoding LVVs with a stable production cell line, one could expect a titer of $\sim 1 \times 10^6$ TU/mL.[14,15] (It must be noted that comparison of titers from different publications is controversial as different titration methods are used in different laboratories. Thus, comparisons must be taken as an approximate guide only.) The reason for this disparity between the modalities is not fully understood, as it could depend on the characteristics of the specific production cell line at hand. For example, we have previously been able to increase packaging cell line titers by supplementing cells during production with additional copies of already-integrated LVV plasmids by transient transfection. In this experiment, different plasmids impacted titer to different extents, suggesting that in this case, production had been limited by the integrated copy numbers of the various constructs (M.M. and M.R., unpublished data). Another factor that could explain the productivity discrepancy between stable cell lines and the fully transient system is the long-term host cell metabolic burden by leaky expression of integrated genes[16,17]: cytotoxic and/or cytostatic effects in production cells due to the expression of vesicular stomatitis virus G protein (VSV-G), Rev, and potentially the cargo gene might be amplified in stable production cell lines compared to the fully transient system. This is expected since there would be more time for accumulation of the gene products when the genes are stably integrated than if plasmids were used transiently. To address this challenge, stable cell lines have been developed with VSV-G and Rev expression controlled by chemically inducible promoters, to limit expression to the production window only. Inducible LVV packaging/producer cell lines have included tetracycline-repressible/inducible, and cumate-inducible expression systems and combinations thereof.[18–24] Other factors limiting the productivity of stable cell lines compared to fully transient systems could include: instability of integrated constructs[25,26]; shorter LVV gene expression window in systems that require induction than in fully constitutive, fully transient production systems; disruption of LVV gene expression by readthrough by host cell factors or, if the construct formed a concatemer before integration, readthrough by neighboring concatemer subunits[27,28]; and silencing of LVV gene expression by host chromatin remodeling.[29]

In addition to the technical challenges described above, a regulatory challenge associated with developing stable LVV production cell lines is the perceived risk of replication-competent lentivirus (RCL) generation. There remains concern that recombination events between stably integrated lentiviral sequences could result in the generation of RCL. However, when Chen and colleagues developed an LVV producer cell line with all LVV genes encoded by a single construct, neither the US Food and Drug Administration (FDA) nor the European Medicines Agency raised concerns beyond what would be expected for a typical LVV production method.[30] Moreover, assays to detect RCL can be performed during development and manufacturing.[31]

Here, we describe two iterations of clonal packaging and producer cell line generation based on suspension HEK293 cells, an easily scalable platform for LVV manufacturing. The first iteration was based on the HEK293BSusp_MCB1 cell line (suspension HEK293 cell line owned by OXGENE, Oxford, UK), and the second was based on the WXATUS0028 cell line (adherent HEK293 cell line owned by WuXi ATU and adapted to suspension in serum-free media). In both cases, the integrated plasmids encoded several safety features to reduce the theoretical risk of RCL generation (described in detail below). Packaging cell line version 1.0 produced on average 3.0×10^7 TU/mL with *EGFP* as the gene of interest (GOI). Producer cell line version 1.0 produced on average 9.0×10^7 TU/mL with *EGFP* as the GOI. Our second generation of packaging cell line (version 2.0) yielded an average of $\sim 1.5 \times 10^8$ TU/mL and the producer cell line version 2.0 produced on average 2.5×10^8 TU/mL (both with *EGFP* as the GOI). For comparison, using the WXATUS0028 cell line for fully transient production typically yields $\sim 1.5 \times 10^8$ TU/mL. Thus, the version 2.0 packaging/producer cell lines are a substantial improvement upon the version 1.0 cell lines and are comparable in terms of LVV production yield with the fully transient system.

Furthermore, we produced LVV preparations encoding various therapeutically relevant GOIs using the fully transient system and the version 2.0 packaging/producer cell lines, demonstrating that the stable systems are consistent with the fully transient system. Finally, we developed a method to screen GOI constructs rapidly in packaging cell lines and to estimate the production titer in an equivalent producer cell line.

RESULTS

Plasmid performance in transient system

The plasmids used to generate stable packaging/producer cell lines were third-generation, self-inactivating lentiviral vector production plasmids (Figure 1). They were designed with the following features: (1) *Gag-Pol* and *VSV-G* gene expression was controlled by Tet-repressible promoters to limit production cell cytotoxicity; (2) *Gag-Pol* and *VSV-G* genes were encoded within separate cassettes on

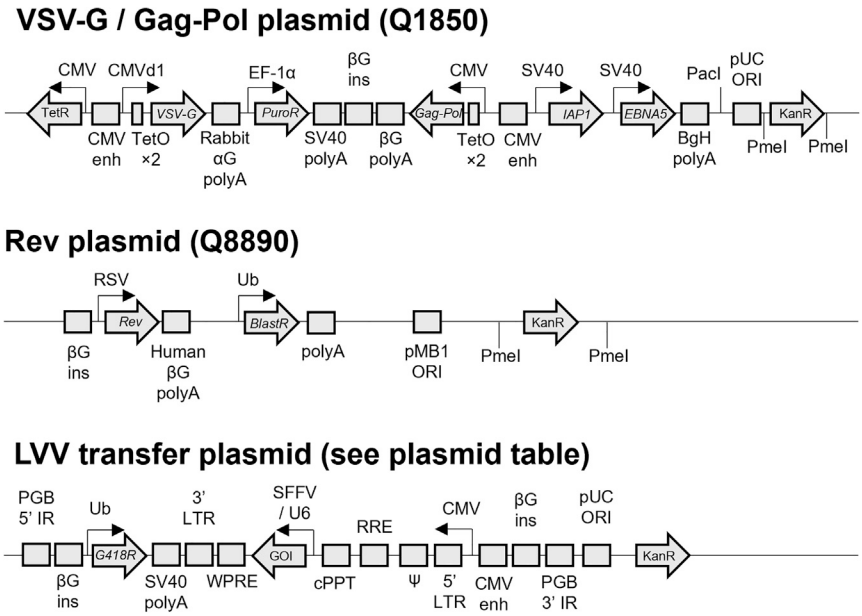

Figure 1. Scheme of plasmid sequences

LVV transfer plasmid identity depends on which GOI is encoded (please refer to Table 1). TetR, tetracycline resistance element repressor; βG ins, β globin insulator; βG polyA, β globin poly-adenylation signal; BgH polyA, bovine growth hormone poly-adenylation signal; BlastR, blasticidin resistance gene; CMV, cytomegalovirus promoter; CMVd1, cytomegalovirus promoter d1; CMV enh, cytomegalovirus promoter enhancer; cPPT, central polypurine tract; *EBNA5*, Epstein-Barr virus-encoded nuclear antigen 5; EF-1α, human elongation factor-1α promoter; G418R, G418 resistance gene; *Gag-Pol*, HIV-1 Gag-Pol gene; *IAP1*, *Bombyx mori* nucleopolyhedrovirus inhibitor of apoptosis 1 gene; KanR, kanamycin resistance gene; 3′ LTR, lentiviral 3′ long terminal repeat; 5′ LTR, lentiviral 5′ long terminal repeat; PGB 5′ IR, piggyBac 5′ inverted terminal repeat; pMB1 ORI, pMB1 origin of replication; ψ, HIV-1 packaging signal; pUC ORI, pUC origin of replication; *PuroR*, puromycin resistance gene; Rabbit αG polyA, rabbit α globin poly-adenylation signal; *Rev*, HIV-1 *Rev* gene; RRE, Rev response element; RSV, rous sarcoma virus promoter; SFFV, spleen focus-forming virus promoter; SV40 polyA, simian vacuolating virus 40 poly-adenylation signal; SV40, simian vacuolating virus 40 promoter; Ub, ubiquitin promoter; U6, U6 promoter; *VSV-G*, vesicular stomatitis virus protein G gene; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

opposing strands to prevent co-transcription and co-packaging of these genes, to reduce the risk of formation of RCL; (3) to limit production cell death during LVV production, *Bombyx mori* nucleopolyhedrovirus inhibitor of apoptosis 1 (*IAP1*) gene and Epstein-Barr virus-encoded nuclear antigen 5 (*EBNA5*) were encoded by the *VSV-G*/*Gag-Pol* plasmid; (4) the *Rev* coding sequence (CDS) was codon optimized for expression in human cells; (5) since a low level of *Rev* expression was required, a β globin insulator was positioned upstream of the gene to prevent distal activation by host cell factors; (6) since high levels of *VSV-G* and *Gag-Pol* expression were required (upon induction), β globin insulators were positioned both upstream and downstream of these genes to limit silencing by heterochromatin.

To limit the risk of transduction of the *IAP1*/*EBNA5* genes, they were encoded within the *VSV-G*/*Gag-Pol* plasmid, rather than on the transfer plasmid. It was thus concluded that the likelihood of IAP1/EBNA5 transduction would be similar to the likelihood of RCL formation, which we have also deemed acceptable. Although we did not test for transduction of these genes here, LVV sequencing and host cell DNA testing against these two genes are part of our release specifications following manufacturing, in line with FDA guidance regarding the control of host cell DNA.[32] Thus, prior to the release of LVV batches, controls would be in place to further diminish the risk of transduction of *IAP1*/*EBNA5* genes.

Since several alterations were made to their sequences, the LVV production performance of the in-house LVV plasmids was assessed in comparison to two commonly used commercial systems in a fully transient production setup using suspension HEK293 cells in the absence of antibiotics. The LVV preparations generated using our in-house plasmids had the highest infectious titer of the three sets tested (mean titer = 2.56×10^7 TU/mL, SD = 7.20×10^4 TU/mL; Figure 2A). In addition, the physical/infectious (P/I) ratio of the LVV preparation generated with our plasmids was lower than that of those generated with sets A and B (in-house = 124 viral particles [VP]/TU [SD = 5 VP/TU], set A = 250 VP/TU [SD = 14 VP/TU], set B = 174 VP/TU [SD = 26 VP/TU]; Figure 2B). Our plasmids were thus used to develop LVV packaging and producer cell lines.

Development of packaging cell line version 1.0: LV001

LVV packaging cell line LV001 was developed by two successive rounds of plasmid integration and clonal cell line isolation. First, linearized Q1850 plasmid (encodes *VSV-G* and *Gag-Pol*; plasmid linearized with PmeI restriction enzyme) was integrated into the genome of HEK293-Ox (HEK293BSusp_MCB1; CD293 media-adapted suspension HEK293 heterogeneous pool acquired from Oxford Clinical Biomanufacturing Facility, University of Oxford). Clonal isolation by fluorescence-activated cell sorting (FACS), then screening for VSV-G expression by immunostaining after the addition of doxycycline and for *VSV-G* and *Gag-Pol* sequence by PCR, yielded a clonal pre-packaging cell line, hereafter referred to as LVPP001. Next, LVPP001 was adapted to BalanCD HEK293 media, then transfected with linearized Q8890 plasmid (encodes *Rev* gene under the constitutive respiratory syncytial virus [RSV] promoter; plasmid linearized with PmeI restriction enzyme).

Clonal packaging cell lines were isolated by FACS. The selection of the top performers involved (1) cell doubling time and lactate production monitoring and (2) in E125 flask format, assessment of transfection efficiency (using Q1365 transfer plasmid: encodes SFFV [spleen

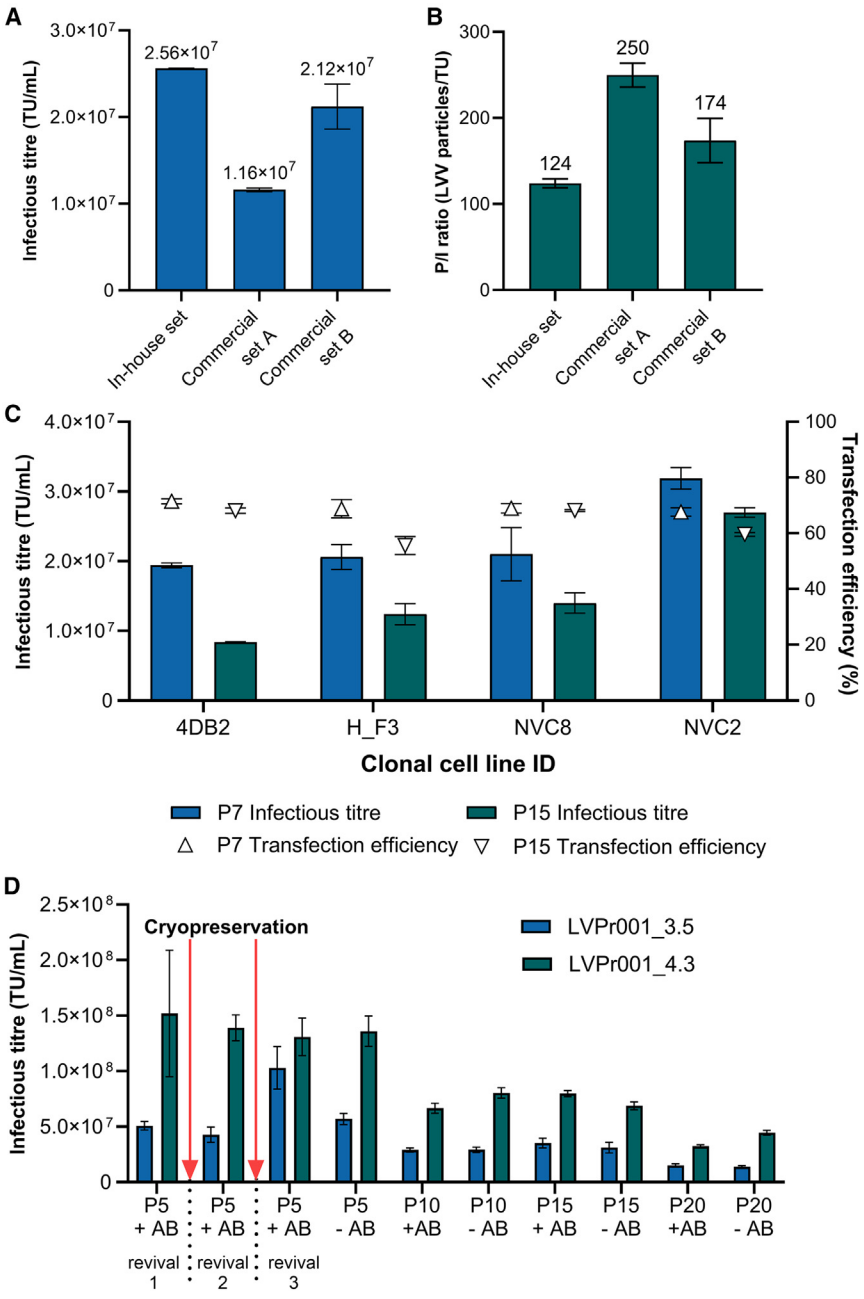

Figure 2. First iteration of stable lentiviral vector production cell line development

Comparison of (A) infectious titers and (B) physical/infectious titer ratios (LVV particles/TU) of in-house LVV plasmids in a fully transient production format compared to two equivalent commercially available plasmid sets. Physical titration by ELISA. Infectious titration by flow cytometry. *n* = two biological replicates; error bars indicate SD. (C) Lentiviral packaging cell line version 1.0 top four clonal cell lines' stability testing. Transfection efficiencies and LVV infectious titers at passage numbers 7 and 15. Infectious titration by flow cytometry. *n* = two biological replicates. Error bars indicate SD. (D) Long-term stability testing of top two LVV producer version 1.0 clonal cell lines. P *x*, passage number *x*. Infectious titration by flow cytometry. *n* = three production replicates. Error bars indicate SD. No AB, no antibiotics present.

focus-forming virus promoter]-*EGFP*-WPRE [woodchuck hepatitis virus posttranscriptional regulatory element] within an LVV genome driven by cytomegalovirus [CMV] promoter with a CMV enhancer) and infectious titer by flow cytometry. The top four clonal cell lines had doubling times of 28.7–34.1 h, had (lactate produced)/(glucose consumed) ratios of 0.83–0.96, had transfection efficiencies of 43.9%–63.5%, and produced 1.14×10^7–3.67×10^7 TU/mL (infectious titer by flow cytometry). These top four cell lines were then tested for stability. Replicate clonal cell lines were passaged both with and without antibiotic supplementation and were tested for LVV production at passage numbers 7 and 15. Transfection efficiencies varied between 55% and 70% across all cell lines (Figure 2C). Clone NVC8 had the most consistent transfection efficiency of 68%–70%. Production titers varied between 8.35×10^6 and 3.19×10^7 TU/mL (infectious titer by flow cytometry), with clone NVC2 producing the highest titers most consistently (2.70×10^7–3.19×10^7 TU/mL; infectious titer by flow cytometry; Figure 2C). LVV packaging cell line clone NVC2 was thus cryopreserved for further use and development and was renamed LV001.

Development of producer cell line version 1.0: LVPr001

An initial attempt to develop an LVV producer cell line used random integration of PmeI-linearized Q8887 plasmid (encodes *Rev* and LVV genome with *EGFP* as the GOI) and yielded a clonal cell line that produced 3×10^6 TU/mL (infectious titer by flow cytometry; data not shown). This titer was deemed insufficient to be competitive with the industry-standard four-plasmid transfection method. Thus, piggyBac transposon technology was used here with the rationale that it should result in a high number of integrations per cell[33] and integrations directed to transcriptionally active loci,[34,35] which thus might result in stable cell lines that produce high LVV titers.

To generate producer cell line pools, LV001 (passage 19) cells were transfected with plasmids R2435 (*EGFP*-encoding LVV genome flanked by transposon inverted terminal repeats; Figure 1, LVV transfer plasmid) and Q9751 (piggyBac transposase). Next, clonal producer cell lines were isolated by FACS. To select the top two clonal

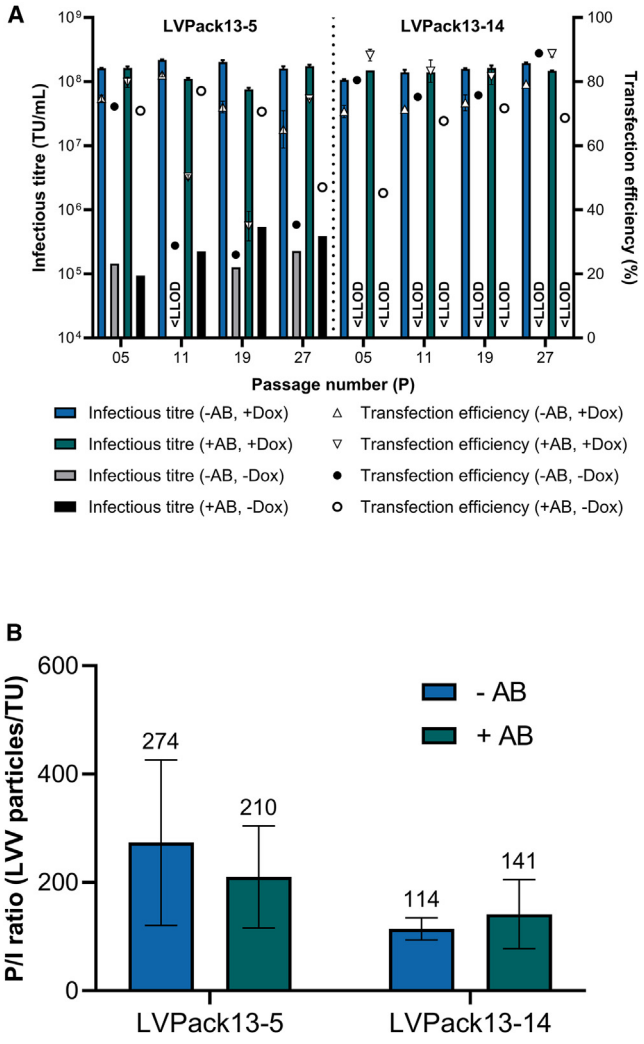

Figure 3. Second iteration of packaging cell line development

(A) Long-term stability testing in the presence and absence of antibiotic selection of top two clonal LVV packaging cell lines derived from WXATUS0028, LVPack13-5, and LVPack13-14. Infectious titration by flow cytometry. *n* = two biological replicates. Error bars indicate SD. (B) Average P/I titer ratios of top two LVPack clonal cell lines throughout long-term stability testing. *n* = four biological replicates. Error bars indicate SD.

cell lines, LVV production was tested. The highest observed titer in this screen was 2.3×10^8 TU/mL (SD = 3.6×10^7 TU/mL) (infectious titer by flow cytometry; data not shown). The top two clonal cell lines were named LVPr001_3.5 and LVPr001_4.3.

To test the long-term stability of the top two LVPr001 clonal cell lines, they were subjected to repeated rounds of cryopreservation and revival and subcultured with and without antibiotics, while LVV production was tested (full description in materials and methods). Clonal cell line LVPr001_4.3 consistently out-performed LVPr001_3.5, but still lost productivity over the course of the experiment (Figure 2D). Cryopreservation did not have a marked impact on productivity. Subculturing without antibiotics did not have a consistent effect on productivity: in only one instance did the non-antibiotic culture perform worse than the corresponding antibiotic-containing culture (Figure 2D, LVPr001_3.5 P5 after second round of cryopreservation and revival). In summary, over 30 passages a negative trend in LVV titer was observed: with 2 rounds of cryopreservation and 3 rounds of revival, clones LVPr001_3.5 and LVPr001_4.3 lost 3.4- to 4.7-fold productivity. This was not altered by the presence of antibiotics in the cell culture medium during routine subculture between productions.

Development of packaging cell line version 2.0: LVPack13-14

To further improve the characteristics of LV001, a new packaging cell line was developed using the WXATUS0028 cell line (clonal, suspension HEK293 cell line, selected for high growth rate and low aggregation; kindly provided by WuXi Advanced Therapies, Philadelphia, PA). To generate a packaging cell line pool, PmeI-/PacI-linearized Q1850 and PmeI-linearized Q8890 plasmids were simultaneously transfected into WXATUS0028 cells (passage number 6). Next, clonal packaging cell lines were isolated by FACS. Fifteen days after sorting, 465 clonal cell lines were transferred to 5 master 96-well plates. To screen for high-producing clones, clonal cell lines were transfected with Q6974 (*EGFP*-encoding LVV transfer plasmid). LVV supernatants were harvested from transfected cells and used to transduce adherent HEK293T cells. EGFP positivity of the transduced HEK293T cells was used to select the top 20 clonal packaging cell lines. Of the 20 chosen clonal cell lines, 16 survived the process of expansion to E125 flask format. LVV production in 24-deep-well plate (DWP) format revealed the top 3 clonal cell lines, all of which produced over 2.15×10^7 TU/mL (LVPack13-1, -13-5, and -13-14; data not shown). LVV production in E125 format over several passages revealed the top 2 clonal cell lines, LVPack13-5 and LVPack13-14, which yielded 2.23×10^8 TU/mL and 2.09×10^8 TU/mL, respectively (data not shown).

LVPack13-5 and LVPack13-14 were then tested for long-term stability. Both cell lines were passaged with and without antibiotics until passage number 27 (~90 generations), with production tests being performed at passage numbers 5, 11, 19, and 27 (Figure 3). When antibiotics were excluded, this was to assess the stability of the cell lines in conditions that mimic typical manufacturing practice. In the presence of antibiotics, LVPack13-5 produced 1.60×10^8–2.17×10^8 TU/mL and had 65%–82% transfection efficiency; in the absence of antibiotics, LVPack13-5 produced 7.61×10^7–1.74×10^8 TU/mL and had 35%–80% transfection efficiency (Figure 3A). Non-induced LVPack13-5 produced up to 5.36×10^5 TU/mL and had 26%–77% transfection efficiency (Figure 3A). In the presence of antibiotics, LVPack13-14 produced 1.07×10^8–1.95×10^8 TU/mL and had 71%–79% transfection efficiency; in the absence of antibiotics, LVPack13-14 produced 1.40×10^8–1.63×10^8 TU/mL and had 82%–89% transfection efficiency (Figure 3A). Non-induced LVPack13-14 did not produce LVV at titers above the lower limit of detection of the titration assay

(5.0×10^4 TU/mL) throughout stability testing and had 45%–89% transfection efficiency (Figure 3A).

To assess P/I titer ratios, viral vector supernatants from stability testing were additionally titrated by p24 ELISA. In the presence of antibiotics, LVPack13-5 produced 210 LVV particles per TU (SD = 94 LVV particles per TU); in the absence of antibiotics, LVPack13-5 produced 274 LVV particles per TU (SD = 153 LVV particles per TU) (Figure 3B). In the presence of antibiotics, LVVPack13-14 produced 141 LVV particles per TU (SD = 64 LVV particles per TU); in the absence of antibiotics, LVPack13-14 produced 114 LVV particles per TU (SD = 21 LVV particles per TU) (Figure 3B).

To further assess stability, at passage numbers 11, 19, and 27, copy numbers of *VSV-G*, *Gag-Pol*, and *Rev* were measured by droplet digital PCR (ddPCR). This analysis indicated no change in copy number of all integrated lentiviral vector constructs throughout the stability testing, regardless of the inclusion of antibiotics in the cell culture media (Figure S1). We did, however, observe a small discrepancy between the copy numbers of *VSV-G* and *Gag-Pol* genes despite the fact that they were co-encoded by the same plasmid (Q1850, Figure 1). We thus cannot exclude the possibility of the presence of partial copies of the Q1850 plasmid in the LVPack13-14 cell line. In addition, it must be noted that instability in production cell lines can be the result of silencing rather than chromosomal deletion; hence, cell line productivity and/or gene expression level should also be considered when assessing cell line stability. Since only minimal differences were observed in LVV production and copy-number retention in the presence or absence of antibiotics, it was concluded that antibiotic supplementation during routine cell line subculture did not impact packaging cell line stability. In addition, it was concluded that antibiotic selection can be removed during manufacturing of clinical LVV batches, in line with regulatory guidance.

Although LVPack13-5 produced the highest titer overall, LVPack13-14 was selected as the top clonal cell line as it produced LVV with the least variability during the long-term stability test, produced the least LVV when not induced (indicating tight repression of packaging genes in the absence of doxycycline), and produced LVV with the lowest P/I ratio (Figures 3A and 3B).

Development of *EGFP*-/anti-CD19 CAR-encoding producer cell line version 2.0

To generate a producer cell line pool, R2435 (Figure 1, LVV transfer plasmid) or R3124 (anti-CD19 chimeric antigen receptor [CAR]-encoding LVV genome flanked by piggyBac inverted terminal repeats; Figure 1, LVV transfer plasmid) and Q9751 (piggyBac transposase) plasmids were simultaneously transfected into LVPack13-14 cells (passage number 5). Next, clonal producer cell lines were isolated by single-cell printing. Fifteen days after single-cell isolation, to screen for high-producing clonal cell lines, LVV production was induced with doxycycline, and then viral vector supernatants were harvested. LVV production was ranked by RT-qPCR probing for Psi packaging element. This screen, alongside the selection of clonal cell lines based on rapid growth in 96-well plates, identified the top 12 *EGFP*-encoding clonal cell lines, and the top 12 anti-CD19 CAR-encoding clonal cell lines, which were then expanded to E125 flask scale. Seven fast-growing *EGFP*-encoding clonal cell lines were expanded and screened for LVV production in 24-DWP format. *EGFP*-encoding LVV production yield varied from 1.82×10^8 TU/mL (SD = 5.39×10^6 TU/mL) to 4.56×10^8 TU/mL (SD = 2.71×10^7 TU/mL) (data not shown). Five anti-CD19 CAR-encoding clonal cell lines survived the expansion process and were screened for LVV production in E125 flask format. Anti-CD19 CAR-encoding LVV production varied from 2.94×10^7 TU/mL (SD = 2.83×10^6 TU/mL) to 9.95×10^7 TU/mL (SD = 1.23×10^7 TU/mL) (data not shown). All clonal cell lines were next tested for growth characteristics (Figures S2 and S3) and then cryopreserved.

To examine their growth profiles, the top seven *EGFP*-encoding LVV producer clonal cell lines were overgrown while cell growth, viability, and metabolites were monitored. This study revealed little difference in growth characteristics between the clonal cell lines (Figure S2) with the following exceptions: clonal cell line EGFP_05 slowed in growth after day 3, when all others continued at the rate observed prior to day 3 (Figure S2A); clonal cell lines EGFP_05 and EGFP_07 in culture exhibited a substantial decrease in lactate between days 2 and 3, indicating that the cells had entered stationary phase (Figure S2D; as reported by Mulukutla and colleagues[36]); clonal cell line EGFP_04 accumulated a higher concentration of ammonium than the other clonal cell lines (Figure S2E), which can increase the death rate in ammonium-sensitive cell lines.[37] Finally, to screen for residual transposase in the top seven *EGFP*-encoding LVV producer clonal cell lines, genomic DNA (gDNA) was extracted and probed with two primer pairs, targeted to the transposase and G418 resistance genes (PGB_F/R and G418_F/R). Gel electrophoresis of PCR products revealed that all samples were positive for the G418 resistance gene (890-bp band), confirming successful gDNA extraction and PCR. Clones EGFP_03 and EGFP_07 were positive for the transposase gene (1,770-bp band) and were thus excluded from further experiments (Figure S4). Based on the outcome of the overgrowth test, the production test in 24-DWP, and the screen for transposase gene integration, clonal cell lines EGFP_01 and EGFP_06 were selected for stability testing.

Next, to examine their growth profiles, the top five anti-CD19 CAR-encoding LVV producer clonal cell lines were overgrown while cell growth, viability, and metabolites were monitored. This study revealed little difference in growth characteristics between the clonal cell lines (Figure S3) with the following exception: clonal cell line CAR_05 in culture had higher lactate concentration than all other cultures throughout the experiment (Figure S3D). However, lactate concentration in CAR_05 cultures decreased 1 day later than all other cell lines. It was thus concluded that this cell line might have the desirable characteristic of remaining in exponential growth for longer than the other clones, which might be beneficial for extended viral vector production. Finally, to screen for residual transposase in the top five anti-CD19 CAR-encoding LVV producer clonal cell lines,

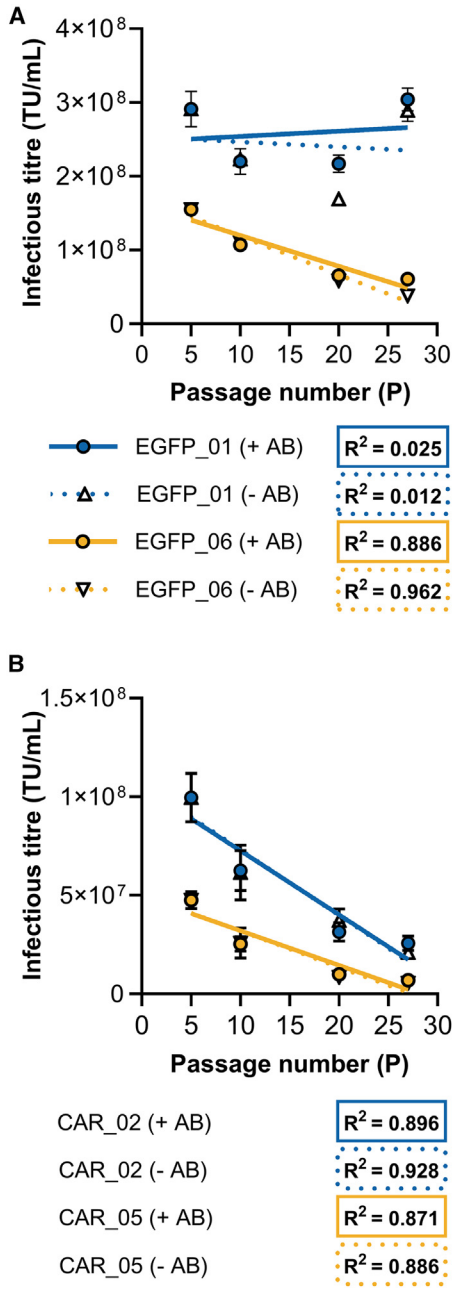

Figure 4. Stability testing of lentiviral vector clonal producer cell lines

(A) *EGFP* encoding (EGFP_01 and EGFP_06). (B) Anti-CD19 CAR encoding (CAR_02 and CAR_05). Production testing in E125 flask format. Infectious titration by flow cytometry and qPCR, respectively. *n* = three biological replicates. Error bars indicate SD.

gDNA was extracted and probed with two primer pairs, targeted to the transposase and G418 resistance genes (PGB_F/R and G418_F/R). Gel electrophoresis of PCR products revealed that all samples were positive for the G418 resistance gene (890-bp band), confirming successful gDNA extraction and PCR. All anti-CD19 CAR-encoding clones were negative for the transposase gene (Figure S4). Based on the outcome of the overgrowth test and the production test in E125 flask format, clonal cell lines CAR_02 and CAR_05 were selected for stability testing.

Next, all clonal producer cell lines were tested for stability of LVV production and stability of genomically integrated LVV genes. Clonal producer cell lines EGFP_01 and EGFP_06 were revived, cultured to passage number 5 in the presence of antibiotics, and tested for LVV production in E125 flask format. Cultures were then split and passaged with and without antibiotics until passage number 27, testing LVV production in E125 flask format at passage numbers 10, 20, and 27. This analysis revealed the following: no correlation between passage number and titer when clone EGFP_01 was subcultured with or without antibiotics (R^2 = 0.0246 and 0.012, respectively; Figure 4A); a strong and negative correlation between passage number and LVV production titer when clone EGFP_06 was cultured with or without antibiotics (R^2 = 0.8858 and 0.9624, respectively; Figure 4A). Although LVV production by clone EGFP_06 declined over time, it remained at approximately 5×10^7 TU/mL at passage number 27. To further assess stability, at passage numbers 5 and 29, copy numbers of *VSV-G*, *Gag-Pol*, *Rev*, and WPRE were measured by ddPCR. This analysis indicated no change in copy number of all integrated LVV constructs throughout the stability testing, regardless of the inclusion of antibiotics in the cell culture media (Figure S5). We again observed a small discrepancy between the copy numbers of *VSV-G* and *Gag-Pol* genes despite the fact that they were co-encoded by the same plasmid (Q1850, Figure 1). We thus cannot exclude the possibility of the presence of partial copies of the Q1850 plasmid in cell lines derived from the LVPack13-14 cell line. It must again be noted that instability in production cell lines can be the result of silencing rather than chromosomal deletion; hence, cell line productivity and/or gene expression level should also be considered when assessing cell line stability. Since only minimal differences were observed in LVV production and copy-number retention in the presence or absence of antibiotics, it was concluded that antibiotic supplementation during routine cell line subculture did not impact *EGFP*-encoding producer cell line stability.

Considering the outcomes of LVV production in 24-DWP format (data not shown), the overgrowth study (Figure S2), and the stability testing (Figure 4A), clone EGFP_01 was selected as the top *EGFP*-encoding LVV producer clonal cell line.

Stability testing was next performed with anti-CD19 CAR-encoding clonal producer cell lines CAR_02 and CAR_05. Passaging and testing were performed exactly as with the *EGFP*-encoding producer cell lines EGFP_01 and EGFP_06. This revealed a strong and negative correlation between passage number and titer for both clones, with and without antibiotics (R^2 = 0.8954 for clone CAR_02 with antibiotics, 0.9297 for clone CAR_02 without antibiotics, 0.8716 for clone CAR_05 with antibiotics, and 0.8864 for clone CAR_05 without antibiotics; Figure 4B). Again, to further assess cell line stability, at passage numbers 10 and 29, copy numbers of *VSV-G*, *Gag-Pol*, *Rev*, and WPRE were measured by ddPCR. This analysis indicated no change

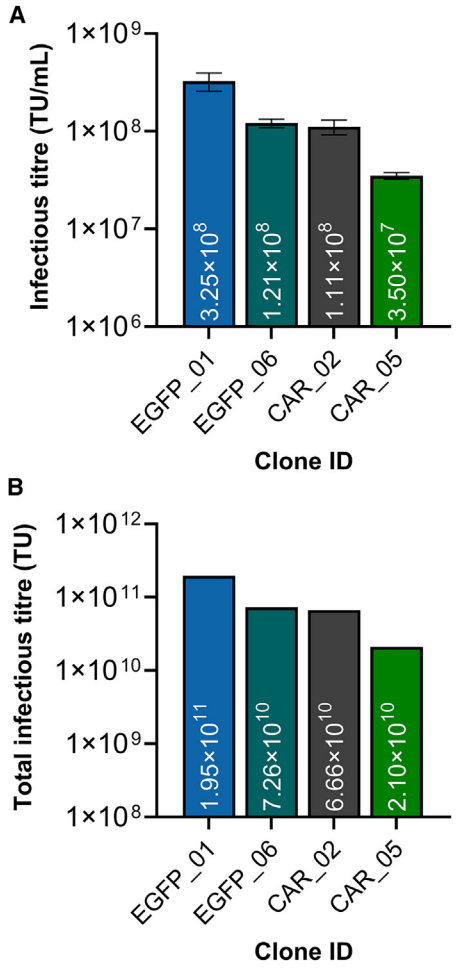

Figure 5. Lentiviral vector production by *EGFP*/anti-CD19 CAR-encoding producer cell line clones in stirred-tank bioreactor

(A) Infectious titer post-clarification. (B) Total infectious titer post-purification by AEX and TFF. *n* = one bioreactor production replicate, three titration technical replicates. Error bars indicate SD of titration technical replicates.

in copy number of all integrated lentiviral vector constructs between passage numbers 10 and 29, regardless of the inclusion of antibiotics in the cell culture media (Figure S6). Since no difference was observed in LVV production or copy-number retention in the presence or absence of antibiotics, it was concluded that antibiotic supplementation during routine cell line subculture did not impact anti-CD19 CAR-encoding producer cell line stability.

Since clones EGFP_01 and EGFP_06 performed differently from each other in the production stability test (Figure 4A), it could not be concluded that the negative correlation between anti-CD19 CAR-encoding producer cell line passage number and production titer (Figure 4B) was due to the identity of the GOI. It is conceivable that increasing the number of clones screened through stability testing could increase the likelihood of identifying an anti-CD19 CAR-encoding producer cell line clone with high production stability.

Considering the outcomes of LVV production in E125 flasks, the overgrowth study (Figure S3), and the stability testing (Figure 4B), clone CAR_02 was selected as the top anti-CD19 CAR-encoding LVV producer clonal cell line.

Lentiviral vector production in stirred-tank bioreactor: producer cell line clones

We next tested LVV production by the version 2.0 producer clonal cell lines in a 1-L stirred-tank bioreactor. The aim was to assess the production performance of the cell lines when cultured in conditions consistent with what would be expected in a manufacturing setting. Producer clonal cell line EGFP_01 produced 3.25×10^8 TU/mL of *EGFP*-encoding LVV post-clarification, and 1.95×10^{11} TU were recovered post-purification by anion exchange chromatography (AEX) and tangential flow filtration (TFF) (Figures 5A and 5B). Producer clonal cell line EGFP_06 produced 1.21×10^8 TU/mL of *EGFP*-encoding LVV post-clarification, and 7.26×10^{10} TU were recovered post-purification by AEX and TFF (Figures 5A and 5B). Producer clonal cell line CAR_02 produced 1.11×10^8 TU/mL of anti-CD19 CAR-encoding LVV post-clarification, and 6.66×10^{10} TU were recovered post-purification by AEX and TFF (Figures 5A and 5B). Producer clonal cell line CAR_05 produced 3.50×10^7 TU/mL of anti-CD19 CAR-encoding LVV post-clarification, and 2.10×10^{10} TU were recovered post-purification by AEX and TFF (Figures 5A and 5B). Production in a stirred-tank bioreactor was thus consistent with production in E125 flask format (post-clarification titers; see the Development of *EGFP*-/anti-CD19 CAR-encoding producer cell line version 2.0 section). Downstream recovery was 37.71%–42.23% ([post-downstream processing titer/post-clarification titer] × 100%).

Primary T cell transduction by lentiviral vector from different production platforms

Following the development of the packaging and producer cell line clones, it was deemed important to compare the function of the LVV produced by these platforms and the fully transient system, specifically, to assess GOI expression in target cells. HEK293T cells and primary T cells (donor 8: 75% $CD4^+$, 25% $CD8^+$; donor 10: 70% $CD4^+$, 30% $CD8^+$) were transduced with *EGFP*-encoding and anti-CD19 CAR-encoding LVV preparations from the three different production platforms at MOI 0.5. All *EGFP*-LVV-transduced cell populations were positive for fluorescence signal when inspected by microscope (Figure 6A). Untransduced cell populations were not fluorescent, confirming that fluorescent signal was due to transduction by *EGFP*-encoding LVV (Figure 6A). Next, GOI expression in primary T cells was quantitatively compared. T cell activation was measured by CD25/CD69 staining, which revealed 99% CD25 positivity and 78%–92% CD69 positivity (data not shown). EGFP expression was measured directly by flow cytometry analysis of transduced cells. Anti-CD19 CAR expression was measured by flow cytometry analysis of transduced cells stained with biotinylated protein L and streptavidin-phycoerythrin (PE). In all cases, transduced cells were significantly more fluorescent than untransduced cells (untransduced-and-stained cells in the case of the anti-CD19

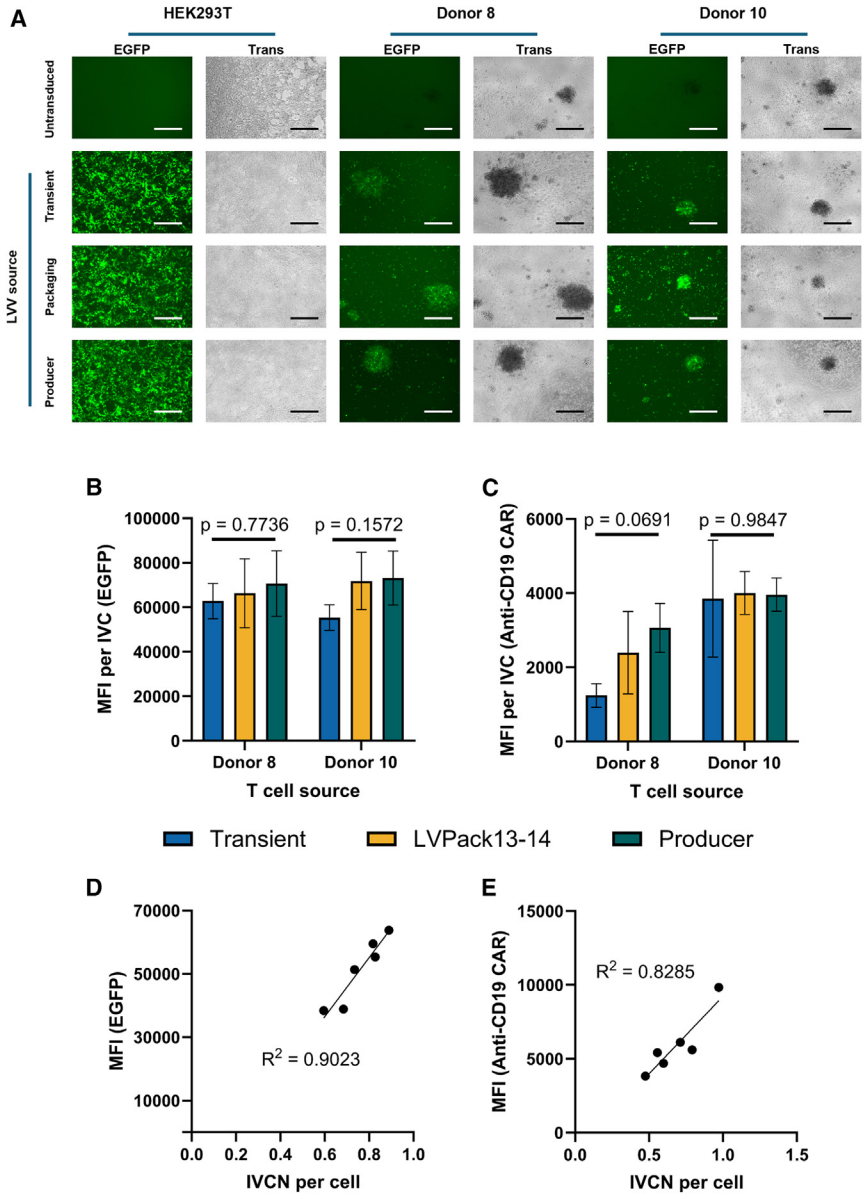

Figure 6. Primary T cell transduction

(A) Microscope images of primary T cells and HEK293T cells transduced with lentiviral vector supernatants prepared with WXATUS0028 cells, LVPack13-14 cells, and EGFP_01 producer cells. Scale bars indicate 750 μm. GOI (B, *EGFP*; C, Anti-CD19 CAR) expression level (MFI) per IVC in donor 8 or donor 10 primary T cells. (D) Linear regression analysis between integrated *EGFP* copy number and EGFP expression level. (E) Linear regression analysis between integrated anti-CD19 CAR copy number and anti-CD19 CAR expression level. *n* = one production replicate. Error bars indicate SD.

CAR experiment; all $p < 0.05$ following a two-tailed t test), confirming cargo gene expression in primary T cells (Figure S7). To determine gene expression per integrated vector copy (IVC), ddPCR was performed to determine the IVC number (IVCN) per cell, then median fluorescence intensity (MFI; of the fluorescence-positive cell population) was divided by IVCN. One-way ANOVA revealed that for a T cell population from a given donor transduced by LVV that encodes a given GOI, there was no significant difference in the GOI expression level per IVC, regardless of the production platform that produced the LVV: $p = 0.77$ with donor 8 and *EGFP* (Figure 6B); $p = 0.16$ with donor 10 and *EGFP* (Figure 6B); $p = 0.07$ with donor 8 and anti-CD19 CAR (Figure 6C); and $p = 0.98$ with donor 10 and anti-CD19 CAR (Figure 6C). Furthermore, linear regression analysis revealed that variation in IVCN accounted for most of the variation in the GOI expression level in primary T cells, indicating that the influence of the production system on GOI expression was minimal: $R^2 = 0.90$ for EGFP (Figure 6D) and $R^2 = 0.83$ for anti-CD19 CAR (Figure 6E). It was thus concluded that the LVV preparations from the different production platforms were consistent with one another in terms of GOI expression level in transduced primary T cells.

Lentiviral vector production with therapeutic cargo genes

We next sought to address two challenges: (1) as EGFP expression is widely known to be well tolerated in HEK293-based cell lines, it would be a more realistic challenge to the cell lines developed here to encode therapeutically relevant GOIs while producing LVV; and (2) during the development of a production process, one would be required to select a production platform modality. This decision might be informed by cost and the performance of the different platforms when producing LVV that encodes the GOI of the user. However, testing the encoding of a new GOI in a producer cell line would require the development of a producer cell line, which requires a commitment of time and resources. We thus tested LVV production by the three production platforms while encoding several different GOIs for two reasons: (1) to examine how effective the platforms are when they encode therapeutically relevant GOIs and (2) to assess the feasibility of a model whereby a GOI could be tested in the packaging cell line to estimate the expected titer in an equivalent producer cell line.

LVV transfer plasmids encoding several therapeutically relevant cargo genes were assembled (Table 1). The various cargo genes were selected to cover a range of packageable sizes and to be representative of various approaches in cell and gene therapy.

Table 1. Summary of lentiviral vector transfer plasmids encoding several cargo genes flanked by piggyBac inverted terminal repeats

Plasmid ID	Gene ID	Gene	Gene size, kbp	Relevant disease
R2435	*EGFP*	EGFP	0.7	no disease, control
R3939	shRNA	anti-α-synuclein shRNA	0.056	Parkinson's disease
R3124	anti-CD19 CAR	anti-CD19 CAR (CD8 leader, scFV (anti-CD19), CD8 hinge, CD8 transmembrane domain, 4-1BB signaling domain, CD3ζ)	1.45	B cell lymphoma
R3941	anti-BCMA CAR	anti-B cell maturation antigen CAR (domains as per anti-CD19 CAR but anti-BCMA scFV domain in place of anti-CD19)	1.5	multiple myeloma
R3943	*PKLR*	pyruvate kinase	1.7	pyruvate kinase deficiency
R3949	*Cas9*	clustered regularly interspaced short palindromic repeats-associated protein 9	4.1	no disease; this was chosen to demonstrate the compatibility of our systems with this frequently used gene editing tool for research purposes
R4132	*STAG2Cas9*	Cas9 with gRNA against stromal antigen 2	4.1	no disease; this was chosen to demonstrate the compatibility of our systems with this frequently used gene editing tool for research purposes
R3947	*FANCA*	Fanconi anemia, complementation group A	4.36	Fanconi anemia
R3945	factor VIII	coagulation factor VIII	4.37	hemophilia A

CAR, chimeric antigen receptor.

To compare the performance of the production platforms when encoding various GOIs compared to when they encode *EGFP*, LVV productions were performed in 24-DWP format with all cargo gene-encoding LVV transfer plasmid variants as per the materials and methods section.

The WXATUS0028 four-plasmid transient system produced 3.08×10^7 TU/mL (SD = 9.49×10^6 TU/mL) of *EGFP*-encoding LVV (Figure 7A). Production titer was not significantly impacted when short hairpin RNA shRNA ($p = 0.9306$), anti-CD19 CAR ($p = 0.3052$), anti-BCMA CAR ($p = 0.5776$), or *PKLR* ($p = 0.0513$) were encoded (two-tailed t test). However, production titer was significantly lower than the *EGFP* titer when *Cas9* ($p = 0.0234$), *STAG2Cas9* ($p = 0.0187$), *FANCA* ($p = 0.0297$), or factor VIII ($p = 0.0227$) were encoded. The average non-*EGFP* titer was 59% of the corresponding *EGFP* titer (Figure 7A).

The LVPack13-14 single transfer plasmid transfection system produced 5.71×10^7 TU/mL (SD = 2.16×10^7 TU/mL) of *EGFP*-encoding LVV (Figure 7A). Encoding the various GOIs had no significant impact on production titer compared to when *EGFP* was encoded (p values: shRNA = 0.9465; anti-CD19 CAR = 0.6041; anti-BCMA CAR = 0.6378; *PKLR* = 0.6874; *Cas9* = 0.1169; *STAG2Cas9* = 0.0979; *FANCA* = 0.2625; factor VIII = 0.0900; Figure 7A). The average non-*EGFP* titer was 84% of the corresponding *EGFP* titer.

LVV production by therapeutically relevant GOI-encoding producer cell lines (heterogeneous populations, also referred to as pools) was performed three times, each with two integration replicates and three production replicates. Thus, producer cell line data are based on 6 integration replicates and 18 production replicates. Producer pools produced 2.60×10^8 TU/mL (SD = 6.01×10^7 TU/mL) of *EGFP*-encoding LVV (Figure 7A). A significant decrease in production was observed when any of the non-*EGFP* GOIs were encoded by producer cell line pools (p values: shRNA = 0.0002; anti-CD19 CAR = 0.0005; anti-BCMA CAR = 0.0010; *PKLR* = 0.0028; *Cas9* = 0.0005; *STAG2-Cas9* = 0.0002; *FANCA* = 0.0005; factor VIII = 0.0003; Figure 7A). The average non-*EGFP* titer was 27% of the corresponding *EGFP* titer.

Overall, producer cell line LVV production was more heavily impacted by the non-*EGFP* GOIs than was production by the WXATUS0028 and LVPack13-14 production systems (Figure 7A). The average WXATUS0028 non-*EGFP* titer was 59% of the corresponding *EGFP* titer. For LVPack13-14, this number was 84%. For the producer cell lines, it was 27%. It is conceivable that in the producer cell lines the constitutively expressed cargo gene products, and thus their impacts on cell health and LVV production, have more time to accumulate than in either of the transfection-dependent cell lines. To test this reasoning, linear regression was performed between infectious titer and cargo gene size. This analysis revealed that 85.3% of the variance in WXATUS0028 infectious titer was due to cargo gene size ($R^2 = 0.853$), 73.3% of the variance in the LVPack13-14 infectious titer was due to cargo gene size ($R^2 = 0.733$), and only 15.7% of the variance in the producer cell line infectious titer was due to cargo gene size ($R^2 = 0.157$). It was thus concluded that most of the variance in the producer cell line titer originated from factors other than cargo gene size—presumably the intracellular function and/or physicochemical properties of the gene products.

To assess the quality of production by the different cell lines, LVV supernatants that encode *EGFP*, shRNA, anti-CD19 CAR, and factor VIII were produced in E125 flask format. This panel of cargo genes was selected to be representative of the range of cargo gene sizes used throughout this study. LVV preparations were then titrated by

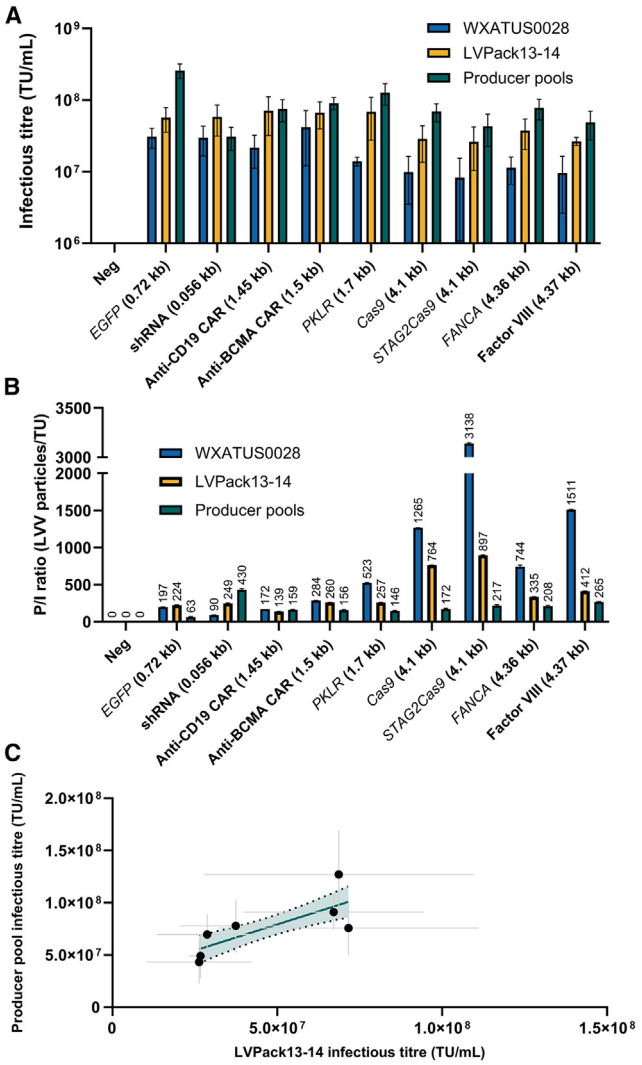

Figure 7. Lentiviral vector titer and composition when encoding several therapeutically relevant GOIs

(A) Infectious titer of lentiviral vector encoding a panel of GOIs, produced by WXATUS0028, LVPack13-14, or producer cell line pools derived from LVPack13-14. Infectious titration by qPCR. *n* = four production replicates, three transfection replicates per production. Error bars indicate SD between production replicates. (B) Physical-to-infectious titer ratio of lentiviral vector preparations encoding various GOIs. Ratio calculated by comparison of qPCR infectious titer and virus-associated p24 ELISA. (C) Linear regression analysis of LVPack13-14 LVV titer against producer cell line LVV titer. Solid line indicates linear regression. Dotted lines and shaded area indicate 95% confidence interval. Each data point indicates the average LVV titer when the cell lines encode a given GOI. LVPack13-14, n = four production replicates. Producer cell lines, n = six production replicates. Error bars indicate SD between production replicates.

qPCR (titration by qPCR analysis of IVCN in transduced HEK293T cells) and p24 ELISA to enable calculation of the P/I ratio. This analysis revealed no consistent trend or difference in the P/I ratio of the preparations from the different cell lines when encoding different cargo genes (Figure 7B).

We next tested whether the LVV titer of the producer cell line with a novel GOI could be predicted by testing said GOI in a packaging cell line, using the data generated above. First, since *EGFP* and shRNA had disproportionate effects on producer cell line LVV titer compared to all other GOIs tested (Figure 7A), these data points were excluded. Second, linear regression was performed between LVPack13-14 titer and producer cell line titer when encoding the remaining GOIs. The data points plotted were the averages of the LVV titers with each of the GOIs; thus, there were seven points. If biological/technical replicate data points had been plotted, then they could have arbitrarily been rearranged. By arranging the data according to GOI, each point can only have one x/y value. This analysis revealed a positive and moderately strong correlation (R = 0.57). In addition, the lower bound of the 95% confidence interval resulted in a positive gradient, indicating a statistically significant correlation between the titers of the LVPack13-14 and producer cell lines (i.e., the 95% confidence interval does not contain a zero-gradient line). The linear regression equation is $y = 1.000x + 2.93 \times 10^7$ and the 95% confidence interval is 0.5324–1.468 (Figure 7C). In the selection of a production platform for LVV manufacturing, this predictive model could act as an additional tool to facilitate the selection of either packaging cell line or producer cell line as follows. The user could rapidly test the production of LVV that encodes their GOI using LVPack13-14, and then use the model to estimate what titer could be expected if a producer cell line were to be developed, thus enabling an informed decision to be made before commitment of time and resources to a producer cell line development campaign.

LVV safety

Since concerns regarding the formation of RCL within LVV producer cell lines are often raised, LVV supernatants prepared using LVPr001_4.3 and LVProEGFP_01 were tested for the presence of RCL (WuXi Advanced Therapies). LVPr001_4.3 was cultured to passage number 20. Then, 310 mL of LVV supernatant (3.07×10^7 TU/mL) were prepared according to the materials and methods section. LVPr001_4.3-derived LVV supernatant (270 mL) was assayed for the presence of RCL by amplification with C8166 cells and then detection of the VSV-G gene by qPCR. No RCL was detected in the LVV preparation derived from LVPr001_4.3 cells.

LVProEGFP_01 was cultured to passage number 26, then 310 mL of LVV supernatant (1.57×10^8 TU/mL) were prepared as above. The LVV preparation (310 mL) was assayed for RCL as above. No RCL was detected in the LVV preparation derived from LVProEGFP_01 cells.

DISCUSSION

We developed LVV packaging and producer cell lines, which reduce or eliminate the need for plasmid transfection during LVV manufacture. We developed a clonal LVV packaging cell line that in E125 flask format consistently produced 1–2 $\times 10^8$ TU/mL, with or without selective antibiotics over 27 passages (~90 generations; Figure 3A; *EGFP* as cargo gene). This platform cell line can either be used directly as a packaging cell line or be developed into a producer cell line by

integration of a cargo gene-encoding LVV transfer plasmid. We next developed *EGFP*- and anti-CD19 CAR-encoding clonal LVV producer cell lines that in E125 flask produced up to 1-3 $\times$ 10^8 TU/mL, with no dependence on selective antibiotics over 27 passages (Figures 4A and 4B, respectively). Finally, we developed a model whereby the LVV production performance of a producer cell line developed with LVPack13-14 and a new cargo gene can be estimated by transiently testing performance using LVPack13-14 as a packaging cell line (Figure 7C). This has the advantage of enabling rapid screening of new cargo genes for optimal LVV production.

Using the titer prediction model, it will now be possible when screening new cargo genes in the packaging cell line to estimate the expected LVV titer from producer cell lines. The model assumes that the new cargo gene does not have a highly toxic impact on the producer cell lines but has at least some negative impact on the production performance of the cell line that encodes it (in contrast to *EGFP*, which was well tolerated by all cell lines here). In addition, the titer prediction model assumes that the packaging cell line titer with the new cargo gene is within the bounds of the model (2.7×10^7–7.2×10^7 TU/mL). Finally, the model assumes that the packaging cell line production is tested in 24-DWP format.

In contrast to the observed trend that stable production cells are less productive than transient production systems,[9] the packaging and producer cell lines presented here were as productive as the transient systems from which they were derived. Moreover, encoding several different cargo genes had little impact on the productivity of the stable cell lines, a finding that is often absent in similar publications.[14,15,18,20,21,23,30,38,39]

Via the transduction of primary T cells, we have demonstrated that LVV produced by the different production platforms are consistent in their ability to express cargo genes in target cells. Combined with the observation that the infectious titers of the LVV produced by the different production platforms are consistent with one another, we conclude that transition from the traditional four-plasmid method to a stable cell line for LVV manufacturing would not alter vector efficacy and thus would be an operationally low-risk undertaking.

In summary, the cell lines developed here produce LVV at titers comparable to the industry-standard four-plasmid transfection method. The advantage is that the requirement for transfection of only one or no plasmids means fewer or no large-scale GMP-grade plasmid preparations would be required for LVV production, reducing operational complexity, variability, and costs, and thereby improving patient access to LVV-based cell and gene therapies.

MATERIALS AND METHODS

Plasmid construction

A guide to the identities of all plasmids used in this study is presented in Table S1.

The Q1850 *VSV-G*/*Gag-Pol*-encoding plasmid was assembled in four restriction cloning stages as follows: vector = OG10 (pUC ori, KanR, and MCS); stage 1: TetR and *VSV-G* inserted by AsiSI and XbaI restriction sites; stage 2: *Gag* and *Pol* fragment inserted by SbfI and PacI restriction sites; stage 3: *Pol* fragment and *PuroR* gene inserted by XbaI and SbfI restriction sites; stage 4: *IAP1* and *EBNA5* genes inserted by BspEI/XmaI and PacI restriction sites.

The Q8890 *Rev*-encoding plasmid was assembled in three restriction cloning stages as follows: vector = OG1 (pUC ori, AmpR, and MCS); stage 1: *Rev* and *HygroR* genes inserted by SbfI and PacI restriction sites; stage 2: AmpR replaced by KanR by PmeI restriction sites; stage 3: *HygroR* replaced by *BlastR* by AvrII and PacI restriction sites.

The R2435 *EGFP*-encoding LVV transfer plasmid was assembled in four restriction cloning stages as follows: vector = R1845 (p15A ori, KanR, and piggyBac inverted terminal repeats); stage 1: p15A ori replaced by pUC ori by SwaI restriction sites; stage 2: *G418R* inserted by BglII and NheI restriction sites; stage 3: *EGFP*-encoding LVV genome inserted by SbfI restriction sites; stage 4: reverse orientation of LVV genome by SbfI restriction sites.

The R3124 anti-CD19 CAR-encoding LVV transfer plasmid was assembled in four restriction cloning stages as follows: vector = stage 3 product in R2435 assembly; stage 1: replace SFFV promoter of stage 3 product with TetO-repressible CMV promoter (R2440); stage 2: reverse orientation of LVV genome by SbfI restriction sites (R2439); stage 3: replace *EGFP* CDS with anti-CD19 CAR CDS by EcoRI and BamHI restriction sites (R2712); stage 4: replace TetO-repressible CMV promoter with SFFV promoter by BstBI and EcoRI restriction sites.

The therapeutic GOI LVV transfer plasmids (R3939, R3941, R3943, R3949, R4132, R3947, R3945) were assembled in one Gibson assembly cloning stage as follows: vector = R2435; all GOI-encoding inserts except factor VIII, *Cas9*, and *Cas9_STAG2* were synthesized externally; stage 1: replace *EGFP* CDS with various GOI CDSs. In the case of R3939, the entire *EGFP* cassette was replaced with an shRNA cassette (U6 promoter).

The Q9751 piggyBac transposase-encoding plasmid was assembled in one Gibson assembly cloning stage as follows: vector = OG10 (pUC ori, KanR, and MCS); codon-optimized piggyBac transposase CDS was synthesized externally; stage 1: insert piggyBac CDS by NotI and NheI restriction sites.

Cell line revival

Cryovials containing 1 mL of 2 $\times$ 10^7 viable cells per milliliter in cell culture media supplemented with 10% (v/v) glycerol were rapidly thawed on a bead bath. Cryovials were transferred aseptically to a biological safety cabinet once only a small amount of ice remained within the cryovials. Cryovial contents were transferred to 24 mL of BalanCD HEK293 media (FUJIFILM Irvine Scientific, Santa Ana, CA) supplemented with 4 mM glutamine in an E125 flask. Cells

were counted and then transferred to an incubator (settings as per the subculture section, below) for 96 h before subculture as per routine). Antibiotics were reapplied once cell viability reached ≥90%.

Transfection

When random integration was used (development of packaging cell lines), HEK293-Ox or WXATUS0028 cell lines were seeded at 3×10^6 viable cells per milliliter in 50% of the final intended volume of antibiotic-free cell culture media. After 30 min of incubation as per the Subculture section, 75 μL of 500-ng/μL linearized plasmid DNA was added to the cell cultures. After 5 min of incubation as per the Subculture section, linear polyethylenimine 25 kDa (Polysciences, Warrington, PA) was added to a final concentration of 3 μL/1 μg DNA. After 24 h of incubation as per the Subculture section, the final 50% of cell culture media was added. After 24 h of incubation as per the Subculture section, transfection efficiency was measured by flow cytometry, and antibiotic selection was applied as per the Subculture section.

When transposase-mediated integration was used (development of producer cell lines), packaging cell lines versions 1.0/2.0 were seeded at 1.11×10^6 viable cells per milliliter in 90% of the final intended volume of antibiotic-free cell culture media. LVV transfer plasmids and a transposase-encoding plasmid were combined at a mass:mass ratio of 9:1 (total plasmid mass in micrograms was equal to the total culture volume in milliliters). Plasmids were combined with PEIpro (Polyplus-Sartorius, Illkirch-Graffenstaden, France) and incubated according to the manufacturer's recommendations before dropwise addition to the cell cultures intended to be transfected. Transfected cell cultures were incubated for 72–96 h according to the conditions in the Subculture section prior to measurement of transfection efficiency by flow cytometry and application of selective antibiotics according to the Subculture section.

Subculture

Cell viability and density were measured using the Vi-Cell XR Cell Viability Analyzer or the Vi-Cell BLU Cell Viability Analyzer. Cell cultures were diluted to 0.3×10^6 viable cells per milliliter in BalanCD HEK293 media preheated to 37°C and supplemented with 4 mM glutamine, and with selective antibiotics depending on the cell line as follows. HEK293-Ox was cultured with no antibiotic selection. Packaging cell line version 1.0 was cultured with 3 μg/mL puromycin (Gibco, Fisher Scientific UK, Loughborough, UK) and 2 μg/mL blasticidin (Gibco, Fisher Scientific UK). Producer cell line version 1.0 was cultured with 3 μg/mL puromycin, 3 μg/mL blasticidin, and 350 μg/mL G418 (Roche, Basel, Switzerland). WXATUS0028 was cultured with no antibiotic selection. Packaging cell line version 2.0 was cultured with 3 μg/mL puromycin and 4 μg/mL blasticidin. Producer cell line version 2.0 was cultured with 3 μg/mL puromycin, 4 μg/mL blasticidin, and 600 μg/mL G418. Stability testing of the cell line from which the Producer cell line version 2.0 was derived indicated that blasticidin and puromycin were not required for stability. However, producer cell line version 2.0 was developed in-parallel with this stability testing. Hence, it was not known at the time whether the producer cell lines would require these antibiotics during routine subculture. Thus, they were included as a matter of prudence. When HEK293-Ox cells and derivatives were incubated in Erlenmeyer flasks, the incubator settings were as follows: 37°C, 85% humidity, 8% CO_2, and 125 rpm with 50-mm orbital diameter. When WXATUS0028 cells and derivatives were incubated in Erlenmeyer flasks, the incubator settings were as follows: 37°C, 85% humidity, 8% CO_2, and 120 rpm with 25-mm orbital diameter. When all cell lines were incubated in 24-DWP, the incubator settings were as follows: 37°C, 85% humidity, 8% CO_2, and 225 rpm with 50-mm orbital diameter. When all cell lines were incubated in 96-well plates, the incubator settings were as follows: 37°C, 85% humidity, and 8% CO_2, stationary.

LVV production: fully transient

In an E125 flask or a 24-DWP, WXATUS0028 or HEK293-Ox cells were seeded at 2×10^6 viable cells per milliliter in an entirely fresh volume of BalanCD HEK293 media supplemented with 4 mM GlutaMAX (Gibco, Fisher Scientific UK). Cell cultures were then incubated for 24 h as per the Subculture section. Cells were counted and transfected using PEIpro transfection reagent with a total mass of DNA according to the production format: E125 flask, 0.3 μg DNA/10^6 viable cells; 24-DWP, and 1 μg DNA/10^6 viable cells. The mass ratio of the four LVV plasmids was as follows: *Rev* plasmid (Q6972), 2; *Gag-Pol* plasmid (Q6975), 5; *VSV-G* plasmid (Q6973), 4; transfer plasmid (Q6974), 5 (all plasmids produced by OXGENE). The transfection reagent:DNA ratio was 2 μL:1 μg. The total culture volume was 25 mL in an E125 flask or 3 mL in a 24-DWP. Transfected cells were then incubated as per the Subculture section for 16–24 h. Sodium butyrate was then added to the cell cultures at a final concentration of 5 mM. Cell cultures were incubated as per the Subculture section for 48 h before clarification by centrifugation at 300 relative centrifugal force (RCF) for 5 min. LVV supernatants were then titrated and finally stored at −80°C.

The two commercial systems used for fully transient LVV production in Figure 2 were the ViraPower Lentiviral Expression Systems (Life Technologies, Carlsbad, CA) and the MISSION Lentiviral Packaging Mix (Sigma-Aldrich, St. Louis, MO). LVV productions were conducted according to manufacturers' protocols. LVV supernatants were then titrated and finally stored at −80°C.

LVV production: packaging cell lines

LVV production using packaging cell lines was performed as per the LVV production: fully transient section with the following deviations: (1) the cell lines were LVV packaging cell lines; (2) the entire mass of transfected DNA comprised LVV transfer plasmid only; and (3) doxycycline was added to the cell cultures to a final concentration of 1 μg/mL 24 h after transfection.

LVV production: producer cell lines

In an E125 flask or a 24-DWP, various producer cell line variants were seeded at 2×10^6 viable cells per milliliter in an entirely fresh volume

of BalanCD HEK293 media supplemented with 4 mM GlutaMAX. The total culture volume was 25 mL in an E125 flask or 3 mL in a 24-DWP. Cell cultures were then incubated for up to 5 h before the addition of doxycycline at 1 μg/mL final concentration. When production was performed in E125 flasks, cell cultures were incubated as per the Subculture section. When production was performed in 24-DWP, cell cultures were incubated as follows: 37°C, 85% humidity, 8% CO_2, and 225 rpm shaking with 50-mm orbital diameter. Cell cultures were then incubated for 24 h before the addition of sodium butyrate at 5 mM final concentration. Cell cultures were then incubated for 48 h as per the Subculture section before clarification by centrifugation at 300 RCF for 5 min. LVV supernatants were then titrated and finally stored at −80°C.

LVV infectious titration by flow cytometry

In a flat-bottomed cell culture-treated 96-well plate, adherent HEK293T cells were seeded at 0.5×10^6 viable cells per milliliter in high glucose DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) (DMEM + FBS). Within 2 h of seeding, HEK293T cells were transduced with LVV supernatants serially diluted in DMEM + FBS. Transduced cell cultures were then incubated as follows for 72 h: 37°C, 85% humidity, and 5% CO_2, stationary. Media was aspirated by pipette. Cells were detached by TrypLE (Gibco, Fisher Scientific UK) and re-suspended in PBS. Detached cells were analyzed for EGFP expression by flow cytometry (Attune NxT Flow Cytometer). EGFP values between 5% and 20% were used to calculate LVV titers using the following equation:

$$Infectious\ titer\ (TU/mL) = \frac{(\%GFP/100) \times number\ of\ HEK293T\ cells\ per\ well}{Neat\ LVV\ input\ volume\ (mL\ per\ well)} \quad \text{(Equation 1)}$$

LVV infectious titration by qPCR

Adherent HEK293T cells were transduced, incubated, and detached as per the LVV infectious titration by flow cytometry section. Detached HEK293T cells were pelleted by centrifugation at 300 × *g* for 5 min, the supernatant was aspirated and discarded, and gDNA was extracted from the cell pellets using a DNeasy Blood and Tissue kit (Qiagen, Manchester, UK). gDNA was used as template in a qPCR reaction using TaqMan Fast Advanced Master Mix (Fisher Scientific UK) and primers/probes against WPRE and albumin. A standard curve was generated by serial dilution of the genomic DNA from a known number of cells known to encode one LVV genome per cell. LVV titer was calculated using the following equation:

$$Infectious\ titer\ (TU/mL) = \frac{(provirus\ copies\ per\ cell \times number\ of\ HEK293T\ cells\ per\ well)}{Neat\ LVV\ input\ volume\ (mL\ per\ well)} \quad \text{(Equation 2)}$$

LVV physical Titration by p24 ELISA

p24 ELISA was performed using a QuickTiter Lentivirus Titer kit (CellBioLabs, San Diego, CA) per the manufacturer's instructions.

LVV physical Titration by RT-qPCR

Viral vector RNA was extracted from LVV supernatants using RNA QuickExtract solution (LGC Biosearch Technologies, Hoddesdon, UK) according to the manufacturer's instructions. Extracted RNA was used as template in a RT-qPCR reaction using TaqMan Fast Virus 1-Step Master Mix (Fisher Scientific UK) and PrimeTime Custom Probe-based qPCR Assay against LVV Psi (IDT, Coralville, IA). A standard curve was generated by RT-qPCR against serially diluted RNA generated by *in vitro* transcription of a linearized LVV transfer plasmid.

Clonal cell line isolation by FACS

Cells were diluted in antibiotic-free cell culture media to 0.5–5.0×10^6 viable cells per milliliter to a total volume of 1–10 mL. Cell suspension was filtered through a 20- to 40-μm cell strainer to remove cell aggregates. Filtered cells were loaded into a Sony SH800 Cell Sorter. To identify live cells, all detection events were filtered according to backscatter area (BSC-A) against forward scatter area (FSC-A). To identify singlet cells, live cell events were filtered according to forward scatter height (FSC-H) against FSC-A. To identify singlet cells with further stringency, singlet cell events were then filtered according to backscatter height against BSC-A. Cells passing the selection criteria (live and twice selected for singlet status) were sorted into sterile, non-treated, flat-bottomed 96-well plates pre-filled with 200 μL per well of 37°C BalanCD HEK293 media supplemented with 4 mM GlutaMAX and 1× InstiGRO HEK (Advanced Instruments, Norwood, MA). Sorted cells were incubated as follows until ≥25% of cell cultures were measured at >4% confluence: 37°C, 85% humidity, and 5% CO_2, stationary. Cell lines identified as clonal were expanded to the E125 flask format.

Clonal cell line isolation by single-cell printing

Cells were diluted to approximately 1×10^6 viable cells per milliliter to a total volume of 1–10 mL of cell culture media. Diluted cell suspension was then filtered through a 20-40-μm cell strainer to remove cell aggregates. Using a Cytena F.SIGHT single cell dispenser, singlet

cells were dispensed according to manufacturer's instructions into sterile, non-treated, flat-bottomed 96-well plates pre-filled with 200 μL per well of 37°C BalanCD HEK293 media supplemented with 4 mM GlutaMAX and 1× InstiGRO HEK. Dispensed cells were incubated as follows until ≥25% of cell cultures were measured at > 4% confluence: 37°C, 85% humidity, 5% CO_2, stationary. Cell lines identified as clonal were expanded to the E125 flask format.

Preliminary LVV production screening in 96-well plate

When packaging cell lines were screened for LVV production, in 96-well plates, 180 μL cell culture was transfected with 20 ng LVV transfer plasmid (*EGFP* as the GOI) complexed with PEIpro transfection reagent to a total volume of 20 μL per the manufacturer's instructions. Transfected cell cultures were then incubated for 3 h per the Subculture section. Doxycycline was then added to the transfected cell cultures at a final concentration of 1 μg/mL, which were then incubated for 24 h per the Subculture section. Sodium butyrate was then added to a final concentration of 5 mM, and cells were incubated per the Subculture section for 48 h. LVV supernatants were harvested by centrifugation at 300 RCF for 5 min. Percentage of EGFP expression in HEK293T cells transduced by LVV supernatants was used to rank packaging cell line clones.

When producer cell lines were screened for LVV production, 96-well plates were scanned by Solentim Cell Metric whole well imager to confirm that the cell confluence was 60%–80%. Cell culture media was aspirated and replaced with cell culture media supplemented with doxycycline at a final concentration of 1 μg/mL. Cells were then mixed by pipetting and incubated per the Subculture section for 24 h. Sodium butyrate was then added to a final concentration of 5 mM, and cells were incubated per the Subculture section for 48 h. LVV supernatants were harvested by centrifugation at 300 RCF for 5 min. LVV supernatants were assayed by Psi (ψ)-directed RT-qPCR. Producer cell line clones were ranked based on RT-qPCR Ct values and 96-well plate cell confluence.

Cryopreservation of cell lines

Cell lines were pelleted by centrifugation at 300 RCF for 5 min and then re-suspended to a final density of 1–2 × 10^7 viable cells per milliliter in antibiotic-free cell culture media supplemented with 10% DMSO (Merck Life Science UK Limited, Gillingham, UK). Cell suspensions were transferred to 2-mL cryovials, which were cooled to −80°C within a CoolCell container. After a minimum of 24 h, frozen cryovials were transferred to liquid nitrogen-cooled cryostorage.

Overgrowth study

Producer cell line clones were seeded at 2 × 10^6 viable cells per milliliter in a final volume of 15 mL BalanCD HEK293 media supplemented with 4 mM GlutaMAX per microbioreactor in an Ambr 15 cell culture bioreactor system. Cell density and viability were measured by the Vi-Cell BLU Cell Viability Analyzer at 24-h intervals. Media concentrations of glucose, lactate, and ammonium, and pH were measured by FLEX2 automated cell culture analyzer at 24-h intervals. Outgrowth and monitoring continued for 4 days in total.

Screen for transposase gene integration

Cells from 1-mL aliquots of cell cultures were pelleted by centrifugation at 6,000 RCF for 2 min. Supernatants were discarded, and cell pellets were frozen at −80°C for 10 min, then thawed on a bead bath set to 42°C (freeze-thaw cycle to aid cell lysis). To extract gDNA, 4 μL of cell pellet was mixed with 36 μL of QuickExtract DNA extraction solution (Lucigen, Middleton, WI) and thermocycled as follows: 8 × (65°C for 6 min; 98°C for 2 min). To each thermocycled sample, 60 μL of nuclease-free water was added to reduce viscosity. Diluted gDNA samples were stored at −80°C before further use. The transposase gene was probed by PCR using the following primers: PGB_F (5′-GGT TCC TCC CTC GAT GAC G-3′) and PGB_R (5′-TTG ACA CAT ATC AAT GTT GTG CTC C-3′). *G418R* was probed by PCR using the following primers: G418_F (5′-GTA AAT TGT CCG CTA AAT TCT GG C-3′) and G418_R (5′-TCT GTG AGC TGA AGG TAC GC-3′). PCR reactions were prepared with the following volumes of components: 10 μL of Q5 High-Fidelity 2× Master Mix (New England Biolabs, Ipswich, MA), 1.25 μL of primer F, 1.25 μL of primer R, 2 μL of diluted gDNA sample (0 μL in negative controls), and 5.5 μL of nuclease-free water (7.5 μL in negative controls). The thermocycle conditions were as follows: 98°C for 30 s, 35 cycles of 98°C for 10 s followed by 66°C for 30 s followed by 72°C for 1 min, and then 72°C for 2 min as a final extension. PCR products were separated by agarose gel electrophoresis (1% agarose and 1:10,000 SYBR Safe DNA gel stain (Thermo Fisher Scientific, Waltham, MA) in 1× Tris-acetate-ethylenediaminetetraacetic acid buffer; 120 V for 50 min) and visualized on a Bio-Rad Molecular Imaging Gel Doc XR + Universal Hood II system.

ddPCR for copy-number variation analysis

Cell pellets were harvested by centrifugation at 300 RCF for 5 min at various passage numbers during stability testing. gDNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) and diluted to 20 ng/μL in nuclease-free water. Copy numbers of the packaging genes and the GOI were determined by ddPCR using QX200 Auto DG Droplet Digital PCR System (Bio-Rad, Watford, UK) with the following primers and probes: HIV *Gag-Pol* primers: 5′-CCT TGG TTC TCT CAT CTG GC-3′, 5′-ATC AAG CAG CCA TGC AAA TG-3′, and probe 5′-FAM-TG CAT CCA GTG CAT GCA GGG CC- IABkFQ-3′; *VSV-G* primers: 5′-GGA CCA AAA TAC ATC ACG CAC AGC-3′, 5′-GCG TGA CTT GCA CGA TCA CG-3′, and probe 5′-FAM-CAG ACT AAG CAG GGA ACC TGG CTG AAC CC- IABkFQ-3′; *Rev* primers: 5′-CTC TGG ATT GCA ACG AAG ACT-3′, 5′-CCG CTT TCC AAG ATG GTA GG-3′, and probe 5′-FAM-AAG CCC ACA GAT CCT GGT GGA ATC- IABkFQ-3′; WPRE primers: 5′-TTG CTT CCC GTA TGG CTT TC-3′, 5′-CGG GCC ACA ACT CCT CAT AA-3′, and probe 5′-FAM- TCT CCT CCT-ZEN-TGT ATA AAT CCT GGT TGC TGT CTC-IABkFQ-3′; human TaqMan Copy Number Reference Assay RNase P TAMRA Quencher

(3′), VIC (5′) (Fisher Scientific UK). No systematic change in the copy numbers of measured genes was observed when comparing LVPack13-14 and the producer cell lines derived from it. Thus, the possibility of a change in the copy number of the reference gene (*RNase P*) during the development of the cell lines was ruled out. Thus, for all copy-number calculations the *RNase P* copy number was assumed to be two, an approximation since HEK293T cells are often triploid or tetraploid. Gene copy number was calculated by dividing the number of copies of the target GOI by the number of *RNase P* copies detected per 22 μL of PCR reaction and multiplying by two.

LVV production in stirred-tank bioreactor: producer cell lines

To prepare seed train cells, ∼90 h prior to bioreactor inoculation, LVV producer version 2.0 cells were seeded at 0.35×10^6 viable cells per milliliter in an Erlenmeyer flask and then incubated as per the Subculture section (EGFP_01 = passage 9; EGFP_06 = passage 11; CAR_02 = passage 11; CAR_05 = passage 11). Twenty-four hours after cell seeding, 5% BalanCD HEK293 feed was added to the seed train. Bioreactors were seeded at 3.0×10^6 viable cells per milliliter at a 1-L volume. Two hours after inoculation, doxycycline was added to a final concentration of 1 μg/mL, and 20–24 h after inoculation, sodium butyrate, anti-clumping agent, and feed were added to final concentrations of 10 mM, 1:5,000, and 5%, respectively. Agitation was set to 400 rpm, pH was set to 7.1 ± 0.2, controlled by CO_2 and 0.5 M NaOH. Dissolved oxygen was set at 40% and temperature was set at 37°C. LVV was harvested 70 h after the addition of doxycycline.

Downstream processing: clarification

Clarification was performed following harvest and Benzonase (Merck Life Science UK Limited) treatment under sterile conditions (5 U/mL; 37°C; 400 rpm agitation for 2 h). To remove cell debris, LVV supernatants were centrifuged for 20 min at 1,000 RCF. A set of two filters was used: a pre-filter for removing cell debris (PALL KA2J100P2S; 0.07 m^2 filter size, 10 μm pore size) and a second filter for removing smaller particulates (PALL KA02EKVP2S; 0.022 m^2 filter size, 0.6 μm/0.22 μm pore size) (both by Pall Corporation, Portsmouth, UK). Filters were flushed and air flushed with PBS prior to use and flushed with 10% PBS v/v after use.

Downstream processing: chromatography

AEX was performed following clarification using either the AKTA Pure or the AKTA Avant system. The Sartobind Q Strong Anion Exchanger was used in three sizes (1, 3, and 7 mL; Merck Life Science UK Limited). The procedure with the Sartobind Q column comprised equilibration (150 mM NaCl), wash (150 mM NaCl), elution, and strip (200 mM NaCl). Elution uses high salt (1,200 mM), which can damage LVV after prolonged exposure; thus, the material was diluted immediately within the fractionation system of the AKTA system. The equilibration, wash, and elution buffers were kept at pH 7.2 with 20 mM Tris for buffering and 1 mM $MgCl_2$. The flow rate was 4 column volumes (CVs)/min, allowing for rapid processing without introducing issues due to high pressure, with the total processing time being 15 min (60 CVs) plus the loading time. The 60 CVs was split into 10 CVs for the equilibration and strip each, and 20 CVs for the wash and elution each.

Downstream processing: TFF

TFF was performed following chromatography to buffer exchange LVV into storage buffer and to concentrate material that was diluted following AEX. The K2Ri system was used in conjunction with an appropriately sized TFF column and a standard shear rate (6,000 s^{-1}) and *trans*-membrane pressure (0.55 bar) (Repligen, Waltham, MA). When processing 1 L bioreactor material, D04-E500-05-N was used, which is a 500-kDa molecular weight cutoff column with a surface area of 40 cm^2. TFF comprised two steps—ultrafiltration, which aimed to reduce the volume of material 10- to 20-fold, and diafiltration, which buffer exchanged the virus into formulation buffer (20 mM Tris HCl, 150 mM NaCl, 2% sucrose w/v, pH 7.3).

Isolation and cryopreservation of human primary T cells

National Health Service (NHS) research ethics committee approval was received for the isolation and use of T cells from human donors (REC ref. 21/NW/0202). Leukocyte cones from two donors were received from NHS Blood and Transplant. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient separation, and any remaining red blood cells (RBCs) were lysed by the addition of RBC lysis buffer (Fisher Scientific UK). PBMCs were resuspended in PBS and passed through a 30-μM filter. Viability and cell count were measured by Bio-Rad TC20. A portion was cultured for later flow cytometry analysis. The remainder were resuspended in MACS buffer (10% FBS [Gibco], 2 μM EDTA [Merck Life Science UK Limited], and PBS [Gibco]) and incubated with $CD3^+$ microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Positive selection of microbead-bound cells was carried out by magnetic separation using autoMACS Pro Separator (Miltenyi Biotec). The resulting $CD3^+$ cell population was cultured at 1×10^6 cells per milliliter in T cell media (RPMI-1640 [Merck Life Science UK Limited] + 10% heat-inactivated FBS [Gibco] + 2 mM Ultraglutamine [Lonza, Basel, Switzerland] + 10 mM HEPES buffer [Gibco] + 0.5 mM sodium pyruvate [Gibco] + 1× MEM Non-Essential Amino Acids [Gibco] + 100 U/mL penicillin + 0.1 mg/mL streptomycin [Merck Life Science UK Limited] + 55 μM 2-mercaptoethanol [Gibco]) supplemented with 50 U/mL interleukin-2 (IL-2) (Fisher Scientific UK) in a humid static incubator at 37°C, 5% CO_2. The following day, PBMCs and $CD3^+$ cells were stained with anti-CD3-PE (clone HIT3a, 1:100; BioLegend, San Diego, CA), anti-CD4-FITC (clone RPA-T4, 1:50; Bio Legend), and anti-CD8-FITC (clone HIT-8a, 1:100; BioLegend) for analysis on the Attune NxT Flow Cytometer. Data analysis was performed using Attune NxT Flow Cytometer software. Cells were cryopreserved at 1×10^7 cells per milliliter in Cryostor CS10 (STEMCELL Technologies, Cambridge, UK) according to the manufacturer's instructions.

Revival and lentiviral transduction of human primary T cells

Cryopreserved $CD3^+$ T cells were revived 72 h before activation. Each 1-mL vial was rapidly thawed, resuspended to 10 mL in

warm T cell media supplemented with IL-2 at 50 U/mL, and centrifuged at 400 RCF for 6 min. The supernatant was discarded and the cells were resuspended to 1×10^6 cells per milliliter in media with IL-2 (50 U/mL). Growth was monitored and media refreshed as required. T cells were activated 16 h prior to lentiviral transduction by incubation with Dynabeads Human T-Activator CD3/CD28 beads (Gibco) at a 2:1 cell:bead ratio. A non-treated, flat bottom 96-well plate was coated with human Fibronectin (Fragment) (Miltenyi Biotec) by the addition of 80 μL per well at 30 μg/mL and overnight incubation at 4°C. The following day, plates were blocked with PBS 2% BSA (Gibco) and cells seeded at 1×10^5 cells per well. For transduction, LVVs were diluted in T cell media supplemented with IL-2 (50 U/mL) for an MOI of 0.5 and a final concentration of 1×10^6 cells per milliliter once added to the wells (completed within 2 h of seeding). Transduction was carried out to produce triplicate samples for multiple assays (flow cytometry, IVCN assay, and RT-qPCR). Following addition of the LVV, the transduction plate was centrifuged at 1,346 RCF for 2 h at room temperature and then placed in a humid static incubator at 37°C, 5% CO_2. At 72 h post-transduction, cells for IVCN and RT-qPCR assays were frozen as cell pellets at −80°C.

EGFP expression analysis of T cells

Dynabeads were removed from T cells by the application of a magnet. Cells were washed twice in MACS buffer before final resuspension in MACS buffer and analysis on Attune NxT Flow Cytometer.

Protein L staining to detect cell surface CAR expression

Dynabeads were removed from all T cell samples by the application of a magnet. T cells were washed twice with PBS 4% BSA, resuspended in 200 μL PBS 4% BSA containing 1.5 μg biotinylated Protein L (Fisher Scientific UK) per well and incubated at 4°C for 30 min. Following incubation, two further PBS 4% BSA washes were carried out, cells resuspended in 200 μL PBS 4% BSA containing 2.5 μg streptavidin-PE (Miltenyi Biotec), and incubated at 4°C for 30 min. Following incubation, cells were washed three times in 200 μL PBS 1% BSA before final resuspension in PBS 1% BSA and analyzed on the Attune NxT Flow Cytometer.

CD25/69 T cell activation marker staining

Dynabeads were removed from T cells by the application of a magnet. Cells were washed once in MACS buffer, resuspended in 18 μL MACS buffer containing both anti-CD25-PerCP-Cy5.5 (BioLegend, clone M-A251, 1:40) and anti-CD69-PE (clone FN-50, 1:40; BioLegend) and incubated at 4°C for 30 min. Following incubation, cells washed twice in MACS buffer before final resuspension in 200 μL MACS buffer and analysis on the Attune NxT Flow Cytometer.

Flow cytometry data analysis

Flow cytometry data analysis was performed using Attune NxT Flow Cytometer software. The cell population was gated to exclude debris. Cell singlets were then gated by comparing FSC-H to FSC-A. Gates to identify cells positive for fluorophore were drawn using unstained/fluorophore-negative cell samples. MFI for gated cells was calculated by the software.

Integrated copy-number assay by ddPCR from LVV-transduced cells

gDNA was extracted from T cell pellets using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. The WPRE copy number was determined relative to known *ALB* copy number (two copies in T cell) by duplex ddPCR assay. Each reaction mixture was prepared to a final volume of 22 μL with 2× ddPCR Supermix for Probes (No dUTP) (Bio-Rad), forward and reverse primers (IDT, 900 nM final concentration), PrimeTime qPCR probes (250 nM final concentration; IDT), and template gDNA (a mass that gave 0.2–1 reference gene copies per droplet, as recommended by Bio-Rad). Primer and probe sequences: WPRE forward 5′-TTG CTT CCC GTA TGG CTT TC-3′, WPRE reverse 5′-CGG GCC ACA ACT CCT CAT AA-3′, WPRE probe 5′-FAM-TCT CCT CCT-ZEN-TGT ATA AAT CCT GGT TGC TGT CTC-3IABkFQ-3′, *ALB* forward 5′-GCT GTC ATC TCT TGT GGG CTG T-3′, *ALB* reverse 5′-ACT CAT GGG AGC TGC TGG TTC-3′, *ALB* probe 5′-5SUN-CCT GTC ATG-ZEN-CCC ACA CAA ATC TCT CC-3IABkFQ-3′. Droplets were generated using AutoDG (Bio-Rad) and PCR carried out in C1000 Touch Thermocycler (Bio-Rad). Droplets were analyzed in QX200 Droplet Reader (Bio-Rad) and the copy number quantified in QX Manager 2.0 (Bio-Rad). The thermocycle conditions were as follows: 95°C for 10 min; 40 cycles of 94°C for 30 s, followed by 60°C for 1 min, 98°C for 10 min, and 10°C for 10 min. The ramp rate was 2°C/s for all steps.

Data processing

All flask and plate titer values were calculated as averages of biological replicates. Thus, error values indicate the population SD between biological replicates. Stirred-tank bioreactor titer values were calculated as averages of analytical replicates. Thus, error values indicate the population SD between analytical replicates. Linear regression and 95% confidence interval were calculated using GraphPad Prism software.

DATA AND CODE AVAILABILITY

The authors declare that the data supporting the findings of this study are included within the article and its supplemental information. The packaging cells and producer cells presented here are available for evaluation subject to the execution of a material evaluation agreement with OXGENE, A WuXi Advanced Therapies Company.

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AUTHOR CONTRIBUTIONS

Conceptualization: M.T., M.M., M.A., R.P.-M., L.D., T.P., C.B., R.C., and Q.L. Formal analysis: M.T., M.M., S.P.J., M.A., M.R., M.P., T.L.C., R.B., S.D., and C.B. Funding acquisition: R.C., W.V., and D.C. Investigation: M.T., M.M., S.P.J., M.A., M.R., C.F., B.G., C.F.-D., I.M., H.A.T., M.K., Z.Z., E.P., R.P.-M., L.D., M.P., J.K., L.H., R.A., L. Montgomery, T.L.C., R.B., I.S., M.T.A., D.H., S.D., H.B.G., C.B., and Q.L. Methodology: M.T., M.M., M.A., H.A.T., E.P., L. McCall, R.P.-M., L.D., S.D., H.B.G., C.B., and Q.L. Supervision: C.B., R.C., W.V., D.C., M.I.P., and Q.L. Writing – original draft: M.T., M.M., M.A., S.D., M.P., and R.A. Writing – review & editing: M.T., C.B., M.I.P., and Q.L.

DECLARATION OF INTERESTS

All listed authors are present or past employees of OXGENE, A WuXi Advanced Therapies Company. W.V. and D.C. are employees and hold stock or stock options within the company. R.P.-M., L.D., T.P., and R.C. are named co-inventors on a patent describing the molecular configuration of the packaging elements (US20200277629A1).

SUPPLEMENTAL INFORMATION

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