

Abstract

Adeno-associated virus (AAV) is currently the most researched viral vector for use in gene therapy. For several decades, human embryonic kidney (HEK293) cells have been the only adenovirus E1-complementing mammalian cell line suitable to produce adeno-associated viral vectors of different serotypes via triple transfection. There is a need for improved methods to address yield, scalability, and ethical concerns regarding rAAV production. To address this demand, we have developed a new cell line derived from baby hamster kidney cells (BHK-21) transformed with a fragment of the Ad5 sequence. We demonstrated this transformed BHK-21 cell line, BHK-[wt E1] stably expresses the E1 proteins. BHK-[wt E1] produced recombinant AAV by triple transfection and was confirmed based on yield, detection of viral proteins, and infectivity. FIG. 1 shows the general scheme of the experiments, and FIG. 4B shows rAAV2 viral genomes from transformed polyclonal BHK-[wt E1] cells compared to parental BHK cells untransformed (measured value below limit of detection) after triple transfection. These data demonstrate the proof of concept for this approach, and continued development and optimization of these cell lines should improve their properties. In addition, for isolating individual clones from a single cell, we used high content imaging system to identify high-quality and pure clones for rAAV producing cell lines. The successful transformation of BHK cells as a new E1 complementing cell line and subsequent expression of high levels of E1 are important factors for rAAV production. We also discuss other strategies for creating E1-complementing cell lines using different E1 sequences. BHK-[wt E1] and other E1-complementing cell lines based on BHK may provide advantages in applications where HEK293 and similar cells are used.

Overview and Approach

Recombinant adeno-associated virus (rAAV) is the current vector of choice for *in vivo* gene therapy. Most rAAV are produced in human embryonic kidney cells (HEK293) which contain the Ad5 E1 region. More scalable cell lines that avoid ethical issues regarding their origin would make gene therapies more accessible. We are developing a new cell line based on BHK-21 (FIG. 1), transformed with Ad5 E1 region and used it to produce rAAV2 by the triple transfection method.

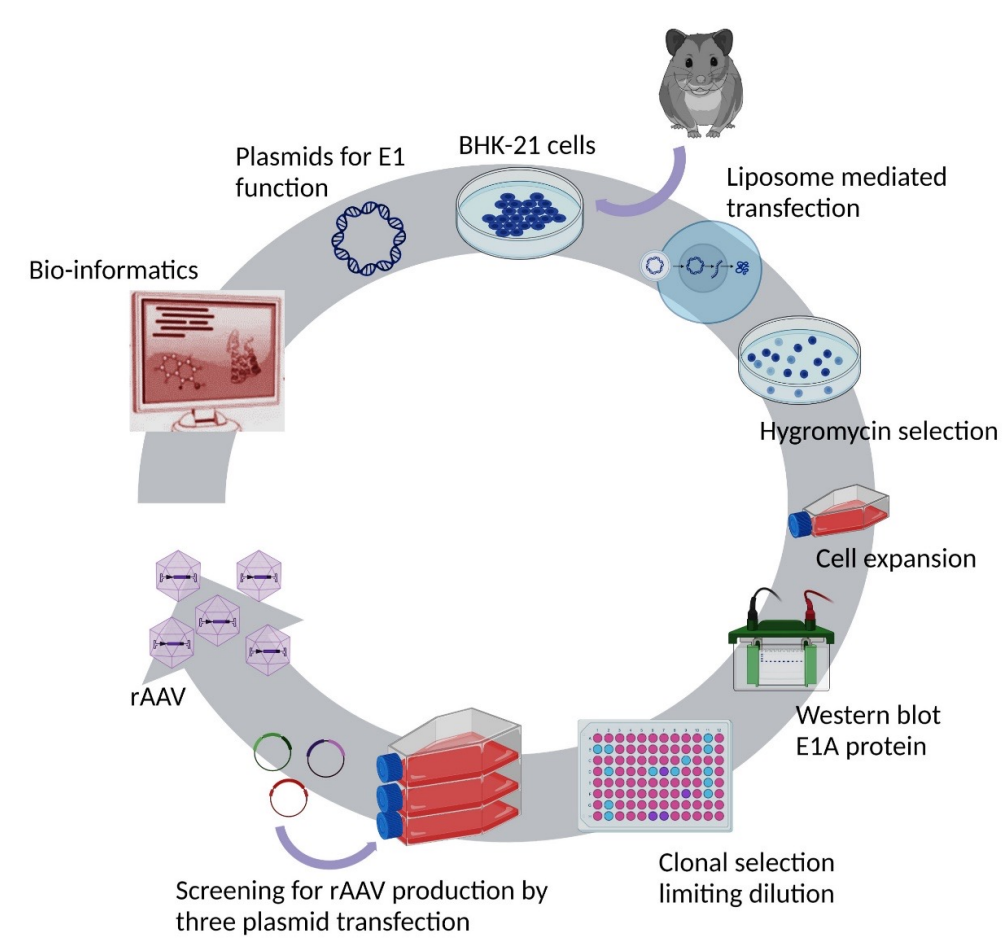


FIG. 1 Process steps for establishing and assessing BHK-21 cells for rAAV production

Methods and Results

We evaluated five different sequences to determine the best option for rAAV2 production (FIG. 2). One of these is the sequence present in HEK293, (wt E1), which is 4344 base pairs. We evaluated sequences with portions of the E1 gene removed including the pIX region. We tested two different promoters, human phosphoglycerate kinase (HuPGK) and human elongation factor 1 alpha (HuEF1a). We constructed plasmids using these sequences with standard bacterial vectors and transformed *E. coli* to generate sufficient quantities for transfection.

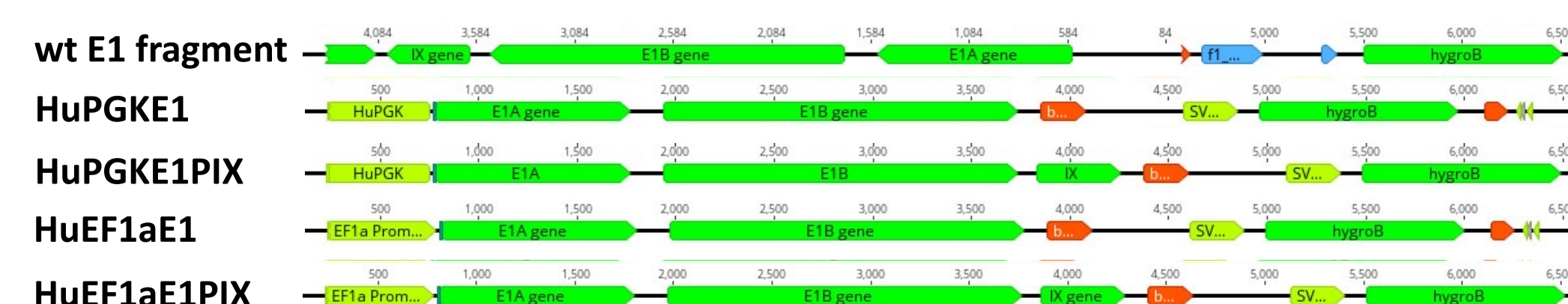


FIG. 2 Schematic representation of fragments of E1 constructs designed to create new E1 cell line. pcDNA3.1/Hygro(+) (GenScript) was used as a backbone for the constructs.

We transfected BHK-21 cells (ATCC) using PEI and all transfected cells expressed E1A (FIG. 3A). Plasmid triple transfection of these constructs showed detectable rAAV2 production only for wt E1 and HuPGKE1 (FIG. 3B). The wt E1 transfected BHK-21, BHK-[wt E1], consistently produced higher rAAV2 capsids, so we used it for further study.

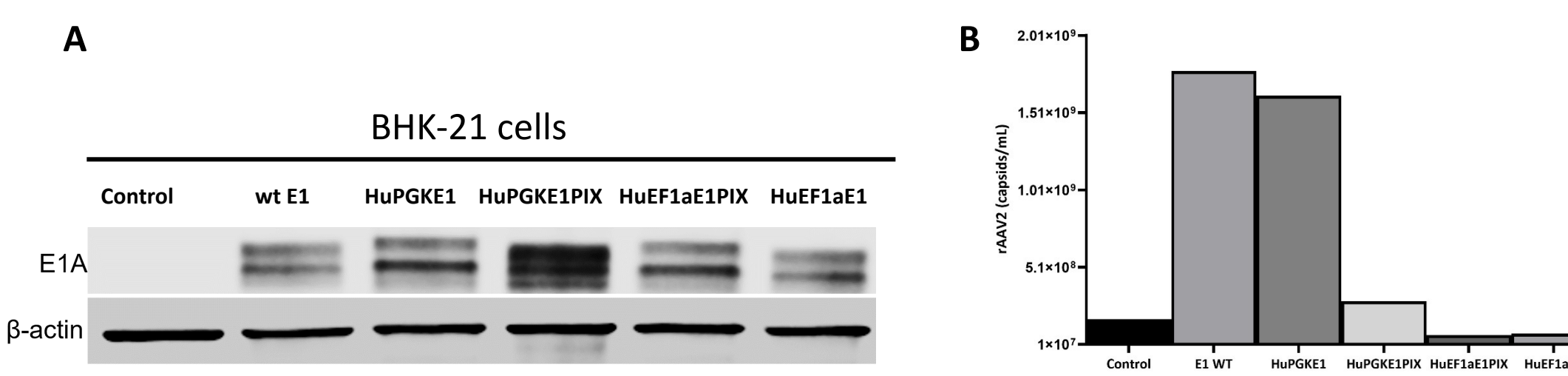


FIG. 3 (A) Western blot analysis of E1A protein expression in BHK cells transfected with E1 constructs designed with appropriate control. β-actin was used as the loading control. (B) ELISA results showing rAAV2 production in BHK cells transfected with wt E1 plasmids by triple transfection.

BHK-[wt E1] transfected cells produced rAAV2 capsids and encapsulated genomes and we also detected VP1, VP2 and VP3 proteins by Western blot (FIG 4). BHK-[wt E1] showed similar growth kinetics as BHK-21 but significant morphological and phenotypical differences (FIG. 5). Transduction of HepG2 cells with rAAV2-FGFP produced from BHK-[wt E1] showed GFP expression (FIG. 6). Using limiting dilution and imaging we isolated clones from BHK-[wt E1] (FIG. 7). Western blots for E1 protein showed varied levels of expression of E1A (FIG. 8) and triple transfection showed various levels of rAAV2 production, with Clone 1 having the highest (FIG. 9).

Results

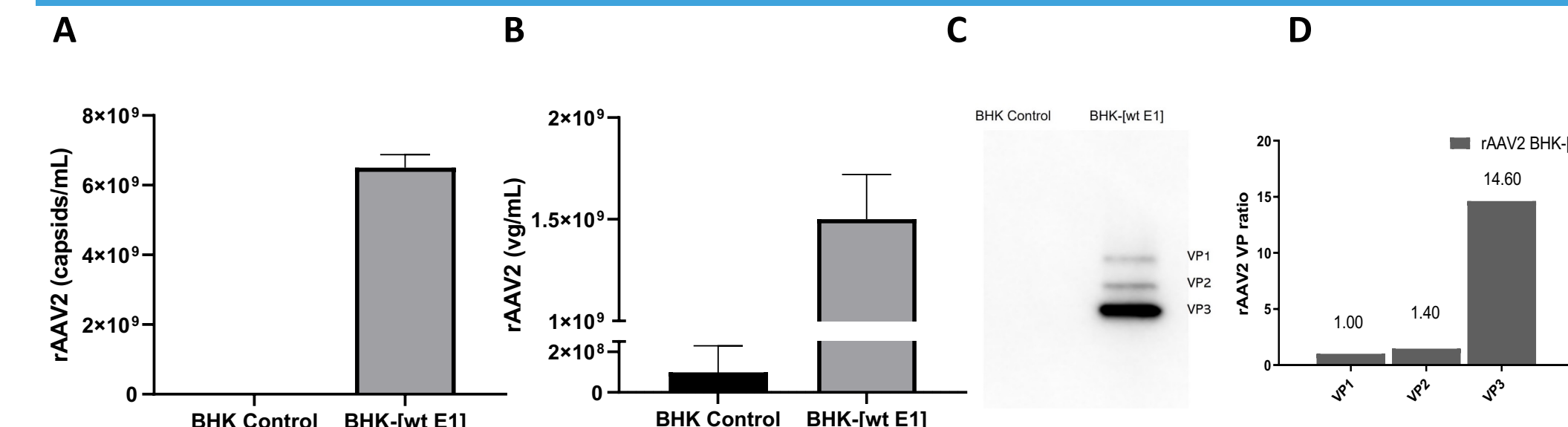


FIG. 4 (A) ELISA results showing rAAV2 production in BHK-[wt E1] by the triple transfection method. Data represent mean ± SD. (B) Digital PCR measurements of rAAV2 viral genomes. BHK-[wt E1] 1.5x10⁹ +/- 2.20x10⁸, BHK-21 Control below level of detection. (C) Western blot analysis of rAAV2 capsid protein (VP1/VP2/VP3). (D) VP protein ratio was quantified by densitometry.

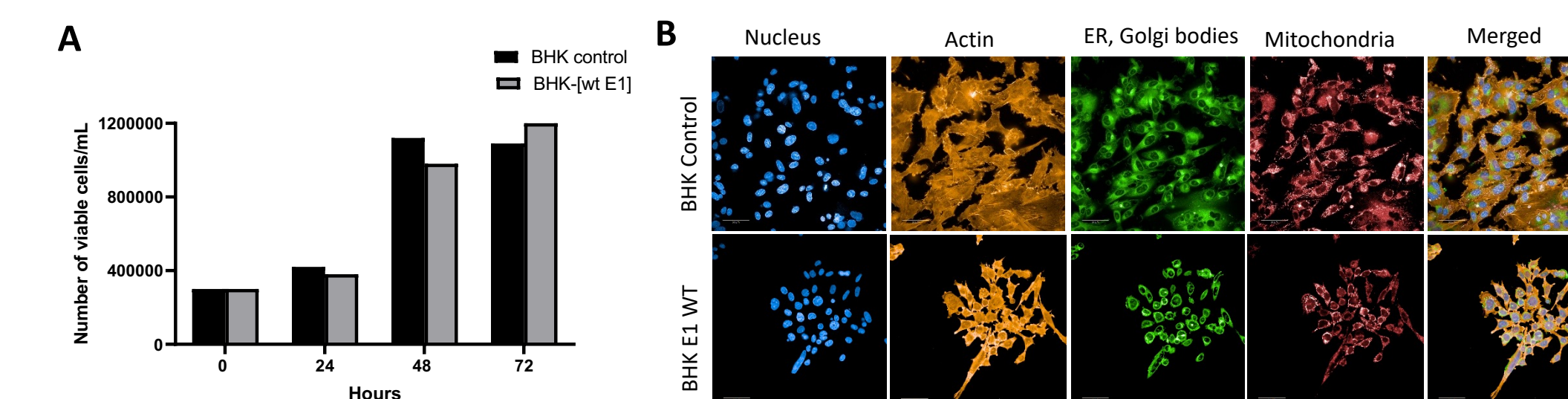


FIG. 5 (A) Growth kinetics of BHK-[wt E1] cells by trypan blue cell viability method. (B) Morphological profiling using cell painting assay on BHK-[wt E1] cells. The column displays four channels imaged using Operetta CLS 40X confocal: Hoechst (DNA), Phalloidin (actin), WGA (Golgi and plasma membrane) and Mito tracker (mitochondria).

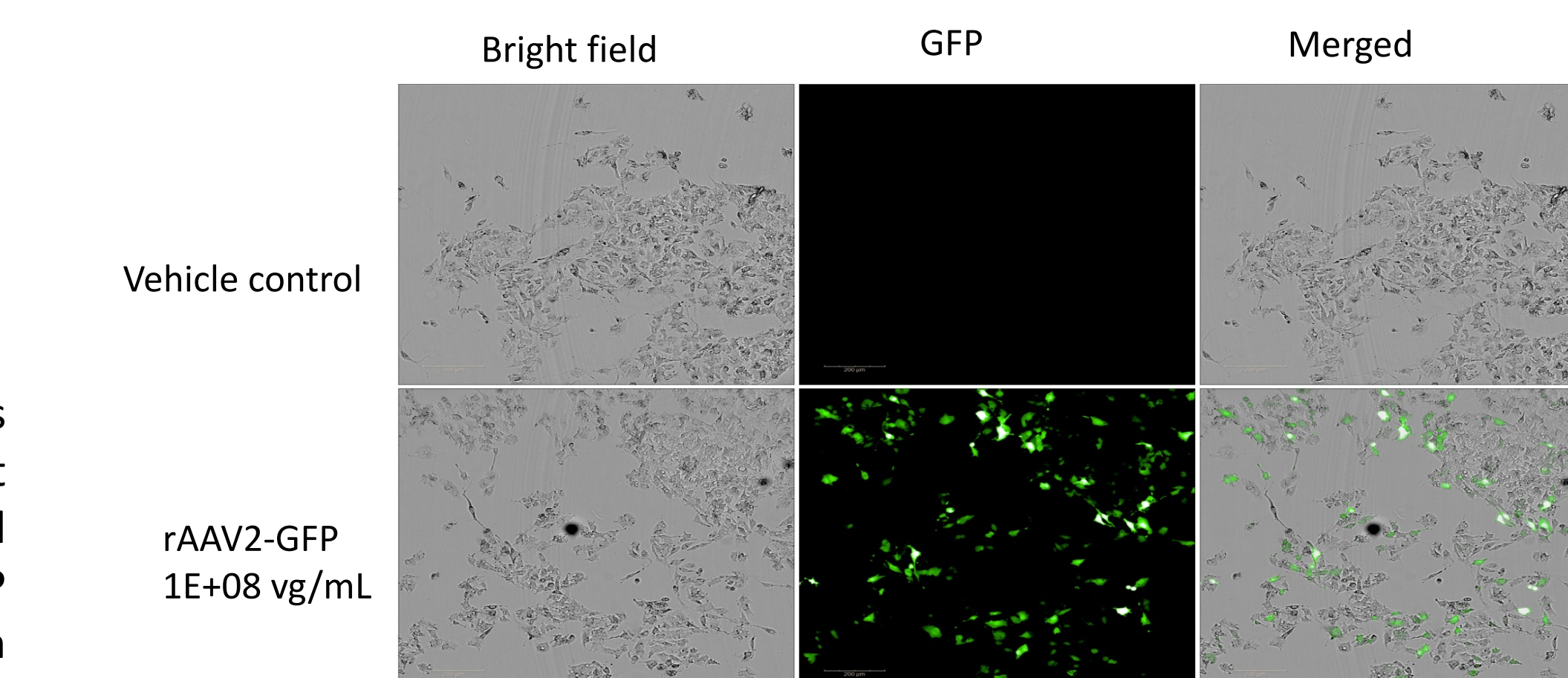


FIG. 6 Analysis of *in vitro* transduction activity of rAAV2 derived from BHK-[wt E1] using HepG2 cell line. Light and fluorescent image of HepG2 cells infected with rAAV2.

Results

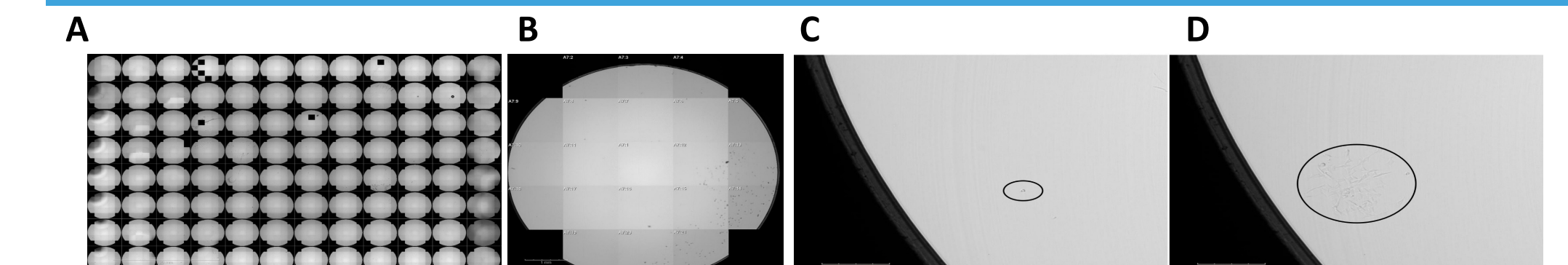


FIG. 7 Operetta CLS (HCS) whole well image of the 96-well plate scanning (A-D). (A) Image from 96-well plate scanned after 6 h incubation by bright phase. (B) Image enlargement of single well segmented of 21 focal regions. (C) The well containing a single cell. (D) The single cell from the well scanned after 5 days proliferating as a monoclonal population.

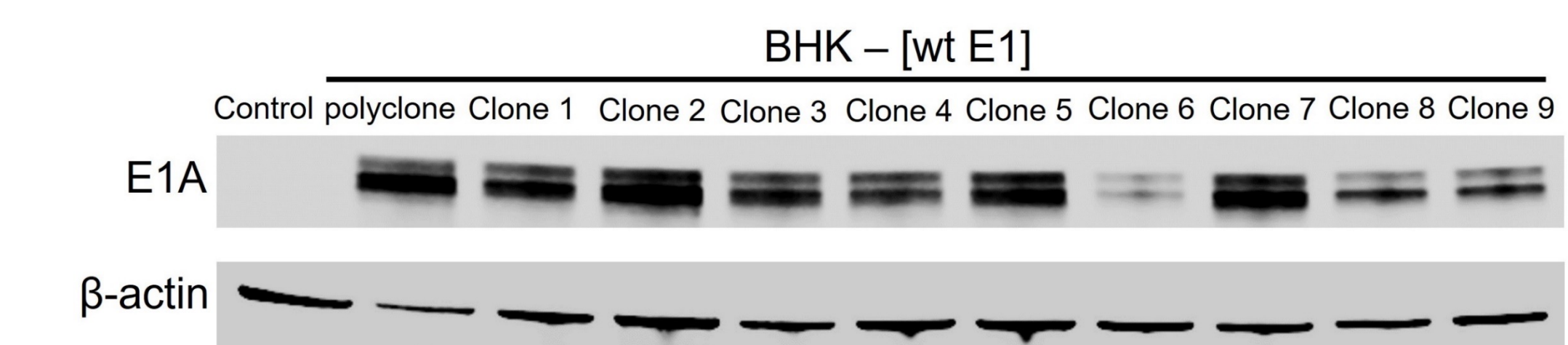


FIG. 8 Western blot analysis of E1A protein expression in BHK-[wt E1] derived clones with appropriate control. β-actin was used as the loading control.

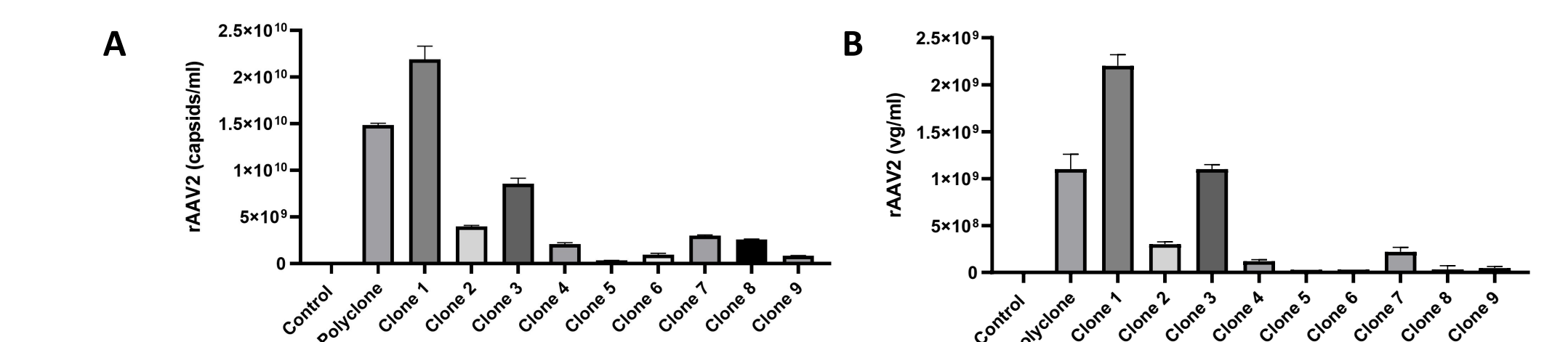


FIG. 9 (A) ELISA results showing rAAV2 production in BHK-[wt E1] derived clones. Data represented by mean ± SD. (B) Digital PCR measurements of rAAV2 viral genomes. BHK-21 control is below the level of detection.

Conclusions

We have designed several constructs coding for the adenovirus E1 gene and shown transfection into BHK-21 cells with hygromycin selection results in E1A expression. Of these E1A-expressing cells, the wild type sequence of E1 (BHK-[wt E1]) and a smaller sequence with the HuPGK promoter was able to produce rAAV2 capsids through triple transfection. rAAV2-GFP produced from BHK-[wt E1] showed encapsulated genomes and HepG2 transduced cells showed GFP expression. Clonal isolation identified a population of rAAV-producing clones and the highest producing clone was selected for further optimization. These results demonstrate the potential of BHK-[wt E1] for rAAV production and other applications that benefit from an E1-expressing mammalian cell line.

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Intellectual Property

This work is covered by U.S. provisional patent applications 63/482,873 filed on February 2, 2023, and 63/487,759 filed on March 1, 2023.