Citation: Molecular Therapy — Methods & Clinical Development (2016) **3,** 16039; doi:10.1038/mtm.2016.39
Official journal of the American Society of Gene & Cell Therapy
**www.nature.com/mtm**

**ARTICLE**

# Helper virus-mediated downregulation of transgene expression permits production of recalcitrant helper-dependent adenoviral vector

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Helper-dependent adenoviral vectors (HDAd) that express certain transgene products are impossible to produce because the transgene product is toxic to the producer cells, especially when made in large amounts during vector production. Downregulating transgene expression from the HDAd during vector production is a way to solve this problem. In this report, we show that this can be accomplished by inserting the target sequence for the adenoviral VA RNAI into the 3′ untranslated region of the expression cassette in the HDAd. Thus during vector production, when the producer cells are coinfected with both the helper virus (HV) and the HDAd, the VA RNAI produced by the HV will target the transgene mRNA from the HDAd via the endogenous cellular RNAi pathway. Once the HDAd is produced and purified, transduction of the target cells results in unimpeded transgene expression because of the absence of HV. This simple and universal strategy permits for the robust production of otherwise recalcitrant HDAds.

*Molecular Therapy — Methods & Clinical Development* (2016) **3**, 16039; doi:10.1038/mtm.2016.39; published online 8 June 2016

## INTRODUCTION

Helper-dependent adenoviral vectors (HDAds) are devoid of all viral-coding sequences and have proven to be excellent vectors for many gene and cell therapy applications because they can mediate high efficiency transduction of many different cells types from many different species *in vivo* and *in vitro* independent of the cell cycle, they have an enormous cloning capacity of 36 kb, do not integrate into the host genome and, provide long-term transgene expression with reduced toxicity.[1] Because HDAd do not contain any viral gene, they must be produced using a helper virus (HV).[2] The HV is an E1-deleted adenovirus whose packaging signal is flanked by loxP sites. To produce HDAd, E1-complementing cells expressing Cre are coinfected with the HDAd and the HV wherein the packaging signal of the HV is excised by Cre-mediated site-specific recombination rendering it unpackagable but still able to undergo DNA replication and trans-complement HDAd production.[2]

Occasionally, we have found that HDAds expressing certain transgenes cannot be produced because the transgene product is toxic to the producer cells, especially when present in large quantities, such as during vector production. For example, $10^5$ to $10^6$ progeny adenoviral genomes are produced postinfection, only about 20% of which are packaged into virions.[3] Because of such high transgene copy numbers during vector production, even transgene products that may not normally be toxic, may have toxic effects when present in such overwhelming quantities. And this problem is further exacerbated by the very strong promoters/enhancers that are normally employed in gene transfer vectors. Therefore, development of strategies to overcome this obstacle is important and necessary for the production of these recalcitrant HDAds.

Adenovirus, including the HV, expressed two RNA polymerase III-dependent noncoding RNAs, called virus-associated (VA) RNAI and VA RNAII.[4,5] VA RNAI functions to ensure high-level adenoviral protein synthesis by binding to and inhibiting protein kinase R (PKR), part of the antiviral interferon response, while the role of VA RNAII is not well known. These short RNA transcripts (~160 nucleotides) have a stem-loop structure similar to pre-miRNA and are expressed throughout the virus life cycle, reaching very high concentrations during the late phase of infection ($10^8$ molecules of VA RNAI/cell and $10^7$ molecules of VA RNAII/cell). Like pre-miRNA, VA RNAI and VA RNAII are exported to the cytosol by Exportin 5 where they are processed by Dicer into functional microRNAs, called mivaRNAs, which are incorporated into the RNA-induced silencing complex.[6–8] Importantly, mivaRNAI derived from VA RNAI has been shown to target the mRNA from reporter transgenes engineered to contain the complementary target sequence in their 3′ untranslated region (UTR), thereby inhibiting their expression.[7,9–11] As well, host cellular mRNAs targeted by VA RNAI-derived mivaRNAI resulting in downregulated expression have been identified.[10,11] In this study, we describe a simple strategy of exploiting expression of VA RNAI from the HV to downregulate transgene expression from the HDAd during its production. In this way, recalcitrant HDAds can be easily produced.

## RESULTS

PiggyBac (PB) is a transposon isolated from cabbage looper moth *Tichoplusia ni* and encodes a transposase that catalyzes PB transposition.[12] Hyperactive PB transposase (hyPBase) bears 7 amino acid substitutions resulting in a 17- and 9-fold increase in excision and

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Received 24 March 2016; accepted 19 April 2016

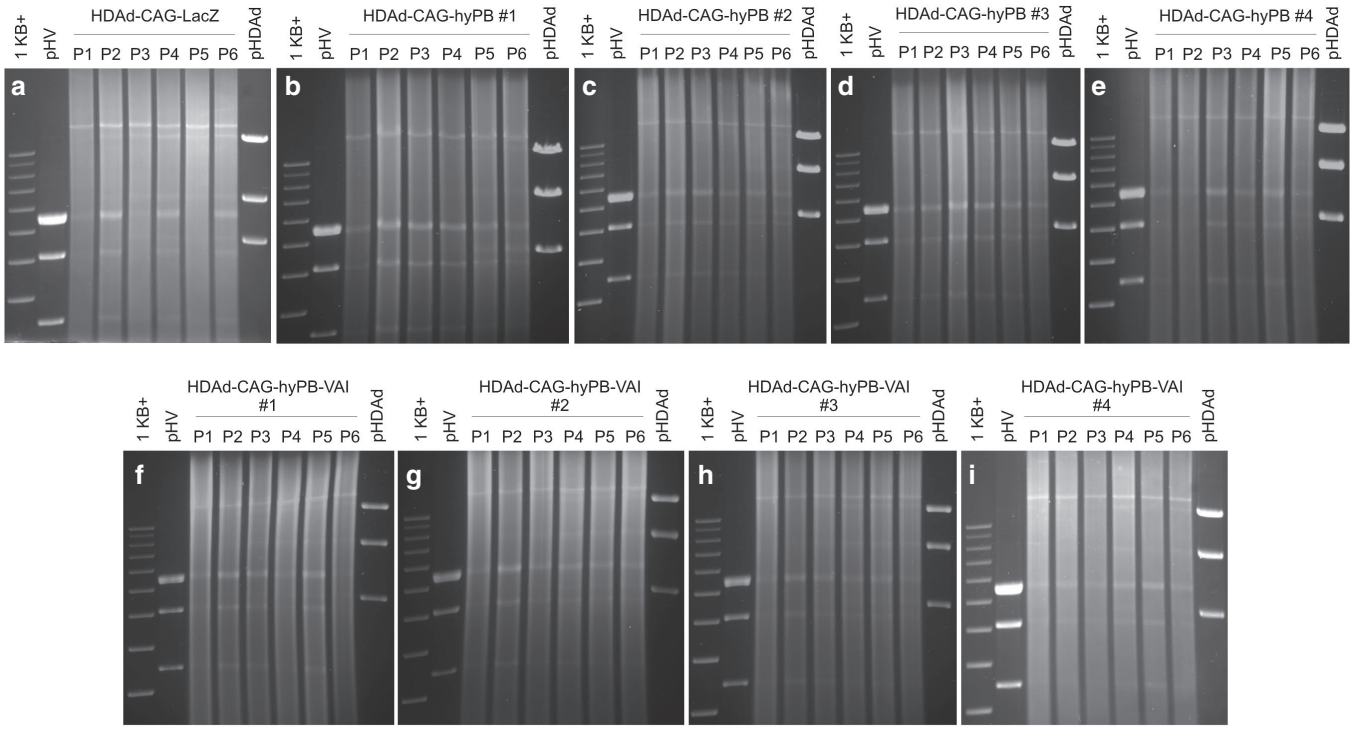


**Figure 1** Amplification of (**a**) HDAd-CAG-LacZ, (**b–e**) HDAd-CAG-hyPB, and (**f-i**) HDAd-CAG-hyPB-VAI. Total cellular DNA extracted from serial passages was digested with ApaLI and analyzed by agarose gel electrophesis to identify those passages containing peak vector titer which is indicated by the visible presence of vector-specific bands in the presence of the background cellular DNA smear. The lanes labeled pHV contain the HV plasmid digested with ApaLI and PacI to serve as a control for HV-specific bands. The lanes labelled pHDAd contain the corresponding HDAd plasmid digested with ApaLI and PmeI to serve as a control for HDAd-specific bands. Passage 0 (the initial transfection with pHDAd and infection with HV of the producer cells) for each amplification was not included in this analysis.

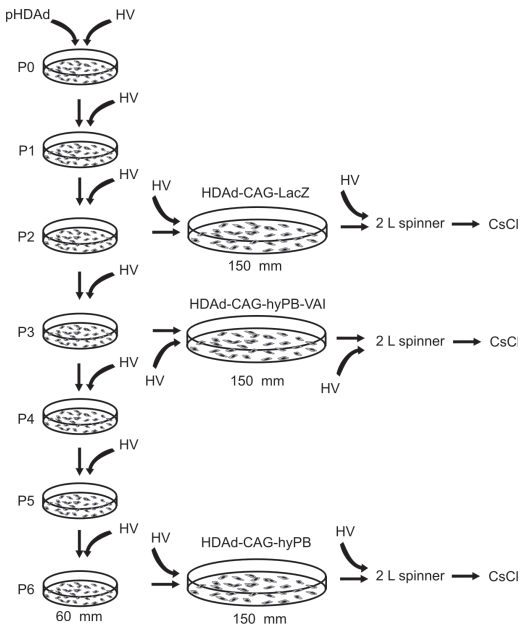


**Figure 2** Schematic of amplification and large-scale HDAd production. As described in the Results and shown in Figure 1, the earliest passage that contains peak vector titer during vector amplification was passage 2 for HDAd-CAG-LacZ, passage 6 for HDAd-CAG-hyPB, and passage 3 for HDAd-CAG-hyPB-VAI. Thus, crude viral lysate from these passages were used to coinfect a single 150 mm dish of producer cells along with HV. The resulting crude viral lysate from this single 150 mm dish was used to coinfect 2 l of producer cells along with HV and the HDAd was subsequently purified by CsCl ultracentrifugation.

integration, respectively, compared to wildtype.[13] We attempted to produce a HDAd expressing hyPBase from the strong CAG promoter (HDAd-CAG-hyPB) using our well-established protocol,[14,15] which entails serial coinfections (called passages) of the producer cells with the HDAd and the HV to increase the HDAd titer, followed by identification of the earliest passage containing peak HDAd titer which would then be used to initiate large-scale vector production. To determine the passages which contain peak HDAd titers, total cellular DNA was extracted from the producer cells at each serial passage, and examined by agarose gel electrophoresis after ApaLI digestion. Serial passages with peak HDAd titers can be identified by the visible presence of HDAd-specific bands in the presence of the background cellular DNA smear, and is typically first attained at serial passage 2 or 3 (refs. [14,15]). As expected, this was indeed the case for the control vector, HDAd-CAG-LacZ, amplified in parallel with HDAd-CAG-hyPB, in which maximum titer was first reached at serial passage 2 (Figure 1a). In contrast, for the four independent amplifications of HDAd-CAG-hyPB, vector-specific bands were not visible until serial passage 5 or 6 (Figure 1b–e).

According to our established protocol, large-scale HDAd production is initiated using the earliest passage containing the highest intensity vector-specific band based on agarose gel analyses.[14,15] Thus, the earliest passage to contain maximum vector titer was passage 2 for the control HDAd-CAG-LacZ (Figure 1a), and passage 6 for the four amplifications of HDAd-CAG-hyPB (Figure 1b–e). Accordingly, crude viral lysate from passage 2 for HDAd-CAG-LacZ and crude viral lysate from passage 6 for HDAd-hyPB were used to coinfect a single 150 mm dish of producer cells along with HV to initiate large-scale vector production (Figure 2). The resulting crude viral lysates from this 150 mm dish was used to coinfect 2 l

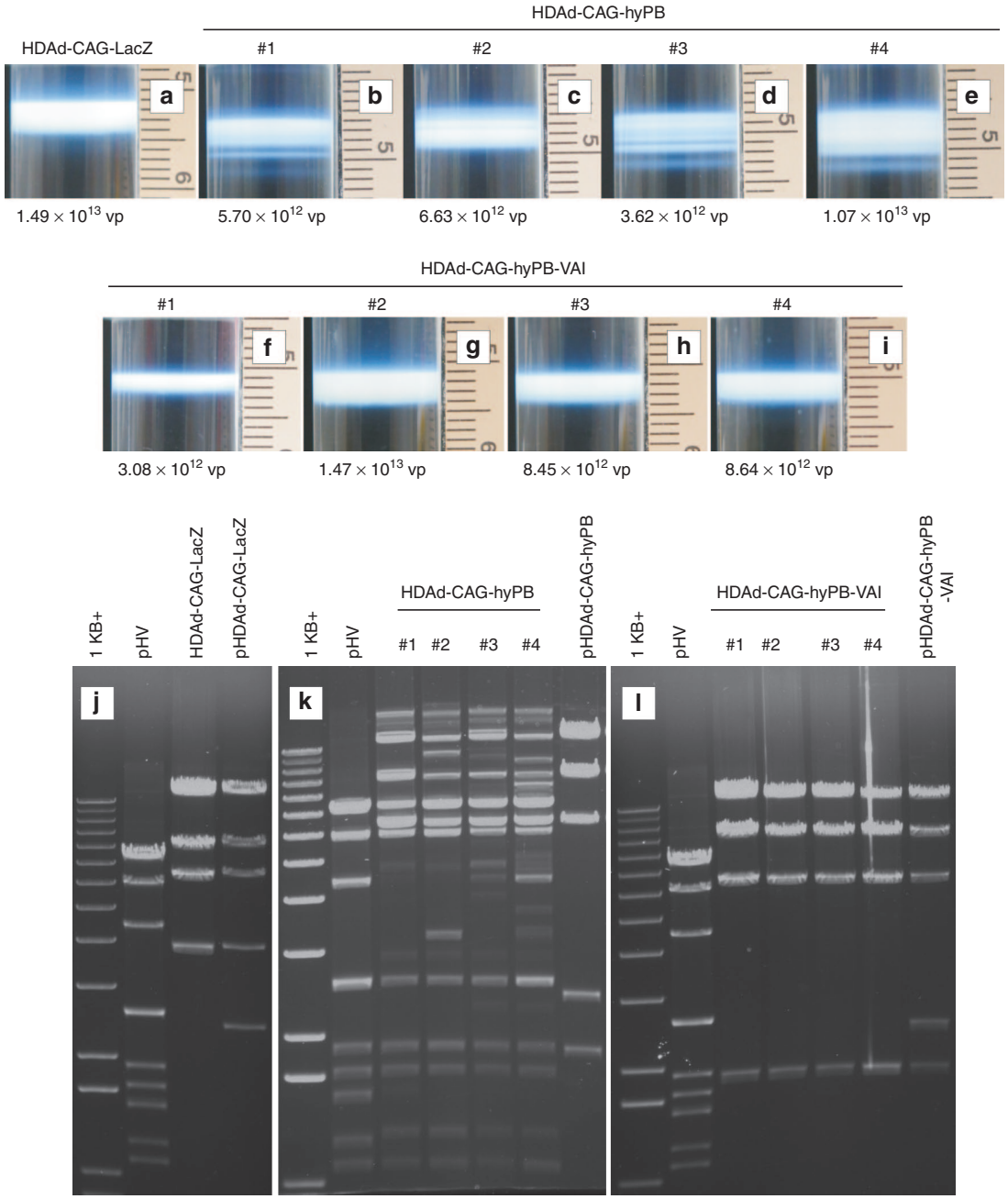


**Figure 3** Appearance of virus band in CsCl gradient and vector genomic structure. (**a–i**) The first continuous CsCl gradient for the indicated vector preparation is shown. (**j–l**) The result of restriction analyses of virion DNA extracted from the indicated vector preparation is shown. The lanes labeled pHV contain HV plasmid digested with ApaLI and PacI to serve as a control for HV-specific bands. The lanes labelled pHDAd-CAG-LacZ, pHDAd-CAG-hyPB, and pHDAd-CAG-hyPB-VAI contain the plasmid form of the indicated HDAd digested with ApaLI and PmeI to serve as a control for the HDAd-specific bands. Total viral particles (vp) obtained from each preparation is indicated.

of producer cells along with HV for large-scale production, and the vectors were purified by CsCl centrifugation (Figure 2). Following CsCl ultracentrifugation, a single band was observed in the gradient for HDAd-CAG-LacZ as expected (Figure 3a). In contrast, multiple bands were observed in the CsCl gradient for the four preparations of HDAd-CAG-hyPB (Figure 3b–e), which is indicative of genomic rearrangement of the HDAd and/or HV. To verify genomic structure, DNA was extracted from the virions obtained from the CsCl gradients, digested with ApaLI and analyzed by agarose gel electrophoresis. As expected, the DNA pattern of HDAd-CAG-LacZ was identical to the plasmid from which it was derived (except for the expected absence of the 2.5 kb fragment containing the bacterial plasmid DNA) indicating no DNA rearrangements (Figure 3j). In contrast, the four preparations of HDAd-CAG-hyPB revealed different DNA patterns with some bands corresponding to the HDAd and some to the HV, as well as novel bands not expected of either, all of which is consistent with a mixed population of various rearranged HDAd and HV DNA (Figure 3k). Because these preparations are useless for their intended purpose, their precise DNA rearrangements were not investigated further.

Given that HDAd-CAG-hyPB and HDAd-CAG-LacZ are identical except for the coding sequence in the expression cassette, it was

reasonable to assume that expression of hyPBase was responsible for the delay in reaching peak titers during vector amplification and HDAd and HV genome rearrangements. Therefore, we reasoned that downregulating hyPBase expression during vector amplification would result in successful vector production. The strategy to accomplish this is presented in Figure 4. Within producer cells coinfected with HV and HDAd, VA RNAI is expressed from the HV which is processed into mivaRNAs by Dicer and incorporated into the RNA-induced silencing complex and, importantly, this has been shown to downregulate expression cassettes bearing the complementary mivaRNAI sequence in the 3′UTR.[7,9–11] Thus, downregulating transgene expression from the HDAd should be achievable during vector production by simply inserting the mivaRNAI target sequence into the 3′UTR of the HDAd's expression cassette. Conversely, once the HDAd is purified, this modification would not inhibit transgene expression by the HDAd in the transduced target cell because of the absence of the HV.

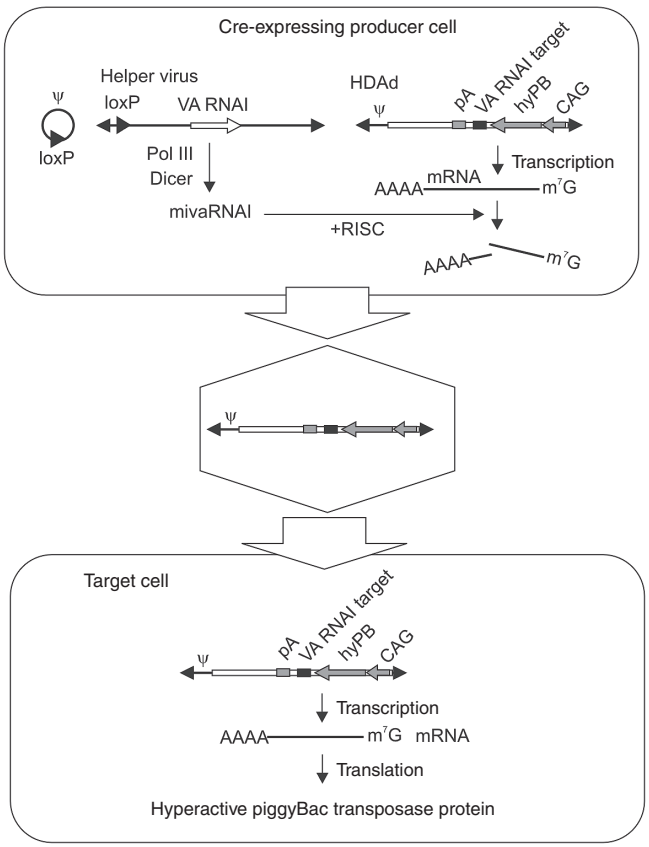


**Figure 4** Strategy to downregulate transgene expression from HDAd during vector production. VA RNAI is expressed from the HV, which is processed into mivaRNAI by Dicer and incorporated into the RNA-induced silencing complex (RISC) and can cleave mRNA expressed from the HDAd which contains the mivaRNAI target sequence in the 3′ UTR. The HV genome cannot be packaged into virions because of excision of the floxed packaging signal (Ψ) by Cre. Thus, following HDAd purification, transduction of target cells result in unimpeded transgene expression from the HDAd due to the absence of the HV.

To evaluate this strategy, HDAd-CAG-hyPB-VAI was created which bears, within the 3′ UTR, nucleotides 119 to 159 from VA RNAI within which resides the mivaRNAI target sequence (Figure 5). In four independent amplifications of HDAd-CAG-hyPB-VAI, peak vector titers were first reached by passage 2 or 3 (Figure 1f–i). For all four amplifications, passage 3 was chosen to initiate large-scale vector production (Figure 2), and in all four cases, a single virus band was obtained in the CsCl gradient (Figure 3f–i). Restriction analysis of the DNA extracted from these four vector preparations revealed the expected pattern, indistinguishable from the plasmid from which the vector was derived (except for the expected absence of the 2.5 kb fragment containing the bacterial plasmid DNA) indicating the absence of DNA rearrangements (Figure 3l).

To confirm that purified HDAd-CAG-hyPB-VAI expresses functional hyPBase, human-induced pluriopotent stem (iPS) cells[16] were coinfected with HDAd-CAG-hyPB-VAI and HDAd-PB-TR. HDAd-PB-TR contains a 2.3 kb segment of DNA flanked by PB terminal repeats (TRs) and is thus excisable in the presence of hyPBase (Figure 6a). As controls, iPS cells were also infected with each vector alone, or mock infected. The next day, total DNA was extracted from the treated cells and subjected to polymerase chain reaction (PCR) analyses. In the absence of hyPBase-mediated excision, a 4.1 kb PCR product is expected, and this is converted to a 1.8 kb PCR product following hyPBase-mediated excision (Figure 6a). The plasmid pHDAd-PB-TR (used to make HDAd-PB-TR), was included in the PCR assay as a control and, as expected, yielded only the unexcised 4.1 kb PCR product (Figure 6b, lane 6). As expected, infection with HDAd-PB-TR alone does not result in excision as evident by the presence of the 4.1 kb PCR product and the absence of the 1.8 kb PCR product (Figure 6b, lane 4). In contrast, the 1.8 kb PCR product, indicative of hyPBase-mediated excision, is only present following coinfection of cells with HDAd-CAG-hyPB-VAI and HDAd-PB-TR (Figure 6b, lane 2). However, the unexcised 4.1 kb PCR product remains visible following coinfection with HDAd-CAG-hyPB-VAI and HDAd-PB-TR (Figure 6b, lane 2) and this may be due to inaccessibility of a fraction of the HDAd-PB-TR genomes to hyPBase (such as some genomes remaining encapsidated) and/or that hyPBase-mediated excision is not 100% efficient and/or some cells were infected with HDAd-PB-TR only. Nevertheless, these results confirm that HDAd-CAG-hyPB-VAI does indeed express functional hyPBase. Subsequently, we have used

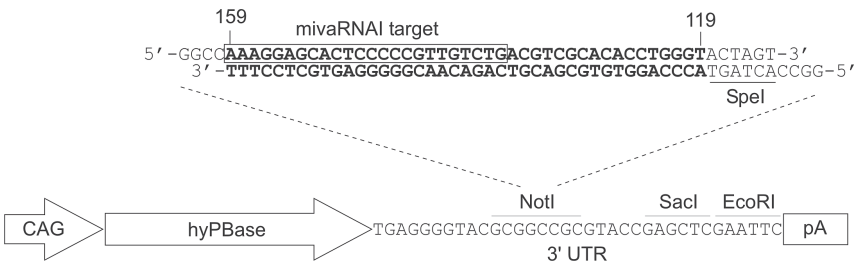


**Figure 5** Modification of hyPBase expression cassette to permit downregulation by the HV. A sequence corresponding to nucleotide 119 to 159 from VA RNAI is inserted into the 3′ UTR of the HDAd expression cassette. The mivaRNAI target sequence is boxed. The 3′ UTR is part of the multiple cloning site of the expression plasmid.

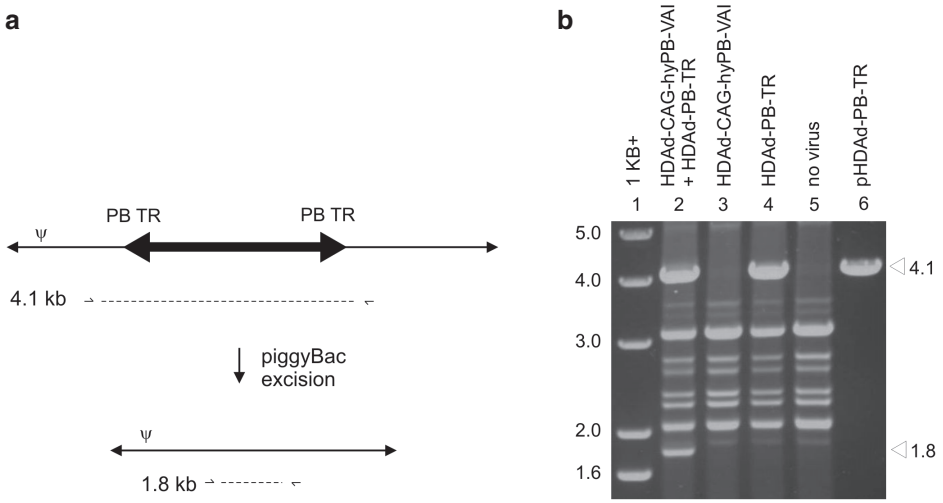


**Figure 6** HDAd-CAG-hyPB-VAI expresses functional hyPBase. (**a**) HDAd-PB-TR contains a 2.3 kb segment of DNA flanked by PB TRs. hyPBase-mediated excision of this DNA segment converts a 4.1 kb polymerase chain reaction (PCR) product to a 1.8 kb PCR product. (**b**) PCR analyses of DNA extracted from human iPS cells infected with the indicated vectors. Lane 5 contains the PCR product obtained from uninfected human iPS cells to provide a control for non-specific amplification products. Lane 6 contains the PCR product from the plasmid used to make HDAd-PB-TR to provide a control for the 4.1 kb unexcised PCR product.

HDAd-CAG-hyPB-VAI to successfully excise DNA segments flanked by PB TRs in other applications (not shown).

## DISCUSSION

During production, the HDAd genome, along with its transgene expression cassette, is replicated to very high copy numbers in the producer cell. The extraordinarily high transgene copy numbers, exacerbated by the use of strong promoter/enhancers, result in very high quantities of transgene product in the producer cells during vector production. At such high amounts, a transgene product that is otherwise benign, may have toxic effects on the producer cells and this could lead to a selection for rearranged vectors with no or reduced transgene expression. HDAd expressing hyPBase from the strong CAG promoter is an example of such a vector; hyBPase is not toxic to mammalian cells[13] but our repeated attempts to produce this vector were unsuccessful resulting in HDAd and HV genome rearrangements.

We have overcome this obstacle by exploiting the fact that the HV expresses VA RNAI, a short noncoding RNA that is processed in functional miRNAs, called mivaRNA, by the endogenous cellular RNAi pathway in the producer cells to downregulate transgene expression from the HDAd. This was accomplished by simply inserting the mivaRNAI target sequence into the 3′ UTR of the HDAd's expression cassette. This simple modification allowed for repeated and robust production of an HDAd expressing the functional hyPBase from the strong CAG promoter. This strategy is straightforward and universal; it does not require the use of a special producer cell line, or drugs to suppress or induce transgene expression, and no special DNA expression control elements need be included in the expression cassette. Once the HDAd is produced and purified, transduction of the target cell results in unimpeded transgene expression from the HDAd because of the absence of the HV.

Recently, Saydaminova *et al*.[17] reported a miRNA-mediated method of downregulating transgene expression from a HDAd during its production. In this case, by miRNA expression profiling, Saydaminova *et al*. identified two endogenous cellular miRNAs, hsa-miR183-5p and hsa-miR218-5p, that were strongly expressed in the producer 293-Cre cells but not in their intended human CD34+ target cells. Thus by inserting the target sequence for hsa-miR183-5p and hsa-miR218-5p into the 3′ UTR of the expression cassette, transgene expression was suppressed during vector production by hsa-miR183-5p and hsa-miR218-5p present in the 293-Cre producer cells. However, transgene expression was unimpeded following HDAd transduction of CD34+ cells due to the absence of hsa-miR183-5p and hsa-miR218-5p in these cells. However, this strategy would be ineffective for target cells, unlike CD34+ cells, that expressed either hsa-miR183-5p or hsa-miR218-5p.

In summary, we have developed a simple and universal strategy to downregulate transgene expression from HDAd during vector production. This permits production of HDAd that otherwise could not be produced because of transgene product-mediated cellular toxicity. Indeed, we have subsequently used this strategy to produce other recalcitrant HDAds which we could not previously produce despite multiple attempts (not shown).

## MATERIALS AND METHODS

An expression cassette containing the CAG promoter, hyPBase coding sequence,[13] and the SV40 polyadenylation signal was inserted into the AscI site of the HDAd genomic plasmid pΔ28E4 (ref. [18]) and the resulting plasmid was used to produce HDAd-CAG-hyPB as described below. The VA RNAI target sequence, created by annealing two oligonucleotide (sequence shown in Figure 5), was inserted into the NotI site in the 3′ UTR of hyPBase expression cassette which was then inserted into the AscI site of pΔ28E4 and the resulting plasmid was used to produce HDAd-CAG-hyPB-VAI as described below. The expression cassette in HDAd-CAG-LacZ is identical to the one in HDAd-CAG-hyPB except that the hyPB coding sequence was replaced with the *Escherichia coli* β-galactosidase coding sequence.

Amplification of HDAd was performed as described in detail elsewhere.[14,15] Briefly, 20 μg of the plasmid form of the HDAd was digested with PmeI and transfected into a confluent 60 mm dish of 116 cells[14] by calcium phosphate co-precipitation (Promega, Madison, WI) and then the cells were infected with the HV AdNG163 (ref. [19]) at an MOI of 2,000 vp/cell (serial passage 0). The HDAd titer was increased by serial coinfections as follows; for each serial coinfection (called a passage), a confluent 60 mm dish of 116 cells was coinfected with HV (200 vp/cell) and 20% of the crude viral lysate containing the HDAd from the previous passage. Total DNA was extracted from each serial passage, digested with ApaLI, and visualized by ethidium bromide staining following agarose gel electrophoresis. Serial passages containing peak titers of HDAd were identified by the visible presence of HDAd-specific bands in the agarose gel. The earliest serial passage containing the most intensely visible HDAd-specific bands was used to initial large-scale vector production as follows; 20% of the crude viral

lysate from the aforementioned passage was used to coinfect a single 150 mm dish of 116 cells along with AdNG163. 48 hours later, 100% of the crude viral lysate from this single 150 mm dish was used to coinfect 2 l of 116 cells ($1 \times 10^9$ cells total) along with AdNG163 at an MOI of 200 vp/cell. HDAd was purified from the coinfected 116 cells 48 later by triple CsCl ultracentrifugation; one step gradient followed by two continuous gradients.

hyPBase-mediated DNA excision was analyzed as follows; feeder free human iPS cells[16] were maintained in mTeSR 1 (STEMCELL Technologies, Vancouver, Canada) on Matrigel (Corning, Tewksbury, MA) coated plates. The iPS cells were infected as follows: $2 \times 10^6$ cells were resuspended in 1 ml mTeSR 1 supplemented with Y27632 (Reagents Direct, Encinitas, CA) to 10 µmol/l in a 1.5 ml microfuge tube and infected with HDAd at an MOI of 350 vp/cell for 1 hour at 37 °C with gentle rocking. Following infection, cells were washed twice with 1 ml mTeSR 1 supplemented with Y27632 to 10 µmol/l and plated in a single Matrigel coated well of a six-well plate in mTeSR 1 supplemented with Y27632 to 10 µmol/l. The next day, DNA was extracted from the infected cells for PCR analysis. PCR was performed with PrimeStar GXL (Takara-Clonetech, Mountain View, CA) using primers 5′ ctcagttttcctggattatgcctggcacc and 5′ gcctgaccaacatggagaaaccccatctc. Thermocycling conditions were as follows; 1 min at 94°C, followed by 30 cycles of 98 °C for 10 seconds and 72 °C for 10 minutes, and a final extension of 10 minutes at 72 °C.

## CONFLICT OF INTEREST

The authors declared no conflict of interest.

## ACKNOWLEDGMENT

This work was support by internal Baylor College of Medicine funds. The iPS cells used in this study were a generous gift from Drs. Ana Crane and Brian Davis (University of Texas).

## REFERENCES

1. Brunetti-Pierri, N and Ng, P (2015). Helper-dependent adenoviral vectors for gene therapy. In: Templeton NS (ed.). *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*, 4th edn. CRC Press: Boca Raton, FL. pp. 47–84.
2. Parks, RJ, Chen, L, Anton, M, Sankar, U, Rudnicki, MA and Graham, FL (1996). A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci USA* **93**: 13565–13570.
3. Kelly, TJ Jr (1984). Adenovirus DNA replication. In: Ginsberg HS (ed.). *The Adenovirus*. Plenum Press: New York. pp. 271–308.
4. Mathews, MB (1995). Structure, function, and evolution of adenovirus virus-associated RNAs. *Curr Top Microbiol Immunol* **199 (Pt 2)**: 173–187.
5. Carnero, E, Sutherland, JD and Fortes, P (2011). Adenovirus and miRNAs. *Biochim Biophys Acta* **1809**: 660–667.
6. Andersson, MG, Haasnoot, PC, Xu, N, Berenjian, S, Berkhout, B and Akusjärvi, G (2005). Suppression of RNA interference by adenovirus virus-associated RNA. *J Virol* **79**: 9556–9565.
7. Aparicio, O, Razquin, N, Zaratiegui, M, Narvaiza, I and Fortes, P (2006). Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production. *J Virol* **80**: 1376–1384.
8. Xu, N, Segerman, B, Zhou, X and Akusjärvi, G (2007). Adenovirus virus-associated RNAII-derived small RNAs are efficiently incorporated into the rna-induced silencing complex and associate with polyribosomes. *J Virol* **81**: 10540–10549.
9. Sano, M, Kato, Y and Taira, K (2006). Sequence-specific interference by small RNAs derived from adenovirus VAI RNA. *FEBS Lett* **580**: 1553–1564.
10. Aparicio, O, Carnero, E, Abad, X, Razquin, N, Guruceaga, E, Segura, V *et al.* (2010). Adenovirus VA RNA-derived miRNAs target cellular genes involved in cell growth, gene expression and DNA repair. *Nucleic Acids Res* **38**: 750–763.
11. Bellutti, F, Kauer, M, Kneidinger, D, Lion, T and Klein, R (2015). Identification of RISC-associated adenoviral microRNAs, a subset of their direct targets, and global changes in the targetome upon lytic adenovirus 5 infection. *J Virol* **89**: 1608–1627.
12. Cary, LC, Goebel, M, Corsaro, BG, Wang, HG, Rosen, E and Fraser, MJ (1989). Transposon mutagenesis of baculoviruses: analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology* **172**: 156–169.
13. Yusa, K, Zhou, L, Li, MA, Bradley, A and Craig, NL (2011). A hyperactive piggyBac transposase for mammalian applications. *Proc Natl Acad Sci USA* **108**: 1531–1536.
14. Palmer, D and Ng, P (2003). Improved system for helper-dependent adenoviral vector production. *Mol Ther* **8**: 846–852.
15. Palmer, DJ and Ng, P (2008). Methods for the production of helper-dependent adenoviral vectors. *Methods Mol Biol* **433**: 33–53.
16. Crane, AM, Kramer, P, Bui, JH, Chung, WJ, Li, XS, Gonzalez-Garay, ML *et al.* (2015). Targeted correction and restored function of the CFTR gene in cystic fibrosis induced pluripotent stem cells. *Stem Cell Reports* **4**: 569–577.
17. Saydaminova, K, Ye, X, Wang, H, Richter, M, Ho, M, Chen, H *et al.* (2015). Efficient genome editing in hematopoietic stem cells with helper-dependent Ad5/35 vectors expressing site-specific endonucleases under microRNA regulation. *Mol Ther Methods Clin Dev* **1**: 14057.
18. Toietta, G, Pastore, L, Cerullo, V, Finegold, M, Beaudet, AL and Lee, B (2002). Generation of helper-dependent adenoviral vectors by homologous recombination. *Mol Ther* **5**: 204–210.
19. Palmer, DJ and Ng, P (2004). Physical and infectious titers of helper-dependent adenoviral vectors: a method of direct comparison to the adenovirus reference material. *Mol Ther* **10**: 792–798.