We describe a strategy to achieve footprintless bi-allelic homology-directed repair (HDR) using helper-dependent adenoviruses (HDAds). This approach utilizes two HDAds to deliver the donor DNA. These two HDAds are identical except for their selectable marker. One expresses the puromycin N-acetyltransferase-herpes simplex virus I thymidine kinase fusion gene (PACTk), while the other expresses the hygromycin phosphotransferase-herpes simplex virus I thymidine kinase fusion gene (HyTk). Therefore, puromycin and hygromycin double resistance can be used to select for targeted HDAd integration into both alleles. Subsequently, piggyBac-mediated excision of both PACTk and HyTk will confer resistance to gancyclovir, resulting in footprintless HDR at both alleles. However, gene-targeting frequency was not high enough to achieve simultaneous targeting at both alleles. Instead, sequential targeting, whereby the two alleles were targeted one at a time, was required in order to achieve bi-allelic HDR with HDAd.

INTRODUCTION
We and others have shown that helper-dependent adenoviruses (HDAds) can efficiently deliver donor DNA to achieve homology-directed repair (HDR) by spontaneous homologous recombination. The HDAd offers a number of advantages as a gene-targeting vector. First, HDR can be achieved without the need to generate an artificial double-strand break (DSB) at the chromosomal target by a designer nuclease like CRISPR/Cas9, thereby eliminating the concerns related to nuclease-mediated off-targeting cleavage, cytotoxicity, and immune responses. Second, because of the HDAds’ tremendous cloning capacity of 37 kb, they can accommodate very long homology arms, and consequently we have shown that multiple genetic alterations up to 22.2 kb apart can be efficiently introduced simultaneously into the chromosomal locus using a single HDAd. This also means that a single HDAd can be used to correct many different mutations from many different individuals. However, HDR at both alleles, so-called bi-allelic gene targeting has not been reported with HDAd. Bi-allelic targeting would be desirable for correcting homozygous dominant mutations or for creating specific isogenic controls. Therefore, in this study, we describe a system to achieve bi-allelic gene targeting with HDAds.

RESULTS
The CF17 induced pluripotent stem cell (iPSC) line was used as a model system to investigate HDAd-mediated bi-allelic targeting in this study. CF17 was derived from a compound heterozygous cystic fibrosis (CF) patient, with one allele bearing the AF508 mutation (a 3-bp deletion resulting in the loss of the phenylalanine at position 508 of the cystic fibrosis transmembrane conductance regulator [CFTR] protein) and the other allele bearing the ΔF508 mutation (a 3-bp deletion resulting in the loss of the isoleucine at position 507 of the CFTR protein). We have previously shown that HDAds can be used to efficiently correct either of these two CF mutations in this iPSC. However, of the 287 total targeted clones analyzed in those studies, none had undergone bi-allelic targeting.

Simultaneous Bi-allelic Targeting
Our first strategy to achieve bi-allelic targeting involved simultaneously co-transducing CF17 cells with two HDAds, named HD-23.8-CFTR-PACTk-DTA and HD-23.8-CFTR-HyTk-DTA (Figure 1). These two HDAds are identical, except one bears the puromycin N-acetyltransferase-herpes simplex virus I thymidine kinase fusion gene (PACTk) to provide positive selection for vector integration by conferring puromycin resistance (puroR) and negative selection for loss of PACTk by conferring sensitivity to gancyclovir (GCVs), while the other HDAd bears the hygromycin phosphotransferase-herpes simplex virus I thymidine kinase fusion gene (HyTk) to provide positive selection for vector integration by conferring hygromycin resistance (hygR) and negative selection for loss of HyTk by conferring sensitivity to gancyclovir (GCVs). Using PACTk and HyTk on different HDAds provides a tool to select for bi-allelic targeted clones by conferring double puroR hygR. A total of six 6-well plates containing 1 × 10⁵ CF17 cells per well were co-transduced with HD-23.8-CFTR-PACTk-DTA and HD-23.8-CFTR-HyTk-DTA at an MOI of 1,000 viral particles (vp) of each vector/cell. 48 h after co-transduction, puromycin and hygromycin were added to the culture media. Unfortunately, no double puroR hygR colonies were obtained, indicating that the frequency of simultaneous bi-allelic targeting was < 3.67 × 10⁻⁸ per cell. Such a low frequency necessitated an alternative strategy to achieve bi-allelic targeting.

Sequential Bi-allelic Targeting
We next turned to a sequential bi-allelic targeting strategy, which involved targeting one allele at a time instead of both at once.
As a first step to determine whether the HDAd had targeted integration into the unmodified allele, genomic DNA was isolated from the puroR hygR colonies, and PCR was used to screen for the presence/absence of the unmodified allele. Targeted HDAd integration into the unmodified allele will result in the loss of the 964-bp PCR product because of the insertion of the HyTk selectable marker between the two PCR primers (Figure 2). 24 of the 39 double puroR and hygR colonies resulting from transduction of clone 27, yielding a puroR hygR frequency of 1.67 × 10−4, and 39 of these were picked for analyses. Similarly, 37 puroR hygR colonies resulted from transduction of clone 27, yielding a puroR hygR frequency of 8.22 × 10−5, and 20 of these were picked for analyses (Table 1).

Footprintless Excision of Selectable Markers

The final step in bi-allelic targeting is excision of PACTk and HyTk, which are flanked by piggyBac (PB) inverted terminal repeats (ITRs) to permit footprintless excision by PB transposase. Two clones (clone 7-12 and clone 27-18, one from each experiment) were selected for PACTk and HyTk excision. This was accomplished by transducing 2 × 106 cells with HDAd-CAG-hyPB-VAI,22 a HDAd expressing the hyperactive PB transposase, at an MOI of 350 vp/mL. The transduced cells were seeded onto 6-well plates at densities of 2 × 105 cells/well, 5 × 104 cells/well, and 2 × 104 cells/well in the absence of puromycin and hygromycin, and 48 h later, gancyclovir was added to the culture media, and the number of gancyclovir-resistant (GCVR)
colonies were enumerated and presented in Table 2. To ascertain if PB-mediated excision of PACTk and HyTk were responsible for GCVR, DNA was extracted from 10 and 22 colonies derived from clones 7-12 and 27-18, respectively, and subjected to southern analyses. PB-mediated excision of either PACTk or HyTk converts the 19-kb and 14.7-kb ApaI fragment back to a 31-kb ApaI fragment (Figure 2) when analyzed with the 5' and 3' probes, respectively, while analyses with the PACTk and HyTk probes are expected to reveal no

Table 1. Gene-Targeting Frequencies at the Unmodified CFTR Locus with HD-23.8-CFTRm-HyTk-DTA

| Cell Line Transduced | Total Cells Transduced | PuroR HygR Colonies | PuroR HygR Frequency | PuroR HygR Clones Analyzed | Correctly Targeted |
|----------------------|------------------------|---------------------|----------------------|---------------------------|-------------------|
| Clone 7a             | 4.5 x 10^6             | 75                  | 1.67 x 10^-4         | 38                        | 24 (63.2%)        |
| Clone 27a            | 4.5 x 10^6             | 37                  | 8.22 x 10^-5         | 20                        | 15 (75%)          |

*Bears targeted integration of HD-23.8-CFTR-PACTk into ΔF508 allele.13
*Bears targeted integration of HD-23.8-CFTR-PACTk into ΔI507 allele.13

Figure 2. Gene targeting at the CFTR locus with HD-23.8-CFTR-HyTk-DTA

A single reciprocal crossover in the right and left homology arms results in integration of the HyTk marker into the CFTR gene, rendering clones hygromycin resistant. PB ITRs flank the HyTk cassette to permit its footprintless excision in the presence of PB transposase. Sizes of the diagnostic ApaI fragments and the locations of the 5' probe, 3' probe, and HyTk probe used for Southern analyses are shown. The positions of PCR primers used to amplify the allele without targeted vector integration is shown. The ΔI507 and ΔF508 mutations are ~0.2 kb from the site of HyTk insertion. The position of the adenoviral packaging signal (i), adenoviral inverted terminal repeat (Ad ITR), and the diphtheria toxin A-fragment gene (DTA)33 are shown for the HDAd. Note that in clones 7 and 27, the other allele (not shown here but is shown in Palmer et al.13) has been targeted by HD-23.8-CFTR-PACTk-DTA.13

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bands. Representative Southern blots are presented in Figure 5, and the results revealed that for the 22 GCV-resistant clones derived from transduction of clone 27-18 with HDAd-CAG-hyPB-VAI, 12 had PACTk and HyTk excised. Likewise, for the 10 GCV clones derived from HDAd-CAG-hyPB-VAI transduction of clone 7-12, five had PACTk and HyTk excised. We do not understand why clones that bore at least one selectable marker escaped gancyclovir counter selection.

For the 17 (12 + 5) clones with excision of both PACTk and HyTk, the next step was to confirm that the ΔF508 and ΔI507 mutations have been corrected in both alleles and that excision of the selectable markers was footprintless. To accomplish this, PCR was used to amplify a 964-bp fragment encompassing these sites (Figure 2) and sequenced. The results revealed that in all cases, only the wild-type sequence was present, indicating that both alleles have been corrected and that excision of PACTk and HyTk was indeed footprintless. Representative sequencing results from two clones, 7-12-1 (derived from clone 7-12) and 27-18-12 (derived from 27-18) are shown in Figures 6 and 7.

In summary, we were unable to achieve simultaneous bi-allelic targeting with HDAd. Instead, we had to sequentially target one allele at a time to achieve footprintless, bi-allelic gene correction.

DISCUSSION
We have found that the frequency of gene targeting by HDAd is not high enough to achieve simultaneous bi-allelic targeting. Consequently, a sequential targeted strategy was used, whereby the two alleles are targeted one at a time. This strategy is greatly facilitated by using two different positive selectable markers, PACTk and HyTk, in the HDAd, because this permits selection for clones with targeted vector integration in both alleles to generate double puromycin and hyromycin resistance. However, having two different positive selectable markers is not absolutely required, because sequential targeting can be accomplished using only one HDAd, but excision would need to be performed after the first targeting so that drug sensitivity is re-established for the second round of targeting. This approach, however, would be more time consuming, requiring an additional selectable marker excision step. As well, one needs to be cognizant of the fact that some of the targeted clones from the second targeting will have had the same allele targeted twice, while the remainder will have both alleles targeted once, and it is only this latter group that is of value. One consideration is that the targeting efficiency of the two alleles may be different based on DNA polymorphisms. However, the HDAd’s long homology arms are able to overcome mismatches to mediate efficiency homologous recombination and negate this possible allele targeting bias. Using a tool like CRISPR/Cas9 in which simultaneous bi-allelic targeting can be achieved would offer an advantage in time savings. However, the tradeoff is that off-target cleavage remains a concern, so time and resources are expended at the backend in this regard. Furthermore, the CRISPR/Cas9-mediated DSB must reside within ~20–50 bp of the desired modification. Thus, if the two modifications are farther apart in the two alleles, then two guide RNAs (gRNAs) will be needed to achieve bi-allelic modification, and
this will increase the chance of off-target cleavage. Also, the requirement that the CRISPR/Cas9-mediated DSBs lie within ~20-50 bp of the modification site can be a significant drawback for bi-allelic HDR, because in some cases, there may be no gRNA and in others, the gRNAs present are suboptimal because they are too inefficient and/or too promiscuous. These problems do not exist with HDAd-mediated bi-allelic targeting, because we have previously shown that modifications spanning large genomic distances of at least 22.4 kb can be simultaneously introduced into the chromosomal target with a single vector due to its long homology arms. Regardless, we present in this study an additional tool and strategies to achieve bi-allelic gene targeting using the HDAd that researchers may employ based on their unique needs and requirements.

**MATERIALS AND METHODS**

**Helper-Dependent Adenoviruses**

HD-23.8-CFTR-HyTk-DTA was derived from HD-23.8-m-PACTk-DTA by replacing the 2,164-bp Sall-ClaI fragment containing the PACTk expression cassette with the 2,677-bp Sall-ClaI fragment containing the HyTk expression cassette from the plasmid pLPBL-13 HyTk. The HyTk coding sequence in pLPBL-13 HyTk was obtained from the 2,115-bp NheI-SalI fragment of RV-L3-HyTk-2L (Addgene plasmid #11684). Further cloning details are available upon request. HDAd-CAG-hyPB-VAI is described elsewhere. HDAds were produced in 116 cells and AdNG163 helper virus as described elsewhere, and titers were determined by absorbance at 260 nm, as described elsewhere.

**Transduction of iPSCs**

CF17, the feeder-free human CF iPSC line used in this study, is described elsewhere and was maintained in mTeSR 1 (STEMCELL Technologies, Vancouver, BC, Canada) on Matrigel (Corning, Tewksbury, MA, USA)-coated plates. Clone 7 was derived from CF17 by targeted HDAd integration into the ΔF508 allele to correct the ΔF508 mutation, as described elsewhere. Clone 27 was derived from CF17 by targeted HDAd integration into the ΔI507 allele to correct the ΔI507 mutation, as described elsewhere. Clones 7 and 27 were maintained in mTeSR 1 (STEMCELL Technologies, Vancouver, BC, Canada) supplemented with puromycin to 0.5 μg/mL (Invitrogen, Carlsbad, CA, USA) on Matrigel (Corning, Tewksbury, MA, USA)-coated plates.

Transduction of iPSCs for gene targeting was performed as follows: iPSCs were seeded onto 6-well plates to reach a density of 5 × 10^4 cells per well the next day, at which time the media was removed and HDAd was added at an MOI of 6,000 vp/cell in 1 mL mTeSR. Following 1 h incubation, the virus was removed, the cells were washed twice with PBS, and 2 mL of mTeSR was added to each well. 48 h later, media was replaced with media containing 0.5 μg/mL puromycin and 100 μg/mL hygromycin. Well-isolated clones were picked and DNA was extracted for analyses. Transduction of iPSCs to achieve PB excision was performed as described previously. In brief, 2 × 10^6 cells were resuspended in 1 mL mTeSR 1 supplemented with Y27632 (R-agents Direct, Encinitas, CA, USA) to 10 μM in a 1.5-mL microfuge tube and incubated overnight at 37°C with gentle rocking. Following infection, cells were washed twice with 1 mL mTeSR 1 supplemented with Y27632 to 10 μM and plated onto 6-well plates at a density of 2 × 10^5, 5 × 10^4, 2 × 10^4, and 1.25 × 10^3 cells/well in nonselective media. 48 h later, the media was replaced with media supplemented with gancyclovir to a final concentration of 2 μM. 220 well-isolated colonies were picked, and DNA was extracted for Southern analysis.

**DNA Analysis**

Genomic DNA from iPSCs was extracted from a single confluent well of a 24- or 12-well plate as follows: Cells were washed once with 0.5 or 1 mL PBS, and 0.4 mL lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA [pH 8.0], 0.5% SDS, 500 μg/mL pronase [Roche, Indianapolis, IN, USA]) was added to the well. The lysate was then transferred into a microfuge tube and incubated overnight at 37°C. The next day, the DNA was precipitated by the addition of 1 mL 95% ethanol, washed once with 1 mL 70% ethanol, and resuspended in 10 mM Tris-HCl [pH 8.0]).

Non-radioactive digoxigenin (DIG)-based Southern blot hybridization was performed as recommended by the manufacturer (Roche, Indianapolis, IN, USA). All Southern probes were DIG-labeled PCR products generated according to manufacturer’s recommendation.
The 5’ probe was generated using the primers 5’-atttcaagtgtcttcgtcgg-3’ and 5’-gttaaggtaagtcaggtgc-3’ and the plasmid pLPBL-1-CFTR-Apal-BamHI. The 3’ probe was generated using the primers 5’-tcattgccctttgtatgtgc-3’ and 5’-catcctccactgccatttc-3’ and the plasmid pLPBL-1-CFTR-BstBI-BstBI. The PACTk probe was generated using the primers 5’-atagagcccaccgcatcc-3’ and 5’-aacggcgacctgtataacg-3’ and the plasmid pLPBL-13-PACTk. The HyTk probe was generated using the primers 5’-cgtctgtcgagaagtttctg-3’ and 5’-caaagcatcagctcatcgag-3’ and the plasmid RV-L3-HyTk-2L (Addgene plasmid #11684).29

Figure 5. Southern Blot Analyses for Selectable Marker Excision
Representative Southern blot analyses of genomic DNA extracted from GCV<sup>+</sup> clones following transduction of targeted clone 27-18 with HDAd-CAG-hyPB-VAI.

Thermocycling conditions were as follows: 2 min at 95°C, followed by 30 cycles of 95°C for 30 s, 58°C for 40 s, and 72°C for 40 s, and a final extension of 7 min at 72°C.

Primers 5’-gcatagcagagtacgtaggacaggg-3’ and 5’-agaataaactgtaaacc-3’ were used to amplify the unmodified CFTR allele(s).
The parental cell line has ΔD1507 mutation in one allele and ΔD508 mutation in the other allele, and this is evident by mixed A/T in the chromatogram. The bi-allelic corrected clones 27-18-2 and 7-12-1 do not show this mixed A/T in the chromatogram; only the wild-type sequence indicated correction at both alleles.

**Figure 7. Sequence Analyses from Bi-allelic Targeting**

The PACTk and HyTk are flanked by PB ITRs and inserted into the 5'-TTAA-3', indicated by the red box. After excision of PACTk and HyTk by PB transposase from clones 27-18-12 and 7-12-1, the sequence is identical to that of the parental CF17, indicating that selectable marker excision was footprintless at both alleles.
encompassing the ΔI507/DF508 mutation and the site of the selectable marker insertion from iPSC genomic DNA using HotStarPlus (QIAGEN, Valencia, CA, USA) according to the manufacturer’s recommendations. Thermocycling conditions were as follows: 5 min at 95°C, followed by 35 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 2 min, and a final extension of 10 min at 72°C. The forward primer was used to sequence the PCR product to determine whether both alleles were corrected after PB excision of the selectable markers and to verify footprintless PB excision of the selectable marker.

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

CONFLICTS OF INTEREST
The authors declare no competing interests.

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