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ARTICLE

Efficient genome editing in hematopoietic stem cells with helper-dependent Ad5/35 vectors expressing site-specific endonucleases under microRNA regulation

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Genome editing with site-specific endonucleases has implications for basic biomedical research as well as for gene therapy. We generated helper-dependent, capsid-modified adenovirus (HD-Ad5/35) vectors for zinc-finger nuclease (ZFN)– or transcription activator-like effector nuclease (TALEN)–mediated genome editing in human CD34+ hematopoietic stem cells (HSCs) from mobilized adult donors. The production of these vectors required that ZFN and TALEN expression in HD-Ad5/35 producer 293-Cre cells was suppressed. To do this, we developed a microRNA (miRNA)-based system for regulation of gene expression based on miRNA expression profiling of 293-Cre and CD34+ cells. Using miR-183-5p and miR-218-5p based regulation of transgene gene expression, we first produced an HD-Ad5/35 vector expressing a ZFN specific to the HIV coreceptor gene *ccr5*. We demonstrated that HD-Ad5/35.ZFNmiR vector conferred *ccr5* knock out in primitive HSC (*i.e.,* long-term culture initiating cells and NOD/SCID repopulating cells). The *ccr5* gene disruption frequency achieved in engrafted HSCs found in the bone marrow of transplanted mice is clinically relevant for HIV therapy considering that these cells can give rise to multiple lineages, including all the lineages that represent targets and reservoirs for HIV. We produced a second HD-Ad5/35 vector expressing a TALEN targeting the DNase hypersensitivity region 2 (HS2) within the globin locus control region. This vector has potential for targeted gene correction in hemoglobinopathies. The miRNA regulated HD-Ad5/35 vector platform for expression of site-specific endonucleases has numerous advantages over currently used vectors as a tool for genome engineering of HSCs for therapeutic purposes.

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INTRODUCTION

Hematopoietic stem cells (HSCs) are an important target for gene therapy. A major task in HSC gene therapy is the site-specific modification of the HSC genome using artificial site-specific endonucleases (EN) that target a DNA break to preselected genomic sites. ENs are employed to knockout genes, correct frame shift mutations, or to knock in a wild-type cDNA into the endogenous site or heterologous sites. There are now a number of different EN platforms to generate site-specific DNA breaks in the genome.[1] One group of ENs contains DNA-binding protein domains. This group includes meganucleases with DNA binding and nuclease properties as well as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) in which the DNA-binding domain is fused with the bacterial endonuclease FokI. Because DNA cleavage by FokI requires two FokI molecules bound to each of the DNA strands, two subunits of the FokI containing ENs have to be expressed. A second group of ENs is based on RNA-guided DNA recognition and utilizes the CRISPR/Cas9 bacterial system. Several approaches have been used to deliver EN expression cassettes to HSCs. Because it is thought that the ENs need to be expressed only for a short time to achieve permanent modification of the target genomic sequence, most of the EN cassette delivery systems allow only for transient expression of ENs without integration of the EN gene into the host genome. Among these delivery systems are: (i) Electorporation of plasmid, minicircle, or mRNA encoding ENs. While avoiding the problems associated with viral gene delivery vectors, electroporation of plasmid DNA can be associated with cytotoxicity in primary cells especially in HSCs.[2,3] This method may not be applicable for *in vivo* HSC transduction, which can be a prerequisite for the application of some EN-based gene-editing approaches. (ii) Infection with integrase-defective, nonintegrating lentivirus vectors.[2] Limitations of this approach can include relatively low

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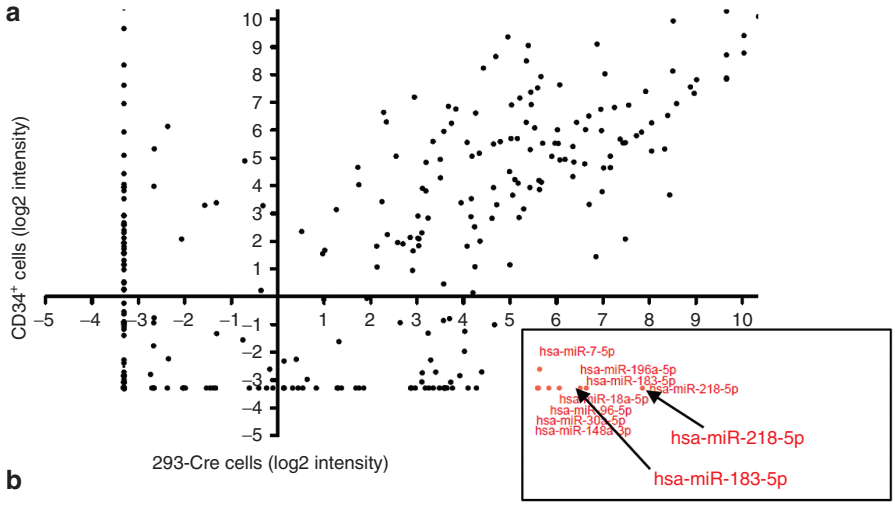
EN expression levels and epigenetic silencing of the EN cassette,[4,5] limited insert capacity (<8 kb) of lentivirus vectors, and the potential risk of recombination between identical sequences when two EN-FokI units are expressed from the same vector. (iii) Transduction with capsid-modified Ad5/35 adenovirus vectors.[6] Ad5/35 vectors contain fibers derived from human serotype Ad35. Ad5/35 vectors target CD46, a receptor that is ubiquitously expressed on 100% of CD34+ cells.[7] Ad5/35 vectors efficiently transduce HSCs, including quiescent primitive HSCs.[6–10] The Ad5/35 vectors platform was recently used in clinical studies to express a ZFN in T cells.[11,12] Ad5/35 vectors used in these studies were first-generation (E1/E3-deleted) vectors. Despite the absence of trans-activating E1 gene products, first-generation vectors express low levels of early (E2A and E4) and late (pIX, fiber, hexon) genes in transduced cells, which may be associated with cytotoxicity in HSCs, especially at elevated doses.[13,14] Toxicity related to leaky viral gene expression can be circumvented by the use of helper-dependent (HD) Ad5/35 vectors that lack all viral genes.[15–18] Growth of HD-Ad vectors depends on coinfection of the producer cells with helper Ad vector, which provides all necessary Ad proteins in *trans*. Removal of helper virus from HD vector preparations is based on Cre-recombinase-mediated excision of the packaging signal flanked by *loxP* sites during coinfection.[19] HD-Ad amplification is done in 293 cells expressing Cre recombinase (293-Cre). cGMP manufacturing of HD-Ad vectors is established (clinical trial locator: NCT01433133, NCT00542568).

We have previously shown that HD-Ad5/35 vectors efficiently transduce human CD34+ cells *in vitro* without significant signs of cytotoxicity.[17,18,20] The HD-Ad5/35 vector platform has major advantages for HSC genome engineering. HD-Ad5/35 vectors transduce primitive subsets of HSCs, have a large capacity (~30 kb) that can accommodate large payloads, including several EN expression cassettes and homologous donor template,[18] and can be used for transducing *in vivo* HSC after intravenous injection.[21] HD-Ad5/35 vectors do not integrate into the genome implying that transgene expression is transient. In the past, it has been challenging to generate HD-Ad5/35 vectors expressing ZFNs or TALENs. During Ad amplification in 293 cells, massive amounts of transgene are produced. This is a problem if overexpression of transgene from the vector is potentially cytotoxic. High levels of EN expression is poorly tolerated in Ad producer 293 cells, which prevents the rescue of vectors or selects for recombined vector genomes and deletion of EN expression cassettes. Notably, Ad replicates through a single-stranded DNA intermediate which enables efficient intramolecular recombination between repeated sequences in the viral genome. Early production of EN-expressing first-generation Ad vectors often involved either the separation of the two EN subunits into two separate vectors which are then coinfected to reconstitute EN activity[22] or the suppression of EN expression in 293 cells using inducible systems.[11,23] Among our attempts to produce CCR5 ZFN-expressing HD-Ad vectors was a vector that allowed for Tet-inducible transgene expression using a fusion of the Krüppel-associated box domain and the tetracycline repressor. We produced green fluorescent protein (GFP) expressing HD-Ad5/35 vectors and showed that background expression in 293 cells with Tet induction was suppressed.[17] However, when we replaced that *GFP* gene with the CCR5 ZFN gene, the resulting HD-Ad genomes isolated from purified particles demonstrated genomic rearrangements and a deletion of parts of the ZFN cassette (Supplementary Figure S1).



	Sequences	CD34+	293-cre
miR-218-5p	UUGUGCUUGAUCUAACCAUGU	39.20 ± 0.31	26.94 ± 0.02
miR-183-5p	UAUGGCACUGGUAGAAUUCACU	n.d.	32.03 ± 0.06
miR-96-5p	UUUGGCACUAGCACAUUUUUGCU	n.d.	34.53 ± 0.32
miR-7-5p	UGGAAGACUAGUGAUUUUGUUGU	35.52 ± 1.28	31.95 ± 0.18
miR-18a-5p	UAAGGUGCAUCUAGUGCAGAUAG	32.34 ± 0.24	28.46 ± 0.08
miR-130a-3p	CAGUGCAAUGUUAAAAGGGCAU	30.70 ± 0.45	31.24 ± 0.07

Figure 1 miRNA expression profiling in 293-Cre versus CD34+ cells. (**a**) MicroRNA log2 intensity scatterplots of CD34+ cells (Y-axis) and 293-Cre cells (X-axis). miRNAs that fulfill our selection criteria (high expression level in 293-Cre cells and absent/low expression in CD34+ cells are labeled in red. 293-Cre and CD34+ cells (pooled from four different donors) were infected with Ad vectors as described in the text. Twenty-four hours after infection, total RNA was isolated and hybridized to an array chip containing >2,000 miRNA probes. (**b**) Confirmation of array results by real-time PCR analysis for selected miRNA using the same RNA samples used for the array study. The C_t value was presented as average and SD from quadruplicate experiments. hsa-miR-130a-3p was selected as a positive control because, based on miRNA array and qRT-PCR assays, it was expressed at high levels in all 293 and CD34+ cell samples. The C_t value correlates inversely with the RNA concentration. n.d., not detectable.

To generate HD-Ad5/35 vectors that express ENs in CD34+ cells, we developed a microRNA (miRNA)-regulated system to suppress expression of the payload in 293-cells while allowing it in CD34+ cells. This enabled us to produce HD-Ad5/35 vectors expressing either a functionally active ZFN or a TALEN at high titers without vector genome rearrangements during production. We demonstrated that an HD-Ad5/35 vector expressing a CCR5 ZFN conferred the expected efficient knock out in primitive human HSCs without affecting the viability and differentiation potential of these cells.

RESULTS

To generate HD-Ad5/35 vectors that express ZFN or TALEN transgenes in human hematopoietic CD34+ stem cells, we used a miRNA-regulated gene expression system. Approaches for miRNA-based regulation were pioneered by Brown *et al.*[24] in 2006. If the mRNA of a transgene contains a target site for a miRNA that is expressed at high levels in a given cell type, the mRNA will be degraded and transgene expression suppressed in this cell type. The power of this approach has been documented in a number of recent studies.[25,26] We set out to establish a miRNA-regulated expression system that would suppress transgene expression in HD-Ad producer cells, *i.e.*, 293-Cre cells, while conferring it in our target cells, *i.e.*, human CD34+ HSCs by establishing the miRNA expression profile in both cell types. Because Ad infection could interfere with the miRNA expression profile, we infected 293-Cre cells with Ad5/35 helper virus at a multiplicity of infection (MOI) of 20 pfu/cell, an MOI used for the amplification of HD-Ad vectors.[27] CD34+ cells from four different adult (G-CSF (granulocyte-colony stimulating factor)-mobilized) donors were pooled and infected with an HD-Ad5/35 vector expressing GFP at an MOI of 2,000 viral particle (vp)/cell, an MOI that confers efficient transduction of CD34+ cells.[18] Total RNA was purified 24 hours after Ad infection and hybridized onto array miRNA chips containing >2,000 different human miRNAs probes (Figure 1a). In total, there were eight candidate miRNAs (Figure 1a, red letters) with high-level expression in 293-Cre cells, but absent or low expression in CD34+ cells. The expression levels of candidate miRNAs were measured by real-time PCR (Figure 1b). Hsa-miR-7-5p and hsa-miR-18a-5p were removed from the candidate list, because they were also expressed in CD34+ cells at relatively high levels. miR-96-5p shared the same seed sequence at the 5′ end of the miRNA (*i.e.*, the sequence which critically determines miRNA target specificity)[28] with other miRNAs. Therefore, we did not include miR-96-5p into our selection. We then selected two miRNAs (hsa-miR-183-5p and hsa-miR-218-5p), which had the highest

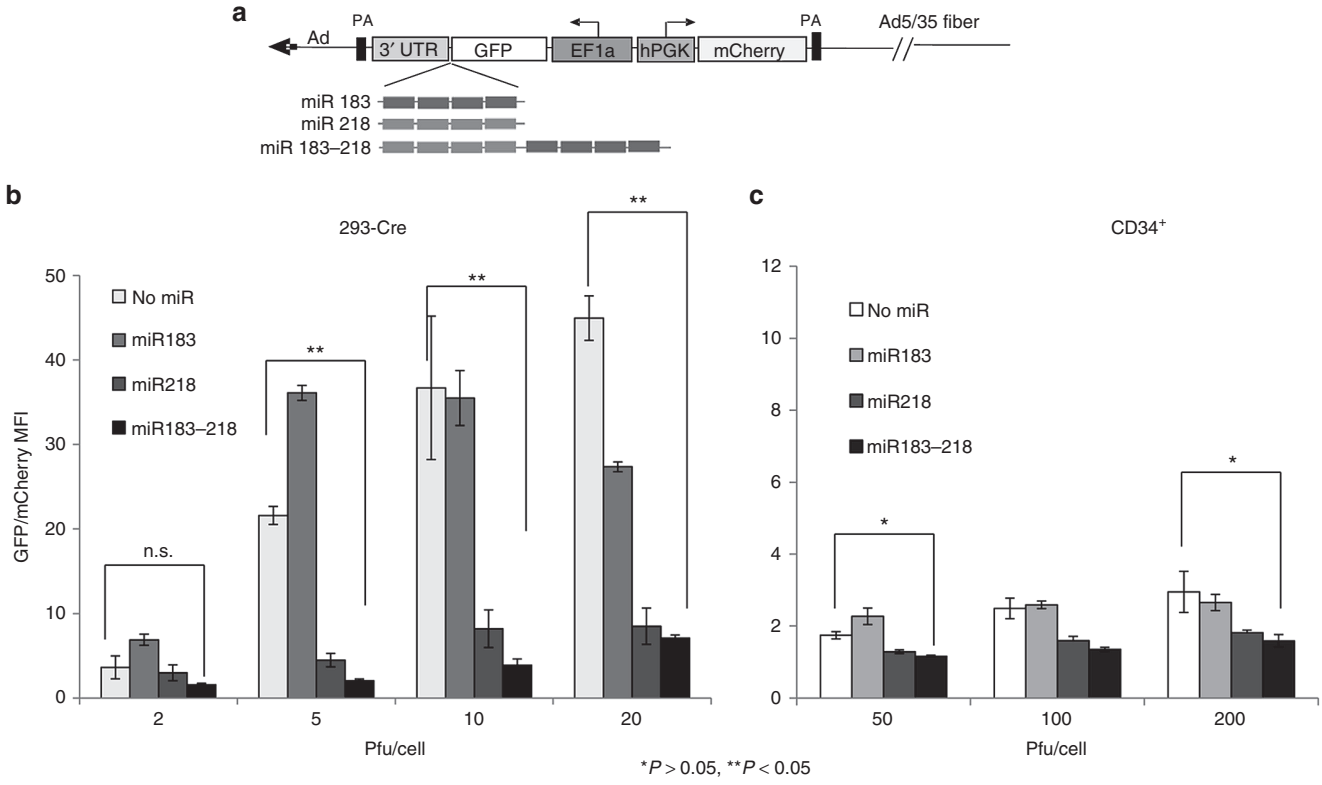


Figure 2 Analysis of miRNA regulated transgene expression. (**a**) Schematic of Ad5/35 vectors used to test miRNA regulated expression. Description is in the text. The 3′ end of the GFP gene is linked to the 3′ untranslated region (UTR) of the globin gene. miRNA target sites were inserted into the 3′UTR. The GFP mRNA transcribed from the EF1a promoter therefore contains miRNA target sites. In contrast, mCherry expression is not regulated by the selected miRNAs. (**b**) Transgene expression in 293-Cre cells. Cells were infected at the indicated MOIs with the Ad5/35 vector that lacks miRNA target sites (no miR) and the vectors containing the miRNA target sites. Shown is the GFP fluorescence intensity divided by the mCherry fluorescence intensity measured by flow cytometry at 48 hours after infection. $N = 3$. P values were calculated using unpaired t-test with unequal variance (GraphPad Prism 5 software GraphPad Software, San Diego, CA). The P values for "no miR" versus "miR218-183" are 0.12; 0.0012; 0.02; and 0.0016 for MOIs 2, 5, 10, and 20 pfu/cell, respectively. Note that the two promoters (PGK and EF1a) are differently regulated and require different transcription factors. For the vector without miR target sites, with increasing MOIs, *i.e.*, transgene copy numbers, GFP levels increase to a much greater degree than mCherry levels. We speculate that the concentration of transcription factors that bind to the EF1a promoter is a limiting factor in 293 cells, particularly if the vector replicates. This is however not relevant for our study and conclusion, because we only compare different vectors at the same MOI. (**c**) Flow cytometry of transduced CD34+ cells 48 hours postinfection. Shown is the GFP/mCherry MFI ratio. $N = 3$. The transduction studies in 293 and CD34+ cells were performed with first-generation vectors. The titers are given in plaque-forming units (pfu). One pfu corresponds to 20 viral particles (vp). GFP, green fluorescent protein; MFI, mean fluorescence intensity; MOI, multiplicity of infection.

expression levels in 293-Cre cells and that were either undetectable (hsa-miR-183-5p) or expressed at the lowest detectable level (hsa-miR-218-5p) in CD34+ cells. To establish the miRNA-regulation system, we inserted four target sites with 100% homology to the selected two miRNAs alone and in combination into the 3′ untranslated region (UTR) of the globin gene. The UTR was linked to the 3′ end of a *GFP* gene, which was under the control of an EF1α promoter, a promoter that is highly active in CD34+ cells (Figure 2a). The GFP expression cassettes were inserted into a first-generation Ad5/35 vector. The vectors also contained a PGK promoter-driven mCherry expression cassette that was not regulated by the selected miRNAs. Normalization of miRNA-regulated GFP expression to mCherry expression allows adjusting for differences in transduction efficiency between different vectors and cell types. We transduced CD34+ cells and 293-Cre cells with the vectors and analyzed GFP and mCherry expression 48 hours later by flow cytometry (Figure 2b,c). In 293-Cre cells, mCherry expression levels were comparable for all four vectors, while GFP expression was suppressed by vectors that contained the miRNA target sites. Based on the mean fluorescence intensity ratio of GFP to mCherry, the greatest suppression was achieved with the vector that contained both the target sequence of miR183 and miR218 (Figure 2b). The difference in normalized GFP levels between the vector that contained the miR218 target sites only and the vector that contained the combination of both miR218 and miR183 target sites is significant, and the *P* values decrease with increasing MOIs (MOI5: $P = 0.047$; MOI10: $P = 0.033$; and MOI20: $P = 0.006$), suggesting that miR183 target sites contribute to suppression of GFP expression.

Considering that first-generation Ad vectors replicate in 293-Cre cells and thus strongly express transgene products, the capability of the miR-183/218-based system to control GFP expression in 293-Cre cells is notable. This is further corroborated by the observation that normalized GFP levels do not increase in an MOI-dependent manner in 293-Cre cells. In contrast, in CD34+ cells both GFP and mCherry expression were comparably high for all vectors (Figure 2c).

Using the miRNA-183/218 regulated gene expression system, we generated an HD-Ad5/35 vector expressing a ZFN under the control of the EF1α promoter (Figure 3a). The ZFN was directed against the gene of the HIV coreceptor CCR5.[11] The two ZFN subunits are linked through a self-cleaving picornavirus 2A peptide and are expressed as a poly-protein that is then cleaved. The miRNA-controlled ZFN expression cassette was inserted into a plasmid that, except the viral inverted terminal repeats (ITRs) and packaging signal, lacked any sequences encoding for viral proteins.[29] The corresponding HD-Ad5/35.ZFNmiR vector (HD-ZFN) was produced in 293-Cre cells at high titers (1.88×10^{12} vp/ml). Restriction analysis of viral DNA isolated from CsCl-gradient purified HD-ZFN particles did not reveal genomic rearrangements (Supplementary Figure S2a). To functionally test the HD-ZFN vector, we first performed transduction studies in MO7e cells, a CD34+ growth factor-dependent erythroleukemia cell line that is often used as a model for HSC gene therapy studies.[30] At day 2 posttransduction, half of the cells were used to analyze ZFN expression by western blot using antibodies against the FokI domain (Figure 3b). Genomic DNA was isolated from the other half

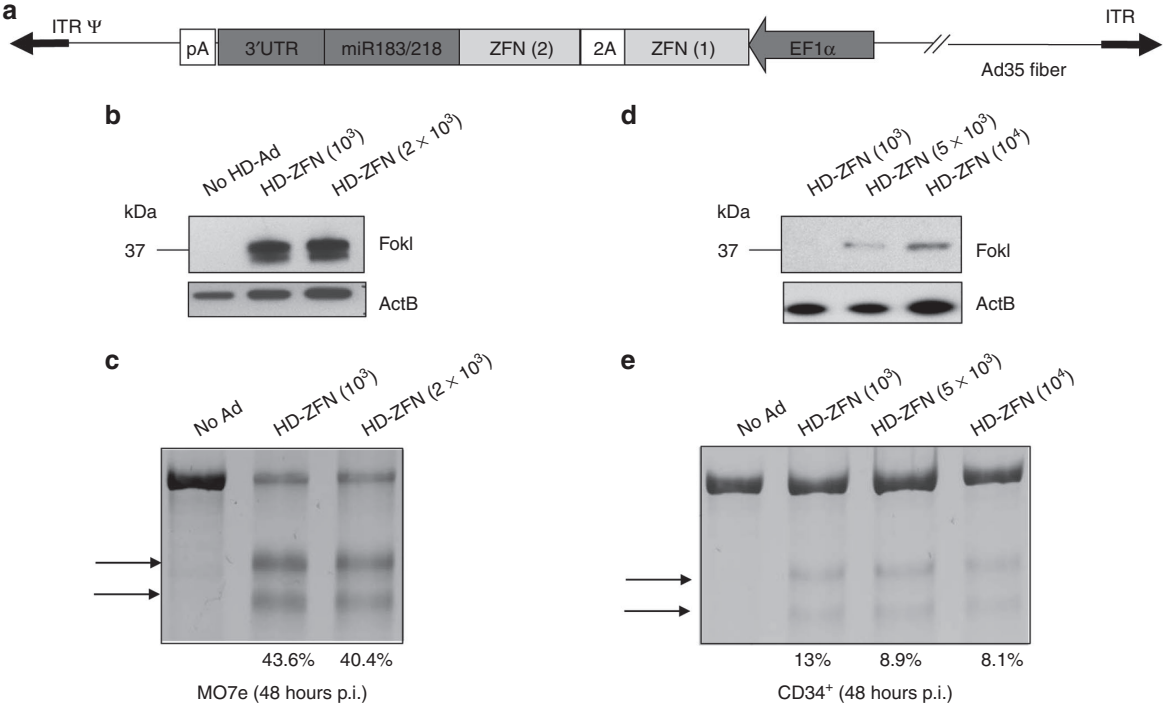


Figure 3 Transduction studies with HD-Ad5/35.ZFNmiR. (**a**) Vector genome structure. The two ZFN subunits are linked trough a self-cleaving viral 2A peptide. The ZFN coding sequence is upstream of miR-183/218 target sites and 3′UTR. Both ZFN subunits are transcribed from the EF1a promoter. In CD34+ cells, the mRNA will not be degraded, and a polyprotein will be expressed which will subsequently be cleaved into the two ZFN subunits at the 2A peptide. (**b**,**d**) Expression of ZFN protein in (**b**) MO7e cells or (**d**) CD34+ cells after transduction with the HD-Ad5/35.ZFNmiR vector (HD-ZFN) at the indicated MOIs. Cells were harvested 48 hours later, and cell lysates were analyzed by western blot with antibodies against the Fok1 domain. Actin B is used as loading control. (**c**,**e**) T7E1 nuclease assay. Genomic DNA from transduced (**c**) MO7e cells or (**e**) CD34+ cells was subjected to a PCR assay based on a T7E1 nuclease that detects mutations.[11] PCR products were separated by polyacrylamide gel electrophoresis. Bands that correspond to disrupted *ccr5* alleles are marked by arrows. The expected size of cleavage products is 141 and 124 bp. The numbers below the lanes indicate the % of disrupted *ccr5* alleles. Studies were done with CD34+ cells from donor A. MOI, multiplicity of infection; ZFN, zinc-finger nuclease.

of cells and analyzed for ZFN cleavage by T7E1 nuclease assay specific for the CCR5-ZFN target site (Figure 3c). This analysis showed that HD-ZFN conferred site-specific DNA cleavage in >40% of *ccr5* alleles in MO7e cells. An analogous study was then performed with human CD34+ cells from two different donors (donor A and donor B). Studies with cells from donor A are shown in Figure 3d,e. In western blot analysis of cells collected 48 hours after infection, detectable FokI signals appeared when cells were infected at MOIs of $\geq 5 \times 10^3$ vp/cell (Figure 3d). Analysis of genomic DNA for *ccr5* modification showed a disruption frequency of 13, 8.9, and 8.1% for MOIs of 10^3, 5×10^3, and 10^4 vp/cell, respectively. Notably, the *ccr5* disruption frequency did not increase with the MOI; it rather decreased most likely due to vector- or ZFN-related toxicity. Furthermore, gene disruption was seen in cells infected at an MOI of 10^3 vp/cell, *i.e.*, an MOI at which ZFN expression was below the western blot detection level. The second study was performed with CD34+ cells from donor B. CD34+ cells from this donor were an aliquot from a CD34+ cell batch that was used for allogeneic HSC transplantation in cancer patients. The transduction efficacy with Ad5/35 and HD-Ad5/35 vectors was comparable to that of CD34+ cells from donor A. These cells can therefore be used to assess potential cytotoxicity of vector transduction. However, the genome of donor B cells contained a small nucleotide polymorphism within the *ccr5* gene close to the ZFN cleavage site (Supplementary Figure S3a). The T7E1 nuclease assay is not able to distinguish between the SNP and ZFN-mediated rearrangements and therefore shows *ccr5* disruption in all samples, including untransduced cells (Supplementary Figure S3b).

To assess whether ZFN expression from HD-Ad5/35 vectors causes cytotoxicity in CD34+ cells at the doses we used, we performed flow cytometry for the apoptosis marker Annexin V at day 4 after transduction. CD34+ cells used for this study were from donors A and B (Figure 4a,b). Although the outcome of the studies slightly differed between the two donors, HD-ZFN transduction did not significantly affect cell viability when compared to untransduced cells and control (HD-bGlob) vector transduced cells. In contrast, transduction of CD34+ with a first-generation Ad5/35 vector expressing the CCR5-ZFN[11] increased the percentage of Annexin V-positive cells in a dose-dependent manner in this experimental set up (Figure 4c).

The next tasks were to show that HD-ZFN mediates CCR5 disruption in primitive HSCs and that transduction and ZFN expression do not affect the ability of these cells to proliferate and differentiate. To assess the latter, we subjected HD-ZFN-transduced CD34+ cells to a long-term culture initiating cell (LTC-IC) assay. This assay measures primitive HSCs based on their capacity to produce myeloid progeny for at least 5 weeks. Committed progenitors initially present in the transduced CD34+ cell population will rapidly mature and disappear during the initial 3 weeks of culture due to their limited proliferative potential. The more primitive cells will be maintained throughout the duration of culture and generate a new cohort of committed progenitors (*e.g.*, colony-forming cells), which can be later detected and enumerated at the end of the assay using progenitor colony assays in semi-solid media. For both the control HD-bGlob and HD-ZFN vectors, transduction of CD34+ cells from donor A decreased the number of colonies compared to untransduced controls whereby the differences were significant only for MOI 5,000 vp/cell (Figure 5a). Transduction of CD34+ cells from donor B with the control vector did not significantly affect colony formation, while transduction with HD-ZFN at an MOI of 1,000 vp/cell significantly decreased it (Figure 5b). Transduction with FG-ZFN vector inhibited colony formation (Figure 5c).

To evaluate CCR5 disruption levels in LTC-IC, cells from all colonies in a plate were combined, genomic DNA was isolated, and subjected to T7E1 nuclease assay. The frequency of HD-ZFN-mediated *ccr5* gene disruption in CFUs at the end of the assay was 23.7% (Figure 5d). This suggested that the vector targeted primitive CD34+ cells and that the gene modification is persistent in HSC progeny. To further support this, we studied whether the HD-ZFN vector is able to mediate CCR5 disruption in NOD/SCID repopulating cells (Figure 6a). This functional HSC assay is thought to potentially be predictive of the ability to repopulate conditioned recipients in human trials.[31] For this assay, we transduced CD34+ cells from donor A with the control HD-bGlob vector or HD-ZFN vector at an MOI of 5,000 vp/cell for 24 hours under low-cytokine conditions to prevent CD34+ cell differentiation. Transduced cells were transplanted into sublethally irradiated NOD/Shi0scid/IL-2Rγnull (NOG) mice. Engraftment of human cells was analyzed 6 weeks after transplantation by flow cytometry for human CD45+ cells in bone marrow, spleen, and peripheral blood mononuclear cells (PBMCs). (Notably, bone marrow engraftment rates with CD34+ cells from adult donors are usually lower than those achieved with umbilical cord-blood derived CD34+ cells). We found that ~6% of bone marrow cells were human CD45+ positive in mice that were transplanted with nontransduced CD34+ cells (Figure 6b). The average bone marrow engraftment rate of HD-ZFN transduced cells was 2.12%, which is about threefold lower than that

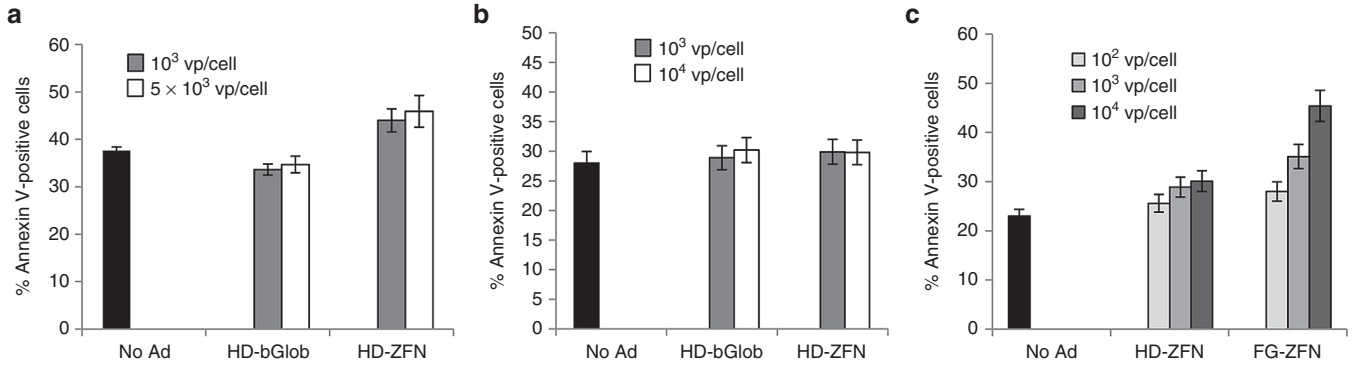


Figure 4 Analysis of CD34+ cytotoxicity associated with HD-ZFN transduction. Studies were performed with CD34+ cells from donor A (**a**) and donor B (**b**). Shown is the percentage of Annexin V-positive cells at day 4 after transduction with an HD-Ad5/35 control vector containing the β-globin LCR (HD-bGlob) or the HD-ZFN vector at the indicated MOIs. Annexin V and 7AAD expression was analyzed by flow cytometry $N = 3$. (**c**) Cytotoxicity after infection of CD34+ cells with first-generation (FG-ZFN) and helper-dependent (HD-ZFN) Ad5/35 vectors expressing the CCR5 ZFN. CD34+ cells from donor B were used. $N = 3$. HD-ZFN versus FG-ZFN (MOI: 1,000): $P = 1.51 \times 10^{-6}$, HD-ZFN versus FG-ZFN (MOI: 10,000): $P = 2.83 \times 10^{-8}$. ZFN, zinc-finger nuclease.

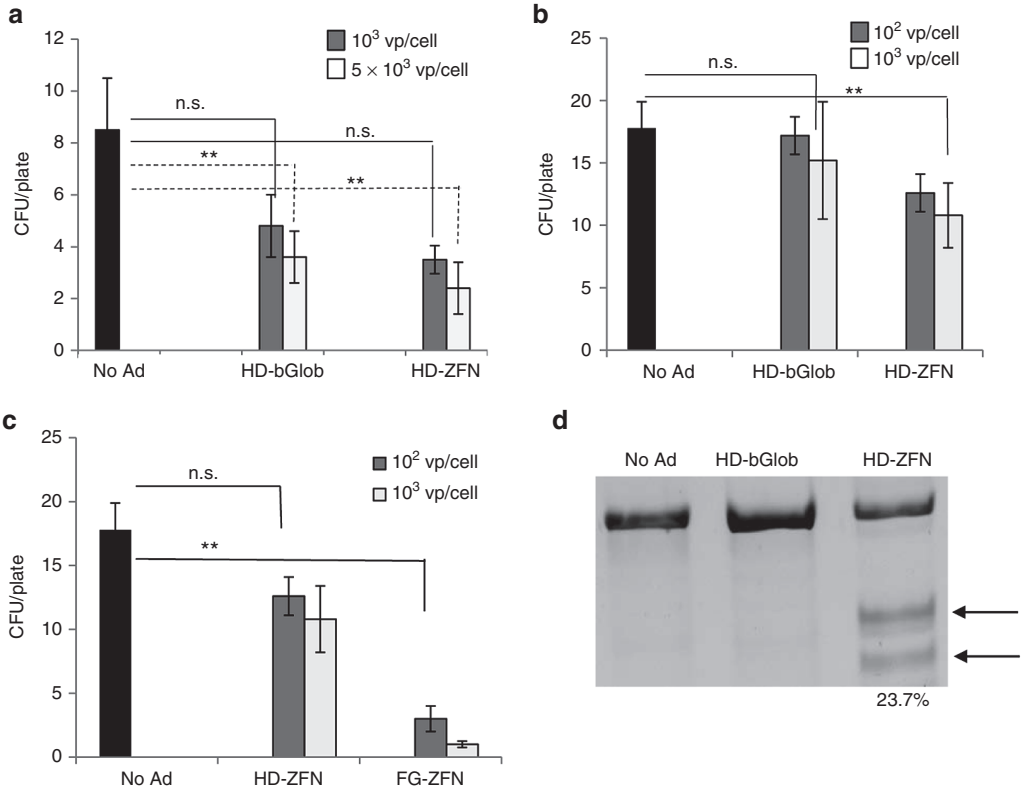


Figure 5 Analysis of LTC-IC. CD34+ cells were transduced with HD-bGlob and HD-ZFN at the indicated MOIs. Three days later, cells were transferred to LTC-IC medium and cultured for 5 weeks. A total of 3,000 LTC-IC cells were then plated in methylcellulose supplemented with growth factors and cytokines. Two weeks later, colonies were counted. Cells from all colonies per plate were combined, genomic DNA was isolated, and subjected to T7E1 nuclease assay. (**a,b**) Numbers of colonies per plate for donor A and B, respectively. There was no difference in the ratio of BFU-E and CFU-GM colonies in the different groups. $N = 3$ plates. n.s., nonsignificant ($P > 0.05$), $^{**}P < 0.05$. (**c**) Number of CFU from donor B cells transduced with FG-ZFN and HD-ZFN. (**d**) T7E1 nuclease assay. CD34+ cells from donor A were used for transduction with HD-bGlob and HD-ZFN at an MOI of 5,000 vp/cell. Genomic DNA was from colonies was isolated and subjected to T7E1 assay. A representative T7E1 nuclease assay of CFU/LTC-IC samples is shown. vp, viral particle; ZFN, zinc-finger nuclease.

of untransduced cells. Interestingly, transduction with the HD-b Glob vector increased the engraftment rate. Analysis of human CD45+ cells in the spleen and PBMC showed similar engraftment rates, although the effect of HD-bGlob transduction was less pronounced in these tissues. For further analyses, human CD45+ cells were purified using magnetic-activated cell sorting. Human CD45+ cells were subjected to progenitor/colony assays to assess the presence of HSCs (Figure 6c). Similar numbers of colonies were found in engrafted CD45+ cells from mice that received untransduced or HD-ZFN transduced CD34+ cells. Colony numbers were higher for the HD-bGlob group, suggesting that this vector improves the survival of HSCs. The reason for this remains elusive at this point. To investigate the frequency of CCR5 modification, human CD45+ cells were analyzed by T7E1 nuclease assay. We found the levels of *ccr5* gene disruption to be 8.4 and 12% in two transplanted mice, respectively (Figure 6d). These data suggest that although HD-ZFN transduction and/or ZFN expression may decrease the engraftment rate of CD34+ cells, *ccr5* gene disruption was achieved in HSCs that persisted in transplanted mice for the time of analysis.

To show the versatility of our miRNA-based approach to regulate transgene expression, we produced a second vector expressing a TALEN targeting the DNase hypersensitivity region 2 (HS2) within the globin locus control region (LCR) (Figure 7a). The site was selected because it is thought that target DNA sequences are better accessible to ENs when they are localized in active chromatin or DNase HS regions.[32,33] We and others have shown in erythroid and HSC lines that the HS2 region is occupied by open chromatin marks.[34–37] We have also previously shown that HD-Ad5/35 vectors carrying a 23-kb fragment of the β-globin LCR preferentially integrated into the chromosomal β-globin LCR through chromatin tethering to the HS2 area.[18,34] The latter studies were done in MO7e cells. As with ZFN-expressing HD-Ad5/35 vectors, our earlier attempts to rescue HD-Ad5/35-TALEN virus vectors (without mRNA-mediated suppression in 293 cells) were unsuccessful.

To generate the HD-Ad5/35.TALENmiR (HD-TALEN) vector, the 3′ end of the TALEN mRNA was modified to contain miR-183/218 binding sites (Figure 7b). The HD-TALEN vector was produced at a high titer (2.5×10^{12} vp/ml) without detectable genome rearrangements (Figure 2b). After infection of MO7e cells with HD-TALEN at an MOI of 1,000 vp/cell, TALEN expression was detected by western blot using an anti-hemagglutinin tag antibody (Figure 7c). T7E1 nuclease assay revealed ~50% modification of the HS2 target site in MO7e cells at day 2 after infection. The ability to place HS2-specific DNA breaks in combination with our globin LCR containing HD-Ad5/35 is relevant for targeted transgene insertion.

Taken together, our studies show the miRNA system is a robust platform for the production of HD-Ad5/35 vectors expressing ZFNs and TALENs.

DISCUSSION

Because ZFNs were the first ENs developed, a substantial amount of data regarding site-specific and off-target activity has been

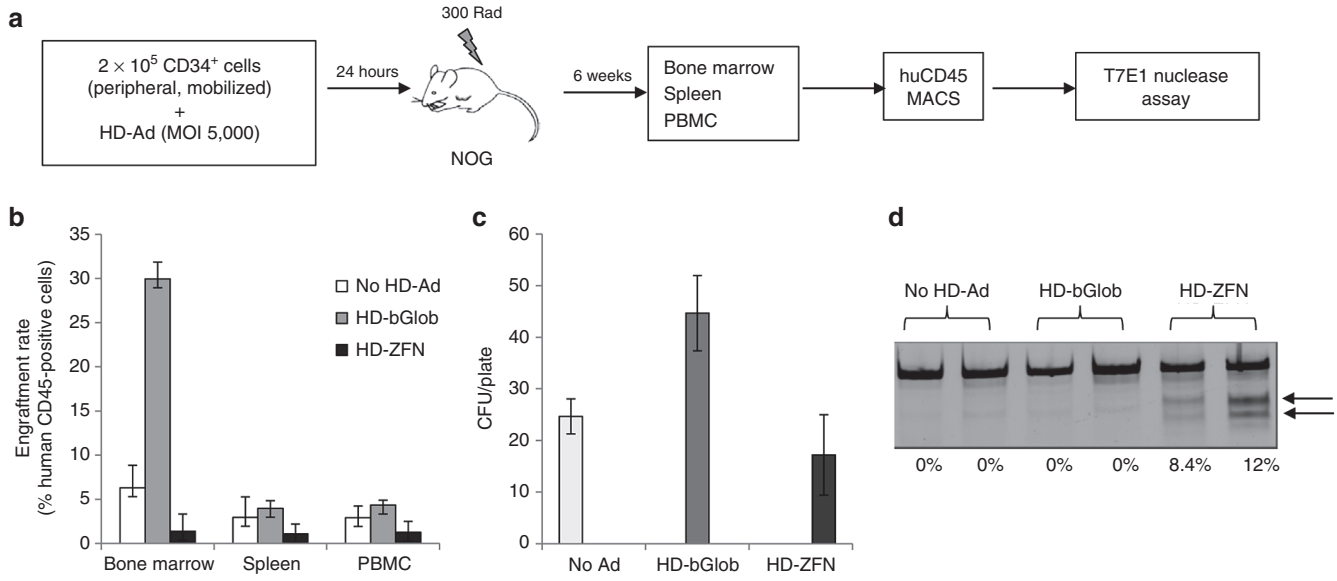


Figure 6 *ccr5* Gene knock out in NOD/SCID repopulating cells. (**a**) Study design. Cryoconserved CD34+ cells from donor A were cultured overnight under low cytokine concentration conditions and transduced with HD-bGlob or HD-ZFN at an MOI of 5,000 vp/cell for 24 hours. Cells were then washed and transplanted into sublethally irradiated NOG mice. Six weeks later, animals were euthanized, and bone marrow cells, splenocytes, and PBMC were collected. The percentage of human cells in collected cells was measured by flow cytometry for the pan-leukocyte marker CD45. Human donor cells were purified by magnetic-activated cell sorting (MACS) using beads conjugated with anti-human CD45 antibodies. CD45+ cells were used for the T7E1 nuclease assay. (**b**) Engraftment rate based on the percentage of human CD45+ cells in total cells from bone marrow, spleen, and PBMCs. $N = 3$. (**c**) Number of colonies from MACS isolated human CD45+ from bone marrow cells in the bone marrow of transplanted mice. $N = 3$. The difference between "no Ad" and "HD-ZFN" is not significant ($P = 0.061$) (**d**) Analysis of *ccr5* gene disruption in human CD45+ cells from bone marrow of transplanted mice. vp, viral particle; ZFN, zinc-finger nuclease.

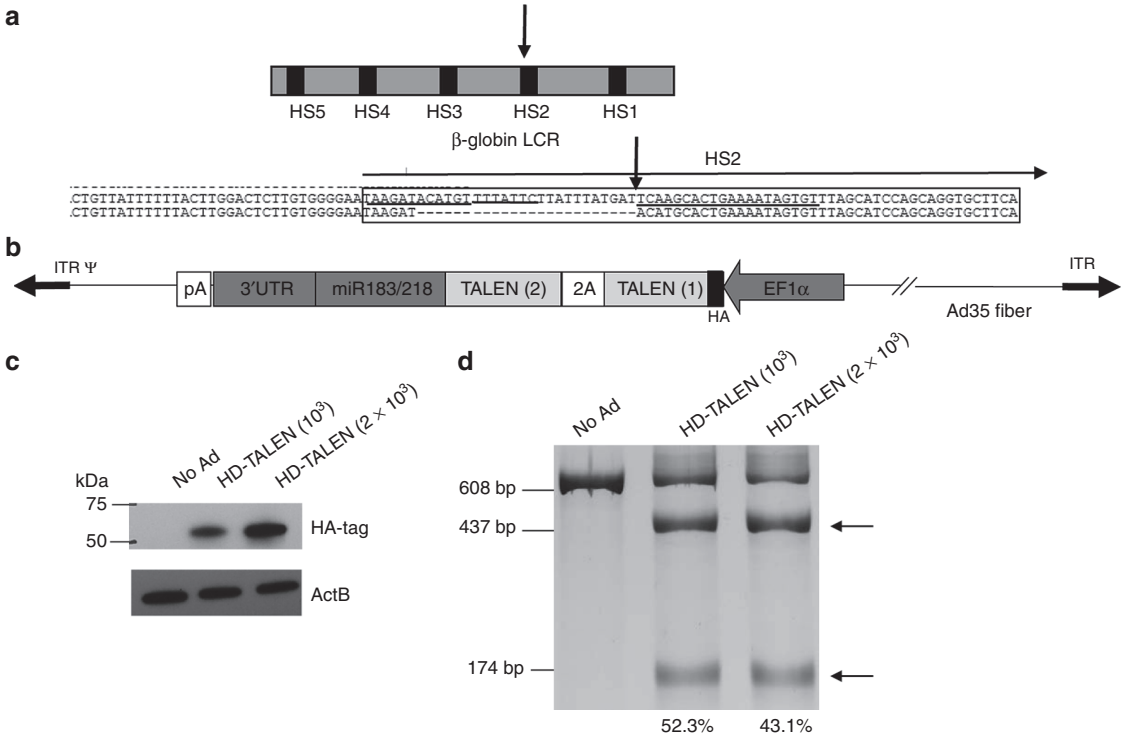


Figure 7 Structure and functional analysis of an HD-Ad5/35 vector expressing a globin LCR-specific TALEN. (**a**) Target site of TALEN. Shown is the structure of the globin LCR with DNase hypersensitivity sites HS1 to HS5. The lower panel shows the 5′ sequence of the HS2 target site labeled by a horizontal arrow. The lines above and below the sequence indicate the binding sites of the two TALEN subunits, respectively. The vertical bold arrow marks the TALEN cleavage site. (**b**) Structure of the HD-Ad5/35.TALENmiR (HD-TALEN) genome. In analogy to the ZFN vector, the two TALEN subunits were linked through a 2A peptide at the 3′ end to the miR183/218 target sequence-containing 3′UTR. The N-terminus of TALEN (1) contained an influenza hemagglutinine (HA) tag. (**c**) Expression of TALEN in MO7e cells. Cells were infected at an MOI of 1,000 vp/cell, and cell lysates were analyzed by western blot with antibodies specific for HA-tag. (**d**) T7E1 nuclease assay analysis. Genomic DNA was isolated from MO7e cells 48 hours after infection at an MOI of 10^3, 2×10^3 vp/cell and subjected to PCR using globin LCR H2-specific primers. The expected length of PCR products is 608, 434, and 174 bp. MOI, multiplicity of infection; vp, viral particle; ZFN, zinc-finger nuclease.

accumulated for these types of ENs. A ZFN targeting the HIV CCR5 coreceptor gene was the first to be tested in clinical trials.[12] This trial involved the *ex vivo* transduction of patient CD4+ T-cells with a CCR5-ZFN expressing Ad5/35 vector. More recent efforts have focused on *ccr5* gene knock out in HSCs. Targeting HSCs versus CD4+ T cells has a number of advantages: (i) As HSCs are a source for all blood cell lineages, CCR5 knock out would protect not only CD4 cells but also all remaining lymphoid and myeloid cell types that are potential targets for HIV infection. (ii) In contrast to CD4+ cell transplants, a single HSC transplant would potentially provide a lifelong source of HIV-resistant cells to allow long-term protection or control of HIV/AIDS. The first successful attempt to achieve ZFN-mediated disruption of *ccr5* gene sequences in HSCs was reported by Holt *et al*.[3] in 2010. This study demonstrated engraftment of the modified HSCs in NOD/SCID/IL2rγ^{null} (NSG) mice resulting in resistance to CCR5-tropic HIV-1 infection. While encouraging, the data also indicated a number of potential problems, including the poor viability of cells transfected with the ZFN-expressing plasmid by electroporation in this experimental system. To address this problem, Li *et al*. used the Ad5/35 vector that was employed for CCR5 knock out in human T-cells. While efficient in T-cells, the Ad5/35 vector achieve less than 5% *ccr5* gene disruption in HSCs.[7] Reasons for this poor efficacy could include suboptimal expression of the ZFN from the promoter used, which was a minimal CMV promoter linked to tet-operator sequences. This promoter, in combination with doxycline induction, was employed by the minCMV-tetO promoter in combination with tet-repressor expressing 293 cells and was used to suppress ZFN expression in adenovirus producer cells. Previously, we showed that this promoter is active in only a very small subset of CD34+ cells after Ad5/35 infection in our experimental system.[17] Poor viability in the study by Li *et al*. could also be due to cytotoxicity associated with first-generation Ad5/35 vectors in HSCs. We attempted to address both of these problems. Instead of the minCMV-TetO promoter we used the EF1a promoter, a promoter that is active in CD34+ and often used in HSC gene therapy studies.[38] We also wanted to replace the first-generation Ad5/35 vector platform with helper-dependent HD-Ad5/35 vectors to avoid potential cytotoxicity associated with leaky viral gene expression from first-generation vectors. Initial attempts to rescue first-generation or helper-dependent Ad5/35 vectors expressing CCR5 ZFN under the EF1a promoter failed or resulted in rearranged genomes with deleted *ZFN* genes in the vectors. This supports the idea that of Ad genomes comprising a pair of ZFN sequences may be unstable, a notion which is further supported by the existence of two identical copies of FokI gene as well as eight ZFN DNA binding motifs in the vector genome, which can serve as substrates for intramolecular recombination. Recombination in Ad genomes is an often occurring event and a major means of Ad evolution. It is mediated by the unique mechanism of Ad DNA replication, which takes place in two steps.[39] In the first stage, DNA synthesis is initiated by viral "terminal protein" and polymerase from both ITRs of the linear genome, resulting in a daughter strand that is synthesized in the 5′ to 3′ direction, displacing the parental strand with the same polarity. The displaced parental strand is a single-stranded molecule that self-anneals through the ITRs, which in turn serves as a primer for the second strand synthesis. We previously demonstrated that intramolecular annealing and recombination between repeat sequences inserted into the Ad genome occurs during the single-stranded stage of the Ad genome.[40]

To guard against the potential cytotoxicity of high level ZFN expression in 293-Cre cells in our system, we established a miRNA-based gene regulation system to suppress the ZFN transgene. The system is based on profiling of miRNA expression in 293-Cre cells and human CD34+ cells pooled from different donors. Studies with reporter genes showed efficient suppression of a transgene that was regulated by hsa-miR-183-5p and hsa-miR-218-5p. While there was background expression of the miRNA-regulated GFP reporter gene, it did not increase in a dose-dependent manner or upon viral replication. The latter could be due to the high levels of miR-183 and -218 in 293-Cre cells and complete saturation of the corresponding target sites. Importantly, the miR183/218-regulation system was sucessful for the generation of HD-Ad5/35 vectors expressing the CCR5 ZFN or the globin LCR TALEN. Potentially, our miRNA-regulated approach is also relevant for the production of lentivirus or rAAV vectors which also use 293 cells as production cells.

In transduction studies, we focused on HD-Ad5/35.ZFNmiR (HD-ZFN). ZFN expression analyzed at day 2 after infection was lower in CD34+ cells than in MO7e cells. This is in agreement with our previous studies with HD-Ad5/35.GFP vectors in which we showed that transduction of CD34+ cells results in GFP expression in ~60% of CD34+ cells and mean GFP fluorescence intensity levels that were about ~10-fold lower than that in MO7e cells. As a limiting factor, we have identified inefficient virus trafficking to nucleus.[41] Li *et al*.[7] showed that this can be, in part, overcome by incubation of CD34+ cells with protein kinase C (PKC) activators which has however the drawback that these compounds might affect HSC biology.

Analysis of *ccr5* gene disruption at day 2 after HD-ZFN transduction did not show a correlation with ZFN expression level at this time point. Analysis at a later time point follow transduction potentially would show a higher level of disruption. It is possible that cellular factors, specifically proteins involved in nonhomologous end joining DNA repair limit the disruption efficiency rearrangement efficacy. Alternatively, considering that CD34+ cells is a highly heterogeneous cell population, it is possible that HD-Ad5/35 transduction, ZFN cleavage, and/or nonhomologous end joining occurs only in fraction of CD34+ cells. Importantly, our subsequent LTC-IC and NOG mice repopulation studies suggested that the targeted CD34+ cells contain primitive stem cells.

We found that HD-ZFN transduction decreased the engraftment rate, survival, and/or expansion of CD34+ cells in NOG mice in our system. This was not necessarily due to HD-Ad5/35 transduction and vector-associated toxicity *per se*, because engraftment rates were actually higher with HD-bGlob transduced CD34+ cells than with nontransduced cells. We therefore speculate that this is related to ZFN expression over an extended time period. Nonintegrating HD-Ad vector genomes are lost after several rounds of cell division, however, persist longer in nondividing cells such as hepatocytes.[42] Because HSCs are low proliferative, HD-Ad5/35 genomes could be maintained for longer time periods and thus express ZFN. For gene engineering purposes, it is sufficient that ZFNs are expressed only for a short time period. Future effort should focus on regulating the duration of ZFN expression from HD-Ad5/35 vectors.

It is noteworthy that we used in our studies CD34+ cells from adult G-CSF mobilized donors, a source that is easier available than fetal liver or cord blood derived CD34+ cells, which were used in previous studies with CCR5 ZFNs.[2,3] A *ccr5* gene disruption frequency of 12% in engrafted HSCs found in the bone marrow of transplanted NOG mice is clinically relevant for HIV therapy considering that these cells can give rise to multiple lineages, including lineages that represent targets and reservoirs for HIV.

Another avenue that we are following is to use the globin LCR-specific TALEN to increase the site-specific integration of a donor HD-Ad5/35 vector through homologous recombination.[18]

The HD-Ad5/35 vector platform for EN gene delivery to HSCs has major advantages over other delivery systems. (i) Most importantly, it allows for efficient targeting of primitive HSCs with less cytotoxicity. (ii) The insert capacity of HD-Ad vectors is 30 kb, which allows the accommodation of several ENs and homologous donor templates. This is important for the simultaneous editing of multiple genes in HSCs for gene therapy purposes or to establish relevant models for multigenic human diseases. The use of HD-AD5/35 vector would also make it possible to combine both the EN expression cassette and the donor DNA sequences with extended homology regions into one vector. In this context is notable that the efficacy of homologous recombination directly correlates with the length of the homology regions.[16] (iii) HD-Ad vectors allow for the transduction of target cells *in vivo*. HD-Ad5 vectors efficiently transduce hepatocytes in mice and nonhuman primates after intravenous injection.[43,44] Our preliminary studies in human CD34+/NOG and human CD46-transgenic mice show that affinity-enhanced Ad5/35 and HD-Ad5/35 vectors can transduce G-CSF/AMD3100 mobilized HSCs after intravenous injection.[21] HSC gene editing approaches involving the *in vitro* culture/transduction, and retransplantation into myelo-conditioned patients are technically complex and expensive. The *in vitro* culture of HSC in the presence of multiple cytokines affects the viability, pluripotency, and engraftment potency of HSCs. Furthermore, the need for myeloablative regimens creates additional risks for patients. Finally, the procedure is expensive and can only be performed in specialized institutions. Therefore, vectors system that allow for *in vivo* HSC genome editing are of relevance.

In summary, we have developed a miRNA-regulated HD-Ad5/35 vector platform for the expression of designed endonucleases in primitive HSCs. This vector system is a new important tool for genome engineering of HSCs for therapeutic purposes.

MATERIALS AND METHODS

Cells

293 cells, 293-C7-CRE[45] cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT) 2 mmol/l L-glutamine, and Pen-Strep. Mo7e cells[30] were maintained in RPMI 1640 medium containing 10% FCS, 2 mmol/l L-glutamine, Pen-Strep, and granulocyte-macrophage colony stimulating factor (G-CSF) (0.1 ng/ml) (Peprotech, Rocky Hill, NJ). Primary human CD34+-enriched cells from G-CSF mobilized normal donors were obtained from the Fred Hutchinson Cancer Research Center Cell Processing Core Facility. We used PBMC-derived CD34+ cells from two different donors, designated as "donor A" and "donor B." CD34+ cells were recovered from frozen stocks and incubated overnight in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% FCS, 0.1 mmol/l 2-mercaptoethanol, stem cell factor (50 ng/ml), DNase I (100 µg/ml), 2 mmol/l L-glutamine, Flt3 ligand (Flt3L, 50 ng/ml), interleukin (IL)-3 (10 U/ml), and thrombopoietin (10 ng/ml). Cytokines and growth factors were obtained from Peptotech.

miRNA array

Array studies were performed using Agilent's human miRNA (8×60 K) V18.0 (Agilent Technologies, Santa Clara, CA) containing 2006 different human miRNA probes. Extraction of miRNA and RNA from Qiagen (Valencia, CA) RNAprotect cell reagent stabilized cells was performed according to the Qiagen miRNeasy kit protocol. RNA samples were frozen at −80 °C. Each slide was hybridized with 100 ng Cy3-labeled RNA using miRNA Complete Labeling and Hyb Kit (Agilent Technologies) in a hybridization oven at 55 °C, 20 rpm for 20 hours according to the manufacturer's instructions. After hybridization, slides were washed with Gene Expression Wash Buffer Kit (Agilent). Slides were scanned by Agilent Microarray Scanner and Feature Extraction software 10.7 with default settings. Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0, Santa Clara, CA.

qRT-PCR for selected miRNAs

RNA prep concentration was measured using ScanDrop (Analytik Jena, Jena, Germany). The reverse transcription was performed using TaqMan miRNA Reverse Transcription Kit with miRNA-specific primers all purchased from Applied Biosystems (Foster City, CA), using 5 ng template, 4 °C 6 minutes, 16 °C 30 minutes, 42 °C 30 minutes, and 85 °C 5 minutes. The real-time PCR was performed in quadruplicate with TaqMan 2× Universal PCR Master Mix with no AmpErase UNG on a 7900HT machine (Applied Biosystems), using 0.27 ng template in 10 µl reaction volume, 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds, 60 °C for 60 seconds. The C_t value was calculated at threshold equals 0.3 and with manual baseline start cycle at 3 and end cycle at 13. miRNA homology in the 5′ seed sequences was analyzed using "R software" (Beijing, China) and "microRNA" bioconductor package (Seattle, WA).[28]

Adenovirus vectors

Ad5/35-RG containing miRNA target sites. The GFP-mCherry cassette from pRG_0[46] was transferred into the adenovirus shuttle plasmid pDeltaE1/Sp1 (Microbix, Ontario, Canada). The following miRNA target sites were synthesized and inserted into the AvrII/SmaI site of the shuttle vectors:

miR-183 target site: 5′ CTAGGATTATGGCACTGGTAGAATTCAC TACTTAT GGCACTGGTAGAATTCACTAC TTATGGCACTGGTAGAA TTCACTACTTATGGCA CTGGTAGAATTCACTATCGCCCGGG

miR-218 target site: 5′ CCTAGGAATTTGTGCTTGATCTAACCA TGTTTCATTG TGCTTGATCTAACCA TGTTTCATTGTGCT TGATCTAACCATGTTTCATTGTGCTTG ATCTAACCATGTATCGCCCGGG

miR-183/218 target site: 5′ CCTAGGATTATGGCACTGGTAGAATTCACTACT TATGGCACTGGTAGAATTCACTACTTATGGCACTGGTAGAATTCACTA CTTATGGCACTGGTAGAATTCACTATCGTTGTGCTTGATCTAACCATGTTTC ATTGTGCTTGATCTAACCATGTTTCATTGTGCTTGATCTAACCATGTTTCATT GTGCTTGA TCTAACCATGTATCGCCCGGG

First-generation Ad5/35 virus vectors were generated and tested as described elsewhere.[6]

HD-Ad5/35-ZFN containing the miR-182/218-regulated CCR5 ZFN under EF1a promoter control. The shuttle plasmid for recombination in HD backbone vector was generated using pBluescript (pBS) plasmid. Briefly, recombination arms were amplified from pHCA plasmid containing stuffer DNA[29] and cloned into pBS generating pBS-Z for ZFN-CCR5 construct and pBS-T for Talen-LCR construct. 3′UTR and pA sequence was synthesized by GenScript (Piscataway, NJ) and cloned into both shuttle vectors via AgeI and XhoI generating pBS-Z-3′UTR-pA and pBS-T-3′UTR-pA. Ef1a promoter was extracted from PJ204-EF1a-pA containing a 1,335 bp fragment of the EF1a promoter with BamHI and NheI, then inserted into respective sites in both shuttle plasmids generating pBS-Z-Ef1a and pBS-T-EF1a. ZFN-CCR5 fragment from pBS-CCR5 (ref. 11) was digested with EcoRI and XbaI and cloned into the shuttle vector generating pBS-Ef1a-ZFN-CCR5. Finally, synthesized miR-183/218 tandem repeats flanked by NotI were cloned into its respective site in pBS-Ef1a-ZFN-CCR5 generating pBS-Ef1a-ZFN-CCR5-miR. The shuttle vector plasmids were linearized with BstBI and recombined with pHCA backbone vector in *Escherichia coli* BJ5183 cells. Recombined pHCA-Ef1a-ZFN-CCR5-miR and pHCA -EF1a-Talen-LCR-miR were then linearized with PmeI and rescued in 293-Cre cells with helper virus (HV-Ad5/35) to generate HD-Ad5/35-EF1a-ZFN-CCR5-miR virus (HD-Ad5/35.ZFNmiR) and HD-Ad5/35-EF1a-Talen-LCR-miR virus (HD-Ad5/35Talen.miR).

HD-Ad5/35-TALEN containing the miR-182/218-regulated HS2-LCR TALEN under EF1a promoter control. The HS2-LCR-specific TALEN was designed by ToolGen (Seoul, South Korea) as described previously.[47] The TALEN recognition sequences are shown in Figure 7a. The DNA-binding domains are fused with FokI. The N-terminus of the DNA-binding domain is tagged with a hemagglutinin-tag and contains a nuclear localization signal. The TALEN cassette was under the control of the EF1a promoter and contained miR sites upstream of 3′UTR. The two TALEN were cloned into pBS-T-EF1a and linked via 2A peptide. Similar to the ZFN-CCR5 construct, miR 183/218 tandem repeats were synthesized and cloned into NotI site of pBS-EF1a-Talen-LCR generating pBS-EF1a-Talen-LCR-miR. For virus rescue, the final plasmid was linearized with PmeI.

HD-Ad5/35.bGlob (HD-bGlob). This vector has been described previously.[18] It contains ~26 kb of the globin LCR. The β-globin promoter controls the expression of a *GFP* gene.

HD-Ad5/35 vectors were produced in 293-Cre cells[27] with the helper virus Ad5/35-helper[41] as described in detail elsewhere.[27] Helper virus contamination levels were determined as described elsewhere and were found to be <0.05%. DNA analyses of HDAd genomic structure were confirmed as described elsewhere.[27]

Flow cytometric analysis

For cytotoxicity analysis, Ad-transduced CD34+ cells were stained with the AnnexinV/7AAD apoptosis kit (eBiosciences, San Diego, CA). For engraftment analysis, cells derived from PBMCs, bone marrow, and spleen were stained with anti-hCD45-PE (BD Biosciences, San Jose CA). The data were then analyzed with FlowJo software (Ashland, OR).

Magnetic-activated cell sorting

Anti-human CD45–conjugated microbeads were from Miltenyi Biotech (Auburn, CA). Cell purification was performed according to the manufacturer's protocol.

LTC-IC assay

Transduced CD34+ cells were incubated in cytokine containing IMDM for 48 hours after which they were transferred to long-term culture initiating conditions. Briefly, adherent murine bone M2-10B4 Fibroblast feeder cell layers were established as described by StemCell Technologies (Vancouver, BC). Transduced CD34+ cells were added to the feeder layer and incubated for 5 weeks in human long-term culture initiating medium with 10^{-6} mol/l hydrocortisone (StemCell Technologies), with weekly half medium changes. After 5 weeks, cells were collected and subjected to colony-forming unit assay.

Colony-forming unit assays

For colony forming unit assay, 2×10^4 cells were transferred from LTC-IC into MethoCult GF H4434 medium (StemCell Technologies) in a humidified atmosphere of 5% CO_2 at 37 °C in the presence of the following cytokines: IL-3, 50 U/ml; SCF, 50 ng/ml; Epo, 2 U/ml; and G-CSF, 6.36 ng.

Western blot

Cell pellets in ice-cold phosphate-buffered saline containing protease inhibitors (Complete Protease Inhibitor Cocktail, Roche, Mannheim, Germany) were sonicated, and the protein containing supernatant stored at −80 °C. A total of 20 µg of total protein was used for the western blot analysis. Proteins were separated by polyacrylamide gel electrophoresis using 4–15% gradient gels (BioRad, Hercules, CA), followed by transfer onto nitrocellulose membranes according to the supplier's protocol (Mini ProteanIII; BioRad). Membranes were blocked in 5% nonfat dry milk (Bio-Rad) and washed in Tris-saline with 0.1% Tween-20. Membranes were incubated with anti-FokI antibody (Sangamo BioSciences, Richmond, CA), anti-hemagglutinin tag (Roche), or anti-β-actin (Sigma Aldrich, St Louis, MO). Membranes were developed with ECL plus reagent (Amersham, Buckinghamshire, UK).

Mismatch sensitive nuclease assay T7E1 assay

Genomic DNA was isolated as previously described.[48] CCR5 or LCR region was amplified. Primers for detection of CCR5 disruption were described previously.[49] Primers for HS-LCR site analysis were: 5′aaatcttgaccattctccactctc and 5′GGAGACACACAGAAATGTAACAGG. PCR products were hybridized and treated with 2.5 units of T7E1 endonuclease (NEB, Ipswich, MA). Digested PCR products were resolved by 10% Tris-borate EDTA buffer (TBE) polyacrylamide gel electrophoresis (Biorad) and stained with ethidium bromide. Band intensity was analyzed using ImageQuant software (Pittsburgh, PA).

Animal studies

All experiments involving animals were conducted in accordance with the institutional guidelines set forth by the University of Washington. Mice were housed in specific pathogen-free facilities. The immunodeficient NOG mice (strain name: NOD/Shi-scid/IL-2Rγnull) were obtained from the Jackson Laboratory (Bar Harbor, ME).

CD34+ cell transplantation

Cryo-conserved CD34+ cells were thawed in phosphate-buffered saline supplemented with 1% heat-inactivated FCS. Freshly thawed cells were cultured overnight in IMDM containing 10% heat-inactivated FCS, 10% bovine serum albumin, 4 mmol/l glutamine and penicillin/streptomycin, as well as human cytokines (TPO, 5 ng/ml; SCF, 25 ng/ml; IL-3, 20 ng/ml; and Flt3L, 50 ng/ml). The next day, cells were infected with HD-bGlob or HD-ZFN at an MOI of 5,000 vp/cell and incubated for 24 hours. Uninfected cells were used as control. The next day, NOG recipient mice received 300 Rad/3 Gy total body irradiation. Twenty-four hours postinfection, 3×10^5 transduced CD34+ cells were mixed with 2.5×10^5 freshly collected bone marrow cells of nonirradiated NOG mice and injected intravenously into recipient mice at 4 hours postirradiation. Six weeks after bone marrow transplantation, the engraftment rate was assayed as follows: blood samples were drawn, red blood cells were lysed, and the remaining cells were stained with PE-conjugated anti human CD45 antibodies and analyzed via flow cytometry. Six weeks after transplantation, bone marrow cells were subjected to double sorting with anti hCD45 (Miltenyi) beads and seeded on methylcellulose. After 2 weeks, colonies were counted and subjected to T7E1 nuclease assay.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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