NOVEL APPROACHES TO RENDER STABLE PRODUCER CELL LINES VIABLE FOR THE COMMERCIAL MANUFACTURING OF RAAV-BASED GENE THERAPY VECTORS

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1. INTRODUCTION

Adeno-associated virus (AAV) is a helper-dependent parvovirus comprising a single-stranded DNA genome. In the last decade, **AAV-based vectors emerged as very promising candidates for gene therapy applications** due to the efficient transduction of non-dividing cells, low vector immunogenicity and long-term transgene expression. Increasing numbers of clinical trials applying AAV-based vectors as well as the first gene therapy drug in the European Union, Glybera®, intensify the **demand for robust and cost-effective manufacturing of these vectors.** Standard production systems involve small scale transient transfection or infection approaches using mammalian or insect cells. However, high production costs combined with considerable regulatory effort and quality concerns gave rise to the **development of stable packaging and producer cell lines.**

In a stable producer cell line with integral helper functions (Fig. 1), all required genetic elements are stably integrated into the genome of the host cell as independent expression constructs: the **recombinant vector** implying a transgene flanked by AAV inverted terminal repeats (ITRs), the **AAV genes rep and cap** required for replication and encapsidation, as well as **adenoviral helper function** delivered by sequences encoding genes E1a, E1b, E2a, E4orf6 and viral associated (VA) I/II RNA. In a timely regulated fashion, viral proteins are expressed and the AAV genome is replicated and encapsidated.

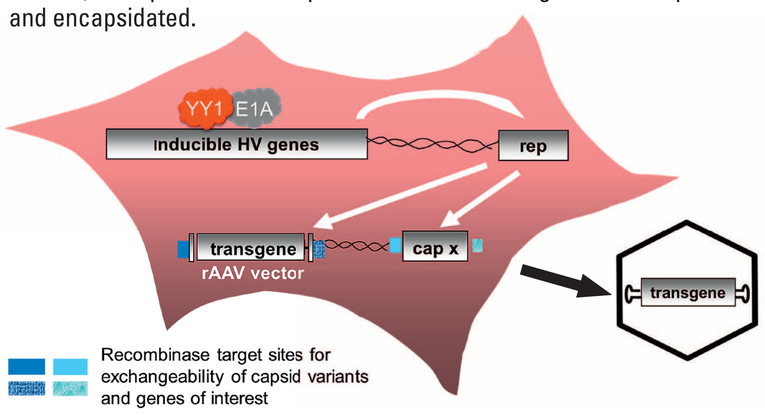

Figure 1: rAAV producer cell with stably integrated adenoviral helper functions

As some of the gene products arising during rAAV production are toxic, an inducible expression of the gene products is indispensable for generation of stable production cells. Thus, we plan to improve an existing inducible expression system for tight and high expression by **integrating temperature-inducible elements** identified by investigation of differential gene expression **(Tab. 1)**. Temperature reduction is widely applied to improve recombinant protein yield in mammalian cell culture (Ref. 1). Making use of a temperature shift as the primary switch for gene expression, we would **combine the induction event with conditions presumably enhancing rAAV production.**

Criteria for using inducible expression systems at industrial scale	Older systems	New systems	*Rentschler improved* system
Costs (caused by life of patents)	+	−	+
Tight expression	−	+	+
Induction event beneficial for recombinant protein expression	−	−	+

Table 1: Comparison of different inducible expression systems regarding the most important criteria for applicability in industrial-scale processes

2. OUTLINE

In a transient approach, we evaluated the feasibility to generate a producer cell line with stably integrated adenoviral helper functions using proprietary plasmids. Different temperatures were tested to produce rAAV. Furthermore, we investigated differential gene expression in response to temperature shifts, with the final goal to enable temperature-inducible rAAV generation at production scale.

3. RESULTS

Generation of rAAV by transient production methods

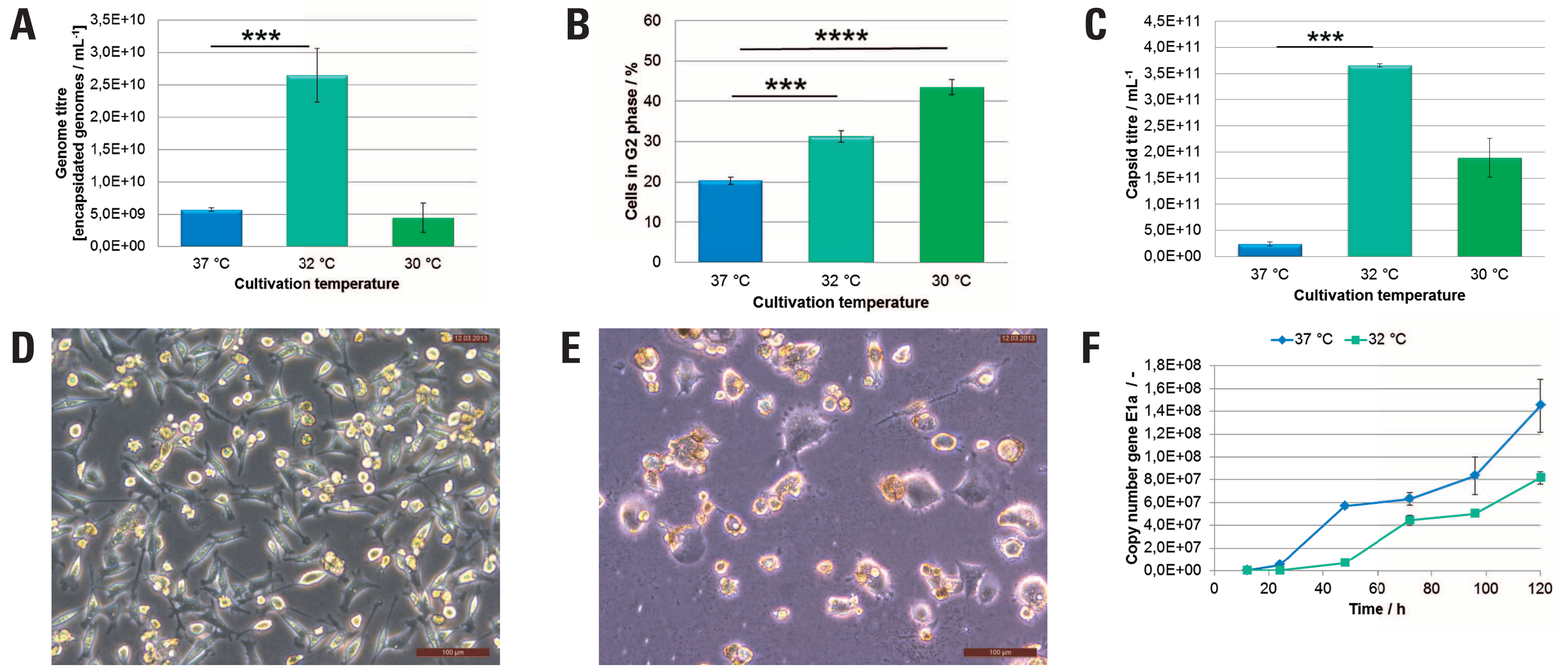

Figure 2: Transfection- and infection-based generation of rAAV in HeLa cells at different temperatures and analysis of temperature-mediated effects on cells and adenovirus kinetics. (A) Cells were transfected by calcium phosphate using three plasmids encoding vector, rep and cap, followed by AdV5 infection and subsequent incubation at three different temperatures. The amount of encapsidated rAAV genomes was determined 96 hours post infection according to (Ref. 2). **(B)** For cell cycle analysis, HeLa cells were stained with 1 mg mL-1 trisodium citrate, 0.05 % Triton X and 10 µg mL-1 propidium iodide 24 hours after temperature shift. **(C)** Recombinant protein synthesis at different temperatures was determined in terms of produced viral capsids during rAAV production using an ELISA A20 Kit (Progen Biotechnik GmbH). **(D+E)** Morphological changes of the cells during rAAV production at 37 and 32 °C were documented 72 hpt (hours post transfection.) **(F)** Adenoviral replication kinetics during rAAV production at 37 and 32 °C were examined by extracting whole DNA from the cells and subsequent analysis of E2a copy number in 50 ng of extracted DNA by qPCR (SYBR Green PCR Kit, QIAGEN). In contrast to replication, infection kinetics were not influenced by temperature (data not shown).

N = 3; two-tailed, unpaired t-test

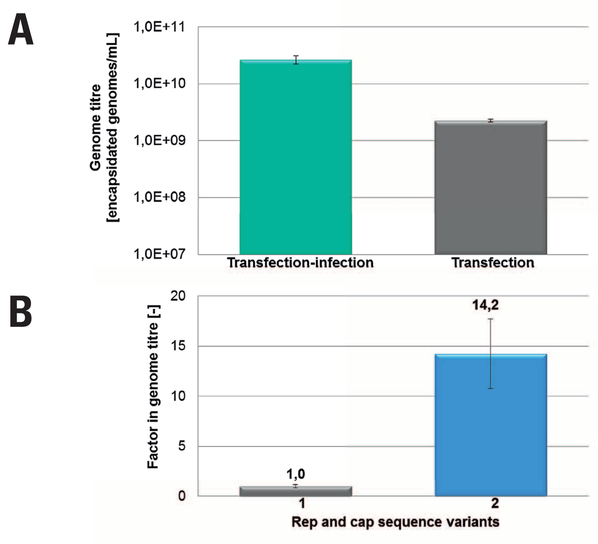

Figure 3: Transient plasmid-based generation of rAAV in HeLa cells at 32°C.
(A) rAAV production by transfection of three plasmids and subsequent AdV5 infection **(Fig. 2A)** compared to exclusively transfection-based rAAV generation using five plasmids encoding vector, adenoviral E1, remaining AdV helper functions, rep and cap. In the latter approach, cell number for seeding and ratio of the five plasmids to be transfected was not yet optimized. The amount of encapsidated rAAV genomes was determined 120 hpt according to (Ref. 2).
(B) Comparison of rAAV generation by plasmids encoding vector, adenoviral E1, remaining AdV helper functions as well as different variants of rep and cap (see below). Genome titre was analyzed according (Ref. 2) 120 hpt.
1: Approach implying functionally separated rep and cap genes on different plasmids, which are devoid of rep78 expression and lack an artificial Rep Binding Site (RBS) in the pUC19 plasmid backbones (Ref. 3).
(standard plasmids used in all preceding experiments)
2: Plasmids from **(1)** modified to avoid the expression of non-functional and truncated viral gene products by deletion of various promoter and potential transcription start sites.

Microarray analysis of differential gene expression in response to temperature shift

Name	Differential expression at	Mode of regulation	Microarray analysis	RT qPCR
Gene *A*	30 C	Up	3.2-fold	6.9-fold
Gene *B*	30 C	Up	2.2-fold	2.6-fold
Gene *C*	30 C	Up	3.3-fold	2.3-fold
miRNA *A*	32 C	Up	3.1-fold	-
miRNA *B*	32 C	Down	3.3-fold	-
miRNA *C*	32 C	Up	3.0-fold	-

Table 2: Microarray analysis of differential gene expression in HeLa at 30 or 32 °C compared to 37 °C. Cells were seeded at two different densities and shifted to 30 or 32 °C 48 hours post seeding. Harvest was carried out 6 hours post shifting. Microarray analysis (GeneChip® Human Exon 1.0 ST Array, Affymetrics) was performed to identify mRNAs differentially expressed more than 2-fold. Validation was done by RT qPCR analysis (EvaGreen® Mastermix, Biorad) and included controls of regulated and non-regulated mRNAs (Ref. 4-6). Differentially expressed miRNAs (>2-fold) were also identified by microarray analysis (GeneChip® miRNA 2.0 Array, Affymetrics). As validation is not yet completed, only an excerpt of most promising miRNAs is shown.

4. SUMMARY AND OUTLOOK

- In a transfection- and infection-based approach to produce rAAV, **cultivation of HeLa cells at 32 °C** resulted in **significantly increased rAAV titres** compared to 37 °C **(Fig. 2)**. This likely arises from an arrest of cells in G2/M phase, causing enhanced growth but decreased proliferation. Hence, cells exhibit enlarged size and elevated protein production, possibly supported by avoided degradation of rDNA as previously described for CHO cells (Ref. 7). Repressed adenoviral replication kinetics may trigger prolonged cellular viability and consequently increase titres, but results also suggest that high copy numbers of helper genes are not essential for efficient rAAV packaging.
- First results suggest that the **adenovirus infection step** can successfully be replaced by transfection of HeLa cells with the known adenoviral helper genes **(Fig. 3A)**. Overall **rAAV yields** could clearly be increased by further improvement of the trans-split AAV helper plasmids rep and cap regarding the avoidance of non-desired products **(Fig. 3B)**.
- Microarray analysis of cells cultivated at different temperatures identified **three genes up-regulated up to 7-fold, and 16 miRNAs likely regulated more than 2-fold at a lowered temperature (Tab. 2).** Underlying genetic switches are the first candidates towards amending inducible expression systems.
- **Pursuing work will focus on the generation and completion of all DNA sequences required for stable rAAV production. Further investigations of temperature-inducible switches will be performed. Once an appropriate switch is identified and validated, it will be used to amend an existing inducible expression system. This system will be applied to drive E1a gene expression in stable HeLa cells.**
- **Our ultimate goal is to provide the tools to generate a versatile and high-titre rAAV production platform that is applicable for industrial scale manufacturing and thus opens AAV-based gene therapy to a high number of potential patients.**

(1) Al-Fageeh M.B. et al. (2005) Biotechnol. Bioeng. 93:829-835; **(2)** Aurnhammer C. et al. (2012) Hum. Gene Ther. Methods 23(1):18-28; **(3)** Bertran J. et al. (2002) Patent WO 02/20748 A2; **(4)** Sonna L.A. et al. (2002) J. Appl. Physiol. 92:1725-1742; **(5)** Nishiyama H. et al. (1997) Gene 204:115-120; **(6)** Ars E. et al. (2000) Nucl. Acids Res. 28(6):1307-1312; **(7)** Tait A.S. et al. (2004) Biotechnol. Bioeng 88(6):707-721

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