Novel tools could give stable producer cell lines their break for commercial manufacturing of recombinant AAV vectors

Verena Emmerling[1, 2], Karlheinz Holzmann[3], Karin Lanz[3], Jessica Wegele[1], Stefan Kochanek[2] and Markus Hörer[1]

[1]Rentschler Biotechnologie GmbH, Erwin-Rentschler-Straße 21, 88471 Laupheim; [2]Division of Gene Therapy, University of Ulm, Helmholtz Str. 8/1, 89081 Ulm; [3]Department of Internal Medicine III, University Hospital of Ulm, Albert-Einstein-Allee 23, 89081 Ulm

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®, raise the **demand for robust and cost-effective manufacturing of those 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.

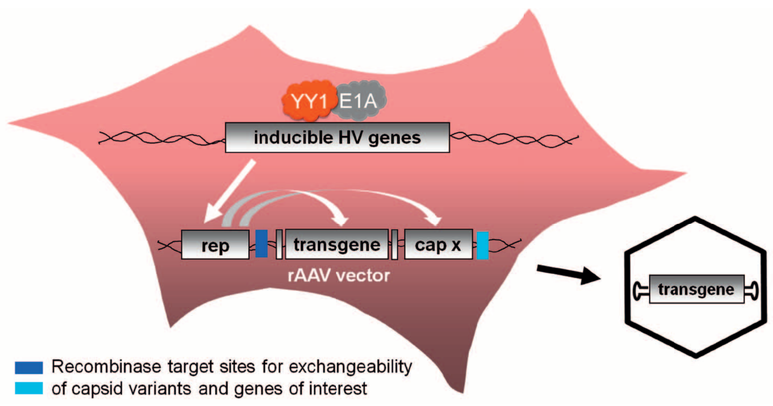

Figure 1: rAAV producer cell with stably integrated adenoviral helper functions

As some gene products arising during rAAV production are toxic, an inducible expression of the gene products is indispensable for generation of stable producer cells. Thus, we plan to improve an existing inducible expression system for tight and high expression by **integrating temperature-inducible elements** identified by differential gene expression analysis **(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

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.

Results

Manufacturing of rAAV by transient production methods

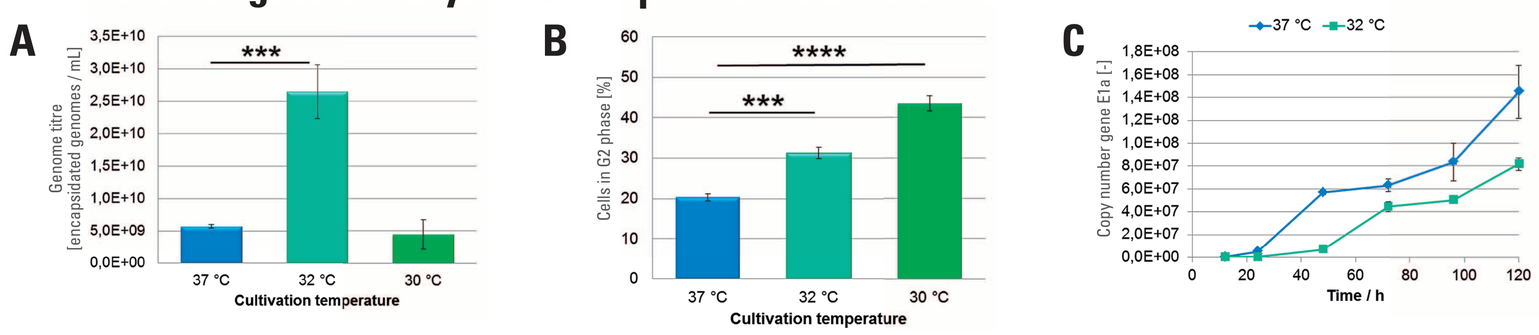

Figure 2: Transfection and infection-based production of rAAV in HeLa cells.
(A) Cells were transfected using three plasmids encoding vector, rep and cap, followed by AdV5 infection and subsequent incubation at three different temperatures. The amount of encapsidated rAAV genomes was determined 96 h post infection according to (Ref. 2).
(B) For cell cycle analysis, cells were stained with 1 mg mL^{-1} trisodium citrate, 0.05 % Triton X and 10 µg mL^{-1} propidium iodide 24 h after shifting.
(C) Adenoviral replication kinetics during rAAV production were examined by qPCR-based quantification of E2a copy number in 50 ng of total extracted DNA.
N = 3; two-tailed, unpaired t-test

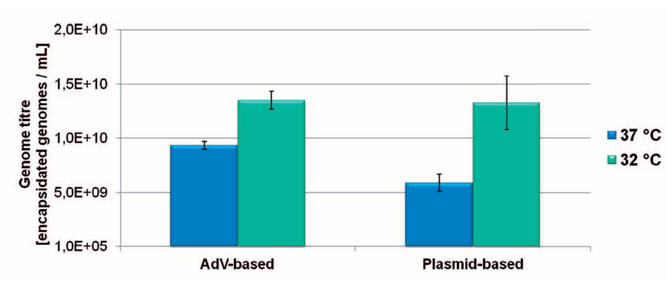

Figure 3: Production of rAAV in HeLa packaging cells stably expressing rep and cap (intermediate step towards a stable producer cell line).
Packaging cells were either transfected using a plasmid encoding vector and infected by AdV5 or were transfected using three plasmids encoding vector and adenoviral helper functions. Subsequent incubation at 37 or 32 °C was followed by determination of the genome titre at different time points according to (Ref. 2). Results for harvest days with highest titers are shown.

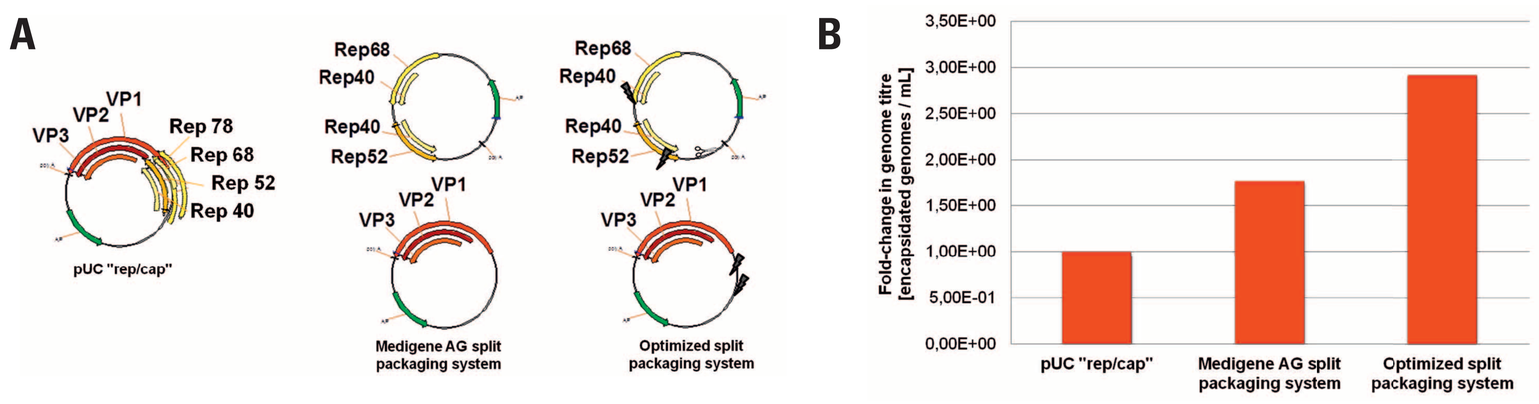

Figure 4: Advancement of Rep and Cap encoding sequences for rAAV production.
(A) A wtAAV2 sequence lacking the ITRs was cloned into a standard plasmid backbone **(pUC "rep/cap")** and optimized in two steps. First, rep and cap genes were functionally separated and cloned on different plasmids. Expression of rep78 was disabled and an artificial Rep binding site (RBS) in the pUC19 plasmid backbones was removed **(Medigene AG split packaging system,** see Ref. 3). These plasmids were then further improved to avoid the expression of non-functional and truncated viral gene products by inactivation of dispensable promoters and potential start codons **(Optimized split packaging system).**
(B) rAAV production applying an exclusively plasmid-based approach. Plasmids encoding vector, adenoviral E1, remaining AdV helper functions as well as the different variants of rep and cap depicted in **(A)** were transfected into HeLa, 293 and 293T cells. Genome titre was determined at different time points according to (Ref. 2). Mean values of all experiments performed are shown.

Microarray analysis of differential gene expression in response to temperature shift

Name	Mode of regulation at 32 °C	Microarray analysis	RT qPCR
gene *A*	up	11.3-fold	ongoing
gene *B*	up	4.8-fold	ongoing
gene *C*	up	2.2-fold	ongoing
gene *D*	down	2.5-fold	ongoing
gene *E*	down	2.4-fold	ongoing
miRNA *A*	up	2.5-fold	ongoing

Table 2: Microarray analysis of differential gene expression in HeLa at 32 °C compared to 37 °C. Cells were seeded at three different densities and shifted to 32 °C 48 hours post seeding. Harvest was carried out 6, 12 and 24 hours post shifting. Microarray analysis (GeneChip® Human Exon 1.0 ST Array and GeneChip® miRNA 2.0 Array, Affymetrics) was performed to identify mRNAs and micro RNAs differentially expressed more than 2-fold at described conditions. Validation is done by RT qPCR analysis (EvaGreen® Mastermix, Biorad and miScript PCR System, Qiagen).
As qPCR validation is not yet completed, only an excerpt of most promising targets from microarray analysis is shown.

Summary and Outlook

- **Transient rAAV packaging at lowered temperatures:**
  - An arrest of cells in the G2 phase of the cell cycle and repressed AdV5 replication kinetics likely trigger increased rAAV titres at 32 °C.
- **rAAV production in HeLa packaging cells:**
  - Using cells stably expressing rep and cap, rAAV production is enhanced at reduced production temperature in both transfection/infection and exclusively plasmid-based protocols, providing the basis for an AAV producer cell line with temperature inducible viral gene expression.
- **Rep and Cap helper plasmids:**
  - Optimization of AAV helper plasmid sequences in terms of avoidance of junk products leads to an improved rAAV productivity in different cell lines.
- **Microarray analysis:**
  - Dozens of genes regulated up to 11-fold and 16 miRNAs likely regulated more than 2-fold were identified in HeLa cells cultivated at lowered temperature **(Tab. 2).**
- **Pending work:**
  - Completion of differential gene expression studies and characterization of underlying regulatory elements.
  - Improvement of commercial inducible expression systems driving the Adenoviral helper genes by the combined use of temperature inducible regulatory sequences.
  - Cloning and transient testing of the final plasmids ultimately used for the generation of stable, temperature inducible AAV producer cells which will be particularly suitable for commercial AAV manufacturing.

(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

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