

REVIEW

Optimizing VLP production in gene therapy: Opportunities and challenges for *in silico* modeling

Leopold Zehetner^{1,2}  | Diana Szélieová²  | Barbara Kraus¹ | Michael Graninger¹ |
Jürgen Zanghellini²  | Juan A. Hernandez Bort¹ 

¹Biotherapeutics Process Development, Baxalta Innovations GmbH, a Part of Takeda Companies, Orth an der Donau, Austria

²Department of Analytical Chemistry, Faculty of Chemistry, University of Vienna, Vienna, Austria

Correspondence

Jürgen Zanghellini, Department of Analytical Chemistry, Faculty of Chemistry, University of Vienna, Vienna, Austria.
Email: juergen.zanghellini@univie.ac.at

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Abstract

Over the past decades, virus-like particle (VLP)-based gene therapy (GT) evolved as a promising approach to cure inherited diseases or cancer. Tremendous costs due to inefficient production processes remain one of the key challenges despite considerable efforts to improve titers. This review aims to link genome-scale metabolic models (GSMMs) to cell lines used for VLP synthesis for the first time. We summarize recent advances and challenges of GSMMs for Chinese hamster ovary (CHO) cells and provide an overview of potential cell lines used in GT. Although GSMMs in CHO cells led to significant improvements in growth rates and recombinant protein (RP)-production, no GSMM has been established for VLP production so far. To facilitate the generation of GSMM for these cell lines we further provide an overview of existing omics data and the highest production titers so far reported.

KEYWORDS

gene therapy, genome-scale metabolic model, HEK-293, omics, virus-like particle

1 | INTRODUCTION

Genetic disorders, such as cancer or rare genetic inherited diseases, may be caused by mutation, alteration, deletion, or differential expression of specific genes. Gene therapy (GT) is a promising approach based on the use of nucleic acids harbored in so-called vectors, which are introduced into somatic cells to correct or prevent pathological processes in patients. The most popular GT-based application might be the replacement of defective genes by others that contain the information to produce the protein of interest correctly.^[1,2] However, this emerging technology still brings some challenges during its application in patients, for example targeting with the appropriate vector the desired organ region. Therefore, numerous viral and non-viral vectors or even hybrids from both were developed or modified in the past to

resolve this aspect. Some examples of vectors may include virus-like particle (VLPs),^[3] artificial polymers,^[4] or extracellular vesicles.^[5]

VLPs form the most prominent group of vectors used in GT and can be produced by engineered host cell lines.^[3] Although production platforms for VLP expression have been established and optimized in the last years, titers and biopotency are still insufficient to meet future demands.^[6] Many efforts have been made to solve these limitations, for example, via cell culture media optimization,^[7] and cell line engineering.^[8] However, some of these efforts are time-consuming trial-and-error approaches; for instance, screening a pool of cells to pick out the best clones or using rational design approaches, like design-of-experiments (design-of-experiments (DoE)), to modify the medium composition to find the best possible medium formulation for boosting titers.

A more sophisticated methodology to shed light on the cellular black box may be based on *in silico* tools. It was already shown that

Abbreviation: CSD, computational strain design; GSMM, genome-scale metabolic model; GT, gene therapy; VLP, virus-like particle.

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media composition could be optimized by analyzing metabolic flux distributions inside host cells and subsequently applying this knowledge to reduce the accumulation of byproducts while increasing cellular productivity.^[9,10] However, additional factors may play a role in how the metabolism works; for example, it was demonstrated that some viruses could reprogram host cells' metabolism. These changes in the metabolism are even more significant than any changes triggered by the expression of recombinant protein (RPs)^[11,12] and may lead to lower production titer due to activation of self-defense pathways of host cells.^[13] This knowledge adds further complexity to elucidate the regulatory mechanisms behind VLP production.

To tackle the resulting derived limitations of low production titers, different strategies have been established in the past, including DoE,^[14] extensive passaging of host cells,^[15] hypothesis-driven genetic engineering,^[8] the implementation of alternative host cell lines,^[16] or media optimization.^[7] It was observed that extensive passaging of Per.C6 cells resulted in an evolved cell line achieving 3-fold higher adenovirus (adenovirus (AdV)) production and a doubled growth rate compared to the parental strain leading to a reduction in the number of production batches, consequently.^[15] In another approach, recombinant adeno-associated virus (recombinant adeno-associated virus (rAAV)) titers could be increased from 2.5×10^9 viral genomes (viral genomes (VG))/mL to 2.7×10^{10} VG/mL in HEK-293F1 cells by media optimization,^[7] although this is not the highest reported rAAV titer so far.^[17] However, despite this progress cost of production are still high, and VLP-based drugs remain top among the list of the most expensive therapeutics in the world.^[18] Consequently, novel strategies must be developed and implemented to increase titers and reduce costs while considering biosafety aspects and maintaining product quality.

In this review, we aim to give a condensed overview of genome-scale metabolic model (GSMM)- and omics-based bioprocess optimization with a strong focus on Chinese hamster ovary (CHO) cell lines since there have been no GSMMs established for cell lines used in GT so far. Nevertheless, similar approaches may be used for these cell lines. In the second part, we summarize cell lines and VLPs, most utilized in the industry for GT and optimization strategies as visualized in Figure 1.

2 | APPROACHES FOR PROCESS OPTIMIZATION USING CONSTRAINT-BASED MODELING

In the last two decades, systems biotechnological approaches have significantly altered the way industrial bioprocesses are designed and operated^[19,20]—at least for microbial systems. A key method to better understand upstream processes is constraint-based modeling (constraint-based modeling (CBM)), which allows for analyzing intracellular metabolic processes. At its heart sits a GSMM, which is a mathematical representation of a cell's metabolism and captures the stoichiometry of all metabolic transformation processes. Since the first GSMM was developed in 1999 for *Haemophilus influenzae*^[21] a large number of metabolic models has been developed and successfully used.^[22] For instance, GSMMs have been used to overcome various

challenges in bioprocess optimization, including media optimization^[23] or engineering target identification.^[19]

Detailed protocols to reconstruct GSMMs from scratch,^[24] supported by several automatic reconstruction pipelines, are available^[25] resulting in several hundred GSMMs, which were reviewed in a recent GSMM-specific phylogenetic study.^[22] Moreover, community-driven, highly manually curated global GSMMs for a few model organisms are currently available. These models include: *Homo sapiens*,^[26,27] *Cricetulus griseus*,^[28] *Mus musculus*, *Drosophila melanogaster*, *Danio rerio*, *Saccharomyces cerevisiae*,^[29] or *Escherichia coli*^[30], among others. Such global models allow cell- or tissue-specific reconstructions based on transcriptomes and metabolic exchange rates. However, the *de novo* reconstruction of global GSMMs for mammalian cells remains challenging and needs extensive manual curation to avoid incorrect transport reactions between organelles, or futile cycles, for example.^[31]

Compared to global reconstructions, cell line-specific GSMMs allow for more accurate predictions^[32] as their construction typically relies on the integration of cell line-specific transcriptomic and fluxomic data. Various algorithms for generating strain, tissue, or cell line-specific models exist.^[33–39] However, certain issues remain, including the selection of transcriptomic thresholds^[40] and the order of constraint introduction.^[41–43] Thus, consensus pipelines remain to be established.

The use of cell line specific GSMMs for studying bioprocesses has been increasing over the last years. However, accurate dry weight measurements are often ignored in these models^[44,45], despite solid evidence that time-resolved extracellular metabolome quantification^[26–28] combined with dry weight determination of biomass to calculate exchange fluxes^[46–49] improves cell line specific models even further. Most often, the measured exometabolome is limited to metabolites with well-established quantification protocols, like amino acids, glucose, lactate, and ammonia. With recent whole metabolome quantification approaches^[50], more exchange fluxes might be available for integration in the future.

In general, bioprocesses can be optimized by improving growth rate or product formation. These cell-specific phenotypes can be assessed by flux balance analysis (flux balance analysis (FBA)), which is based on the stoichiometric matrix S , including metabolites as rows, reactions as columns, and the corresponding fluxes as a vector v being constrained by lower (l) and upper (u) boundaries. Under a steady-state assumption of the system, so that influx and efflux of metabolites are in equilibrium:

$$Sv = 0 \quad (1a)$$

$$l \leq v \leq u \quad (1b)$$

The higher number of reactions compared to the number of metabolites in large networks results in a solution space with various possible solutions. Therefore, optimizing for a specific reaction, most often an artificial biomass reaction or a product-related reaction, leads to a specific solution.^[51] GSMMs have been used as platforms to

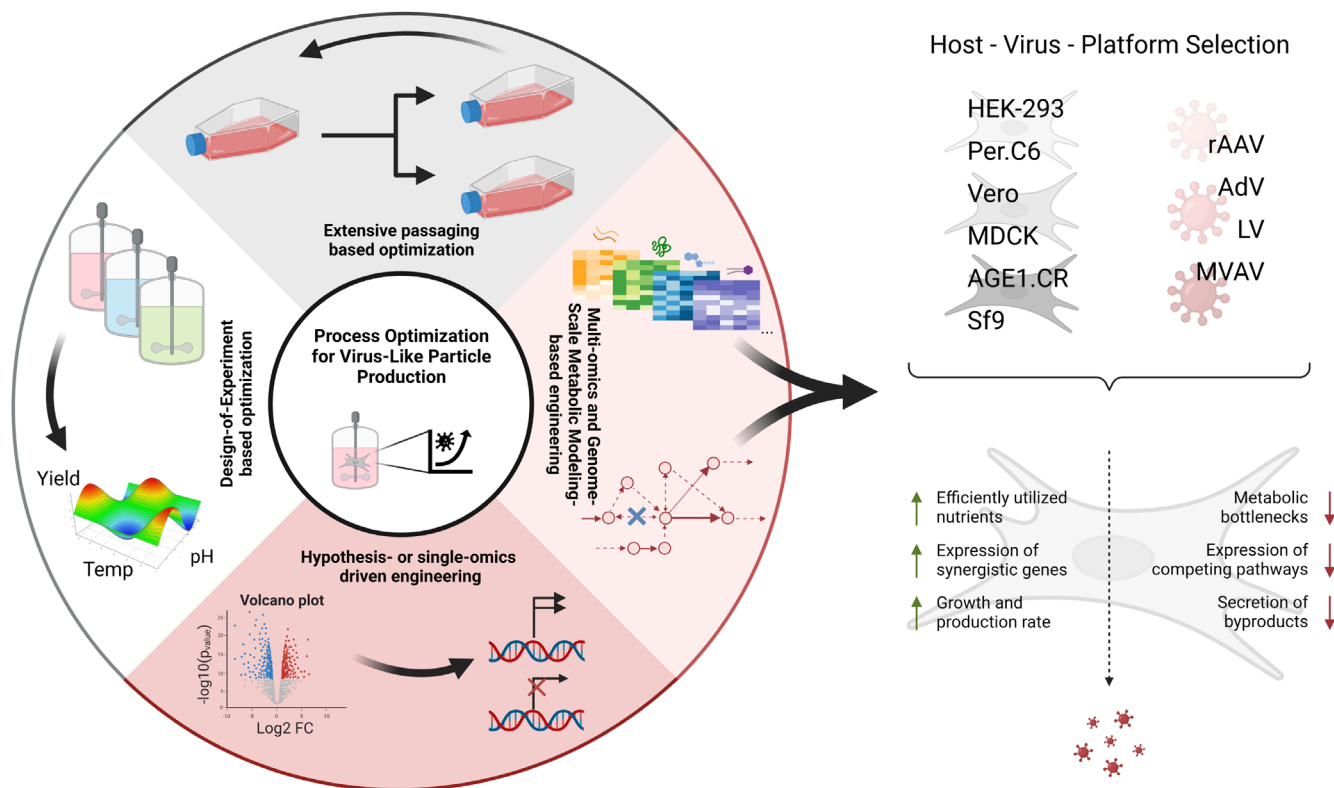


FIGURE 1 Overview of current strategies for bioprocess optimization and host-virus platforms in gene therapy. Implementing genome-scale metabolic models for these cell lines based on multi-omics data may result in higher titers.

integrate experimentally generated multi-omics data and run simulations to identify metabolic bottlenecks under different conditions.^[28] The most common constraints are metabolite exchange rates, calculated from concentration changes of extracellular metabolites.^[46] Although FBA is still the most common approach for optimization of objective functions, it has been shown that FBA predicts growth rates in linear dependence on uptake rates without considering overflow metabolism.^[52,53] Consequently, alternative strategies have been developed to reproduce more accurate growth rates, such as hybrid-FBA,^[54] or enzyme-constrained FBA.^[52] For example, the overestimation of growth rates could be decreased from approx. 4-fold to 1.05-fold by applying hybrid-FBA.^[54]

Alternative or additional constraints may be introduced to obtain more reliable simulation results. Several types of constraints can be used, including enzyme constraints,^[55] thermodynamic constraints,^[56] regulatory constraints^[57] or hybrid versions thereof.^[53,58] However, all constraining methods include certain disadvantages, which need to be considered when integrating them. Common types of CBM were summarized in an extensive review, discussing available tools, applications, and challenges in microbial cell factory design.^[59]

In particular, enzyme-constrained genome-scale metabolic model (enzyme-constrained genome-scale metabolic models (ecGSMMs) s) have been shown to improve accuracy tremendously and allow condition-specific constraining of models since they rely on integrating quantitative proteomics measurements into models. Recently,

software tools to introduce enzyme-constraints^[55,60,61] have been published following the general equation: $v_i \leq k_{cat,i} \cdot [E_i]$ where v_i is the flux of a certain reaction i , catalyzed by an enzyme E_i , $k_{cat,i}$ is the corresponding turnover number of the enzyme, obtained from databases, such as BRENDA^[62] and $[E_i]$ is the concentration of enzyme(s) catalyzing the reaction.

Despite valuable insights and promising results from ecGSMMs, the lack of catalytic values of enzymes in databases limit the accuracy of these models. Several approaches have been developed to predict or improve the estimates of turnover numbers of enzymes. These include prediction of turnover numbers based on quantitative proteomics and fluxomics data under different conditions,^[63] machine learning^[64] and deep learning approaches,^[65] and an approach based on metabolic control analysis to improve the turnover number estimates.^[66] Until now, no comparative study has been published to determine which approach results in the most accurate ecGSMMs.

Recently, a new modeling framework – resource balance analysis (resource balance analysis (RBA)) – has been gaining popularity. In addition to a metabolic network, RBA-based models contain reactions for the synthesis of molecular machines (e.g., enzymes, ribosomes) and predict metabolic fluxes as well as the concentrations of the corresponding catalysts.^[67] RBA-based models can be used to study resource reallocation in different media or after knock-out or differential expression and may lead to more realistic strain or medium designs in the future. Recently, a tool has been published that automatically builds RBA-based models from GSMMs.^[68]

To efficiently design strains and predict engineering targets *in silico*, numerous tools for computational strain design (computational strain design (CSD)) have been developed as reviewed recently,^[69] and condensed in workbenches.^[70,71] CSD tools have been successfully applied to improve titers in microbial cell factories, such as *S. roseosporus*; three genes were predicted in the GSMM to have synergistic effects on daptomycin production and led to a titer improvement of 43.2%.^[72] However, CSD tools have not been applied to mammalian cell lines so far.^[69]

Numerous examples of successful bioprocess optimization using GSMMs are available. However, they typically focus on producing small molecules in well-studied microbial cells, such as *E. coli*^[20] or *S. cerevisiae*,^[19] which have less complex genomes or metabolic networks, and a higher amount of annotated genes than mammalian or insect cells. Moreover, due to the structural simplicity of small molecules, their production is less challenging than the production, secretion, and purification of RPs or VLPs. Additionally, host cell metabolism is influenced to a higher degree when synthesizing complex products compared to small molecules.

Surprisingly, GSMM of human tissues have been successfully used in disease modeling,^[73] and drug target discovery,^[74] whereas GSMMs of human cell lines are currently not used in bioprocess development. Except for small-scale models utilized for metabolic flux analysis (metabolic flux analysis (MFA)),^[47] there are currently no GSMMs available for any cell line used for VLP production, even though highly curated reference models are available for certain species.^[26,27] This is certainly a missed opportunity to optimize manufacturing in GT. Due to the lack of studies, we focus on the most prominently studied mammalian cell line in industry, CHO, even though production in these cells is limited to RPs so far and not to GT products.

2.1 | CHO models as opportunity to reconstruct mammalian cell models

Recently, comprehensive reviews summarized the efforts to optimize CHO-based bioprocesses utilizing *in silico* tools.^[75,76] Therefore, we focus only on recent approaches to improve these bioprocesses.

The generation of a community-driven reference model for CHO cells,^[28] iCHO1766, formed the basis for numerous studies, primarily focusing on optimizing media composition under batch,^[10] fed-batch,^[77] and continuous culturing conditions,^[9] as well as for monoclonal antibody (monoclonal antibody (mAB)) production improvements,^[78,79] and by integrating time-resolved transcriptomics data.^[80]

For example, to optimize the medium composition, Huang et al.^[23] integrated metabolite exchange rates collected during a fed-batch process into iCHO1766 and validated predicted fluxes with transcriptomics data. Based on the knowledge generated during their simulations, the amino acid concentration of leucine and valine was dramatically increased in the media leading to a production increase of 11.8%. Although their model was not specifically tailored to the cell

line clone (neither cell-specific model nor cell-specific biomass composition), it was a promising example of how GSMMs facilitated process optimization and avoided time-consuming DoEs. Even though artificial biomass reactions are commonly used for growth simulation as an objective function,^[46] this concept might be questionable: Schinn et al.^[81] showed much more accurate growth predictions when using reactive oxygen species (reactive oxygen species (ROS)) synthesis or mitochondrial reduced nicotinamide-adenine dinucleotide (reduced nicotinamide-adenine dinucleotide (NADH)) regeneration as objective function for example. This aspect needs to be studied in more detail since selecting an appropriate objective function is one of the most fundamental pillars of the simulation of GSMMs.

Apart from metabolism, protein secretion machinery is an additional bottleneck for producing RPs in CHO cells. Hence, Gutierrez et al.^[82] coupled a model of a secretory pathway to a CHO-GSMM and estimated the energy cost of secretion in terms of adenosine triphosphate (adenosine triphosphate (ATP)) consumption. Furthermore, the model was verified by replicating experimental data of mAB titer improvement by knockdown of the selection marker gene *neoR*. In contrast, Morrissey et al. identified that secretion of proteins has no significant impact on the energy metabolism in CHO-GSMMs.^[32] However, Kol et al., showed experimentally that mAB titers, purity, and growth rates increased by knocking out sets of secreted host cell proteins in different CHO clones.^[83] The most recent version of CHO-GSMM^[32] was generated by combining previous models. By comparing the updated global CHO-GSMM, two cell line specific CHO-GSMMs and a recently published reduced CHO-model,^[84] it was shown that the reduced models predict core metabolic functions more precisely, whereas essential gene identification is more accurate in the cell line specific models.^[32] Despite the comprehensive comparison, the impact of selecting the most suitable objective function for phenotype prediction remains to be investigated.

Currently, only one ecGSMM for CHO cells has been published based on iCHO2296, which resulted in more accurate prediction of overflow metabolism.^[52] Additionally, lactate consumption and utilization as energy source after entering the tricarboxylic acid cycle (tricarboxylic acid cycle (TCA)) were identified,^[52] as already examined for HEK-293 cells.^[49] Despite the availability of various updates^[32,52,82] and omics datasets available for different CHO cell lines,^[28,85] CSD-tools have so far not been applied to CHO-GSMMs, which limits improvement strategies.

Besides *in silico* strain optimization, GSMMs may be a cornerstone in the next decade as part of the digitalization, and computational optimization of bioprocesses as described for HEK-293 cells during rAAV production^[86] and for CHO cells.^[87] To realize the full potential of digital tools for process control and monitoring, robust biomarkers for cellular states throughout the process need to be identified. Established concepts for biomarker identification for diagnosis can be adapted for bioprocesses based on multi-omics data.^[88] For example, comprehensive quantitative metabolomics analyses, as performed for CHO^[45] and HEK-293 cells,^[44] could form starting points to more efficient and sustainable bioprocesses.

2.2 | Specific difficulties in reconstructing VLP-producing cell lines

After GSMM reconstruction, the target production reactions need to be added to the model. While the addition of biosynthesis pathways for small molecules is usually straightforward, there is currently no standardized way to add VLP production into a GSMM. Several approaches are possible, depending on the desired level of detail.

The simplest approach is to add an artificial VLP production reaction with an average composition, similar to a biomass reaction. While for some VLPs the main components are proteins and nucleic acids (rAAV, AdV), for VLPs with a membrane (lentivirus [LV], modified vaccinia ankara virus [MvaV]), lipids need to be included as well. The average composition of nucleic acids can be calculated from the genome sequence and the protein composition from the sequences and stoichiometries of the capsid proteins. However, the lipid composition needs to be measured as it was shown that viruses can reprogram the host cell's lipid metabolism and may have a different membrane composition than the host.^[89,90]

We can also model the VLP production in more detail and add separate reactions for the expression, assembly, modifications and secretion of the VLPs (similarly to the mammalian secretory model^[82]). This would allow the integration of enzyme constraints for each reaction (i.e., kinetic constants and enzyme concentrations). If available, kinetic models can be used to obtain the necessary parameters. For example, Nguyen et al.^[6] generated a mechanistic model for rAAV 5 production in HEK-293 cells consisting of 14 reactions, and estimated kinetic parameters using data from literature and their transient transfection experiments. However, mechanistic models still need to be developed for other viruses and host cells. A detailed overview of the biology of commonly used viruses in GT was recently published by Bulcha et al.^[3] which can serve as a starting point for model construction. However, additional experiments are needed to obtain the necessary parameters (plasmid uptake rates, VLP production rates etc.)

3 | POTENTIAL CELL LINES USED IN GENE THERAPY SUITABLE FOR FUTURE GSMMs

Numerous cell lines have been screened for VLP production as alternative production platforms with improved biosafety profiles.^[16] However, only a few are currently used in industrial processes for producing the most commonly used VLPs, rAAVs, AdVs, LVs^[3], or MvaV.^[91,92] Therefore, only selected cell lines, listed in Table 1, are discussed in the following sections.

3.1 | Advantages of generating cell line-specific genomes

While reference genomes are available for most cell lines listed in Table 1, generating cell line specific genomes and other omics datasets

is needed to tackle common challenges in VLP production. Long-term passaging or cross-contaminations may lead to wrongly packaged VLPs or decrease biosafety profiles.^[93,94] Furthermore, stably integrated viral genes necessary for VLP expression may lose their function after numerous passaging steps which reduces VLP titers.^[95] These obstacles can be caused by changes in the genome, but could be detected by *de novo* sequencing of the production cell lines. For example, *de novo* sequencing of three Vero genomes led to the discovery of 60 potentially intact retroviral genes, which were not identified in a reference genome.^[95] Although these genes have not been characterized as harmful to humans, they comprise potential tumorigenicity.^[96] Furthermore, epigenomic changes in cell lines have been shown to occur after a certain number of passages, which might be responsible for altered metabolism and production profiles over time. With the invention of third-generation sequencing platforms, it is possible to detect these changes over time and to guarantee the long-term reproducibility of bioprocesses.^[97]

Other than detecting the challenges described above, *de novo* sequencing can provide new information that might be useful for genetic engineering. For example, assembled genome can be used to predict unannotated genes' functions. In a recent study, the concept of identifying gene functions is described in detail by combining different databases. The identification of novel enzymes allows the expansion of metabolic networks, which is especially important for mammalian cell lines, and offers novel targets for genetic engineering.^[98] Additionally, updated genomes allow more reliable genetic engineering through more accurate primer design and avoiding incorrect cleavages.

Finally, cell line-specific genomes (and transcriptomes) can be used to reconstruct cell line-specific GSMMs. These form a basis for more accurate predictions of bioprocess and cell line engineering strategies, as discussed in the previous section. Currently, no GSMMs are available for the cell lines in Table 1. However, several small-scale metabolic models for MFA have been generated and used for process optimization.^[48,99]

3.2 | HEK-293

HEK-293 cells and derived clones are the workhorses for producing VLPs in GT. Several strains have been optimized to grow in suspension and under serum-free conditions, the most appropriate conditions in industrial bioprocesses. To achieve these settings, adherent cell lines have been adapted through numerous passaging steps by reducing the presence of serum by each passage^[115] and optimized for the production of rAAV^[116,117] or RPs which are difficult to express in CHO cells.^[118] However, the highest rAAV-titers in HEK-293 cells have been reported using adherently grown HEK-293H in fetal bovine serum (FBS)-containing media,^[17] which is not the best choice for manufactured products as recommended by the health authorities due to potential presence of prions or any other compounds, and complicates scale up. Additionally, these cell lines often lack a deep metabolic investigation.

TABLE 1 Selected cell lines utilized for VLP production in industrial bioprocesses. Currently highest titers reported in literature are presented, as well as genomes, reference-GSMMs, and omics-based optimization approaches for VLP production.

Cell line	Genome	Reference-GSMM	VLP	Titer (VG/mL)	Omics Data
HEK-293	PRJNA565658, ^[100] PRJEB3209 ^[101]	Human-Gem, ^[26] Recon3D ^[27]	rAAV	10 ¹⁵ ^[17]	Proteome ^[102]
			AdV	2 × 10 ¹¹ ^[103]	–
			LV	4.5 × 10 ⁹ ^[104]	Proteome ^[105]
PER.C6	–	Human-Gem ^[26] , Recon3D ^[27]	AdV	10 ⁵ VG/cell ^[106]	–
Vero	PRJNA847024, ^[107] PRJDB2865 ^[95]	–	AdV	(not quantified) ^[108]	–
			rAAV	75,000 VG/cell ^[109]	–
MDCK	PRJNA905218 ^[110]	–	AdV	2.1 × 10 ⁸ ip/mL ^[111]	–
AGE1.CR.pIX	PRJNA669953	–	MvaV	2.5 × 10 ¹⁰ TCID50/1/day ^[91]	–
Sf9	PRJNA380964 ^[112]	–	rAAV	2.4 × 10 ¹² ^[113]	Proteome ^[114]

Abbreviations: AdV, adenovirus; GSMM, genome-scale metabolic model; LV, lentivirus; MvaV, modified vaccinia ankara virus; rAAV, recombinant adeno-associated virus; VG, viral genome; VLP, virus-like particle.

3.2.1 | Growth optimization of HEK-293 cells

This issue may be addressed by investigating the cellular behavior under various conditions using omics experiments to allow the identification of engineering targets for faster optimization and metabolic analyses.^[100,101] Recently, a differential analysis of three adherent strains, including HEK-293E and HEK-293T, and two suspended strains, HEK-293H, and HEK-293F, was performed based on transcriptomic and genomic data. After pairwise comparison, five potential target genes were identified to be consistently upregulated in adherent cell lines, with all involved in cellular adhesion, and DHRS3 and LOX among them.^[100] Since adherently grown cell lines complicate scale up, dispensable transmembrane proteins, such as DHRS3, have become promising engineering targets. Although DHRS3 consists of fewer amino acid (amino acids (AAs) s) (302), fewer transmembrane domain (4), and can be expected to be expressed only in small amounts than other transmembrane proteins, such as the monosaccharide transporter GLUT1 (492 AA, 12 TMD),^[119] it should be considered additionally, that DHRS3 consumes NADPH,^[120] which decreases reduction potential for metabolic redox reactions and for maintaining a reducing environment.^[121] Though this is just exemplary, and the influence of one enzyme may be negligible, metabolic profiling and how resources are allocated and utilized by cells is one of the most important aspects during both early process development and optimization attempts of established processes.

Therefore, a model for HEK-293SF-3FS cells was established and used to analyze the formation of lactate based on 13C-labeled glucose and glutamine substrates during exponential growth.^[122] Interestingly, the proportion of lactate generated from glutamine is negligible, whereas 77% of glucose is catabolized to lactate and 15% of glucose is metabolized in the pentose phosphate pathway. The main problem arises from the amount of lactate reintroduced (only 22%) into the TCA cycle as substrate.^[123] Based on these findings, Dietmair et al.^[124] examined the influence of RP production in HEK-293F cells using time-resolved transcriptome, metabolome, and fluxome data. Despite

similar growth rates of producing and non-producing clones, glucose consumption and lactate production were 20% higher in non-producer cells, whereas AA transporters were upregulated in producer cells. Supported by differential expression analysis, the authors concluded that protein assembly and -folding might be bottlenecks during RP production instead of metabolic limitations. Nevertheless, a more efficient strategy to utilize lactate as a carbon source remained unresolved until recently: Martinez-Monge et al.^[49] investigated the influence of pH-controlled and -uncontrolled growth phases based on metabolic changes of HEK-293 cells. Besides the two main growth phases, consuming glucose first, followed by lactate consumption, a third phase was observed where glucose and lactate were co-consumed under non-pH-controlled conditions. During this phase, a 3-fold increase in the growth rate was recorded. Despite these promising results, pH-control of good manufacturing practice (good manufacturing practice (GMP))-compliant bioprocesses is one of the cornerstones, especially with a focus on batch-to-batch consistency and reproducibility.

Furthermore, it must be considered that engineering metabolic enzymes is often more challenging. On the other hand, it is still difficult and laborious to diminish gene expression or enzymatic activity, though possible.^[125] One opportunity to expand the number of potential metabolic engineering targets is the implementation of HEK-specific GSMMs. Surprisingly, the most recent and most extensive model for HEK-293 cells was curated by manual reduction of the Recon2.0,^[126] and consists of only 357 reactions.^[47] Although reduced models were recently shown to capture core metabolic functions more precisely,^[32] the number of potential targets for engineering is limited.

3.2.2 | Adeno-associated virus production in HEK-293 cells

Recently, reviews have been published describing the production cycles of rAAVs, AdVs, and LVs in general,^[3] and in HEK-293 cells, specifically.^[8]

rAAV has been one of the most prominent vectors for gene delivery in recent decades. There is a large number of studies that describe various strategies to increase productivity. Zhao et al.^[14] chose a DoE approach to identify the best parameter setting during the production phase in HEK-293T cells for multiple serotypes, and lead to a maximum of $> 10^{14}$ VG/mL. However, DoE-approaches are often laborious and are limited to specific process parameters. In contrast, the identification of engineering targets is so far based on only one or a few omics datasets under a limited number of conditions.^[8] Nevertheless, recently published studies based on omics techniques could form a starting point for omics-based process optimization on a cellular level.

Strasser et al.^[102] used FreeStyle 293-F as host cells for rAAV 5 production and examined the metabolic differences based on proteomics data. The pathway analysis revealed changes in endosomal-lysosomal protein levels, translation machinery, and energy metabolism between transfected and not-transfected cells. Based on their investigation, chloroquinone was added to alter endocytosis, which led to a titer improvement of 35%. In addition, by generating double knockouts of STAT1, and BAX genes, which are involved in the immune response to viral infection, titers for rAAV production could be increased by 80% compared to a wild-type strain.^[13] Despite different study designs, both omics-data-based and hypothesis-driven approaches significantly improve rAAV titers. However, these studies only report improvements based on a limited number of genetic targets and neglect computational optimization approaches.

In contrast, Nguyen et al.^[6] predicted improvements of rAAV 5 production by temporally staggering transfection of the plasmids due to different expressions of the corresponding viral genes. The established mechanistic model of plasmid uptake and viral particle production allows fundamental insights into time-staggered bottlenecks of rAAV production in HEK-293 cells. Though these results remain to be verified, it could potentially overcome the premature depletion of capsid proteins.^[127,128] Furthermore, secretion rates of rAAVs remain dependent on serotype. Certain rAAVs interact with host cell proteins and are not secreted, which complicates purification and bears the risk of potential contaminations.^[129] However, this issue needs to be addressed by alterations on a molecular level of viral protein since the cellular interaction proteins are essential for host cells.

3.2.3 | Adenovirus production in HEK-293 cells

Although AdV has been extensively used for gene delivery in GT, or vaccination,^[103] only a few studies investigated the production process in HEK-293 cells: Negrete et al.^[130] adapted a HEK-293 substrain to grow in suspension and compared adenoviral vector titers in dependency of different multiplicity of infection (multiplicity of infection (MOI)), with a maximum productivity of 3×10^{10} plaqueforming units (PFU/mL) at a MOI of 10. Joe et al.^[103] further decreased the MOI, resulting in 2×10^{11} VG/mL at a 1000 L scale for the production of an AdV-based Covid-19 vaccine. Using a DoE approach, Chen et al.^[131] could identify optimized parameters for the production of AdV in a perfusion reactor.

A model for MFA studies, covering 40 reactions, was generated by Henry et al.^[48] based on 293SF-3F6 cells. By comparing six different infection states, including different cell densities and perfusion rates, it was possible to identify the best settings for ATP and AdV production. To the best of our knowledge, there are currently no studies describing process optimization for AdV production in HEK-293 cells based on omics data. However, since AdVs are reliable and safe vectors for gene delivery and were used as vaccines during the recent Covid-19 pandemic, more effort should be put into optimizing these bioprocesses.

3.2.4 | Retrovirus production in HEK-293 cells

Retrovirus-like particles are of particular interest in GT due to their ability to stably integrate target genes into patients' genomes. However, these vectors can potentially cause tumors, as was shown in several clinical trials, using MvaV.^[132] Therefore, despite their limited application in vivo, these vectors are commonly used for ex vivo GT applications, such as the generation of CAR-T cells.^[133] Besides gamma-retrovirus (gamma-retrovirus (gRV)), LVs became more important during the last decade, particularly HIV-1-based vectors.^[134]

LV production processes and optimization strategies have been established using transient transfection,^[135] stable expression,^[104,136] flow electroporation,^[137] various media supplements,^[138] or DoE approaches^[139] to reach maximum titers of 4.5×10^9 VG/mL.^[104] An important milestone was set by Lavado-Garcia et al.^[105] by investigating metabolic changes between transfected, mock-transfected, and non-transfected HEK-293SF-3F6 cells in suspension during HIV-1 production based on quantitative, labeled time-resolved proteomes. Besides changes caused by virus production, cell density effects were examined to find the optimal cell density for transfection. By considering both growth and production phases, it was possible to identify more engineering targets to overcome bottlenecks such as cell density effects. Based on these data transfection efficiency was increased by overexpression of UGCG by 17%, and VLP production was raised 3.3-fold by overexpressing NEDD4L, which is involved in endosomal sorting.^[140] Nevertheless, it would have been possible to generate time-resolved ecGSMMs for each state by adding measurements for usually measured metabolites for bioprocess control, such as AA, glucose, lactate, and comparatively inexpensive ammonia. Combining proteomics and fluxomics data would allow the prediction of catalytic rates of enzymes under different conditions.^[63]

3.3 | Per.C6

An adherent Per.C6 cell line, derived from human fetal retinoblasts, was adapted to grow in suspension and under serum-free conditions. Additionally, the adenoviral E1-gene was inserted to produce E1-deficient recombinant AdV-like particles.^[141] Metabolic behavior of Per.C6 cells was studied after infection with AdVs and media exchange,^[142] following numerous passages of the original cell line,^[15]

under various pH conditions,^[143] and under low levels of glucose and glutamine.^[144] Based on these studies, the authors identified a pH of 7.3 as the optimum for AdV production in Per.C6 cells. Additionally, they found that cell growth was reduced after media exchange and production decreased. In the same study, the authors described the increased consumption of glucose, oxygen, and certain aliphatic AAs.

In contrast to other producing cells, such as HEK-293 cells, Per.C6 cells produce high titers at different cell densities, although the cell-specific productivity is lower. Based on that, AdV production could be increased by adapting the cell line to suspension culture. In contrast to the original cell line, the high-producer cells grew after infection with AdVs, which increased the available amount of cells for infection.^[15] Currently, the highest titer of AdV particles in Per.C6 cells was reported by Vellinga, et al.^[106] Despite the availability of numerous exchange rate data under different conditions, no metabolic network or omics-data-based optimization studies for Per.C6 cells have been published. In recent years, the platform was used to produce an AdV-based Covid-19 vaccine. Besides AdV production, Per.C6 cells were used to manufacture poliovirus-based vaccines.^[145] Although these candidates have not yet been used for GT, they offer potential alternatives for future gene delivery vectors.

3.4 | Vero

Vero cells are derived from *C. sabaeus* and have been used as a model to investigate metabolic influence after viral infection and as production hosts for antigen-presenting VLPs, or deliver genes, as recently summarized.^[146] Despite lower growth rates and cell densities in manufacturing processes compared to other cell lines, Vero cells can be considered for high-yield viral vector production.

Recently, the genomes of substrains were sequenced, assembled, and annotated^[95,107] as a basis for strain design. Furthermore, differences between adherent and suspended Vero cells on transcriptome level were investigated and revealed differentially expressed pathways as potential engineering targets.^[147] As in HEK-293 and MDCK cells, knocking out STAT1 increased titers of Dengue 2 virus vaccines 30-fold.^[13] Metabolic analyses during cell culture processes lead to the identification of essential metabolites for growth,^[148] under various conditions, including batch,^[149] or fed-batch.^[150] In the latter study, three kinetic models were established for flux analysis by measuring specific uptake and secretion rates to enable control strategies for fed-batch processes. However, further omics-based, time-resolved studies would allow more detailed analyses and may address problems such as low cell densities. Although Vero cells are mainly used for antigen-presenting VLPs, they can be used as a production platform of various VLPs for gene delivery, including AdVs,^[108] rAAVs,^[109] or vesicular stomatitis virus^[151] for example.

3.5 | MDCK

MDCK cells, derived from kidney cells from *C. familiaris*, were extracted and adapted for cultivation.^[152] By integrating the human SIAT7E

gene, the cell line could be converted to grow in suspension and under serum-free conditions for influenza vaccine production.^[153] Alterations between adherent and suspension cultures were investigated based on label-free proteomes and metabolomes in combination with enzyme activity assays.^[154] The generated data were integrated into a MFA model, which was generated based on different media compositions.^[155] Besides differentially expressed intracellular proteins, alterations in catalytic activities were observed for enzymes, including Pirin, CAV1, or AMPK, which are known to influence cellular metabolism directly. Furthermore, glucose uptake rate and lactate secretion rate decreased by 20% and 40%, respectively, after adaptation to suspension. Consequently, growth rates in suspension culture were reduced by up to 30%.^[154] A proteome-based comparative study of adherent and suspension cell lines revealed differently expressed proteins involved in intercellular adhesion, junction, and adhesion to the extracellular matrix.^[156] These data may form the basis for enzyme-constrained MDCK cell modeling on genome scale as soon as a reference model is available. However, experimental biomass composition should be measured additionally to improve predictions.

Besides metabolic flux profiling during growth and influenza production,^[157] MDCK cells are mainly used for AdV production, such as canine AdV vectors type 2.^[111,158] Carinhas et al.^[159] performed a label-based metabolic flux analysis to observe changes in E1-transformed MDCK cells during growth, and production phases, by comparing infected and mock-infected cells. After infection with AdV, central metabolic fluxes were increased. Based on that, several strategies for titer improvement were suggested, such as supplementation of lipids, cholesterol, or nucleic acid precursors. However, a more extensive set of engineering targets could be identified by measuring changes in transcriptome or proteome levels.

Currently, there is a need for GSMM established for MDCK cells, and genome, transcriptome, or proteome-based studies investigating VLP production for GT. However, Tao et al.^[160] compared whole transcriptomic profiles after infection with different influenza strains, but not with a focus on productivity, and Capellini et al.^[161] investigated differential expression of 41 genes by mimicking bacterial infection, and cadmium exposure. Although the cell line-specific genome has not been sequenced so far, a reference genome from the original species is available and could serve as a starting point for developing a GSMM.^[110] However, the scientific community should consider closing these knowledge gaps further to increase the production of influenza and AdV vectors.

3.6 | AGE1.CR.pIX

AGE1.CR cells are derived from the fetal retina of *C. moschata*. The cell line was adapted to grow in suspension and to produce different VLPs, including MvaV.^[92] After adaption to suspension growth under serum-free conditions, a substrain was developed by integrating the adenoviral *pIX* gene to increase the capsid stability during production.^[162] In a detailed study, these two cell lines were ana-

lyzed regarding growth, metabolism, and VLP production from the same research group.^[163] Lohr et al.^[164] observed that these clones behave the same way as other mammalian cell lines regarding the Warburg effect. However, AGE1.CR cell lines showed a higher independency from glutamine compared to mammalian cell lines, which was also confirmed by MFA simulation. In recent years, these cell lines were optimized for high-density cultivation, using perfusion or continuous processes for MvaV production.^[91,92,165,166] However, neither genomes nor transcriptomes or proteomes are currently published to our knowledge to generate a cell line specific GSMM. Nevertheless, a sequenced genome of *C. moschata* was provided, which may serve as a starting point to generate a reference GSMM (GenBank Assembly Accession: GCA_018104995.1).

3.7 | Sf9

Sf9 cell lines, derived from ovarian cells of *S. frugiperda*, have gained much attention during the last century due to the well-established baculovirus-system for stable and high-yield production of RPs and VLPs.^[167,168] The cell line was adapted to grow in suspension culture, under serum-free conditions, and on a large scale.^[169] Recently, the genome was sequenced and annotated.^[112] Quantitative proteomics studies of Sf9 cells during growth and 6 hours post-infection with baculoviruses shed light on cellular behavior during growth and early-stage infection. Differential expression analysis revealed lower expression of viral proteins at higher cell densities and differentially expressed enzymes involved in metabolism, translation, protein processing, and signaling.^[114] Currently, the highest titer of 2.4×10^{12} VG/mL rAAV particles produced in Sf9 cells was reported, in addition to a decreased contamination rate with baculovirus genes.^[113]

Moreover, the metabolic potential was examined during growth,^[170] and production of VLPs.^[171] Both studies compared the metabolic behavior of Sf9 cells to other insect-derived cell lines. They mentioned a lower lactate and ammonia production and a higher VLP production in Sf9 cells. For further specification and simulation, a small-scale metabolic model for MFA analyses was developed, containing 51 reactions.^[99]

One of the most critical issues when using different platforms for VLP production was reported in different studies recently.^[172,173] By comparing rAAV production in HEK-293 and Sf9 cells, it was identified that posttranslational modifications of capsids and methylation patterns of VGs differed between the two platforms.^[172] Consequently, the change of production platform should be done carefully since influences in biopotency and safety profiles may be likely due to these changes.

Most of the mentioned studies end with suggestions for genetic engineering but need to verify their findings. Furthermore, there is currently no GSMM established, despite transcriptomes and proteomes available. Combined with the sequenced genome, these resources may form a sufficient starting point to generate a draft-GSMM.

4 | CONCLUSION

VLP-based GT emerged as one of the most promising approaches to cure genetic diseases. However, numerous challenges remain, even for FDA-approved drugs, including safety profiles, or titers. Despite the availability of high-throughput techniques to generate multi-omics datasets of host cells, optimization approaches still rely mainly on long-term passaging,^[15] hypothesis-driven genetic engineering,^[8] or DoE.^[14] In recent years, a few studies initiated omics-based strain design approaches for VLP production in GT.^[17,102] Currently, HEK-293 cell lines are the most commonly used platforms for VLP production, though other cell lines surpass HEK-293 cells in growth rates,^[15] auxotrophy,^[164] or productivity.^[106] In turn, available global GSMMs^[26,27] of human cells are advantageous for CSD approaches, while GSMMs need to be established first for non-human cell lines used in GT. The lack of global GSMM reconstructions for these cell lines is surprising, even though tremendous advances based on GSMMs have been reported in disease modeling^[73] or bioprocess optimization.^[23] Nevertheless, numerous challenges must be addressed when implementing GSMMs for these cell lines, including experimental and computational approaches.

AUTHOR CONTRIBUTIONS

Leopold Zehetner: Conceptualization-equal, formal analysis-equal, investigation-equal, visualization-equal, writing-original draft-equal, writing-review & editing-equal. Diana Széliová: Conceptualization-equal, investigation-equal, visualization-equal, writing-review & editing-equal. Barbara Kraus: Writing-review & editing-equal. Michael Graninger: Writing-review & editing-equal. Jürgen Zanghellini: Conceptualization-equal, supervision-equal, writing-review & editing-equal. Juan Antonio Hernandez Bort: Conceptualization-equal, funding acquisition-equal, project administration-equal, supervision-equal, writing-review & editing-equal.

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CONFLICT OF INTEREST STATEMENT

The author declares no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable-no new data generated.

ORCID

Leopold Zehetner  <https://orcid.org/0009-0003-2677-3685>

Diana Széliová  <https://orcid.org/0000-0002-9885-9758>

Jürgen Zanghellini  <https://orcid.org/0000-0002-1964-2455>

Juan A. Hernandez Bort  <https://orcid.org/0000-0003-2348-4864>

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