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Development of cell lines with increased susceptibility to diverse adeno-associated viral vectors to enable *in vitro* potency assays

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Vectors based on adeno-associated viruses (AAVs) are promising therapeutic modalities used in gene therapy. Robust cell-based assays that demonstrate and quantify the potency of AAV vectors in expressing their transgene are needed for clinical development. However, many AAV clinical serotypes poorly transduce cells *in vitro* and often contain cell-type-specific promoters inactive in commonly used cell lines. Here, we enhance the efficiency of *in vitro* AAV transduction by overexpressing the AAV receptor (AAVR/KIAA0319L), preventing transcriptional silencing by the HUSH complex, and using CRISPR activation (CRISPRa) to drive transgene expression. For the latter, we utilized guide RNAs targeting the conserved AAV2 inverted terminal repeat (ITR) sequence present in most AAV transfer vectors. Using this strategy, we engineered cell lines that showed marked increases in transduction by AAV vectors across a wide range of clinically relevant serotypes and containing cell-type-specific promoters. These improvements enabled the efficient determination of AAV functional titers (also referred to as transducing titers), which can be used to robustly monitor potency across diverse AAV preparations. The strongly enhanced susceptibility of these cell lines to transduction by a variety of divergent AAV vectors could facilitate the development of standardized *in vitro* quantitative assays for AAV-based gene therapy products.

INTRODUCTION

Adeno-associated virus (AAV)-based vectors are leading candidates for gene therapy applications with several approved products and many more in clinical trials. An important aspect of the development of clinical gene therapy vectors is the characterization and quantification of vector potency.[1,2] Regulatory agencies in the United States and Europe have drafted guidelines that call for potency assays that are precise, accurate, specific, and robust, while minimizing the use of animals in testing.[3,4] Robust cell-based assays that quantify the ability of a clinical gene therapy AAV vector preparation to transduce cells and express the desired transgene are crucial for product quality control. Patients with high levels of neutralizing antibodies against AAV can also be refractory to treatment with AAV gene therapy products.[5] Determining neutralizing antibody levels in patients before treatment with an approved drug or inclusion in a gene therapy trial can be an important predictor of the success of a drug.[6] A major roadblock in the development of cell-based assays, including *in vitro* potency assays and neutralization assays, is the inability of multiple clinically relevant AAV serotypes to transduce cells *in vitro*,[7] which is likely due to differential engagement of glycan attachment factors.[8] Moreover, the usage of cell-type-specific promoters in gene therapy vectors further complicates the development of *in vitro* assays that are applicable across different AAV vectors.

Here, we report a multi-pronged strategy to overcome these obstacles by developing cell lines with dramatically enhanced susceptibility to functional transduction by a wide variety of AAV vectors that differ in their serotype and in their promoter driving the transgene. This was achieved by combining overexpression of the multi-serotype AAV receptor[9,10] (AAVR, also called KIAA0319L) with genetic knockout (KO) of SETDB1, a histone methyltransferase involved in the transcriptional repression of recombinant AAV genomes.[11–13] To enable promoter-independent AAV expression, we used CRISPR activation (CRISPRa) targeting sequences in the AAV inverted terminal repeat (ITR). While current quality control and dosing is largely dependent on determining the vector genome titer and other physical parameters of the AAV preparation, the engineered cell lines facilitate the functional titration of AAV gene therapy products. We expect that the broad susceptibility of these cell lines to different AAV vectors will be useful for the development of standardized quantitative potency and neutralization assays for AAV-based gene therapy products.

RESULTS

To develop an *in vitro* assay for quantifying AAV vector-mediated transgene expression, we used HeLa cells (H1HeLa lineage) as parental cells due to the relatively broad transduction of HeLa cells by AAV at high multiplicity of infection (MOI).[14] For the vast

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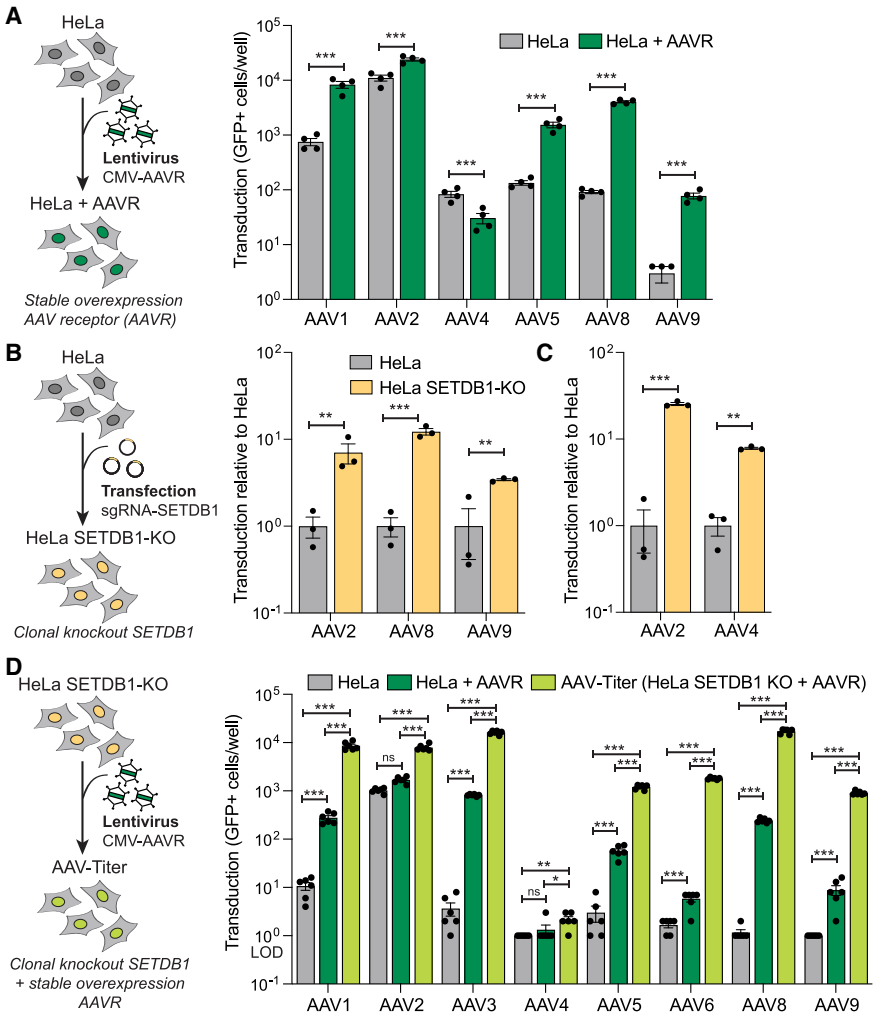

Figure 1. Development of the AAV-Titer cell line, with enhanced susceptibility to *in vitro* transduction by multiple serotypes

(A) Transduction of parental HeLa and HeLa cells overexpressing AAVR (HeLa + AAVR) by different AAV serotypes encoding GFP at an MOI of 20,000 vg/cell. Transduction was measured by live cell imaging at 24 h post-transduction. A two-way ANOVA was performed on log transformed data with a Sidak's multiple comparison test (n = 4). (B) Transduction of HeLa cells and HeLa cells with *SETDB1* knockout (KO) mutations. Cells were transduced with AAV2, AAV8, and AAV9 encoding luciferase at an MOI of 20,000 vg/cell, and luciferase activity was assessed at 48 h post-transduction. A two-way ANOVA was performed on log transformed data with a Sidak's multiple comparison test (n = 3). (C) Transduction of HeLa and SETDB1-KO HeLa cells with AAV2 (MOI = 20,000 vg/cell) or AAV4 (MOI = 100,000 vg/cell) encoding luciferase. Transduction was assessed by luciferase activity at 72 h post-transduction. A two-way ANOVA was performed on log transformed data with a Sidak's multiple comparison test (n = 3). (D) Transduction of parental HeLa, HeLa + AAVR, and AAV-Titer (HeLa SETDB1 KO + AAVR) cells by various AAV serotypes encoding GFP. Cells were transduced at an MOI of 1,000 vg/cell, and transduction was assessed by live cell imaging at 24 h post-transduction. A two-way ANOVA was performed on log transformed data with a Tukey's multiple comparisons test (n = 6). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not significant.

majority of natural and synthetic AAV serotypes used in clinical gene therapy, AAVR expression is rate limiting for the entry of AAV in cells and subsequent transgene expression.[9,10,15,16] Only one evolutionary lineage, comprising AAV4 and AAVrh32.33, is AAVR independent.[15] To increase the susceptibility of HeLa cells to AAV transduction, we stably overexpressed AAVR through a lentivirus-based approach. These cells were transduced with AAV reporter vectors encoding the GFP transgene with capsids from distinct serotypes that are AAVR dependent (AAV1, AAV2, AAV5, AAV8, and AAV9) or independent (AAV4). Compared to the parental cell line, AAVR overexpression resulted in markedly higher numbers of GFP-expressing cells for AAV1, AAV5, AAV8, and AAV9 (Figure 1A). The increase was less pronounced for AAV2, which inherently has a high transduction efficiency *in vitro*.[7] AAV4 did not benefit from AAVR overexpression, as expected. To further enhance transduction, we used CRISPR-Cas9 to generate a clonal HeLa cell line that contains a KO mutation in *SETDB1* (Figure S1). In line with the human silencing hub (HUSH) complex's role in transcriptional silencing of AAV vectors,[12,13] *SETDB1* KO resulted in significant increases in AAV vector transgene expression, as measured using AAV reporter vectors encoding luciferase (Figures 1B and 1C). These increases were observed for the AAVR-dependent (AAV2, AAV8, and AAV9) and -independent (AAV4) serotypes consistent with a role of SETDB1 on the transcriptional level, rather than related to AAV cellular entry mediated by AAVR.

Subsequently, we combined the two approaches by stably overexpressing AAVR through lentiviral transduction in the background of HeLa cells containing the *SETDB1* KO mutation, followed by clonal selection (Figure S1). This cell line, which was engineered to be optimally susceptible to AAV transduction, was named AAV-Titer to reflect its potential in determining the functional titer of AAV vector preparations. We compared the transduction of AAV-Titer cells with parental HeLa cells and HeLa cells overexpressing AAVR using an MOI of 1,000 viral genomes (vg)/cell (Figure 1D). At this MOI, transduction in parental HeLa cells with AAV2 was readily apparent, while the transduction of the other serotypes was negligible (less than 10 cells per well). In stark contrast, for all serotypes except AAV4, transduction in the AAV-Titer cell line resulted in a dramatic increase in transduced cells. Although for AAV2, which efficiently transduces the parental cells, this increase was modest, for

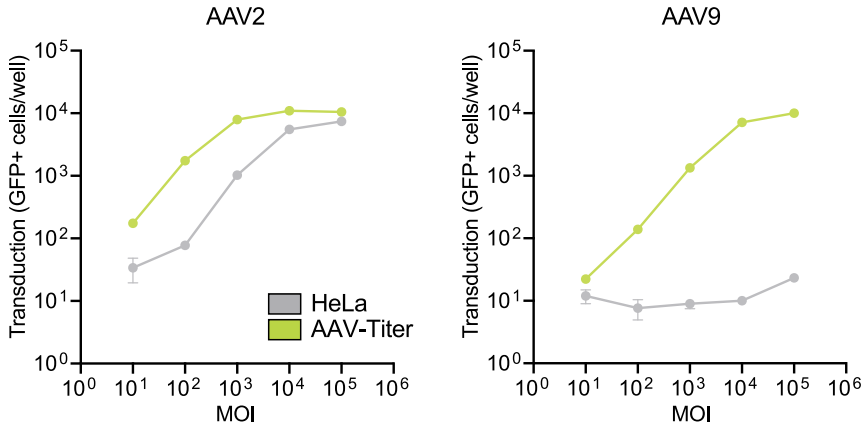

Figure 2. Transduction of HeLa and AAV-Titer cells by AAV2 and AAV9 encoding GFP at a range of MOIs
Transduction (GFP⁺ cells) was measured by live cell imaging at 24 h post-transduction. Points represent the mean of three replicates, and error bars represent SEM. Error bars smaller than the points are not shown.

AAV1, AAV3, AAV5, AAV6, AAV8, and AAV9 the increases amounted to around 1,000-fold. The AAVR-overexpressing cell line was intermediately susceptible to AAV transduction, suggesting that the combined effect with *SETDB1* KO resulted in remarkable susceptibility of the AAV-Titer cell line.

We further assessed the difference in transduction of HeLa and AAV-Titer cells at a range of MOIs for two selected AAV serotypes—AAV2 and AAV9 (Figure 2). For AAV2, linear increases in the number of transduced cells were observed with increasing MOI, eventually reaching saturation for both cell lines. At MOIs within the linear range, AAV-Titer cells exhibited a marked increase in transduction compared to parental HeLa cells. For AAV9 transduction in HeLa cells, we did not reach MOIs that display a linear increase in transduction, with very few cells transduced even at the highest MOI tested (100,000 vg/cell). In stark contrast, linear increases started between 10 and 100 vg/cell in AAV-Titer cells and reached saturation at 10,000 vg/cell. This experiment further demonstrates that AAV-Titer cells are markedly more susceptible to functional transduction by distinct AAV serotypes and highlights the utility of testing a range of MOIs while establishing a quantification protocol using this cell line.

In recombinant AAV preparations, the large majority of genome-containing particles are not infectious and are unable to functionally transduce cells (i.e., leads to transgene expression).[17] Therefore, solely determining the genome titer (vg/mL) during quality control does not reflect vector potency, which can differ due to variation during production or storage conditions. Except for AAV2, which has an unusually high transduction efficiency *in vitro*, it has been challenging to determine the functional titer of AAV stocks. To show the utility of the AAV-Titer cell line in determining the functional titer (here, measured as transducing units (TU)/mL) for a non-AAV2 serotype in clinical use, we exposed an AAV9 reporter virus to increasing temperatures and determined the genome titer and functional titer over time. We determined the functional titer (also referred to as transducing titer) by assessing the expression of fluorescent proteins encoded by the AAV transgenes. The genome titer remained stable over the time course at all tested temperatures (Figure 3A). The functional titer remained stable at 25°C, but treatment at higher temperatures resulted in a loss of functional titer, which was most pronounced at the highest temperature tested (42°C) (Figure 3B). These experiments demonstrate the utility of AAV-Titer cells in determining vector potency in a quantitative manner under different conditions, revealing changes in potency that were not detected by relying solely on genome titer.

Currently, about 50% of AAV gene therapy vectors in clinical trials include a promoter that is cell type specific.[18] The ongoing research in identifying and characterizing additional promoter elements that can increase the specificity of AAV vectors beyond what can be achieved with capsid modification will likely increase this percentage in the future.[19] This complicates the development of uniform *in vitro* potency assays because cell-type-specific promoters often require the use of specific primary cells rather than more standard, robustly growing, cancer cell lines. A prior report demonstrated that this can be overcome by leveraging CRISPRa in cancer cells and recruiting the transcriptional machinery to the cell-type-specific promoter contained in the AAV vector.[16] This allows for robust transgene expression from the AAV vector even with a promoter that is inactive in the cancer cell line. While this method greatly facilitates the determination of transgene expression, it uses a guide RNA (gRNA) that is specific to the cell-type-specific promoter and therefore needs to be customized for different AAV vectors. To generate cell lines that are more broadly applicable, we employed a strategy that uses gRNAs that target an invariant region of recombinant AAVs, its ITRs (Figure 4A). We designed three gRNAs that target distinct regions of the AAV2 ITR (Figure 4A). Most AAV transfer vectors used in research and clinical settings use AAV2 ITRs in conjunction with AAV2 rep expressed in *trans*, regardless of the capsid used in the final packaged product. We first introduced transcriptional elements required for CRISPRa[20] in the AAV-Titer cell line and named this modified cell line AAV-Titer-dCas9-VP64. We next introduced the three ITR guides resulting in three distinct cell lines (AAV-Titer-ITR1, AAV-Titer-ITR2, AAV-Titer-ITR3). To assess the functionality of our approach, we used AAV8-hSyn-mCh, which encodes the mCherry (red fluorescent protein) transgene expression driven by the neuron-specific hSyn promoter. As expected for the non-neuronal HeLa cellular background, transduction with AAV8-hSyn-mCh led to undetectable red fluorescence in the AAV-Titer-dCas9-VP64 cells (Figure 4B). In stark contrast, expression of the ITR1 gRNA resulted in efficient transduction by AAV8-hSyn-mCh

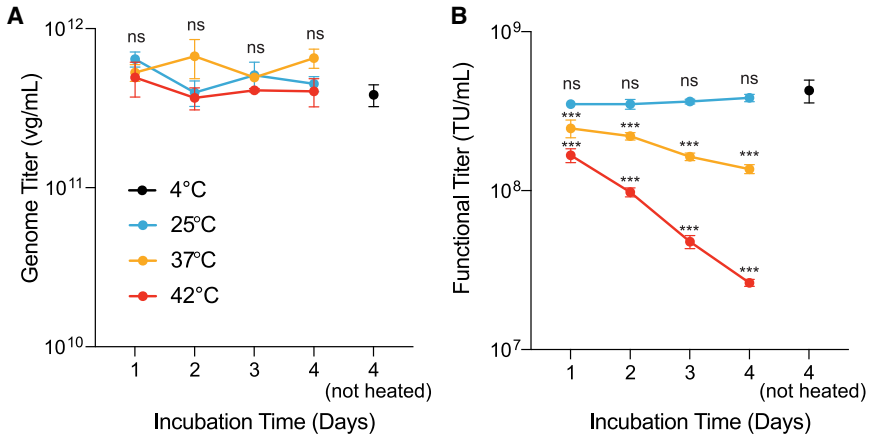

Figure 3. Functional titration on AAV-Titer cells to monitor the effect of high-temperature storage on the potency of an AAV9 vector batch

(A) AAV9 encoding GFP was kept at 25°C, 37°C, or 42°C for 4 days, with samples collected each day. We also kept a sample (n = 3 biological replicates) at 4°C for 4 days as a control that was not heat treated ("not heated"). Genomic titer (vg/mL) was measured for each sample (n = 3 biological replicates with two technical replicates each). A two-way ANOVA was run on log transformed data using biological replicates with a Dunnett's multiple comparison test (n = 3). No statistically significant differences were found between any of the heat-treated samples versus the control. (B) The functional titer in transducing units (TU) per milliliter was determined for each sample using AAV-Titer cells by live cell imaging at 24 h post-transduction (mean of n = 3 biological replicates with three technical replicates each; error bars represent SEMs). A two-way ANOVA was run on log transformed data using biological replicates with a Dunnett's multiple comparison test (n = 3) between each temperature's three time points and the control. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

as detected by red fluorescence imaging (Figure 4B). Transduction by a vector containing the ubiquitous CMV promoter was efficient regardless of the presence of the ITR1 gRNA. To assess the effect of the CRISPRa approach more quantitatively and to compare the different gRNAs, we used live cell imaging on AAV-Titer-dCas9-VP64 cells without gRNA expression or expressing the distinct ITR1, ITR2, and ITR3 guides. Compared to the parental AAV-Titer-dCas9-VP64 cells, expression of the ITR gRNAs resulted in a strong increase in the number of mCherry^{+} cells from AAV8 and AAV9 vectors containing the neuron-specific hSyn promoter, demonstrating the functionality of the approach (Figure 4C). To determine the broader utility of the approach, we assembled a panel of AAV vectors containing promoters with cell type specificity, including hSyn (neurons), CaMKII (primarily excitatory neurons), D2SP (dopamine receptor type-2 neurons), and hMOR (human Mu opioid receptor neurons). The vectors contained capsids from diverse natural serotypes or synthetic capsids, including capsids known to be refractory (AAV1, AAV5, AAV8) or tractable (AAV2, AAV-DJ) to *in vitro* transduction of unmodified cell lines.[7] Functional transduction was determined quantitatively by live cell imaging and flow cytometry on HeLa, AAV-Titer, and AAV-Titer-ITR1 cells (Figures 4D–4F). While all three cell types were efficiently transduced by a vector containing the universal CAG promoter, there were clear differences in transduction by the other AAVs tested. For all cell-type-specific promoters, we observed strongly enhanced transduction in the AAV-Titer-ITR1 cell line. For the hMOR promoter, this was only detectable using flow cytometry but not with live cell imaging, likely because the latter was less sensitive in fluorescence detection. These results show that the AAV-Titer-ITR1 cell line could be a useful tool for *in vitro* functional testing of AAVs using cell-type-specific promoters.

DISCUSSION

We have engineered cell lines with greatly enhanced susceptibility to *in vitro* transduction by a wide variety of AAV vectors of different natural and engineered serotypes carrying distinct promoter elements. These cell lines can be used for quantitative *in vitro* potency assays and allow for the functional titration of AAV vectors used in clinical gene therapy and research. In addition, we foresee utility of the AAV-Titer cell line for antibody neutralization assays. We expect the AAV-Titer cell line to be useful in quality control and assurance processes for AAV gene therapy products. The AAV-Titer cell line combines the overexpression of AAVR with inactivation of the HUSH complex, which represses the expression of AAV-encoded transgenes in the nucleus. This allows for efficient transduction by most AAV serotypes while increasing transgene expression compared to standard cell lines.

In our proof-of-principle experiments, we measured AAV transduction by assessing the expression of reporter transgenes encoded by AAV through flow-based assays and live cell imaging. These methods are commonly used to assess functional transduction due to their accuracy and convenience, as reported before.[21] However, the AAV-Titer cell line can also be used to assess the transduction of AAV with non-reporter transgenes by designing assays specific for the transgene of interest, which could have utility for *in vitro* potency assays. Assays could include immunofluorescence microscopy with transgene-specific antibodies, activity assays specific for the transgene product, or fluorescence *in situ* hybridization using probes complementary to the mRNA of the transgene of interest.

The ability to determine the functional titer in addition to the genome titer will allow for more accurate determination of lot-to-lot variation or product stability. For example, we have shown a decrease in functional titer due to heat treatment, which was not captured with the genome titer. We have determined the functional titer based on transduction units, a commonly used method.[21] Another common method to determine AAV infectious titers utilizes the HeLa rep-cap cell line that allows for replication of the AAV genome when co-infected with replication-competent adenovirus. The replicated virus in the nucleus

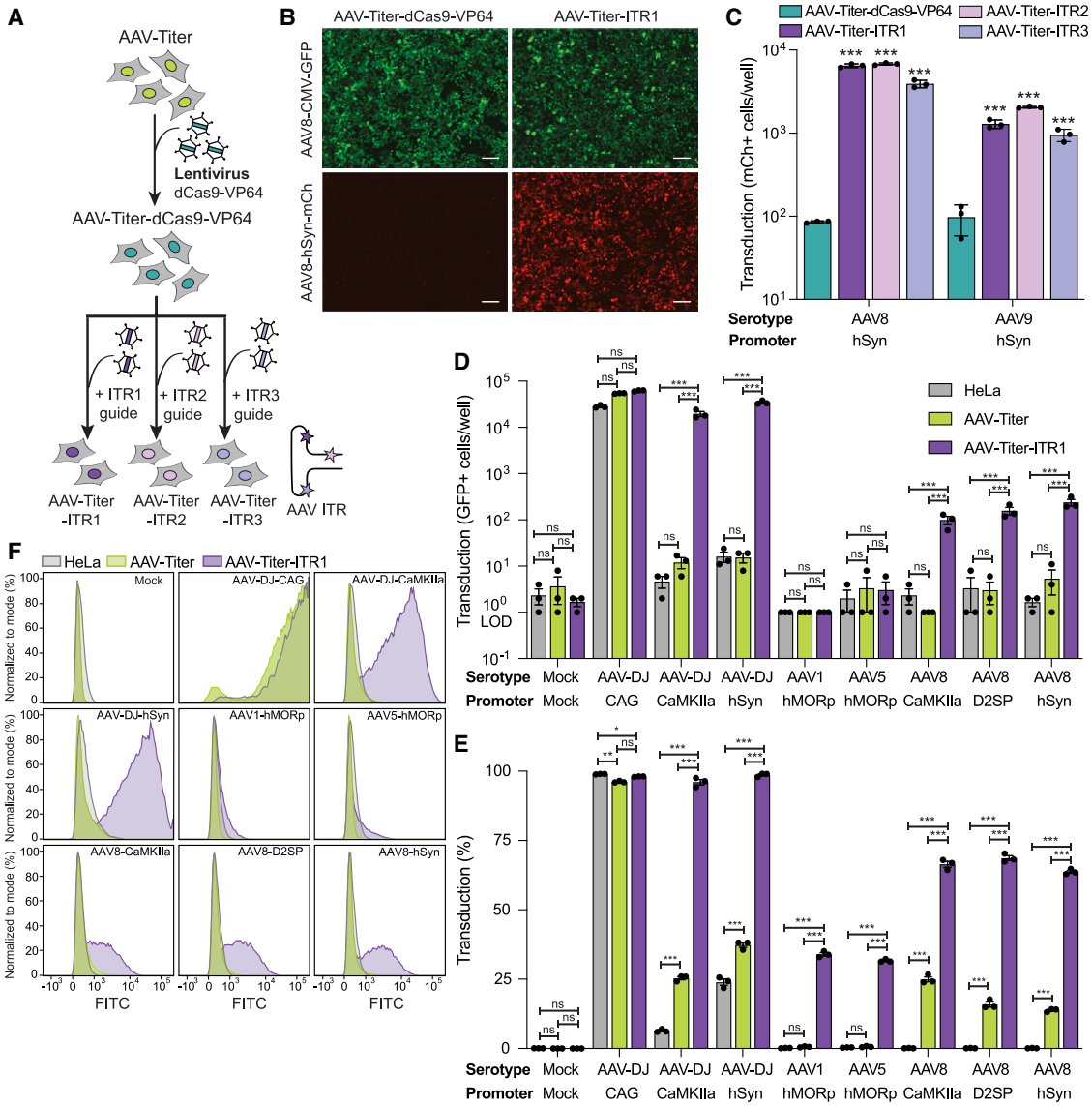

Figure 4. Development of the AAV-Titer-ITR cell lines with enhanced susceptibility to *in vitro* transduction by AAV vectors containing cell-type-specific promoters

(A) Schematic of the utilization of CRISPR activation to drive expression of the transgene independent of the promoter contained in the AAV vector by targeting the conserved ITR of AAV. AAV-Titer cells were modified to express dCas9-VP64 and PP7-P65-HSF1 (AAV-Titer-dCas9-VP64), along with a guide RNA (gRNA) targeting various areas of the AAV ITR. Multiple guide-targetable sequences are present in the conserved AAV ITR, which allows for the use of different gRNAs, including the three shown and tested. (B) AAV-Titer-dCas9-VP64 cells expressing ITR guide no. 1 (AAV-Titer-ITR1) or parental AAV-Titer-dCas9-VP64 cells were transduced with AAV containing a universal promoter (AAV8-CMV-GFP) or a cell-type-specific promoter (AAV8-hSyn-mCh). Transduction is shown at 48 h post-transduction. Scale bar, 200 μm. (C) Transduction of AAV-Titer-dCas9-VP64 cells with or without ITR guides at an MOI of 10,000 vg/cell using AAV8-hSyn-mCh or AAV9-hSyn-mCh. Transduction was measured at 48 h post-transduction by live cell imaging. A two-way ANOVA was performed on log transformed data with a Sidak's multiple comparison test ($n = 3$). (D) HeLa, AAV-Titer, and AAV-Titer-ITR1 cells were transduced at an MOI of 10,000 vg/cell, with AAV vectors carrying capsids from different serotypes (AAV-DJ, AAV8, AAV1, AAV5, AAV9) and containing different cell-type-specific promoters predicted to be inactive in the HeLa cellular background (hSyn, CaMKIIa, D2SP, hMORp). Transduction was assessed at 72 h post-transduction by live cell imaging (D) or flow cytometry (E). For the live cell imaging data, a two-way ANOVA was performed on log transformed data, followed by Tukey's multiple comparisons test ($n = 3$). For the flow cytometry data, a two-way ANOVA was performed, followed by Tukey's multiple comparisons test ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not significant. (F) Representative flow cytometry plots of HeLa, AAV-Titer, and AAV-Titer-ITR1 cells transduced with AAV vectors of different serotypes. Data is one of three replicates averaged in (E).

is then quantified by a subsequent qPCR or hybridization of probes to part of the AAV genome.[22] However, many AAV serotypes do not transduce the HeLa rep-cap cell lines well. While the determination of functional titer has been possible for the few serotypes that efficiently transduce *in vitro*,[7] including AAV2 and AAV-DJ, our engineered cell lines facilitate this for many other serotypes used in clinical settings, including AAV5, AAV8, and AAV9. Our cell lines have direct utility in functional titration based on transduction. In future research they can be modified to express rep-cap, if an infectious titer assay based on AAV genome replication is desired.

A substantial proportion of AAVs used in research[19,23] and in the clinic[18] use cell-type-specific promoters to target transgene expression. Previously, CRISPRa targeting was used as strategy to drive transgene expression from photoreceptor-specific promoters using gRNA specific for the promoter.[16] Here, we adapted this strategy by targeting an invariant region contained in the AAV vector. This allows transgene expression independent of the identity of the cell-type-specific promoter. We incorporated this strategy in the AAV-Titer background and demonstrated that the resulting cell line displayed a robust transduction when challenged with a variety of distinct cell-type-specific AAV vectors. Together, we expect that the strategy described in our study and the developed cell lines will facilitate *in vitro* potency assays and functional titration for a broad range of AAV vectors used in research and in clinical development.

MATERIALS AND METHODS

Construction of plasmids

The construction of pLenti-AAVR-FLAG-PURO was previously described.[9]

A single-guide RNA (sgRNA) (5′-CGTCCTCAGAGCTACTGTCC-3′) was selected to target the *SETDB1* gene on chromosome 1 at position 150930029 on the negative strand. This sgRNA was cloned into the pSpCas9(BB)-2A-GFP. pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid no. 48138; http://n2t.net/addgene:48138; RRID: Addgene_48138).[24] This produced the plasmid PX458-sgRNA-SETDB1.

The CRISPR activation plasmid lenti-dCAS9-VP64_Blast was used to generate AAV-Titer-dCas9-VP64. Lenti-dCAS-VP64_Blast was a gift from Feng Zhang (Addgene plasmid no. 61425; http://n2t.net/addgene:61425; RRID: Addgene_61425).[20] The additional transcription factors and gRNA were delivered using the pXPR_502 plasmid. pXPR_502 was a gift from John Doench and David Root (Addgene plasmid no. 96923; http://n2t.net/addgene:96923; RRID: Addgene_96923).[25] The puromycin resistance gene was replaced by the BleoR resistance gene, which confers resistance to the bleomycin/phleomycin family of antibiotics including Zeocin. Cloning was performed using Gibson assembly using a synthetic DNA construct (Integrated DNA Technologies) as an insert.

Guide RNAs targeting the AAV2 ITR (ITR1: 5′-CCGACGCCC GGGCTTTGCCC-3′, ITR2: 5′-AGTGGCCAACTCCATCACTA-3′, and ITR3: 5′-CACTGAGGCCGGGCGACCAA-3′) were cloned into pXPR_502.

Cell lines production

H1HeLa cells were obtained from American Type Culture Collection (CRL-1958), and 293FT cells were obtained from Thermo Fisher (R70007). Cells were grown in DMEM + 10% fetal bovine serum (FBS) + penicillin-streptomycin (D10) (Thermo Fisher, 11995073; Sigma, 4333).

AAVR overexpressing cells were previously generated as described.[9] Lentivirus was produced using pLenti-AAVR-FLAG-PURO and 293FT cells and utilized to transduce the respective cell lines overnight. Cells stably expressing AAVR were selected by treatment with 1–3 μg/mL puromycin over 2 days (InvivoGen). This population of cells is referred to as HeLa + AAVR.

SETDB1 KO cells were generated through transfection of the PX458-sgRNA-SETDB1 plasmid into H1HeLa cells. After 2 days, GFP^+ cells were single-cell sorted into individual wells of a 96-well plate. After individual cell clones were isolated, *SETDB1* KO was confirmed by western blot and by Sanger sequencing an amplified genome fragment around the target site followed by TIDE (Tracking of Indels by Decomposition) indel analysis.[26] One clonal cell line (clone 2-7) was chosen and is referred to as HeLa SETDB1-KO.

To generate H1HeLa SETDB1-KO cells stably overexpressing AAVR, SETDB1-KO (clone 2-7) was transduced with lentivirus generated using pLenti-AAVR-FLAG-PURO and 293FT cells. After 48 h, cells were selected using puromycin (InvivoGen). To generate clonal cell lines, cells were single-cell sorted into three 96-well plates using flow cytometry. After single colonies grew, cells were replica plated. Clones were selected for high transducibility by transduction with scAAV9-GFP (University of North Carolina at Chapel Hill Gene Therapy Center Vector Core [UNC Vector Core]) at an MOI of 1,000 vg/cell. Clones with a high percentage of GFP transduction were identified and the untransduced replica was expanded. One clonal cell line (clone 4) was selected based on high transduction and general growth characteristics. This clonal cell line, which stably overexpresses AAVR and contains the *SETDB1* KO mutation, is referred to as AAV-Titer.

H1HeLa SETDB1 KO + AAVR clone no. 4 (AAV-Titer) was used as the progenitor cell line for all cell lines used for ITR activation cell line development. Cells were first transduced with lentivirus generated with plent-dCAS9-VP64_Blast plasmid. Cells were selected using blasticidin for ∼1 week. We refer to this population of cells as AAV-Titer-dCas9-VP64. These cells were subsequently transduced with lentivirus generated using pXPR_502 + ITR guide nos. 1, 2, or 3, which contain a Zeocin resistance gene. Cells were selected using Zeocin for ∼2 weeks, followed by single-cell cloning using flow cytometry. After testing cell clones for transduction efficiency using AAV8-hSyn-mCh, a single clonal cell line for each of the distinct gRNAs was chosen. We refer to these three clonal cell lines as

AAV-Titer-ITR1, AAV-Titer-ITR2, and AAV-Titer-ITR3. They overexpress AAVR, contain *SETDB1* KO, express the CRISPRa machinery, and express individual gRNAs.

Transduction experiments

Purified, titered stocks of recombinant AAV1, AAV2, AAV3, AAV4, AAV6, AAV8, and AAV9 vectors encoding reporter genes under the CMV promoter were purchased from the UNC Vector Core. AAV vectors encoding EGFP were self-complementary and those encoding firefly luciferase single stranded. All input titers were determined by the supplier using qPCR to detect vg in the sample. Purified, titered stocks of AAV were used for all experiments. AAV8-hSyn-mCh and AAV9-hSyn-mCh were obtained from the UNC Vector Core. AAV-DJ-hSyn1-eYFP (GVVC-AAV-016), AAV8-hSyn-eYFP (GVVC-AAV-122), AAV-DJ-CaMKIIa-eYFP (GVVC-AAV-008), AAV8-CaMKIIa-eYFP-NRN (GVVC-AAV-123), AAV8-D2SP-eYFP-WPRE (GVVC-AAV-114), AAV1-hMORp-eYFP (GVVC-AAV-258-1), AAV5-mMORp-eYFP (GVVC-AAV-251-5), and AAV-DJ-CAG-hrGFP (GVVC-AAV-021) were obtained from the Stanford Gene Vector and Virus Core (RRID: SCR_023250).

AAV transduction experiments were carried out in 96-well plates, with cells plated in 200 μL DMEM + 10% FBS + penicillin-streptomycin at day −1 to obtain an approximate density of 50%–70% confluence at the time of transduction. The approximate number of cells at the time of transduction was 2×10^4 cells/well. AAV was diluted in 50 μL DMEM at the specified MOI based on vg and added to the 96-well plates. Confluence and GFP and mCherry fluorescence were tracked over time using live cell imaging with the Incucyte S3 Live Cell Analysis Instrument (Sartorius). The number of GFP^+ or $mCherry^+$ cells in each well was calculated using Incucyte Analysis software. Vector genome titers (Figure 3A) were determined using digital polymerase chain reaction (QIAcuity One, Qiagen) using QIAcuity Probe Master Mix (Qiagen, catalog no. 250102) and primer/probe sets targeted to the CMV segment of the recombinant AAV genome. Functional titers (Figure 3B) were calculated by determining the number of transduction events (GFP^+ cells) at 24–72 h post-transduction. One TU is defined as one GFP^+ cell. Functional titers (TU/mL) were determined by dividing the number of GFP^+ cells by the input volume of the AAV stock virus. For the firefly luciferase experiment, luciferase activity was assessed using the Luciferase Assay System (Promega, E1500). Luciferase activity was read using a Glomax luciferase plate reader (Promega). In some experiments, transduction was assessed by flow cytometry on a LSRFortessa X-20 (BD Biosciences), and the percentage of GFP^+ singlet cells was calculated based on gating of non-transduced cells.

DATA AND CODE AVAILABILITY

The data generated in this study are available upon request from the corresponding author.

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AUTHOR CONTRIBUTIONS

J.Z., E.S.E., N.R.W., and J.E.C. designed the studies. J.Z., E.S.E., and A.P. performed the experiments and analyzed the data. J.Z., E.S.E., and J.E.C. wrote and revised the manuscript, with assistance from additional co-authors. J.E.C. acquired the funding for the study.

DECLARATION OF INTERESTS

J.E.C. and J.Z. are inventors on a patent application by Stanford University regarding the described methods.

SUPPLEMENTAL INFORMATION

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