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.

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INTRODUCTION

Helper-dependent adenoviral vectors (HDAd) 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 cell 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 HDAd 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, 10⁵ to 10⁶ 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 HDAd.

Adenovirus, including the HV, expressed two RNA polymerase III-dependent noncoding RNAs, called virus-associated (VA) RNAI and VA RNAII.⁴ 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 (10⁴ molecules of VA RNAI/cell and 10⁵ 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.⁵⁻⁶ 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.⁷⁻¹¹ 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 HDAd 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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We attempted to produce a HDAd expressing hyPBase from the strong CAG promoter (HDAd-CAG-hyPB) using our well-established protocol, 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. 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 2 l 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 of producer cells along with HV and the HDAd was subsequently purified by CsCl ultracentrifugation.
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.

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 the purification of HDAd-CAG-hyPB-VAI expressing functional hyPBase, human-induced pluripotent stem (iPS) cells16 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 pHAd-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 coinfec- tion 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 coinfec- tion 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
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 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 mivaRNA 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. 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, and the SV40 polyadenylation signal was inserted into the Ascl 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 sequences (shown in Figure 5), was inserted into the Noti site in the 3′ UTR of hyPBase expression cassette which was then inserted into the Ascl site of pΔ28E4 and the resulting plasmid was used to produce HDAd-CAG-hyPB-VAl 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. Briefly, 20 μg of the plasmid form of the HDAd was digested with Pmel and transfected into a confluent 60 mm dish of 116 cells by calcium phosphate co-precipitation (Promega, Madison, WI) and then the cells were infected with the HV AdΔNG163 (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 RNA was extracted from each serial passage, digested with ApaLI, and visualized by ethidium bromide staining following agarose gel electrophoresis. Serial passages containing peak titer 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...
lysat 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 × 10⁶ cells total) along with AdNG163 at an MOI of 200 vp/cell. HDAd was purified from the coinfected 116 cells 48 hours later by triple CsCl ultracentrifugation; one step gradient followed by two continuous gradients.

hyPBase-mediated DNA excision was analyzed as follows; feeder free human iPSCs 19 were maintained in mTeSR 1 (STEMCELL Technologies, Vancouver, Canada) on Matrigel (Corning, Tewksbury, MA) coated plates. The iPSCs were infected as follows: 2 × 10⁶ 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’ ctgatttctctggatatctgcctggacc and 5’ gcctgaccaaatatggagaaaccccatctc. 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.

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