Production of CRISPR/Cas9-Mediated Self-Cleaving Helper-Dependent Adenoviruses

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Prolonged expression of CRISPR/Cas9 raises concerns about off-target cleavage, cytotoxicity, and immune responses. To address these issues, we have developed a system to produce helper-dependent adenoviruses that express CRISPR/Cas9 to direct cleavage of the vectors’ own genome after transduction of target cells. To prevent self-cleavage during vector production, it was necessary to downregulate Cas9 mRNA as well as inhibit Cas9 protein activity. Cas9 mRNA downregulation was achieved by inserting the target sequences for the helper-virus-encoded miRNA, mivaRNAi, and producer-cell-encoded miRNAs, hsa-miR183-5p, and hsa-miR218-5p, into the 3’ UTR of the HDAd-encoded Cas9 expression cassette. Cas9 protein activity was inhibited by expressing anti-CRISPR proteins AcrIIA2 and AcrAI4 from both the producer cells and the helper virus. After purification, these helper-dependent adenoviruses will perform CRISPR/Cas9-mediated self-cleavage in the transduced target cells, thereby limiting the duration of Cas9 expression and thus represent an important platform for improving the safety of gene editing by CRISPR/Cas9.

INTRODUCTION
CRISPR/Cas9 has revolutionized genome editing because of its efficiency and ease of use. However, the prolonged expression of Cas9 in target cells raises important safety concerns regarding off-target cleavage, cytotoxicity, and immune responses.1–3 To maximize safety, Cas9 expression should be transient, ceasing after the desired DNA double-strand break has been accomplished. In this study, we have achieved this by developing a system for producing helper-dependent adenoviruses (HDAds) that express Streptococcus pyogenes CRISPR/Cas9, which directs self-cleavage of the vectors’ own genome following transduction of target cells. HDAds have no viral genes and are excellent 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, they do not integrate into the host genome, and they provide long-term transgene expression with reduced toxicity.4 Thus, this system can be used to improve the safety of CRISPR/Cas9 gene editing, and its utility and implications are discussed.

RESULTS
Producing a CRISPR/Cas9-mediated self-cleaving HDAd was not our original objective. Instead, we were originally interested in producing an all-in-one HDAd for homology-directed repair (HDR) by gene targeting (Figure 1). This is because we38 and others7–18 have shown that HDAds can efficiently deliver donor DNA into cells to achieve gene targeting by spontaneous homologous recombination. We hypothesized that the efficiency of gene targeting by HDAd could be further improved by incorporating CRISPR/Cas9 to introduce a recombinogenic double-strand break (DSB) at the chromosomal target (Figure 1). Thus, we constructed an all-in-one HDAd (called HD+donor+sgRNA+Cas9) that contained the donor DNA and expressed Cas9 and single-guide RNA (sgRNA) to target the chromosome (Figures 1 and 2A). To avoid self-cleavage of the all-in-one HDAd during vector production, the canonical 5′ CA 3′ protospacer adjacent motif (PAM) in the donor was mutated to 5′ TCA 3′ (Figures 1 and 3A). However, HD+donor+sgRNA+Cas9 could not be produced with our standard 116 producer cells19 and our standard helper virus AdNG163;20 multiple viral bands were present after cesium chloride (CsCl) ultracentrifugation (Figure 3C) and restriction analysis of the virion DNA (Figure 4, lane 3) revealed that it did not match the expected pattern of the plasmid from which it was derived (Figure 4, lane 4). These results indicate that the vector had undergone genomic rearrangement. Sequencing of the HD+donor+sgRNA+Cas9 virion DNA revealed site-specific Cas9 cleavage (Figure 3C) compared to the expected sequence of its parental plasmid (Figure 3B); the sequence is interpretable and as expected up to the Cas9 cleavage site, but afterward it becomes uninterpretable, and this can be attributed to the formation of insertions and deletions (indels) due to non-homologous end joining (NHEJ) as a consequence of site-specific Cas9 cleavage (Figure 3C). Together, these results indicate that during production of HD+donor+sgRNA+Cas9, CRISPR/Cas9-mediated site-specific self-cleavage occurred despite mutating the PAM in the donor from 5′ CA 3′ to 5′ TCA 3′. That CRISPR/Cas9 can cleave at sgRNA target sites without a canonical 5′ CCN 3′ PAM has been observed, albeit at much reduced efficiencies.21–23 And this is likely exacerbated during vector production because the HDAd genome containing the Cas9 expression cassette is replicated to 105 to 106 copies,24 which results in an enormous amount of Cas9 in the producer cells leading to efficient site-specific cleavage of the donor DNA, even with the 5′ CA 3′ to 5′ TCA 3′ PAM.
mutation. It was our desire to produce this all-in-one HDAd without genomic rearrangement that led to the successful development of a system to produce a CRISPR/Cas9-mediating self-cleaving HDAd as detailed below.

One possible strategy to overcome CRISPR/Cas9-mediated self-cleavage is to downregulate Cas9 mRNA during vector production by miRNA. Two such strategies have been described to downregulate transgene expression from the HDAd during its production. The first strategy makes use of two microRNAs (miRNAs), hsa-miR183-5p and hsa-miR218-5p, expressed exclusively by the HDAd producer cells. The second strategy makes use of a miRNA expressed by the helper virus called mivaRNAI. Because these miRNAs are only present during vector production, transgene expression from the HDAd is unimpeded in the transduced target cells. We utilized both strategies by inserting the target sequence for these three miRNAs in the 3′ UTR of the Cas9 expressed to create HD-donor-sgRNA-Cas9miR (Figure 2B) and attempted to produce this vector in 116 cells with AdNG163. Unfortunately, multiple viral bands were observed following CsCl ultracentrifugation, indicating vector genome rearrangement (Figure 3D).
Encouragingly, the number of virus bands was reduced compared to that observed for HD+donor+sgRNA+Cas9, suggesting the miRNA strategy reduced but did not eliminate CRISPR/Cas9-mediated self-cleavage. Restriction analysis of the HD+donor+sgRNA+Cas9miR virion DNA (Figure 4, lane 5) revealed that it did not match the expected pattern of the plasmid from which it was derived (Figure 4, lane 6). These results indicate that the vector had undergone genomic rearrangement. Sequence analyses of the HD+donor+sgRNA+Cas9miR virion DNA revealed site-specific Cas9 cleavage (Figure 3D) compared to the expected sequence of its parental plasmid (Figure 3B); the sequence is as expected up to the Cas9 cleavage site, after which it becomes uninterpretable because of indel formation due to NHEJ (Figure 3D). Again, it was encouraging that the sequence of HD+donor+sgRNA+Cas9miR was more “interpretable” than that of HD+donor+sgRNA+Cas9 (Figure 3C), again indicating that the miRNA strategy was able to reduce but not eliminate CRISPR/Cas9-mediated self-cleavage during HDAd production even with the PAM mutated from 5’ CCA 3’ to 5’ TCA 3’.

Encouragingly, production of HD+donor+sgRNA+Cas9miR in 116 cells with AdNG163Acr yielded a single virus band following CsCl ultracentrifugation (Figure 3E). Also encouraging was that restriction analysis of the HD+donor+sgRNA+Cas9miR virion DNA (Figure 4, lane 9) revealed a pattern that was indistinguishable to the plasmid from which it was derived (Figure 4, lane 10). Finally, sequence analysis of HD+donor+sgRNA+Cas9miR virion DNA revealed the expected sequence with no evidence of indel formation at the Cas9 cleavage site (Figure 3E). Taken together, these results indicate that the all-in-one HD+donor+sgRNA+Cas9miR could be successfully produced in 116 cells with AdNG163Acr.

Although the combination of miRNA and anti-CRISPR proteins expressed from the helper virus permitted for the production of HD+donor+sgRNA+Cas9miR, it should be emphasized that the CRISPR/Cas9 target in the vector contained the 5’ CCA 3’ to 5’ TCA 3’ PAM mutation. Would this same strategy permit successful production of CRISPR/Cas9-mediated self-cleaving HDAd with a bona fide 5’ CCN 3’ PAM? The significance of producing such a
vector will be addressed in the Discussion. To answer this question, we created two new HDAds designed to self-cleave their own reporter LacZ transgene, called HD+LacZ+sgRNA633+Cas9miR and HD+LacZ+sgRNA86+Cas9miR (Figures 2D and 2E), and proceeded to produce them in 116 cells with AdNG163Acr. HD+LacZ+sgRNA633+Cas9miR and HD+LacZ+sgRNA86+Cas9miR differ in the sgRNA sequence used to self-cleave the LacZ transgene, and both contain targets for mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p in the 3′ UTR their Cas9 expression cassettes. As shown in Figures 5A and 5D, only a single virus band was visible following CsCl ultracentrifugation. Restriction analyses of virion DNA from both vectors (Figure 4, lane 14 for HD+LacZ+sgRNA633+Cas9miR and lane 17 for HD+LacZ+sgRNA86+Cas9miR) revealed a pattern indistinguishable from their parental plasmids (Figure 4, lanes 13 and 19, respectively), and with additional faint bands consistent with the AdNG163Acr (see Figure 4, lane 16). Sequence analyses of the virion DNA revealed indel formation precisely at the site of Cas9 cleavage for both vectors (Figures 5A and 5D). Therefore, the method that successfully produced HD+donor+sgRNA+Cas9miR (bearing 5′ CCA 3′ to 5′ TCA 3′ PAM mutation) by preventing CRISPR/Cas9-mediated self-cleavage, did not permit production of HD+LacZ+sgRNA633+Cas9miR and HD+LacZ+sgRNA86+Cas9miR, indicating that suppression of CRISPR/Cas9 by mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p, and AcrIIA4 and AcrIIA2 when expressed from the helper virus, were not enough to prevent cleavage in the presence of a bona fide 5′ CCN 3′ PAM.

Therefore, we sought next to further inhibit Cas9 activity during vector production by engineering the producer cells to constitutively express AcrIIA4 and AcrIIA2. This was accomplished by stably transfecting the producer cell line 116 with the plasmid pNG191 (Figure 2F), and G418-resistant colonies were screened by their ability to amplify HD+LacZ+sgRNA633+Cas9miR using the ANG163Acr. Several G418-resistant clones were able to amplify HD+LacZ+sgRNA633+Cas9miR, and one such cell line, 116Acr3, was chosen for large-scale production of HD+LacZ+sgRNA633+Cas9miR and HD+LacZ+sgRNA86+Cas9miR using AdNG163Acr. Following CsCl ultracentrifugation, only a single virus band was visible for of HD+LacZ+sgRNA633+Cas9miR (Figure 5B) and HD+LacZ+sgRNA86+Cas9miR (Figure 5E). Restriction analyses of virion DNA from both vectors (Figure 4, lane 15 for HD+LacZ+sgRNA633+Cas9miR and lane 18 for HD+LacZ+sgRNA86+Cas9miR) revealed a pattern indistinguishable from their parental plasmids (Figure 4, lanes 13 and 19, respectively), and with additional faint bands consistent with the AdNG163Acr (see Figure 4, lane 16). Sequence analyses of HD+LacZ+sgRNA633+Cas9miR and HD+LacZ+sgRNA86+Cas9miR virion DNAs revealed the expected sequence with no evidence of indel formation at the site of Cas9 cleavage (Figures 5B and 5E, respectively). Finally, because HD+LacZ+sgRNA633+Cas9miR and HD+LacZ+sgRNA86+Cas9miR contain the reporter LacZ transgene, their infectivity (the proportion of physical viral particles that are infectious expressed as the viral particle to blue-forming unit ratio [vp:BFU]) can be determined by titration.20 HD+LacZ+sgRNA633+Cas9miR and HD+LacZ+sgRNA86+Cas9miR produced in 116Acr3 cells with AdNG163Acr without genome rearrangement (Figures 5B and 5E) had vp:BFU ratios of 24:1 and 18:1, respectively. These ratios are comparable to the parental HD28E4LacZ from which they were derived, which ranged from 12:1 to 22:1,19,20 and all of these ratios are below the FDA recommended ratio of 30:1 for clinical-grade adenoviruses.28 In contrast, HD+LacZ+sgRNA633+Cas9miR and HD+LacZ+sgRNA86+Cas9miR produced in 116Acr cells with AdNG163Acr that showed evidence of indel formation within the LacZ transgene (Figure 5A and 5C) had much higher vp:BFU ratios of 44:1 and 87:1. This higher vp:BFU was expected because a subpopulation of these vectors will have had their LacZ transgene inactivated by CRISPR/Cas9-mediated indel formation.

With a producer cell expressing AcrIIA2 and AcrIIA4 along with a helper virus expressing AcrIIA2 and AcrIIA4, we wondered if down-regulation of Cas9 mRNA by mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p was still required to prevent self-cleavage during vector production. To determine this, we tried to produce HD+LacZ+sgRNA633+Cas9 (which lacks the target sites for mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p; see Figure 2A) in 116Acr3 cells with AdNG163Acr. The results revealed that self-cleavage had
sgRNA633 sequence CCGACGGGTGTACTCGCTCAC

A  HD+LacZ+sgRNA633+Cas9miR produced in 116 cells with AdNG163Acr
Vp:BFU = 44:1

B  HD+LacZ+sgRNA633+Cas9miR produced in 116Acr3 cells with AdNG163Acr
Vp:BFU = 24:1

C  HD+LacZ+sgRNA633+Cas9 produced in 116Acr3 cells with AdNG163Acr
Vp:BFU = 62:1

gRNA86 sequence CCGCAGATAGAGATTCGGATT

D  HD+LacZ+sgRNA86+Cas9miR produced in 116 cells with AdNG163Acr
Vp:BFU = 87:1

E  HD+LacZ+sgRNA86+Cas9miR produced in 116Acr3 cells with AdNG163Acr
Vp:BFU = 18:1

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occurred during vector production; as shown in Figure 5C, more than one viral band was visible following CsCl ultracentrifugation, sequence analyses of the virion DNA reveal indel formation at the Cas9 cleavage site, and titration of the vector revealed a reduction in infectivity with a vp:BFU ratio of 62:1. These results indicate that downregulation of Cas9 mRNA by mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p is necessary to prevent self-cleavage during vector production.

In summary, we were successful in producing CRISPR/Cas9-mediated self-cleavage of the HDAd even when the sgRNA target within the vector possessed a bona fide 5’ CCN 3’ PAM, by exploiting mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p to downregulate Cas9 mRNA and by inhibiting Cas9 protein activity through expression of AcrIIA2 and AcrIIA4 from the producer cells and helper virus. The ability to produce a CRISPR/Cas9-mediated self-cleaving HDAd is significant because long-term Cas9 expression is undesirable due to safety concerns associated with off target cleavage, cytotoxicity, and immune responses. A CRISPR/Cas9-mediated self-cleaving HDAd would solve these problems. For example, the desired gene editing can be performed with a CRISPR/Cas9-mediated self-cleaving HDAd, which also limits the duration of CRISPR/Cas9 expression (Figure 7).

Recently, Li et al. reported toxicity following transduction of human hematopoietic stem cells with HDAd expressing CRISPR/Cas9. This CRISPR/Cas9-mediated toxicity was alleviated by transduction with a second HDAd expressing AcrIIA2 and AcrIIA4. However, the requirement for sequential co-transduction by two HDAd adds cost and complexity and reduces efficiency, and transduced cells continue to express immunogenic Cas9, AcrIIA2, and AcrIIA4. Instead, the CRISPR/Cas9-mediated self-cleaving HDAd described herein represents an improved approach able to accomplish the same objective but without the drawbacks noted. Finally, the strategies described here may be adapted to produce CRISPR/Cas9-mediated self-cleaving vectors based on other viruses.
MATERIALS AND METHODS

Adenoviruses

HD+donor+sgRNA+Cas9 was derived from HD-23.8-CFTRm-PACTk-DTA by replacing the LacZ expression cassette with a Cas9 expression and by changing the PAM in the donor from 5'CCA 3' to 5'TCA 3'. HD+donor+sgRNA+Cas9 was derived from HD+donor+sgRNA+Cas9 by inserting the target sequences for mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p (shown in Figure 2) into the NotI site present in the 3' UTR of Cas9 expression cassette in HD+donor+sgRNA+Cas9. The helper virus AdNG163Acr was derived from AdNG16320 by inserting the AcrIIA4 and AcrIIA2 expression cassette into the unique ClaI sites of the stuffer sequence in the adenoviral E3 region. HD+LacZ+sgRNA663+Cas9miR was constructed by inserting the 5,896-bp AscI fragment containing the Cas9 expression cassette from HD+donor+sgRNA+Cas9 into the unique Ascl site of HDΔ28E4LacZ followed by inserting the sgRNA633 expression cassette into the unique ScI site. HD+LacZ+sgRNA86+Cas9miR was constructed the same way, except the Cas9 expression and by changing the PAM in the donor from 5'CCA 3' to 5'TCA 3'. HD+donor+sgRNA+Cas9 by inserting the target sequences for mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p (shown in Figure 2) into the NotI site present in the 3' UTR of Cas9 expression cassette in HD+donor+sgRNA+Cas9. The helper virus AdNG163Acr was derived from AdNG16320 by inserting the AcrIIA4 and AcrIIA2 expression cassette into the unique ClaI sites of the stuffer sequence in the adenoviral E3 region. hd+LacZ+sgRNA663+Cas9miR was constructed by inserting the 5,896-bp AscI fragment containing the Cas9 expression cassette from HD+donor+sgRNA+Cas9 into the unique Ascl site of HDΔ28E4LacZ followed by inserting the sgRNA633 expression cassette into the unique ScI site. HD+LacZ+sgRNA86+Cas9miR was constructed the same way, except the Cas9 expression cassette in HD+donor+sgRNA+Cas9 was derived from HD-23.8-CFTRm-PACTk-DTA by replacing the LacZ expression cassette with a Cas9 expression and by changing the PAM in the donor from 5'CCA 3' to 5'TCA 3'. HD+donor+sgRNA+Cas9 was derived from HD+donor+sgRNA+Cas9 by inserting the target sequences for mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p (shown in Figure 2) into the NotI site present in the 3' UTR of Cas9 expression cassette in HD+donor+sgRNA+Cas9. The helper virus AdNG163Acr was derived from AdNG16320 by inserting the AcrIIA4 and AcrIIA2 expression cassette into the unique ClaI sites of the stuffer sequence in the adenoviral E3 region. HD+LacZ+sgRNA663+Cas9miR was constructed by inserting the 5,896-bp AscI fragment containing the Cas9 expression cassette from HD+donor+sgRNA+Cas9 into the unique Ascl site of HDΔ28E4LacZ followed by inserting the sgRNA633 expression cassette into the unique ScI site. HD+LacZ+sgRNA86+Cas9miR was constructed the same way, except the Cas9 expression cassette in HD+donor+sgRNA+Cas9 was derived from HD-23.8-CFTRm-PACTk-DTA by replacing the LacZ expression cassette with a Cas9 expression and by changing the PAM in the donor from 5'CCA 3' to 5'TCA 3'. HD+donor+sgRNA+Cas9 was derived from HD+donor+sgRNA+Cas9 by inserting the target sequences for mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p (shown in Figure 2) into the NotI site present in the 3' UTR of Cas9 expression cassette in HD+donor+sgRNA+Cas9. The helper virus AdNG163Acr was derived from AdNG16320 by inserting the AcrIIA4 and AcrIIA2 expression cassette into the unique ClaI sites of the stuffer sequence in the adenoviral E3 region. HD+LacZ+sgRNA663+Cas9miR was constructed by inserting the 5,896-bp AscI fragment containing the Cas9 expression cassette from HD+donor+sgRNA+Cas9 into the unique Ascl site of HDΔ28E4LacZ followed by inserting the sgRNA633 expression cassette into the unique ScI site. HD+LacZ+sgRNA86+Cas9miR was constructed the same way, except the Cas9 expression cassette in HD+donor+sgRNA+Cas9 was derived from HD-23.8-CFTRm-PACTk-DTA by replacing the LacZ expression cassette with a Cas9 expression and by changing the PAM in the donor from 5'CCA 3' to 5'TCA 3'. HD+donor+sgRNA+Cas9 was derived from HD+donor+sgRNA+Cas9 by inserting the target sequences for mivaRNAI, hsa-miR183-5p, and hsa-miR218-5p (shown in Figure 2) into the NotI site present in the 3' UTR of Cas9 expression cassette in HD+donor+sgRNA+Cas9. The helper virus AdNG163Acr was derived from AdNG16320 by inserting the AcrIIA4 and AcrIIA2 expression cassette into the unique ClaI sites of the stuffer sequence in the adenoviral E3 region.

HDAd Producer Cell

The plasmid pNG191 (Figure 2F) contains the expression AcrIIA4 and AcrIIA2 from AdNG163Acr but with a synthetic DNA sequence bearing the neomycin-resistance coding region inserted between AcrIIA2 and the polyadenylation signal. The 116Acr3 producer cell line was generated as follows: plasmid pNG191 was digested with ApaLI, and 2 µg was transfected into 60-mm dishes of 116 cells cultured in 100 µg/mL hygromycin (Sigma, St. Louis, MO, USA). Forty-eight hours later, G418 (InvivoGen, San Diego, CA, USA) was added to the culture media at a concentration of 400 µg/mL. Well-isolated drug-resistant colonies were picked and expanded to assess their ability to amplify HD+LacZ+sgRNA663+Cas9miR as described previously.19

AUTHOR CONTRIBUTIONS

D.J.P, D.L.T., and P.N. conducted the experiments. P.N. designed the experiments and wrote the paper.

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