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RESEARCH ARTICLE

Exploring nutrient supplementation and bioprocess optimization to improve the production of lentiviral vectors in serum-free medium suspension cultures

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Abstract

The use of lentiviral vectors (LV) in gene therapy has been growing in recent years. To meet the increasing clinical demand, LV production platforms will benefit from improved productivity and scalability to enable cost-effective manufacture of LV-based therapies. Here we report the adaptation of 293T cells to serum-free suspension cultures and the improvement of LV yields through transfection parameters optimization, process intensification and medium supplementation with nutrient boosters. Cells were sequentially adapted to different serum-free culture media, transfection parameters were optimized and the two best-performing conditions were selected to explore process intensification by increasing cell density at the time of transfection. LV production at higher cell densities increased volumetric titers up to 12-fold and lipid supplementation was the most efficient metabolic optimization strategy further enhancing LV productivity by 3-fold. Furthermore, cell concentration was identified and validated as an important source of transfection variability impairing cellular uptake of DNA polyplexes, impacting transfection efficiency and reducing LV titers down to 6-fold. This work contributes to improving LV-based gene therapy by establishing new scalable manufacturing platforms and providing key metabolic insights, unveiling important bioreaction parameters to improve vector yields.

KEYWORDS
bioprocess engineering, gene therapy, lentiviral vectors, serum-free medium suspension culture, transient transfection

1 | INTRODUCTION

Lentiviral vectors (LV) are recombinant viral particles widely used as gene transfer vehicles in research and clinical applications.[1] LV are currently the vectors of choice for gene therapy applications requiring long-term transgene expression due to their ability to transduce and integrate into both dividing and non-dividing cells.[2] The potential of LV-based gene therapy materialized in over 200 clinical trials and market approval of nine products.[3]

The most used method for LV production is based on transient co-transfection of plasmid DNA into adherent HEK 293 cells or, most commonly, their 293T derivates.[4] However, transient transfection of adherent cells at a large scale is challenged by high batch-to-batch variability, cost- and labor-intensive scale-out processes and burdensome downstream processing due to the presence of animal-derived serum in the culture medium.[4] More recently, platforms for LV production in suspension cultures have been developed yielding titers from 10^6 to 10^8 transducing units per milliliter (TU/mL).[5] These

platforms are typically based on serum-free chemically defined medium formulations, which facilitates scale-up to larger volumes, downstream processing, and bioprocess standardization. Previous works generally started with the adaptation of 293T clones to serum-free suspension culture and rely on polyethylenimine (PEI) complexation [6,7] or electroporation [8] for plasmid transfection. Stable producer cell lines expressing LV components under the regulation of inducible promoters have also been adapted to serum-free suspension culture.[9,10] However, clone-based systems rely on finding rare clones combining high transfectability and LV production potential, limiting the reproducibility of these methods. Alternatively, establishing new platforms using available cell populations allows to circumvent clone-screening steps, offering a reliable and accessible source to establish serum-free suspension cultures for LV production. Transient transfection protocols for LV production are challenged by tremendous variability, often exacerbated when using suspension cultures. This variability can be partially attributed to changes in the physicochemical properties of the DNA/PEI complexes, namely particle size distribution and charge. These properties have been described to be affected by different factors, in particular DNA/PEI incubation times and the ion content of the transfection medium.[11,12] However, little is known about other bioprocess parameters influencing transfection efficiency and limiting the robustness and standardization of transient LV production. To this end, process intensification and nutrient supplementation strategies can be explored to improve LV production in serum-free suspension cultures. Nutrient supplementation is a relatively cost-effective and fast approach to improve cell culture yields, including the production of virus-based biopharmaceuticals.[13] For example, it was demonstrated to improve the production of gammaretroviral vectors,[14] influenza virus-like particles [15] and herpes simplex virus 1 vectors.[16] Some of the newly established LV production platforms use nutrient boosters or culture adjuvants to supplement serum-free medium formulations.[8,17] However, only a small set of supplements has been evaluated for LV production in suspension culture, most of them without a clear metabolic target (e.g., peptone extracts).[18,19] A more comprehensive study of the metabolic requirements for LV manufacture in serum-free suspension cultures is still missing.

In this work, we established a new scalable LV production platform by adapting the original 293T cell population (ATCC CRL-3216) to a serum-free suspension culture. Process intensification and nutrient supplementation strategies were explored to improve vector titers. Furthermore, we took advantage of the high number of LV production runs performed to search for sources of variability in the transfection process and identified a new parameter exerting extreme influence in transfection efficiency.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culture media

Human embryonic kidney 293 cells, expressing the simian virus 40 (SV40) large T antigen, 293T cells (ATCC CRL-3216), were obtained from the American Type Culture Collection (Manassas, VA). These cells were used for LV production and titration. Adherent cells were maintained in Dulbecco Modified Eagle Media (DMEM; Corning, NY) supplemented with 10% v/v fetal bovine serum (FBS; Gibco, Paisley, UK) in an incubator with a humidified atmosphere of air with 8% v/v CO_2 at 37°C. Additionally, these cells were also adapted to serum-free suspension culture using one of three different medium formulations: LV-Max production medium (Gibco), BalanCD HEK293 (Fujifilm Irvine Scientific, Tokyo, Japan) supplemented with 4 mM GlutaMAX (Gibco) and FreeStyle F17 expression medium (Gibco) supplemented with 4 mM GlutaMAX. Suspension cells were maintained in polycarbonate Erlenmeyer flasks with vented cap on an orbital shaker plate at 125 rotations per minute (RPM).

2.2 | Adaptation of 293T cells to suspension culture

293T cells were sequentially adapted to one of the three different serum-free medium formulations starting from serum-containing adherent culture followed by transfer to suspension culture. Cells were sub-cultured every 3 or 4 days over the course of 10 passages. Every two passages cells were cultured in increasing amounts of the serum-free formulation until transfer to suspension cell culture conditions in the respective serum-free medium formulation.

After transfer to suspension, cells were sub-cultured every 3 or 4 days at an inoculum of 3×10^5 cells per milliliter (cells mL^{-1}) and were considered successfully adapted when reaching over 1×10^6 cells mL^{-1} and over 90% viability at 96 h post-seeding.

2.3 | Cell number and doubling time

Cell concentration and viability was determined by trypan blue exclusion method using Cedex HiRes Analyzer (Roche, Basel, Switzerland). Doubling time was calculated at each passage using the following equation:

$$\text{DT (hours)} = \frac{t \times \ln(2)}{\ln\left(\frac{C}{C_0}\right)}$$

where *t* represents time between passages (hours), C represents final cell density (cell mL^{-1}) and C0 represents initial cell density (cell mL^{-1})

2.4 | Plasmids

Plasmid pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene #12252) contains a self-inactivating (SIN) LV transgene[20] carrying a green fluorescent protein (GFP) reporter. Plasmid pMDLg/pRRE (Addgene #12251) codes for the human immunodeficiency virus 1 (HIV-1) gag-pro-pol polyprotein. Plasmid pRSV-REV (Addgene #12253) codes for the second and third exons of HIV-1 REV.[21] Plasmid pMD2.G (Addgene

#12259) codes for the envelope glycoprotein of vesicular stomatitis virus (VSV-G). All plasmids were kindly provided by Dr. Didier Trono through Addgene plasmid repository (Cambridge, MA).

2.5 | Transfection of 293T in suspension and LV production

Suspension-adapted 293T cells were amplified and pre-inoculated at 7 × 10^5 cell mL^{-1} at 24 h before transfection to ensure an active growth phase at the time of transfection. In the following day, cells were confirmed to be at 1 × 10^6 cell mL^{-1}, or adjusted otherwise, in a 20 mL culture volume. Transfection was performed using PEIpro (Polyplus-Transfection, Strasbourg, France) transfection reagent, using different transfection conditions. At 24-, 48- and 72-hours post-transfection, cells were assessed for growth and viability. Transfection efficiency was determined by quantifying GFP positive cells by flow cytometry analysis using Gallios flow cytometer (Beckman Coulter, Brea, CA).

Production of LV with suspension-adapted 293T cells was performed by transient co-transfection of GFP-encoding SIN LV transgene (pRRLSIN.cPPT.PGK-GFP.WPRE), third-generation LV packaging plasmids (pMDLg/pRRE and pRSV-REV) and VSV-G envelope plasmid (pMD2.G), using different amounts of total DNA mass per millions of cells and different mass ratios of DNA to PEI. The mass proportions of each plasmid in transient LV production per every 1 µg of total DNA were: 0.55 µg vector genome, 0.21 µg gag-pro-pol, 0.06 µg REV and 0.18 µg envelope. These proportions were adjusted to the total amount of DNA mass per millions of cells. Cells were harvested at 48- and 72-hours post-transfection and assessed for cell growth and viability. Transfection efficiency was determined by quantifying GFP positive cells by flow cytometry analysis using Gallios flow cytometer. Viral supernatants were harvested at 48- and 72-hours post-transfection, clarified and stored at −80°C until further use.

For high cell density production settings, the same protocol described above was applied for three different inoculums at the time of transfection: 1, 2 and 3 × 10^6 cells mL^{-1}. Cells were concentrated by centrifugation for 5 min at 300 *g* and resuspended in appropriate volumes to achieve the desired concentration.

Nutrient supplementation experiments followed the transfection protocol described above using the highest cell density condition (3 × 10^6 cell mL^{-1} at transfection). The production medium was supplemented with one or a combination of the following supplements: (i) vitamins (Sigma, Steinheim, Germany); (ii) nucleosides (Merck Millipore, Billerica, MA); (iii) antioxidants (Sigma); (iv) polyamines (Sigma); (v) reduced glutathione (Sigma); (vi) LipiMAX bovine lipoprotein (Selborne Biological Services, Selborne, UK). Further details on the supplements used and final concentrations are provided in supplementary material (Table S1).

2.6 | Lentiviral vector titration

For LV titration, adherent 293T cells were seeded at 5 × 10^4 cells cm^{2-1} in 24-well plates in DMEM supplemented with 10% v/v FBS and incubated overnight. After 24 h, culture medium was removed, and cells were transduced with 200 µL of serial dilutions of viral supernatant. Dilutions were performed in DMEM supplemented with 10% v/v FBS containing 8 µg mL^{-1} of polybrene (Sigma). After overnight incubation at 37°C, 300 µL of DMEM supplemented with 10% v/v FBS were added to each well. Two days after transduction, cells were harvested and analyzed for GFP fluorescence by flow cytometry. The number of LV transducing units per volume (T.U./mL) was calculated using the following equation:

$$\text{Titer}\left(\frac{\text{T.U.}}{\text{mL}}\right) = \frac{\%\ \text{of GFP positive cells} \div 100}{\text{volume of transduction}} \times \text{dilution factor} \times \text{number of cells at infection}$$

LV productivity (T.U./cell·day) was calculated as the average of transducing units per viable cell per day between 48- and 72-h post-transfection, using the following equation:

$$\text{LV productivity} = \frac{\text{Average T.U. titer between 48 and 72 h post} - \text{transfection (T.U./mL)}}{\text{Average viable cell density between 48 and 72 h post} - \text{transfection (cell/mL)}}$$

2.7 | Polyplex tracking assays

Suspension-adapted 293T cells were transfected with either stained or unstained reporter plasmid using the highest cell density condition (3 × 10^6 cell mL^{-1}). Reporter plasmid was stained with YoYo-1 Iodine (Invitrogen, Waltham, MA), a green, fluorescent nucleic acid intercalant agent. Reporter labelling was performed by mixing 1 µL of 1 mM

YoYo-1 per 25 μg of plasmid DNA followed by an incubation of 2 h in the dark at room temperature. Cultures were sampled at different time-points: 0-, 5-, 15-, 30-, 45-, 60-, 75-, 90-minutes and 2-, 4-, 8-, 24-, 48- and 72-hours post-transfection. To quantify DNA polyplex internalization, extra-cellular YoYo-1 signal was quenched using a trypan blue solution at a final concentration of 0.1% w/v in phosphate-buffered saline (PBS). Polyplex uptake was measured at different time-points by analysing the percentage of YoYo-1 positive cells by flow cytometry using BD FACSCelesta flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.8 | Statistical analysis

Statistical analysis was performed using Student's t-test. Differences were considered significant for $p < 0.05$.

3 | RESULTS

3.1 | Adaptation of 293T cells to suspension

To establish a new scalable LV production system, we started by adapting 293T cells (ATCC CRL-3216) to serum-free suspension culture. Cells were sequentially adapted to three different commercially available serum-free medium formulations: BalanCD HEK293, Free Style F17, and LV-Max production medium. The adaptation process was successful for all serum-free medium formulations and adapted cells were amplified and used to establish master cell banks.

The growth of 293T adapted cells was characterized by daily monitoring of cell concentration (Figure 1A) and viability (Figure 1B) over the course of 9 days. Adapted cells displayed a typical cell growth curve featuring a stationary phase during the first day in culture, followed by an exponential growth phase of up to 6 days and entered a declining phase toward the end of the culture (Figure 1A). From the three culture media evaluated, LV-Max production medium sustained higher cell densities. All media supported high cell viability (above 80%) up to 5 days after inoculation (Figure 1B). Adapted cells were subcultured every 3 or 4 days and monitored for growth for 9 weeks. Cells cultured in the different media presented similar growth profiles over time reaching cell densities of approximately 1.5×10^6 at 3 days after subculture and generally above 2.5×10^6 cell mL^{-1} at 4 days after subculture (Figure 1C). Adapted cells showed a tendency to reach higher cell densities at later passages, which became more evident from passage 9 onward (Figure 1C). Cells adapted to the different media showed similar doubling times ranging between 31 and 33 h (Table S2, supplementary material) which is in accordance with the values reported by the serum-free media manufacturers.

3.2 | Optimization of LV production in suspension cultures

After adaptation to serum-free suspension culture, 293T cells were first used to optimize the parameters for transient transfection. To this end, cells were transfected with the LV transgene plasmid pRRLSIN.cPPT.PGK-GFP.WPRE serving as fluorescence reporter, using three different amounts of total DNA mass per millions of cells, each with three different mass ratios of DNA to transfection reagent (PEIpro). For all media tested, the highest transfection efficiencies were obtained using 1 μg of DNA per million cells with a 1:3 DNA to PEI mass ratio or 2 μg of DNA per million cells, with a 1:2 DNA to PEI mass ratio. These conditions yielded similar transfection efficiencies for each medium tested, up to 75% GFP positive cells in BalanCD HEK293, 70% in LV-Max and 60% in FreeStyle F17 (Figure S1A, supplementary material). These transfection parameters were selected for further studies, namely LV production, and designated as transfection conditions A and B, respectively. Additionally, the impact of transfection on cell growth was assessed by measuring viable cell density and cell viability at 48 h post-transfection. All transfected cultures showed reduced cell growth and viability when compared to those of non-transfected cultures (Figure S1B, supplementary material). These effects were more evident in cells transfected with higher amounts of DNA.

To evaluate the potential of the newly adapted 293T cells for transient LV production, cells were transfected using the two best performing conditions determined previously, transfection conditions A and B (Figure S1A, supplementary material), and assessed for growth, transfection efficiency and transducing units titers. Transfection efficiencies were above 70% at 48 h post-transfection in all serum-free media formulations tested (Figure 2A). Additionally, no significant differences in transfection efficiencies were observed between transfection condition A and B for each medium (Figure 2A). Moreover, and in accordance with the results from the reporter gene transfection assay (Figure S1B, supplementary material), transfected cells showed reduced cell growth and viability when compared to non-transfected cultures (Figure 2B). Both transfection conditions led to decreased cell viability to approximately 70% (Figure 2B).

LV production in BalanCD HEK293 medium delivered the highest transducing units titers, up to 6.5×10^6 T.U. mL^{-1}, followed by Free Style F17 medium, up to 1.2×10^6 T.U. mL^{-1}, while in LV-Max production medium, titers were below 2.3×10^5 T.U. mL^{-1} (Figure 2C). There were no significant differences in transducing units titers between transfection conditions A and B, for the respective medium and time of harvest (Figure 2C).

The two media supporting higher LV production, BalanCD HEK293 and Free Style F17, were selected to proceed to process intensification studies, aiming at further improving LV titers, by increasing cell density at the time of transfection. To achieve high cell density at the time of transfection, a cell concentration step was included prior to the transfection procedure. Cells were amplified and pre-inoculated at 1×10^6 cell mL^{-1} 24 h before transfection to ensure that they would be in an active growth state. Then, prior to transfection, cells were concentrated by centrifugation and resuspended to defined cell densities: standard (1×10^6 cell mL^{-1}), medium (2×10^6 cell mL^{-1}) and high (3×10^6 cell mL^{-1}) cell density.

For each media tested, no significant differences in transfection efficiencies were observed between the different cell densities at the time of transfection and between transfection condition A and B (Figure 3A). Performing transfection at high cell density conditions resulted in LV

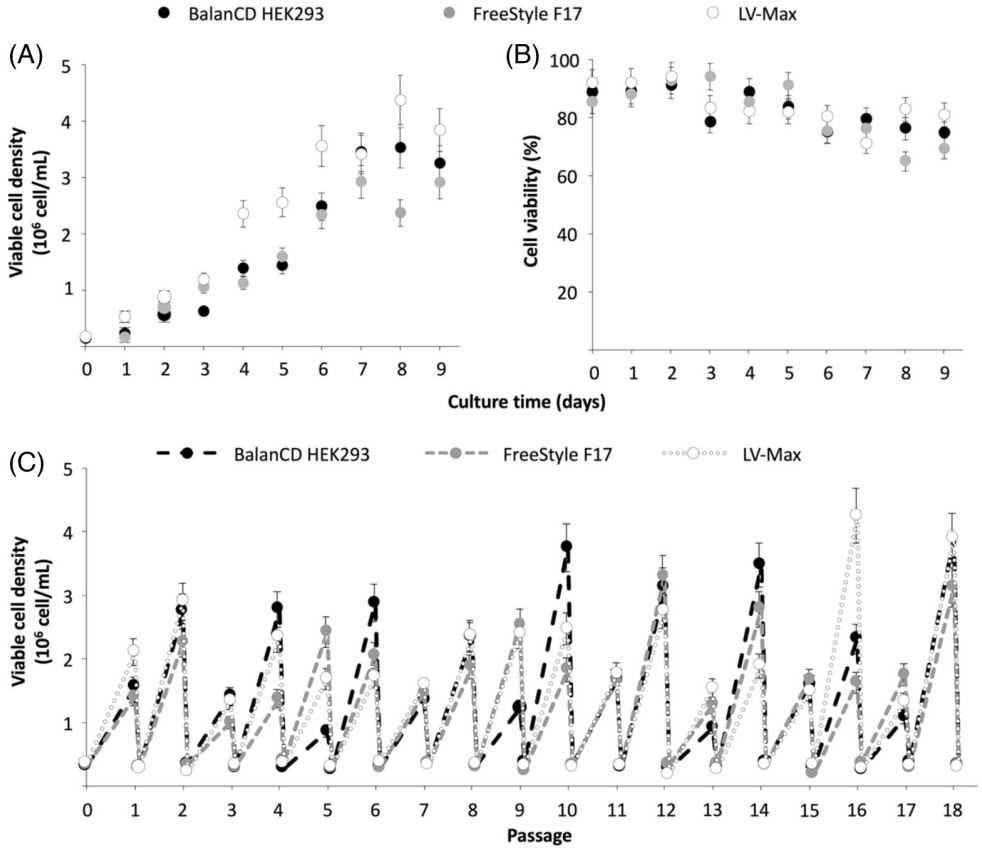

FIGURE 1 Growth of 293T cells adapted to serum-free suspension culture. Viable cell density (A) and viability (B) of newly adapted 293T cells, two passages after transfer to suspension culture, growing in three different serum-free medium formulations: BalanCD HEK293, FreeStyle F17, and LV-Max. Monitoring of growth (C) of 293T cells adapted to the three different medium formulations over 18 passages (9 weeks) after transfer to suspension culture. Cells were sub-cultured every 3 or 4 days, alternately. Values given in supplementary material (Table S3). Error bars represent ± 10% variation associated to cell counting by trypan blue exclusion method ($n = 1$).

titer increases. The highest volumetric titers obtained were 2.4×10^7 T.U. mL^{-1} and 1.5×10^7 T.U. mL^{-1} using transfection condition A in BalanCD HEK293 and Free Style F17, respectively (Figure 3B). Increasing cell density at transfection improved transducing unit titers up to 5-fold and 12-fold for BalanCD HEK293 and Free Style cultures, respectively. These improvements were obtained using transfection condition B when compared to the corresponding standard cell density condition (Figure 3B).

3.3 | Nutrient supplementation for improved LV production

To further improve LV titers, a set of nutrient boosters and medium supplementation strategies was evaluated. These were chosen based on previous evidence of their relevance in the context of enveloped viral vector production.[14] To maximize the availability of the nutrient boosters during the LV production period, cells were resuspended in freshly supplemented medium prior to transfection. Since transfection conditions A and B delivered similar LV production performance in the process intensification experiments (Figure 3B), transfection condition A (1 μg total plasmid DNA per millions of cells and 1:3 DNA to PEI mass ratio) was selected. This condition requires less plasmid DNA which represents an important factor for cost-effectiveness of the manufacture process.

Transducing units titers up to 4×10^7 T.U. mL^{-1} and 2×10^7 T.U. mL^{-1} were obtained when supplementing BalanCD HEK293 cultures with nucleosides or vitamins and FreeStyle F17 cultures with lipids, respectively (Table S4, supplementary material). Lipid supplementation in Free Style F17 medium significantly improved specific LV productivity by up to 3-fold (Figure 4A). These improvements were obtained using higher concentrations of the LipiMAX supplement (0.75% v/v or 1.25% v/v) regardless of the presence of Insulin-Transferrin-Selenium-Ethanolamine (ISTE) as a lipid carrier. On the other hand, high doses of reduced glutathione (10 mM) in BalanCD HEK293 medium reduced LV productivity by up to 2-fold (Figure 4A). The effect of the different supplementation strategies on transfection efficiency was also evaluated. In both media, lipid supplementation and reduced glutathione supplementation led to decreased transfection efficiencies in a dose-dependent manner, while for the remaining supplements, no differences in transfection efficiencies were observed (Figure 4B).

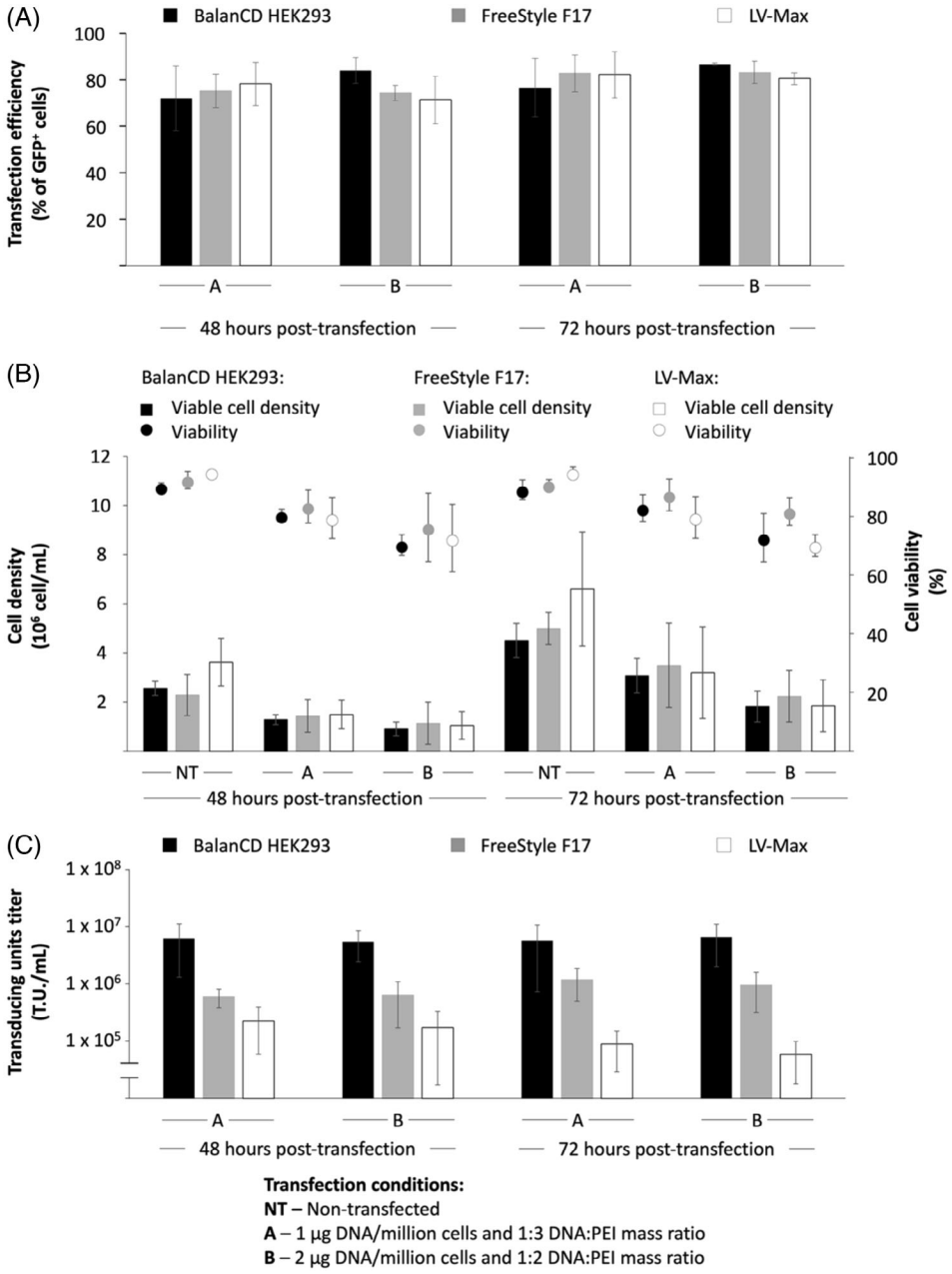

FIGURE 2 Transient lentiviral vector production with adapted 293T cells. Transfection efficiency (A), viable cell density and viability (B) and transducing units titers (C) of suspension-adapted 293T cells were assessed in the context of transient LV production. Cells adapted to one of three different serum-free medium formulations (BalanCD HEK293, FreeStyle F17 and LV Max production medium) were transfected using two different transfection conditions (A: 1 µg of total plasmid DNA per millions of cells and 1:3 DNA to PEI mass ratio, or B: 2 µg of total plasmid DNA per millions of cells and 1:2 DNA to PEI mass ratio). Cultures were sampled at 48 and 72 h post-transfection. Transducing units titers were quantified by flow cytometry analysis of transduced 293T cells. Transfection efficiency is given by percentage of GFP positive cells at 48 or 72 h post-transfection. Viable cell density (bars) and viability (circles) were determined by cell counting using trypan blue exclusion method. Values are shown as average ± standard deviation ($n = 3$).

3.4 | Cell concentration at transfection as a variability source in LV production in serum-free suspension cultures

Batch-to-batch variability has been described as one of the biggest challenges in LV production.[22,23] Also in this work, high variability in transfection efficiency and titers was observed throughout the different production runs, even for similar conditions. This variability was particularly evident at the process intensification stage and nutrient supplementation assays. During the nearly 80 LV production runs conducted during this work, it was noticed that long time periods occurring between cell concentration and resuspension in fresh medium would lead to low transfection efficiencies and, consequently, low titers.

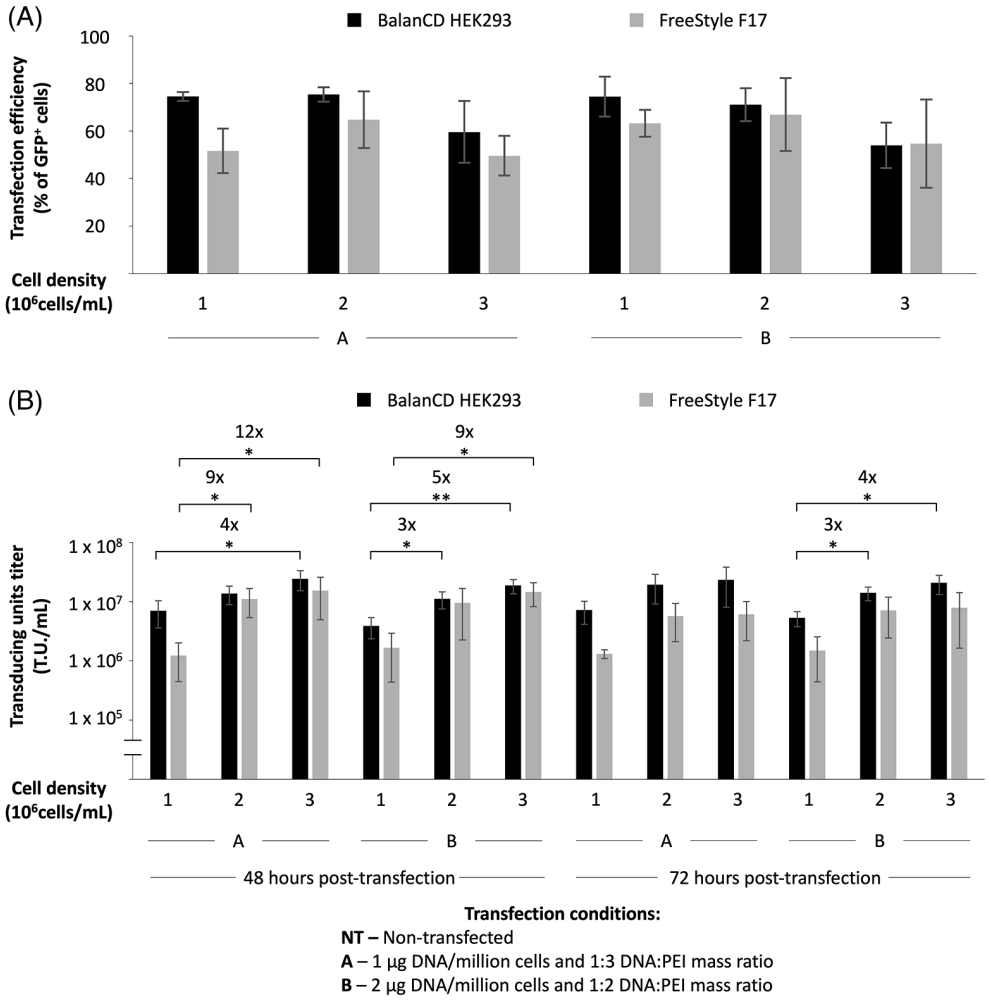

FIGURE 3 Lentiviral vector production at different cell densities at the time of transfection, using 293T adapted cells. Transfection efficiency (A) and transducing units titer (B) of 293T cells adapted to BalanCD HEK293 or to FreeStyle F17, transfected at three different cell densities at the time of transfection (1×10^6, 2×10^6, and 3×10^6 cells mL^{-1}) using transfection conditions A (1 µg of total plasmid DNA per millions of cells and a 1:3 DNA:PEI mass ratio) or B (2 µg of total plasmid DNA per millions of cells and a 1:2 DNA:PEI mass ratio). Transfection efficiency is given by the percentage of GFP-positive cells at 48 h post-transfection. Viral supernatant was harvested at 48 and 72 h post-transfection. Infectious titers were quantified by flow cytometry analysis of transduced 293T cells. Values above the bars represent significant titer fold-change (*$p < 0.05$ given by a Student's t-test) relative to standard cell density condition (1×10^6 cells mL^{-1} at transfection) at the respective time of harvest and transfection condition. Values are shown as average ± standard deviation ($n = 3$).

Following these observations, the impact of cell concentration on LV production was studied. To this end, the conditions of high cell density transfection were re-created for suspension cultures in BalanCD HEK293, FreeStyle F17, and for cells grown in adherent cultures in DMEM with 10% v/v FBS. Then, cells were kept concentrated for different time periods before re-suspension in fresh medium. Increasing the time cells spend concentrated led to lower transfection efficiencies in suspension cultures, with this effect being more pronounced in cells cultured in Free Style F17 medium (Figure 5A). This effect was absent in cells cultured in adherent and serum-containing conditions (Figure 5A).

Lower transfection efficiencies occurring when increasing the time cells spend concentrated were also associated with decreased LV titers in both suspension culture media (Figure 5B). This effect was more evident in Free Style F17 medium while, again, no differences were observed with cells cultured in adherent conditions (Figure 5B). LV productivity in BalanCD HEK293 was significantly impaired after a 30-minute incubation period, decreasing by up to 4-fold when compared to the cells that were immediately resuspended (Figure 5B). For cells cultured in Free Style F17 the effect seems to be exerted at earlier time points with productivity losses of 4-fold after 15 min maintained concentrated and further down to 6-fold productivity loss after 30 min (Figure 5B).

To further understand the effect of the time cells spend concentrated in transfection performance, a polyplex tracking assay was conducted comparing cells concentrated by centrifugation that were either immediately resuspended or kept concentrated for 30 min before resuspension. In line with the previous results of transfection efficiency (Figure 5A), polyplex uptake rate was decreased in cells maintained concentrated for 30 min (Figure 6A). This effect was more

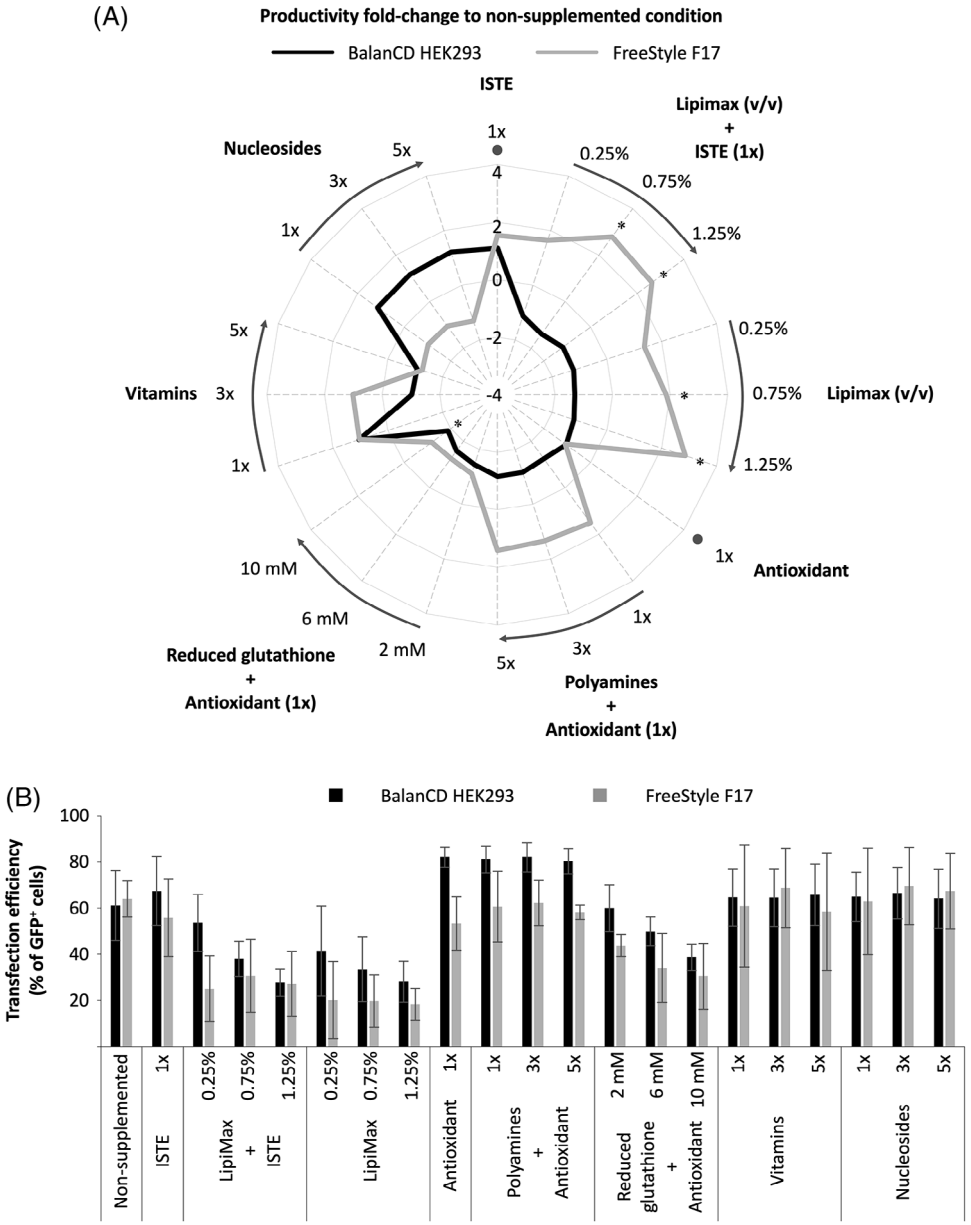

FIGURE 4 Evaluation of nutrient supplementation strategies in lentiviral vector production. Cell specific LV productivity (A) and transfection efficiency (B) of adapted 293T cells cultured in media supplemented with different nutrient boosters (Table S1, supplementary material). 293T cells adapted to BalanCD HEK293 or to Free Style F17 were transfected at high cell density (3×10^6 cells mL^{-1} at the time of transfection) using 1 µg of total plasmid DNA per millions of cells and a 1:3 DNA:PEI mass ratio. The production medium was supplemented as described in the Materials and Methods section. At 48 and 72 h post-transfection cells were assessed for viable cell density, viability, transfection efficiency, and viral supernatant was harvested. Cell specific LV productivity was calculated as the average of transducing units between 48 and 72 h post-transfection per viable cell, as described in the materials and methods section 2.6, and is represented as fold-change relative to the non-supplemented condition. Values represent an average of 4 independent experiments, error bars were omitted for simplicity; values ± standard deviation are given in supplementary material (Table S4, supplementary material). Transfection efficiency at 48 h post-transfection is given by the percentage of GFP positive cells and values are shown as average ± standard deviation ($n = 4$). $^*p < 0.05$ given by Student's t-test.

evident in cells adapted to FreeStyle F17 where polyplex uptake rates in cells maintained concentrated for 30 min were 2-fold lower than the respective immediate resuspension condition (Table S5, supplementary material). Impaired polyplex uptake rates resulted in reduced intracellular accumulation of polyplexes for cells kept concentrated for 30 min when compared to cells immediately resuspended for the respective medium and time-point (Figure 6B).

4 | DISCUSSION

The increasing use of LV for gene therapy applications in recent years has pressed the need for scalable and robust production platforms. In this work we aimed at improving LV manufacture in serum-free suspension cultures by exploring process intensification and nutrient supplementation strategies.

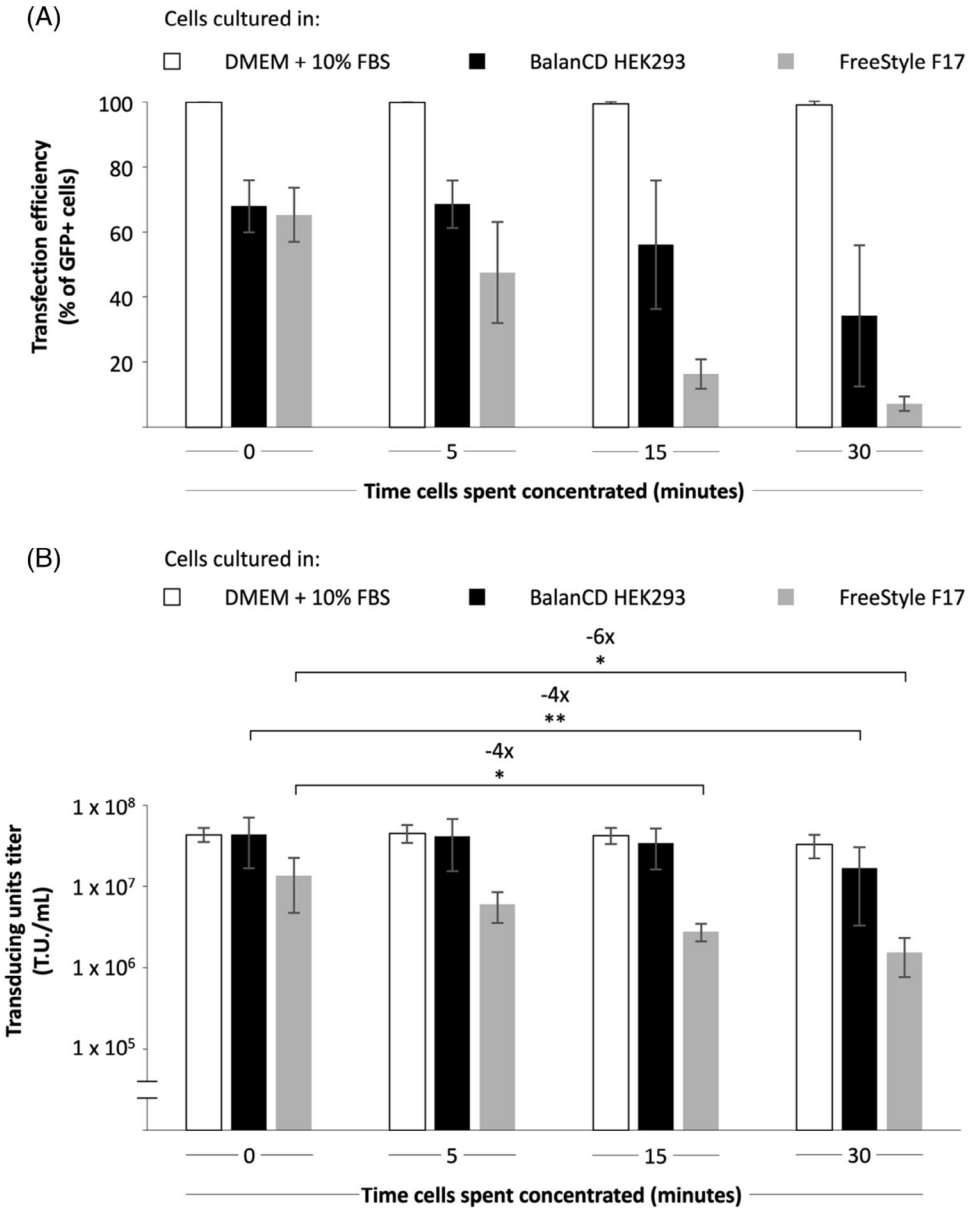

FIGURE 5 Effect of time that cells spent concentrated on lentiviral vector production. Transfection efficiency (A) and transducing units titers (B) of 293T cells resuspended and transfected immediately after concentration or kept concentrated for 5, 15, or 30 min prior to resuspension and transfection. Suspension-adapted 293T cells cultured in BalanCD HEK293 or in Free Style F17 were transfected at high cell density (3×10^6 cells mL^{-1}) using 1 μg of total plasmid DNA per millions of cells and a 1:3 DNA:PEI mass ratio. Adherent 293T cells cultured in DMEM with 10% FBS v/v were transfected following a procedure that mimics the transfection protocol used for suspension cells. Briefly, cells were harvested by trypsinization, concentrated by centrifugation then resuspended and transfected immediately after concentration or kept concentrated for 5, 15, or 30 min prior to resuspension and transfection. Cells were transfected using the same transfection conditions (1 μg of total plasmid DNA per millions of cells and a 1:3 DNA:PEI mass ratio) and inoculated at 1.5×10^5 cells cm^{2-1}. At 48 and 72 h post-transfection, cells were assessed for viable cell density, viability, transfection efficiency, and viral supernatant was harvested. Transfection efficiency is given by the percentage of GFP-positive cells at 48 h post-transfection. Transducing units titers were quantified by flow cytometry analysis of transduced 293T cells. Values above bars represent significant fold-change in specific productivity ($^*p < 0.05$ and $^{**}p < 0.01$ given by a Student's t-test) relative to the respective non-incubated condition. Values are shown as average ± standard deviation ($n = 3$).

To establish a new LV production platform using suspension cultures, the 293T cell population (ATCC CRL-3216) was adapted to three different serum-free medium formulations (Figure 1). We chose to adapt the original the 293T cell population (ATCC CRL-3216) from where most 293T clones are derived, including clone 293T/F17 (ATCC CRL-11268) which has been previously used for LV production in suspension cultures.[7,24] To our knowledge, this was the first time the parental 293T population (ATCC CRL-3216) was adapted

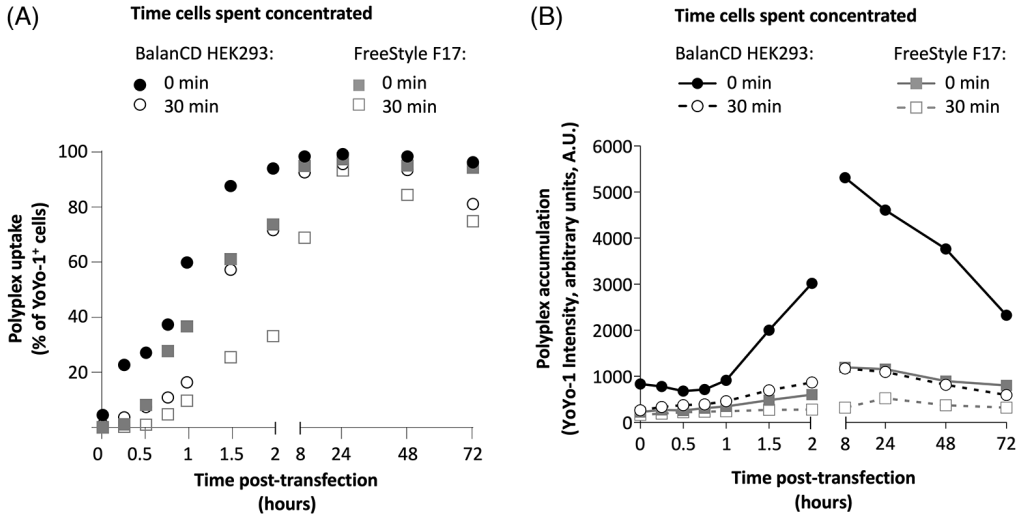

FIGURE 6 Effect of time that cells spent concentrated on polyplex internalization. Monitoring of polyplex uptake (A) and accumulation (B) over time. Suspension-adapted 293T cells cultured in BalanCD HEK293 or in Free Style F17 were transfected at high cell density (3×10^6 cells mL^{-1}) using 1 μg of reporter plasmid DNA per millions of cells and a 1:3 DNA:PEI mass ratio. Reporter plasmid was stained with green fluorescent DNA intercalant agent YoYo-1. The extra-cellular YoYo-1 signal was quenched using trypan blue solution. Cells were harvested at different time-points post-transfection and analyzed by flow cytometry. Polyplex internalization and accumulation were measured by the percentage of YoYo-1 positive and fluorescence intensity, respectively.

to serum-free suspension culture for LV production. The adaptation was performed using an accessible cell source and commercial materials to facilitate the implementation and reproducibility of this new LV production system by other groups and laboratories. All the selected serum-free medium formulations for suspension culture are chemically defined and suited for LV production in 293T cells and compatible with PEI transfection. Cells were successfully adapted to three different serum-free medium formulations and maintained in culture for over nine weeks (Figure 1). At later passages cells achieved increasingly higher cell densities in culture (Figure 1C). This effect occurred in all media tested and is likely due to the selection of faster-growing subpopulations along passages and had no effect on LV productivity (Figures S2). Transient LV production using these cells yielded titers up to 1×10^7 T.U. mL^{-1} (Figure 2C), consistent with what has been reported for similar approaches described by other authors.[6,7,10,24] Therefore, we show that it is possible to achieve similar titers by adapting the parental cell population, reducing the time and effort needed to search for highly transfectable clones. This also provided a suspension-adapted cell population, with a more diverse genetic background which can be advantageous for future cell line establishment.

To increase LV titers, we first pursued a process intensification strategy by increasing cell density at transfection. This method has been successfully used for recombinant protein expression [25] and LV production [26] in 293T cells. Higher cell densities at the time of transfection improved vector yields by up to 12-fold (Figure 3B). These results show improvements above the linear cell density increase, indicating that process intensification was achieved not only by increasing producer cells in culture but also by improving specific productivity.

Then, a set of nutrient supplementation strategies was evaluated, seeking to maximize LV productivity. Medium manipulation was performed using nutrient supplementation since this provides a fast and cost-effective approach to improve the manufacture of biopharmaceutical products. Different nutrient supplementation strategies were explored, guided by our previous work on identifying metabolic pathways recruited in the production of enveloped viral vectors.[14] To facilitate the implementation of the different supplementation strategies to clinical-grade LV production, all nutrient boosters are chemically defined and compliant with good manufacturing practices (GMP) except LipiMAX, which is not chemically defined but is available in GMP-certified versions. The experiments were performed at high cell density conditions (3×10^6 cell mL^{-1} at transfection) where medium nutrient consumption rate is higher, and the benefits of supplementation would be more likely to occur.

Lipid supplementation was found to significantly improve vector productivity in serum-free suspension cultures by up to 3-fold, when compared to non-supplemented conditions (Figure 4A). Lipids are uptaken by the cell through lipoproteins thus, lipid supplementation was performed with or without ISTE as a carrier. However, since LipiMAX is a highly purified lipid fraction of bovine serum, it already contains serum lipoproteins which may explain the beneficial effects of this supplement regardless of the presence of ISTE carrier. In previous work on retroviral vectors (RV), lipid metabolism was identified as one of the major metabolic pathways recruited in

vector production and found to play a crucial role in RV titers under serum-deprivation.[27,28] Given the similarities between lentiviruses and gammaretroviruses, LV production in serum-free suspension cultures may face similar metabolic bottlenecks. Indeed, enveloped viral vector production imposes specific metabolic requirements on cells, particularly affecting the lipid biosynthesis pathways due to vector budding from the cellular membrane. In this context, lipid supplementation may help compensate for the lipid imbalance caused by LV budding. Moreover, these previous studies were performed in HEK 293 derived cells suggesting that these supplementation strategies might also be applied to improve LV production in HEK 293, although further optimizations could be required to address metabolic challenges specific to these cells.

Lipid supplementation proved to be beneficial for FreeStyle F17 cultures but not for BalanCD HEK293 cultures, suggesting that these requirements depend on the serum-free medium formulation. It is also noteworthy that the effect of nutrient supplementation on LV productivity is conditioned by the impact of the supplements on transfection efficiency, namely, in the case of using glutathione and lipids which impaired transfection efficiency (Figure 4B). The presence of these supplements may impact DNA-PEI complexation by shifting physicochemical properties of the reaction, namely pH, or interfering with cellular uptake pathways by altering the composition or fluidity of the cell membrane. Future approaches using biophysical methods and polyplex uptake assays similar to those described in this work could shed light on specific mechanisms leading different nutrients to impact DNA-PEI complexation and cellular uptake pathways. Despite the detrimental effect of lipid supplementation on transfection efficiency (Figure 4B) this strategy significantly increased vector productivity (Figure 4A). This suggests that lipid supplementation may provide even higher LV productivity improvements in processes not relying on transient transfection, namely when using stable producer cell lines.[13] Moreover, the benefits of nutrient supplementation may have been diluted by working with cell populations with better results being expected if working with cell clones or stable producer cells, which present more defined metabolic requirements.

Batch-to-batch variability is one of the main challenges in transient LV production. Previous studies have described the effect of different physicochemical properties of DNA polyplexes on transfection variability.[11,12] However, little is known about other sources of variability, namely those related to cell physiology. In this work, high transfection efficiency variability was observed in the multiple LV production runs. This was found to be particularly exacerbated in experimental designs requiring cell concentration steps and frequently associated with production runs where, due to experimental handling conditions, cells would spend more time concentrated before resuspension. Our results confirmed that the cell concentration step and, particularly the time that cells spend concentrated, impairs transfection efficiency and consequently vector titers (Figure 5). This effect was more evident in cells cultured in FreeStyle F17 than in BalanCD HEK293 medium, and it was not observed with cells cultured in adherent and serum-containing conditions, indicating this effect to be related to the adaptation of cells to suspension cultures (Figure 5). Indeed, the adaptation of cell populations to suspension may result in the selection of cell subsets within the parental population with specific physiologic factors that were not evident before adaptation, namely, reduced tolerance to cell-to-cell proximity.[29] This may explain the susceptibility of adapted 293T cells to concentration steps in transfection protocols, although the biological mechanisms underlying this phenotype are, at this point, unknown. Considering these results, it is of major importance to standardize and optimize LV production protocols requiring cell concentration steps such as high-cell density perfusion.

The effect of cell concentration steps on transfection efficiency was further evaluated in polyplex tracking assays. DNA polyplex uptake was found to be slower in cells kept concentrated for longer periods of time (Figure 6A), resulting in lower accumulation of polyplexes, ultimately reducing the amount of intracellular DNA (Figure 6B). Also here, the molecular mechanisms underlying this phenomenon are unclear. However, if selected cells are indeed less tolerant to cell-to-cell proximity, they are more likely to activate signaling pathways to slowdown metabolism in response to contact with neighboring cells. Further studies to evaluate adapted cells physiology and signaling could be of relevance to fully understand this effect, particularly in comparison to the non-adapted parental population.

With this work we established a new cell system and optimized transient transfection protocols for scalable LV production in serum-free suspension cultures delivering titers up to 4×10^7 T.U. mL^{-1}. LV productivity was further improved by exploring nutrient supplementation strategies, providing valuable information on the metabolic requirements for LV production in serum-free suspension conditions. Additionally, we identified, for the first time, cell concentration steps as a key factor strongly affecting the variability of LV production batches in serum-free suspension cultures. These findings will help advancing the state-of-the-art of LV manufacture for gene therapy applications.

AUTHOR CONTRIBUTIONS

Conceptualization, T.A.V., A.F.R and A.S.C.; Methodology, T.A.V., A.F.R, A.S.C.; Investigation, T.A.V. and A.F.R.; Data curation, T.A.V; Formal analysis, T.A.V. and A.F.R.; Writing original draft, T.A.V and A.F.R.; Draft reviewing and editing, A.F.R. and A.S.C.; Supervision, A.F.R. and A.S.C.; Funding acquisition, A.S.C.; Project administration, A.S.C. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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