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# The Nontoxic Cell Cycle Modulator Indirubin Augments Transduction of Adeno-Associated Viral Vectors and Zinc-Finger Nuclease-Mediated Gene Targeting

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**Abstract**

Parameters that regulate or affect the cell cycle or the DNA repair choice between non-homologous end-joining and homology-directed repair (HDR) are excellent targets to enhance therapeutic gene targeting. Here, we have evaluated the impact of five cell-cycle modulating drugs on targeted genome engineering mediated by DNA double-strand break (DSB)-inducing nucleases, such as zinc-finger nucleases (ZFNs). For a side-by-side comparison, we have established four reporter cell lines by integrating a mutated *EGFP* gene into either three transformed human cell lines or primary umbilical cord–derived mesenchymal stromal cells (UC-MSCs). After treatment with different cytostatic drugs, cells were transduced with adeno-associated virus (AAV) vectors that encode a nuclease or a repair donor to rescue EGFP expression through DSB-induced HDR. We show that transient cell-cycle arrest increased AAV transduction and AAV-mediated HDR up to six-fold in human cell lines and ten-fold in UC-MSCs, respectively. Targeted gene correction was observed in up to 34% of transduced cells. Both the absolute and the relative gene-targeting frequencies were dependent on the cell type, the cytostatic drug, the vector dose, and the nuclease. Treatment of cells with the cyclin-dependent kinase inhibitor indirubin-3′-monoxime was especially promising as this compound combined high stimulatory effects with minimal cytotoxicity. In conclusion, indirubin-3′-monoxime significantly improved AAV transduction and the efficiency of AAV/ZFN-mediated gene targeting and may thus represent a promising compound to enhance DSB-mediated genome engineering in human stem cells, such as UC-MSCs, which hold great promise for future clinical applications.

## Introduction

The precise manipulation of complex genomes has been a hallmark of studying gene function *in vivo* for more than two decades (Capecchi, 1989). Gene targeting, the underlying technology, is based on the homologous recombination (HR) pathway and describes the targeted transfer of genetic information from an exogenous donor DNA to the host genome. Meanwhile, this technology has been increasingly employed in biotechnology, and therapeutic applications are tangible, as epitomized by the introduction of targeted alterations in the genome of multi- and pluripotent human stem cells (Chamberlain *et al.,* 2004; Hockemeyer *et al.,* 2009; Zou *et al.,* 2009; Benabdallah *et al.,* 2010). Two major platforms have been described to overcome the low HR frequency in such cells: a viral vector platform based on adeno-associated virus (AAV) (Chamberlain *et al.,* 2004; Mitsui *et al.,* 2009; Khan *et al.,* 2010) and DNA double-strand break (DSB)-inducing agents, such as meganucleases (Silva *et al.,* 2011; Takeuchi *et al.,* 2011), zinc-finger nucleases (ZFNs) (Urnov *et al.,* 2010; Carroll, 2011; Rahman *et al.,* 2011), and transcriptional activator-like effector (TALE) nucleases (Bogdanove and Voytas, 2011; Mussolino and Cathomen, 2012).

Early studies involving the natural homing endonuclease I-*Sce*I, which recognizes an 18-bp target site, demonstrated that the creation of a DSB in the target gene stimulates recombination with an exogenous donor DNA up to 10,000-fold (Rouet *et al.,* 1994; Choulika *et al.,* 1995). The enhancing

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effect of DSBs is mainly due to activation of the cellular DNA damage response. In the presence of high concentrations of an appropriately designed donor molecule, sequence information from the donor DNA can be transferred to the target locus by HR-based homology-directed repair (HDR). When employing designer ZFNs, gene conversion frequencies of up to 50% were reported in human cell lines in the absence of selection (Lombardo *et al.,* 2007; Maeder *et al.,* 2008). In combination with AAV donor vectors, gene-targeting frequencies of up to 65% in transduced human cell lines were reported (Miller *et al.,* 2003; Porteus *et al.,* 2003; Gellhaus *et al.,* 2010; Hirsch *et al.,* 2010 ; Händel *et al.,* 2012), suggesting that a combination of the two platforms leads to synergistic effects.

Gene targeting in human stem cells has remained challenging. Although ZFN-mediated targeted integration has been achieved in up to 40% of a mesenchymal stromal cell (MSC) population (Benabdallah *et al.,* 2010), such high frequencies have remained unmatched in other therapeutically relevant human stem cells, including embryonic stem or induced pluripotent stem cells (Mitsui *et al.,* 2009; Zou *et al.,* 2009). Hence, gene targeting in human stem cells has been dependent on the co-integration of a selection marker to enrich for targeting events (Hockemeyer *et al.,* 2009; Mitsui *et al.,* 2009; Zou *et al.,* 2009; Khan *et al.,* 2010). Importantly, MSCs, which bear differentiation potential to multiple lineages, such as bone, neurons, and cardiac tissues, have been effectively transduced with AAV serotype 2 vectors (Kumar *et al.,* 2004; Chng *et al.,* 2007). In concordance with the differential availability of the cellular DNA repair factors that mediate HDR or nonhomologous end-joining (NHEJ) throughout the cell cycle, AAV-mediated gene targeting has been shown to be dependent on the cell-cycle phase (Trobridge *et al.,* 2005). Consequently, it has been speculated that transient cell-cycle arrest can affect the efficiency of ZFN- and AAV-mediated genome editing. A few studies have reported that a transient arrest in the G2 phase using the microtubule inhibitors vinblastine or nocodazole augment ZFN-mediated HDR up to seven-fold (Urnov *et al.,* 2005; Potts *et al.,* 2006; Maeder *et al.,* 2008; Olsen *et al.,* 2010). However, these compounds are highly toxic to cells and therefore not useful in a therapeutic context.

The present study was designed to assess the effect of the cell cycle on DSB-induced gene targeting and to identify compounds that stimulate HDR activity with minimal cytotoxicity. We identified the cyclin-dependent kinase (CDK) inhibitor indirubin-3′-monoxime as a promising candidate to increase AAV transduction and AAV/ZFN-mediated gene targeting by transiently arresting the cell cycle. Because indirubin-3′-monoxime is a well-tolerated anti-cancer drug (Eisenbrand *et al.,* 2004), it may present a promising compound to enhance DSB-dependent genome engineering in future clinical applications.

## Materials and Methods

### *Plasmids and vectors*

Plasmid pRK5.mCherry was generated by subcloning the mCherry cassette from pRSET-BmCherry (gift of Dr. Roger Y. Tsien) into vector pRK5 (Alwin *et al.,* 2005). All other plasmids have been described before: plasmids encoding AAV vector in Gellhaus *et al.* (2010); ZFN expression plasmids in Alwin *et al.* (2005) and Szczepek *et al.* (2007); and control vector pRK5.LHA-*Sce*IΔ in Szczepek *et al.* (2007). Maps and sequences of all plasmids are available upon request.

### *Cell-cycle profiling*

HeLa, HT-1080, and U-2 OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biochrom AG) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen). Umbilical cord–derived mesenchymal stromal cells (UC-MSCs) were isolated as previously described (Hatlapatka *et al.,* 2011) and cultured in α-MEM Glutamax (Invitrogen) supplemented with 10% FCS selected for MSC (Stem Cell Technologies), 100 IU/ml penicillin, and 10 mg/ml streptomycin (PAA Laboratories GmbH). The polyclonal target cells HeLa.696, HT1080.696, U2OS.696, and UC-MSC.696 were generated by lentiviral transduction as previously described (Cornu and Cathomen, 2007; Gellhaus *et al.,* 2010). The phenotype of UC-MSC was evaluated by flow cytometric analysis of surface antigens typical for MSC (CD105, CD73, CD90) and absence of hematopoietic stem cell markers (CD45, HLA-DR, CD14, CD34, CD19). All antibodies had been purchased from Becton Dickinson and staining procedure was performed as previously described (Hatlapatka *et al.,* 2011). For cell-cycle profiling, cells were kept in serum-free conditions for 24 h before 200,000 cells were seeded per well of a 6-well plate. Four hours later, cells were incubated for 4 h (vinblastine) or 24 h (hydroxyurea, indirubin-3′-monoxime, L-mimosine or etoposide) with the indicated components (Sigma-Aldrich). Then, cells were fixed overnight in ethanol 24, 48, or 72 h after treatment. Before staining with propidium iodide solution (3.8 m*M* sodium citrate, 25 μg/ml of PI, 0.2 mg/ml of RNase A), low molecular weight DNA was released by incubating samples in DNA extraction buffer (200 m*M* $Na_2HPO_4$, 100 m*M* citric acid pH 7.8). At least 10,000 cells were assessed by flow cytometry (FACSCalibur, BD Biosciences), and cell cycle profile was evaluated by plotting FL3-W versus FL3-A (see Supplementary Fig. S1 for details, available online at www.liebertonline.com/hum). The number of cells containing a fragmented genome due to apoptosis was quantified by determining the percentage of subG1 events (see Supplementary Fig. S2 for details).

### *Recombinant AAV and cell transduction*

Recombinant AAV vectors (rAAV) were produced and titrated as previously described (Gellhaus *et al.,* 2010). HeLa, HT-1080, or U-2 OS cells were incubated in serum-free DMEM for 24 h before 50,000 cells per well of a 12-well plate were seeded and treated with cytostatic drugs as described above. To assess AAV transduction, cells were infected with rAAV.donor-REx at 20 gc/cell (HeLa and U-2 OS) or 50 gc/cell (HT-1080). For AAV-based gene targeting, polyclonal target cell lines were infected with rAAV.donor-*Sce*I at 2,000 gc/cell alone or with rAAV.donor at 2,000 gc/cell in combination with I-*Sce*I or ZFN expression vectors at 2000, 500, or 200 gc/cell. For AAV-mediated gene correction in human primary cells, $5 \times 10^4$ UC-MSC.696 were seeded in 24-well plates coated with 0.1% porcine gelatin (Sigma-Aldrich) 6–8 h before transduction with 10,000 gc/cell of AAV-ZFN and 2,000 gc/cell of AAV-donor. The frequencies of gene targeting and transduction were determined 4 or 11 days after transduction by flow cytometry, respectively.

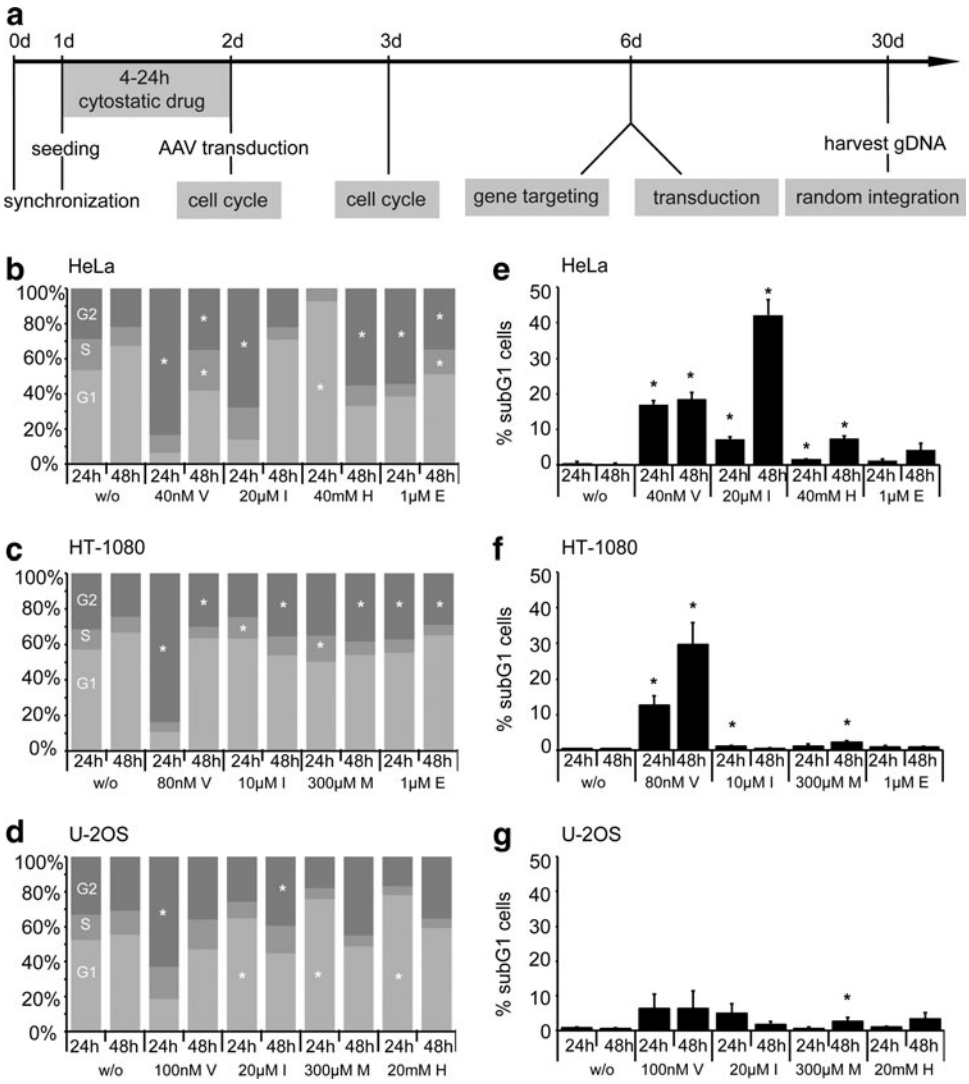


**FIG. 1.** Cell-cycle profile and toxicity. **(a)** Experimental setup. Cells were synchronized by serum starvation for one day prior to incubation with cytostatic drugs for 4 h (vinblastine) or 24 h (all others). Cell-cycle profiles were measured 24 h and 48 h later by flow cytometry. For gene targeting, cells were transduced on day two with the AAV vectors, and the extent of gene targeting and transduction was determined by flow cytometry on day six. After 30 days in culture, cells were harvested and genomic DNA extracted for analysis of AAV random integration. **(b–d)** Cell-cycle profiles of HeLa **(b)**, HT-1080 **(c)**, and U-2 OS **(d)** cells. At 24 h and 48 h after treatment with cytostatics, cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. The percentage of cells in G1 (light gray), S (gray), and G2/M (dark gray) phase is indicated with a bar graph. Asterisks mark a significant difference ($p < 0.05$) from nontreated cells. **(e–g)** Cytostatic treatment-induced toxicity in HeLa **(e)**, HT-1080 **(f)**, and U-2 OS **(g)** cells. At 24 h and 48 h after treatment, cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. The percentage of cells in subG1 phase is indicated. Asterisks mark significant differences ($p < 0.05$) from nontreated cells. E, etoposide; H, hydroxyurea; I, indirubin-3′-monoxime; M, L-mimosine; V, vinblastine; w/o, without cytostatics.

### *Transfection-based gene targeting*

100,000 serum-starved HeLa.696 cells were seeded into each well of a 12-well plate and transfected using polyethylenimine (PEI) as previously described (Söllü *et al.*, 2010). Twenty-five ng of nuclease expression vectors (ZFN or I-*Sce*I) were co-transfected with 1.6 $\mu$g of donor DNA (pTR.dGFPiN) and 100 ng of pRK5.mCherry as an internal transfection control. After 24 h, cells were rinsed and incubated with 40 m*M* hydroxyurea for 24 h or 40 n*M* vinblastine for 4 h. At 3 and 7 days post-transfection, the percentage of EGFP and mCherry-positive cells was determined by flow cytometry. Relative gene targeting is indicated as EGFP positive cells in the mCherry positive cell fraction.

### *Western blotting*

Western blots were basically performed as described before (Händel *et al.*, 2009). Cells were harvested 72 h post-infection with AAV vectors and resuspended in lysis buffer. Equal amounts of proteins (60 $\mu$g) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membrane (Millipore). Proteins were detected using

antibodies recognizing the HA tag (Novus Biologicals; 1:5,000) and visualized after incubation with an IRDye-800CW–labeled secondary antibody (Li-Cor Bioscience; 1:20,000) using an Odyssey® Infrared Imaging System (Li-Cor Bioscience).

### *Quantitative PCR*

Total DNA from polyclonal cells was isolated 30 days after AAV transduction using the Invisorb Spin Cell Mini Kit (Invitek). For quantitative real-time polymerase chain reaction (PCR), SYBR Green (DyNAmo™ Capillary SYBR Green, Finnzymes) and template concentrations of 100 ng genomic DNA were used. The PCR cycling conditions were as follows: Denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, 60°C for 20 sec, and 72°C for 20 sec. Random integration (RI) #538 RIfw (5′-tgcttacatccgttctcgtg) and #539 RIrv (5′-cgcagacccttaaccaggta); target locus (TL) #361 TLfw (5′-gaggagctgttcaccggg) and #237 TLrv (5′-cgtaggtc agggtggtcac); gene targeting (GT) #136 GTfw (5′-caagggc gaggagctgtt) and #559 GTrv (5′-ctcggcgcgggtcttgtag). The samples for detection of gene targeting were pre-amplified using 0.125 U of Taq polymerase (Peqlab Biotechnologie GmbH) using 300 ng of genomic DNA, 300 $\mu M$ of each primer (#361 TLfw and #559 GTrv), 200m*M* dNTPs, and 1x reaction buffer. Cycling conditions were as follows: denaturation at 94°C for 4 min, followed by 13 cycles of 94°C for 30 s, 60°C for 30 s, 2 min at 72°C, and a final extension of 7 min at 72°C. The product of this first PCR reaction was purified with the Invisorb PCR Purification Kit (Invitek), and 1 out of 20 $\mu$l was used as a template in the qualitative PCR (qPCR).

### *Genotyping*

Allele-specific PCR using primer pair #136 GTfw and #559 GTrv was employed to detect gene correction on the genome level, as described previously (Händel *et al.*, 2012). Briefly, cells were harvested 16 days after transduction, and 300 ng of genomic DNA was used for PCR. Control reaction was performed with *PTBP*2 gene-specific primers (#1274 PTBPfw

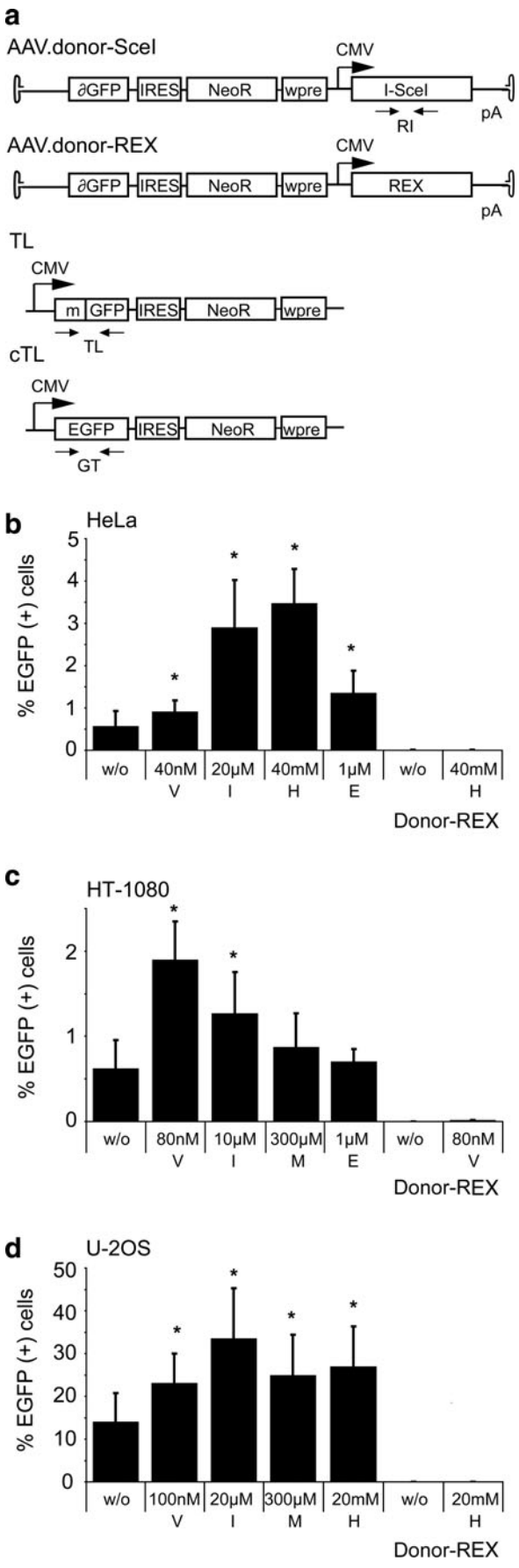


**FIG. 2.** Effect of cytostatic drugs on DNA double-strand break-stimulated gene targeting. **(a)** Setup of DSB-mediated gene correction. The target locus (TL) contains a disrupted *EGFP* (mGFP) gene with binding sites for I-*Sce*I and zinc-finger nucleases (ZFNs). After infection with vector AAV.-donor-*Sce*I, the I-*Sce*I–induced DSB stimulates homologous recombination with the AAV donor to restore *EGFP* (cTL). AAV.donor-REx expresses DsRed-Express and served as a control vector. Small arrows indicate primer binding sites for qPCR analysis (Fig. 4). **(b–d)** Rescue of EGFP expression in HeLa **(b)**, HT-1080 **(c)**, and U-2 OS cells **(d)**. A total of 50,000 cells were treated with cytostatic drugs, as indicated, before infection with AAV.donor-*Sce*I (2,000 gc/cell). The extent of gene correction was measured by counting EGFP-positive cells by flow cytometry four days after transduction. Graphs indicate average and standard deviation of three independent experiments. Asterisks mark significant differences ($p < 0.05$) from nontreated cells. IRES, internal ribosome entry site; NeoR, neomycin resistance gene; wpre, woodchuck hepatitis post-transcriptional regulatory element; cTL, corrected target locus; E, etoposide; H, hydroxyurea; I, indirubin-3′-monoxime; M, L-mimosine; V, vinblastine; w/o, without cytostatics.

5′-tctcattccctatgttcatgc and #1275 PTBPrv 5′-gttcccgcagaa tggtgaggtg). PCR products (328 bp and 150 bp, respectively) were visualized by electrophoresis on a 2% agarose gel.

### *Statistical analysis*

All experiments were independently performed at least three times. Error bars represent standard deviation, and statistical significance was determined using a two-sided Student's *t*-test with unequal variance.

## Results

### *Optimizing transient cell-cycle arrest*

An initial set of experiments was performed to determine the optimal drug concentration to transiently arrest the cell cycle with minimal toxicity in three different human cell lines. To this end, HeLa (aneuploid adenocarcinoma), HT-1080 (quasi-diploid fibrosarcoma), and U-2 OS (hypertriploid osteosarcoma) cells were serum starved for one day prior to incubation with a broad range of concentrations of etoposide, hydroxyurea, indirubin-3′-monoxime, L-mimosine, and vinblastine. Cell-cycle profiles and drug-induced apoptosis were measured at day 1 and 2 after treatment with cytostatics by staining fixed cells with propidium iodide and ensuing analysis of DNA content by flow cytometry (Fig. 1a and Supplementary Fig. S1). The cell cycle profiles in response to optimal concentrations of the chosen chemicals are depicted in Figure 1b–d. The microtubule inhibitor vinblastine induced the strongest G2 arrest in the three tested cell lines. Incubation of cells with the CDK inhibitor indirubin-3′-monoxime affected the cell cycle with different kinetics and induced a significant G2/M arrest in HeLa cells at day 1 after treatment and in HT-1080 and U-2 OS cells after two days. On the other hand, some cytostatic compounds induced significant effects only in a subset of the tested cell lines. For instance, treatment with hydroxyurea induced a robust G1 arrest in HeLa and U-2 OS cells but did not affect HT-1080 cells (not shown).

To assess the cytotoxicity associated with the treatment regiment, the extent of apoptosis was determined by quantifying the subG1 fraction (Supplementary Fig. S2). Vinblastine was associated with high toxicity in all cell lines tested (Fig. 1e–g). The anti-cancer drug indirubin-3′-monoxime was described as a potent inducer of caspase-mediated apoptosis in HeLa cells (Shi and Shen, 2008), but the substance revealed no or only mild toxicity in HT-1080 and U-2 OS cells. The other compounds, hydroxyurea, etoposide, and L-mimosine, provoked no or only mild cytotoxicity in all tested cell lines at the concentrations used.

In summary, we have compared five drugs for conditions to induce a transient cell-cycle arrest in the G1 or G2/M phase with the lowest possible toxicity in three different cell lines. The observed differences with regard to the degree of cell-cycle arrest and cytotoxicity is most likely a reflection of the different genetic background and chromosomal alterations of the used cell lines.

### *Transient cell-cycle arrest augments AAV-mediated gene targeting*

In order to assess quantitatively whether a transient G2 arrest enhances gene targeting after induction of a DSB in the target locus, we employed a previously described enhanced green fluorescent protein (EGFP) rescue assay (Gellhaus *et al.,* 2010). Target cell lines that carry a mutated EGFP target locus were generated by lentiviral transduction and subsequent selection in the presence of neomycin. To ensure that results are comparable between the different cell lines, we worked with polyclonal cell populations, and the parental cells were transduced with less than 0.1 transducing units per cell to make sure that individual cells are unlikely to contain more than one target locus. The EGFP open reading frame in the target locus is disrupted by binding sites for I-*Sce*I and a ZFN. Upon delivery of a donor DNA in the form of an AAV vector, EGFP expression can be rescued by HDR between the target locus and the AAV donor (Fig. 2a). As shown previously, creation of a DSB in the target locus by expression of I-*Sce*I stimulates gene correction significantly (Miller *et al.,* 2003; Porteus *et al.,* 2003; Gellhaus *et al.,* 2010). HeLa, HT-1080, and U-2 OS-based target cell lines were treated with cytostatic drugs and then infected with 2,000 genome copies (gc) of AAV.donor-*Sce*I or AAV.donor-REx per cell. Treatment with hydroxyurea and indirubin-3′-monoxime–enhanced AAV-based gene targeting in HeLa cells by a factor of 5–6, while vinblastine and etoposide had significant but more modest effects (Fig. 2b). In HT-1080 cells, treatment with vinblastine and indirubin-3′-monoxime revealed the strongest effects with a 2- to 3-fold stimulation of EGFP rescue (Fig. 2c). While AAV-based gene targeting was able to rescue EGFP expression in about 0.6% of infected HeLa and HT-1080 cells, U-2 OS cells were much more susceptible to gene correction with AAV vectors (Fig. 2d). In the absence of cytostatics, 14% of cells were EGFP-positive upon infection with AAV.donor-*Sce*I. Treatment of cells with indirubin-3′-monoxime could stimulate gene correction more than 2-fold to 34% of corrected cells. Of note, after infection with the control vector AAV.donor-REx, the number of EGFP-positive cells was not significantly above background for all target cell lines, confirming that efficient AAV-mediated gene targeting is dependent on the creation of a DSB in the EGFP target locus.

In conclusion, transient cell-cycle arrest induced by particular cytostatics enhanced AAV-mediated gene correction in a cell-type–specific manner. Only the CDK-inhibitor indirubin-3′-monoxime showed at least a 2-fold stimulatory effect in all three tested cell lines.

### *Cytostatic drugs augment AAV transduction*

Reagents that induce DNA damage have been described to enhance the transduction of AAV in cultured cells and this effect has been mainly attributed to their properties of activating the DNA damage response (Alexander *et al.,* 1994; Russell *et al.,* 1995). Using the donor vector containing the REx expression cassette, we compared the effects of the different drugs on AAV transduction in the three different cell lines. Upon infection with 20 or 50 gc/cell of AAV.donor-REx, 1–4% of cells were scored REx-positive by flow cytometry four days later (Fig. 3). Preincubation of cells with the examined cytostatic drugs increased AAV transduction significantly between 1.5 to 8.3-fold, depending on the cell line and the specific drug used. However, only indirubin-3′-monoxime and vinblastine showed a stimulatory effect in all three cell lines. Infection with AAV.donor-*Sce*I served as a

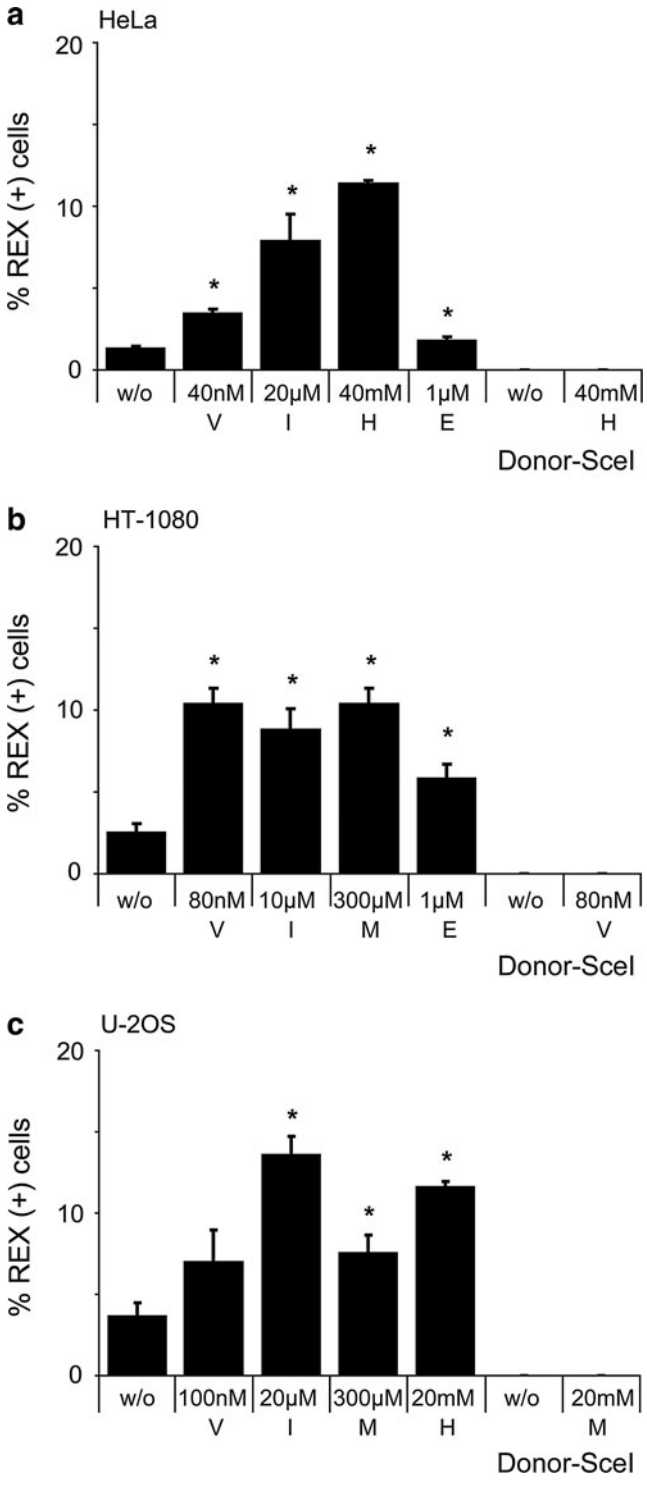


**FIG. 3.** Effect of cytostatic drugs on AAV transduction. Transduction frequency in HeLa **(a)**, HT-1080 **(b)**, and U-2 OS **(c)** cells. After treatment with cytostatic drugs, 50,000 cells were transduced with AAV.donor-REx at 20 gc/cell (HeLa, U-2 OS) or 50 gc/cell (HT-1080) and assessed by flow cytometry four days later. Graphs represent average percentage of REx-positive cells with standard deviation, and asterisks mark significant ($p < 0.05$) differences in transduction frequency of treated vs. nontreated cells. Control infections were performed with AAV.donor-*Sce*I. E, etoposide; H, hydroxyurea; I, indirubin-3′-monoxime; M, L-mimosine; V, vinblastine; w/o, without cytostatics.

negative control. These results show that the beneficial effects of the cytostatic drugs on AAV transduction parallels the increase in AAV-mediated gene targeting (Fig. 2), suggesting that transduction is a rate-limiting step in AAV-mediated gene targeting.

### *Targeting ratio is cell-type dependent*

The cellular proteins mediating HDR are mainly available in the G2 phase of the cell cycle, while NHEJ factors are more prominent in G1 (Shrivastav *et al.,* 2008). Changing the cell-cycle profile during AAV infection could therefore affect the fate of the donor DNA in terms of whether it will be used as a template for HDR-mediated gene targeting or for NHEJ-mediated illegitimate recombination, respectively. To answer this question, the gene targeting frequency of cells transduced with AAV.donor-*Sce*I was offset against the random integration frequency of the vector, as determined by flow cytometry and quantitative PCR on extracted genomic DNA. The targeting ratio was defined here as the quotient between the gene targeting frequency and the illegitimate integration frequency. Similar to the diverse responses to treatment with cytostatic drugs in terms of cell-cycle profile, the different cell lines dealt differently with the exogenous vector DNA. While in HeLa cells the AAV vector was ~7 times more likely to integrate into the host genome than to serve as a template for HDR, the ratio was about 1:1 in HT-1080 cells, and 33:1 in favor of gene targeting in U-2 OS cells (Fig. 4). However, none of the cytostatic drugs had a significant effect on the targeting ratio in any of the tested cell lines (Supplementary Fig. S3). In brief, while the targeting ratio itself was highly cell-type dependent, probably reflecting the cells' distinct ability to cope with DSBs and exogenous DNA, treatment of cells with cytostatic drugs did not significantly affect the balance between gene targeting and random integration.

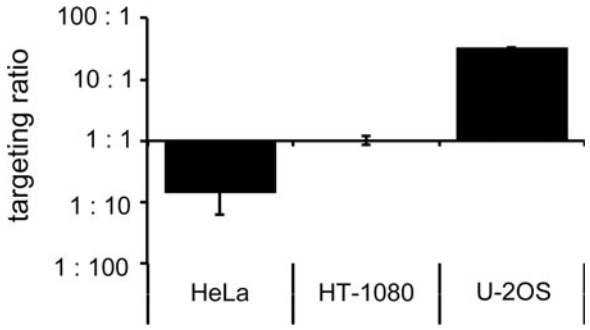


**FIG. 4.** Targeting ratio. I-*Sce*I-induced AAV-mediated gene targeting was performed as described in Figure 1. Quantitative PCR was performed on genomic DNA extracted 30 days post transduction with primer pairs (see Fig. 1a) to quantify the number of randomly integrated AAV vector genomes (RI) or target locus (TL) as an internal control. The targeting ratio is indicated as the quotient between the number of gene-targeting events (percent EGFP-positive cells at day six) and RI events (I-*Sce*I copies per TL at day 30). Graph represents average targeting ratio with standard deviation.

*Transient cell-cycle arrest augments zinc-finger nuclease-mediated gene targeting in cell lines*

While I-*Sce*I has been widely used as a benchmark in the genome engineering field, DSB-enhanced gene targeting in a therapeutic context is only possible when employing customizable designer nucleases, such as zinc-finger nucleases (ZFNs). Here we used previously characterized ZFNs with heterodimeric *Fok*I domains that recognize a target site in the EGFP reporter (Szczepek *et al.*, 2007). To directly compare I-*Sce*I with ZFN-induced gene targeting in an AAV context, the respective nucleases were expressed from separate vectors and independently from donor administration (Fig. 5a). As expected, transduction of the U-2 OS target cell line with increasing amounts of AAV vector revealed that both I-*Sce*I (Fig. 5b) and ZFN-mediated gene targeting (Fig. 5c) was strictly dose-dependent. Depending on the individual cytostatic and the vector dose, a transient cell-cycle arrest improved AAV-mediated gene targeting up to 5.3-fold. Also, there was no considerable difference between cell-cycle–dependent stimulation of AAV.*Sce*I and AAV.ZFN-induced gene targeting, suggesting that these cytostatic drugs can be used to enhance any kind of DSB-mediated gene targeting.

The increase in AAV transduction after treatment of cells with particular cytostatic drugs implied that the observed augmentation in gene targeting (Fig. 2) was exclusively due to enhanced transduction efficiency (Fig. 3). Consistent with this implication was the observation that AAV-mediated I-*Sce*I and ZFN expression was enhanced by treating cells with hydroxyurea, vinblastine, and indirubin-3′-monoxime (Fig. 5d). To demonstrate that transient cell-cycle arrest has a direct stimulatory effect on gene targeting and not only on

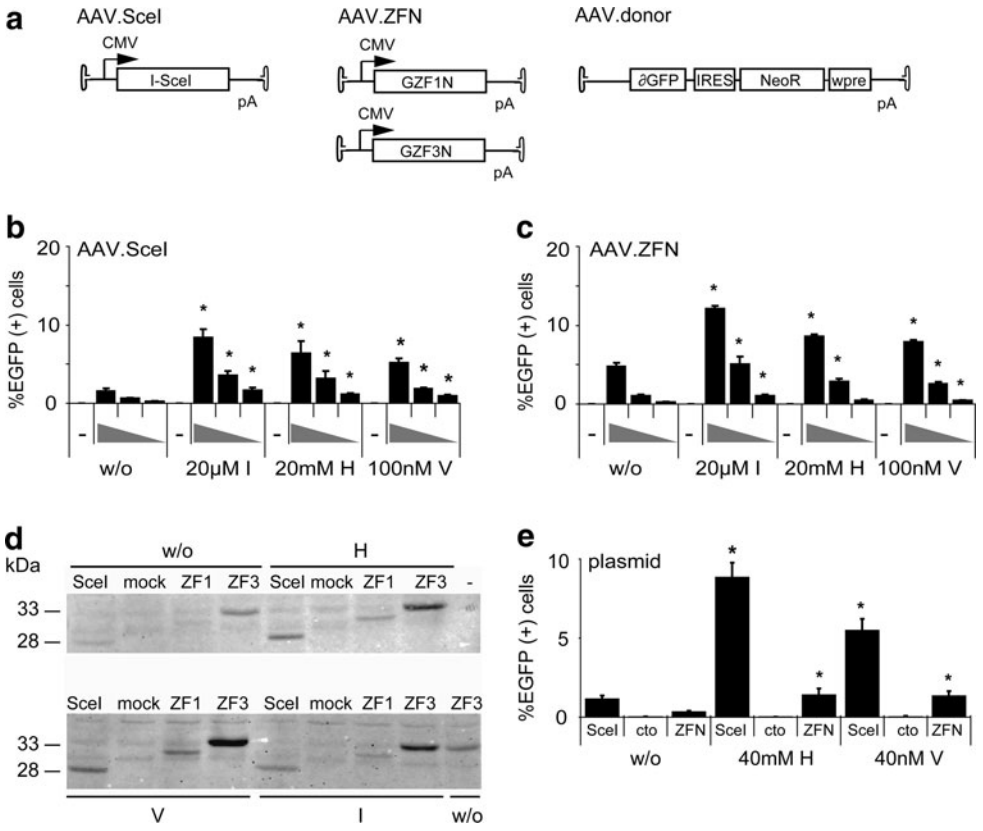


**FIG. 5.** AAV-mediated gene targeting with zinc-finger nucleases. **(a)** Experimental setup. Target cell lines contain a disrupted EGFP (mGFP) gene with binding sites for ZFN and I-*Sce*I. A DSB to stimulate gene targeting between target locus and AAV.donor was created by transduction with either two AAV vectors encoding ZFN subunits GZF1N and GZF3N or with an I-*Sce*I AAV expression vector, respectively. **(b, c)** AAV-mediated gene targeting in U-2 OS target cells induced by I-*Sce*I **(b)** or ZFN expression **(c)**. After pretreatment with cytostatic drugs, cells were transduced with various AAV vector amounts (AAV.donor 2,000 gc/cell in all samples; AAV.ZFN or AAV.*Sce*I 2,000, 500, or 200 gc/cell, respectively) and assessed after four days by flow cytometry. Graphs represent average percentage of EGFP-positive cells with standard deviation and asterisks mark significant ($p < 0.05$) differences between treated and nontreated cells. **(d)** Expression analysis. U-2 OS cells were transduced with AAV.ZFN or AAV.*Sce*I (2,000 gc/cell). Cells were harvested after 72 h and I-*Sce*I, GZF1N (ZF1), and GZF3N (ZF3) expression levels detected using an HA tag-specific antibody. Mock transduced cells served as a control. **(e)** Plasmid-based gene targeting in HeLa target cells. Cells were transfected with nuclease expression plasmids, donor plasmid, and pRK5.mCherry as a transfection marker. After 8 h, transfected cells were treated with cytostatic drugs and evaluated by flow cytometry three days later. Graphs indicate percentage of EGFP-positive cells with standard deviation, normalized for transfection efficiency. Asterisks mark significant ($p < 0.05$) differences in gene correction in treated vs. untreated (w/o) cells. Transfection with a nonfunctional nuclease is indicated as "cto." H, hydroxyurea; I, indirubin-3′-monoxime; V, vinblastine; w/o, without cytostatics.

AAV transduction, the HeLa target cell line was transfected with the AAV vector–encoding plasmid DNA. As expected, expression of either I-*Sce*I or the ZFN improved gene correction at the target locus significantly and resulted in 1.2% or 0.3% of EGFP-positive cells, respectively (Fig. 5e). A further significant increase (up to 7.6-fold) in gene targeting was observed upon treatment of cells with either hydroxyurea or vinblastine. These results verify that transient cell-cycle arrest is a general means of improving DSB-mediated gene targeting.

### *Indirubin-3′-monoxime enhances AAV/ZFN-mediated gene targeting in human mesenchymal stromal cells*

To demonstrate that transient cell-cycle arrest improves gene targeting also in human stem cells, the defective EGFP reporter was introduced into umbilical cord–derived mesenchymal stromal cells (UC-MSCs). These primary cells were transduced with the same AAV vectors, previous to treatment with cytostatic drugs (Fig. 6a). Notably, UC-MSCs did not exhibit cell cycle changes in response to any of the drugs tested before (not shown) with the exception of indirubin-3′-monoxime, which induced a significant, dose-dependent G1 arrest (Fig. 6b). At the highest concentration, treatment with indirubin-3′-monoxime led to a 10-fold increase in EGFP-positive cells compared to untreated cells, as measured by flow cytometry 11 days post-transduction (Fig. 6c). PCR-based genotyping, which was used to verify gene correction on the genome level (Händel *et al.,* 2012), confirmed the stimulatory effect of indirubin-3′-monoxime on AAV/ZFN-mediated gene targeting. Of note, treatment with the cytostatic drug did not lead to profound cell toxicity (Supplementary Fig. S4) and did not alter MSC properties. After initial cell-cycle arrest, the cells regained their high proliferative capacity and displayed typical morphology as well as characteristic surface markers (Supplementary Fig. S5).

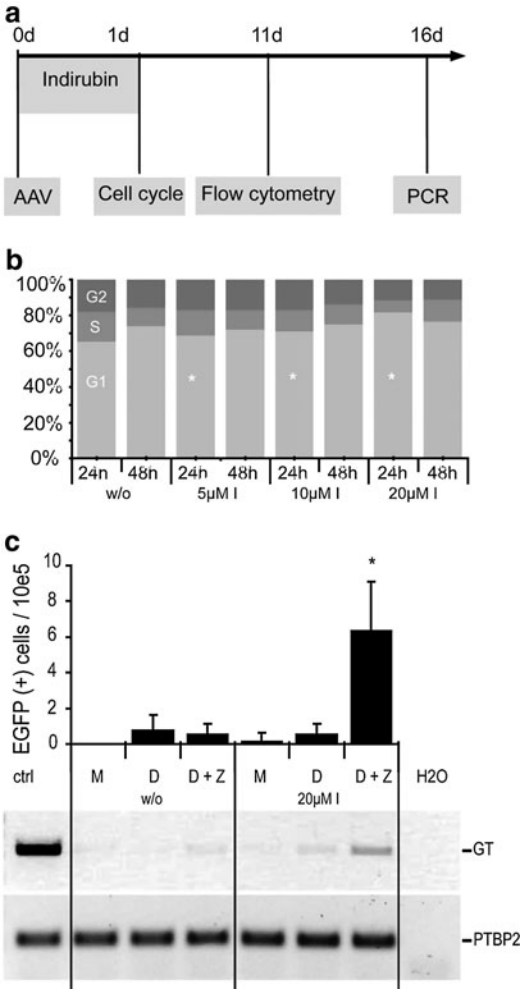


**FIG. 6.** ZFN-mediated gene targeting in human mesenchymal stromal cells. **(a)** Experimental setup. UC-MSC.696 cells were seeded in a 24-well plate (50,000 cells/well) 6 h before transduction with 10,000 gc/cell of AAV-ZFN and 2,000 gc/cell of AAV-donor and then treated with indirubin-3′-monoxime. **(b)** Cell-cycle analysis of UC-MSC.696 cells after treatment with indirubin-3′-monoxime. At 24 h and 48 h after treatment with cytostatics, cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. The percentage of cells in G1 (light gray), S (gray), and G2/M (dark gray) phase is indicated as a bar graph. Asterisks mark a significant difference ($p < 0.05$) from nontreated cells. **(c)** Gene correction in UC-MSC. The graph shows the numbers of EGFP-positive cells per 100,000 cells measured by flow cytometry. Asterisk indicates a significant increase ($p < 0.05$) over nontreated cells. To verify the presence of faithfully corrected EGFP gene on the genome level, allele-specific PCR was performed. A PCR encompassing a fraction of the *PTBP2* gene was used as a control ($N = 5$). I, indirubin-3′-monoxime; M, mock; D, donor; D + Z, donor and ZFN; GT, gene targeting; *PTBP2*, locus-encoding polypyrimidine-binding protein 2.

## Discussion

Targeted genome engineering, such as gene correction or the integration of a therapeutic transgene cassette into a "neutral site" in the human genome, will be safer and more efficient if NHEJ-mediated random integration of the donor DNA can be suppressed and HR-mediated gene targeting enhanced. Conventional gene targeting, *e.g.,* the generation of gene knockouts in murine ES cells, likely depends on the occurrence of a natural DSB at the target locus to mediate HR between the targeting vector and the gene of interest. The intentional creation of a DSB by a designer nuclease, on the other hand, has been shown to significantly enhance HR-based genome engineering if sufficient amounts of appropriately designed donor molecules are available (Lombardo *et al.,* 2007). Since illegitimate integration is only mildly (if at all) increased by the created DSB (Miller *et al.,* 2003; Gellhaus *et al.,* 2010), the net balance between the two repair pathways strongly shifts toward HR. One way to shift the balance further is the ectopic expression of HR factors (Yanez and Porter, 1999) or, *vice versa*, the suppression of factors involved in NHEJ (Fattah *et al.,* 2008) or sister chromatid HR (Potts *et al.,* 2006). For instance, the genetic knockout of one *Ku70* allele, a gene encoding a protein that forms an active complex with Ku86 and protein kinase catalytic subunit (DNA-PKcs) at DSBs, increased AAV-mediated gene targeting 6-fold (Fattah *et al.,* 2008). Whereas this effect could be further enhanced by siRNA-mediated knockdown of the second *Ku70* allele (Fattah *et al.,* 2008), knockdown of DNA-PKcs did not have an effect on AAV-mediated gene targeting (Vasileva *et al.,* 2006). On the other hand, although it has been shown that Rad51 and Rad54 are essential for both ZFN

and AAV-mediated gene targeting (Vasileva *et al.,* 2006; Bozas *et al.,* 2009), human cells stably over-expressing RAD51 revealed only a two-fold increase in conventional, plasmid-based gene targeting (Yanez and Porter, 1999). On the whole, it remains open to discussion whether a genetic intervention to shift the balance in AAV or ZFN-mediated gene targeting from NHEJ to HR is meaningful in a therapeutic context.

The availability of NHEJ and HR repair factors is strongly cell-type dependent. For example, we and others have shown previously that DSB-induced gene targeting works particularly well in U-2 OS cells (Händel *et al.,* 2009; Gellhaus *et al.,* 2010; Chen *et al.,* 2011). Moreover, the balance between the pathways changes during progression through the cell cycle (Shrivastav *et al.,* 2008). Even though NHEJ seems to be active throughout the whole cell cycle, the DSB repair shifts from NHEJ toward HR as the cell progresses from G1 to S/G2, as for instance, evidenced by a lower activity of the NHEJ factor DNA-PKcs (Lee *et al.,* 1997) and increased expression of the HR factors RAD51 and RAD52 (Chen *et al.,* 1997) in the S phase. Hence, the cell-cycle status is an important factor in HR-based genome engineering. The balance between HDR and NHEJ can be shifted toward HDR by chemical or genetic inhibition of NHEJ, which led to up to 10-fold increase of AAV HDR-based gene targeting (Paulk *et al.,* 2012). A few compounds used in chemotherapy regiments have been described to enhance ZFN-mediated gene targeting, such as vinblastine (Urnov *et al.,* 2005; Potts *et al.,* 2006; Maeder *et al.,* 2008) and nocodazole (Olsen *et al.,* 2010). Our results extend this observation to AAV/ZFN-mediated gene targeting and confirm that the stimulatory potential on gene targeting is conveyed by transiently arresting the cells in G1 or G2. However, microtubule inhibitors are rather cytotoxic, as substantiated by this study, and their application in a therapeutic context is not advisable. Here, we have identified indirubin-3′-monoxime as a robust enhancer of AAV/ZFN-mediated gene targeting in three different cell lines and in primary human MSC. The anti-cancer drug indirubin has been characterized as a potent CDK-inhibitor (Hoessel *et al.,* 1999), and more active derivatives, such as indirubin-3′-monoxime, have been shown to induce apoptosis in a number of cancer cells, including HeLa (Shi and Shen, 2008), and to inhibit the growth of tumors in clinical studies and in animal models without major side effects (Eisenbrand *et al.,* 2004). Of note, while the stimulatory potential of the other cytostatic drugs to enhance AAV/ZFN-mediated gene correction was limited to certain cell types, treatment with indirubin-3′-monoxime showed a significant activity in all cells tested here.

It has been known for a while that substances that induce DNA damage, such as cis-platinum, hydroxyurea, camptothecin, or etoposide increase AAV transduction in cultured cells (Alexander *et al.,* 1994; Russell *et al.,* 1995). This effect has been attributed to activation of the cellular DNA damage response, which in turn is important to process the vector genome to generate a transcription-competent template (Cervelli *et al.,* 2008). The development of capsid variants optimized for cell targeting and/or trafficking (Büning *et al.,* 2008; Li *et al.,* 2008), as well as the use of compounds that do not directly damage the DNA, such as proteasome inhibitors (Jennings *et al.,* 2005; Monahan *et al.,* 2010), have further improved AAV transduction efficiencies both in cultured cells (Ellis *et al.,* 2012) and *in vivo* (Paulk *et al.,* 2012). The effect might be linked to the nuclear trafficking of the viral particles. In combination with sodium butyrate, a histone deacetylase inhibitor, ZFN-mediated gene targeting could be increased up to six-fold in a human cell line (Ellis *et al.,* 2012). These drugs are FDA-approved, which might facilitate integration into clinical protocols for rAAV-based gene therapy; however, cytotoxic side effects on relevant primary target cells need to be investigated. Here we show that AAV transduction can also be augmented with a CDK inhibitor that arrests the cell cycle without obvious cytotoxicity. The fact that the stimulatory effect of indirubin-3′-monoxime on AAV transduction parallels the increase in AAV-mediated gene targeting identifies transduction as another rate-limiting step in AAV-mediated gene targeting. Interestingly, depending on the cell type, indirubin-mediated cell-cycle arrest occurred in the G1 or G2 phase of the cell cycle, yet in both instances we found an increase in gene targeting. This suggests that the transient cell-cycle arrest had a dual effect in AAV/ZFN-mediated gene targeting: (i) an increase in AAV transduction that leads to an augmented nuclease expression and (ii) a longer transition time through the cell cycle that may facilitate gene targeting.

In conclusion, we have identified and characterized the application of small molecule drugs for improving AAV/ZFN-mediated gene targeting by transiently slowing transition through the cell cycle. In particular, incubation of cells with indirubin-3′-monoxime, a well-tolerated CDK inhibitor with minimal toxicity in normal cells, augmented the gene-targeting frequency up to 10-fold to correct a mutated reporter gene in up to 34% of infected human cells. Given the recent success in AAV-mediated gene targeting in other multipotent (Chamberlain *et al.,* 2004; Jang *et al.,* 2011) or pluripotent stem cells (Mitsui *et al.,* 2009; Khan *et al.,* 2010; Asuri *et al.,* 2012), respectively, as well as *in vivo* (Papadopoulos *et al.,* 1997; Paulk *et al.,* 2012), these findings present a promising strategy to enhance AAV/ZFN-mediated genome engineering for potential clinical applications.

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## Author Disclosure Statement

The authors declare no potential conflict of interest.

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