Differential Transgene Silencing of Myeloid-Specific Promoters in the *AAVS1* Safe Harbor Locus of Induced Pluripotent Stem Cell-Derived Myeloid Cells

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Targeted integration into a genomic safe harbor, such as the *AAVS1* locus on chromosome 19, promises predictable transgene expression and reduces the risk of insertional mutagenesis in the host genome. The application of gamma-retroviral long terminal repeat (LTR)-driven vectors, which semirandomly integrate into the genome, has previously caused severe adverse events in some clinical studies due to transactivation of neighboring proto-oncogenes. Consequently, the site-specific integration of a therapeutic transgene into a genomic safe harbor locus would allow stable genetic correction with a reduced risk of insertional mutagenesis. However, recent studies revealed that transgene silencing, especially in case of weaker cell type-specific promoters, can occur in the *AAVS1* locus of human pluripotent stem cells (PSCs) and can impede transgene expression during differentiation. In this study, we aimed to correct p47^{phox} deficiency, which is the second most common cause of chronic granulomatous disease, by insertion of a therapeutic p47^{phox} transgene into the *AAVS1* locus of human induced PSCs (iPSCs) using CRISPR-Cas9. We analyzed transgene expression and functional correction from three different myeloid-specific promoters (miR223, CatG/cFes, and myeloid-related protein 8 [MRP8]). Upon myeloid differentiation of corrected iPSC clones, we observed that the miR223 and CatG/cFes promoters achieved therapeutically relevant levels of p47^{phox} expression and nicotinamide adenine dinucleotide phosphate oxidase activity, whereas the MRP8 promoter was less efficient. Analysis of the different promoters revealed high CpG methylation of the MRP8 promoter in differentiated cells, which correlated with the transgene expression data. In summary, we identified the miR223 and CatG/cFes promoters as cell type-specific promoters that allow stable transgene expression in the *AAVS1* locus of iPSC-derived myeloid cells. Our findings further indicate that promoter silencing can occur in the *AAVS1* safe harbor locus in differentiated hematopoietic cells and that a comparison of different promoters is necessary to achieve optimal transgene expression for therapeutic application of iPSC-derived cells.

Keywords: induced pluripotent stem cells, genome editing, genomic safe harbor, CRISPR-Cas9, chronic granulomatous disease

INTRODUCTION

THE DISCOVERY OF THE CRISPR-Cas9 system and its development as a gene editing tool have accelerated and broadened the application of designer nucleases to study gene functions in basic biology or to treat genetic diseases in clinical trials.¹ Designer nucleases allow site-specific correction of a point mutation or insertion of a therapeutic minigene. These strategies reduce the risk of insertional

mutagenesis, which has been observed in the past using long terminal repeat (LTR)-driven gamma-retroviral vectors for gene therapy.^{2–6} In these studies, gamma-retroviral vectors semirandomly integrated into the host genome and caused transactivation of adjacent proto-oncogenes, which caused acute leukemia in some cases. In contrast to retroviral vectors, a gene correction approach that directly targets the mutated gene or a safe harbor

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locus, for example, the *AAVS1* locus or the *CCR5* gene, could avoid insertional mutagenesis as well as unpredictable transgene silencing, which has also been observed for retroviral vectors.^{7,8} A genomic safe harbor locus is defined by two major criteria: (1) the inserted gene expression cassette functions predictably and (2) it does not alter the host genome by means of gene disruption or activation of neighboring genes.^{7,9} Another advantage of targeting a safe harbor locus for gene therapy is that the complete cDNA is inserted into the patient genome, so that the same correction strategy could be applied for all patients independent of their underlying disease-causing mutation.

In contrast, approaches that directly target the mutated gene may require development of specific strategies for different mutations, including a costly and time-consuming detailed risk evaluation for each approach. However, similar to a retroviral gene addition approach, a tailored and optionally lineage/tissue-specific promoter to drive cell type-specific expression of the transgene is also needed in a genomic safe harbor locus. Although many compact cell type-specific promoters have been developed in the past years and are already clinically used in gene therapy trials, they do not completely recapitulate the endogenous promoter in terms of spatiotemporal expression and could be epigenetically silenced in human induced pluripotent stem cells (iPSCs) during lineage-specific differentiation. In addition to their potential to differentiate into cells of almost all lineages, iPSCs provide an unlimited autologous stem cell source for future cell therapies. Moreover, iPSCs can be generated through reprogramming patient-derived somatic cells and can be used as models to study disease pathophysiology and to develop novel gene and cell therapies.

In this study, we used CRISPR-Cas9 to correct p47^{phox} deficiency, an autosomal-recessive form of chronic granulomatous disease (CGD), which is caused by mutations of the p47^{phox} subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The NADPH oxidase is found in the membrane of phagosomes in phagocytic cells, where it transfers electrons from NADPH to molecular O₂ to generate superoxide anions and other reactive oxygen species (ROS) upon stimulation, for example, the phagocytosis of microbes.^{10–12} ROS generation results in a change of the phagosome milieu, which leads to the release of different granules into the phagosome. These granules contain antimicrobial proteins, for example, myeloperoxidase and defensins, which synergize with the produced ROS to mediate killing of the encountered microbes. Owing to the lack of NADPH oxidase activity, CGD patients suffer from severe bacterial and fungal infections and the formation of granulomas in various organs, which can be life threatening.¹³ Only hematopoietic stem cell (HSC) transplantation and potentially gene therapy are curative treatment options.

The p47^{phox} subunit is encoded by *NCF1*, which has two highly homologous pseudogenes *NCF1B* and *NCF1C* on the same chromosome.^{14–18} Over 90% of p47-CGD patients share the same disease-causing mutation, namely the dinucleotide deletion c.75_76delGT (Δ GT) in exon 2.¹⁹ This Δ GT mutation is also found in the two pseudogenes of healthy individuals. It is hypothesized that the Δ GT mutation is copied into the functional *NCF1* gene from one of the two pseudogenes through gene conversion and consequently causes the disease.¹⁸ Merling *et al.* demonstrated the feasibility of a gene editing approach to directly correct the Δ GT mutation in patient-derived p47^{phox}-deficient iPSCs.²⁰ They showed that even the correction of the Δ GT mutation in the pseudogenes can restore p47^{phox} expression. However, in a gene editing approach, the presence of the two pseudogenes could be problematic because of multiple DNA double strand breaks (DSBs) that are introduced into the genome due to the sequence homology between *NCF1* and its pseudogene. The introduction of multiple DSBs increases the risk of unwanted off-target effects, for example, larger deletions or inversions. Moreover, the pseudogenes could compete with the therapeutic donor template for homology-directed repair (HDR) and could reintroduce the false sequence into the host genome, which could potentially reduce the overall gene editing efficiency.

To avoid potential difficulties with gene editing in the *NCF1* locus, we targeted the *AAVS1* safe harbor locus on chromosome 19 to correct p47^{phox} deficiency. Recently, Ordovàs *et al.* demonstrated that the *AAVS1* locus is not as “safe” as previously thought to be.²¹ The authors observed transgene silencing of different cell type-specific promoters that were inserted into the *AAVS1* locus in embryonic stem cells and differentiated hepatocytes. As p47^{phox} expression is restricted to the myeloid lineage, we tested three different myeloid-specific promoters (the miR223 promoter,²² the chimeric *CathepsinG* and *cFes* [CatG/cFes]²³ promoter, and the myeloid-related protein 8 [MRP8] promoter²⁴) and analyzed which promoter provided reliable transgene expression in the *AAVS1* locus of differentiated iPSCs.

Upon myeloid differentiation of corrected iPSCs, we observed that the miR223 and CatG/cFes promoters yielded similar or slightly lower p47^{phox} expression levels and NADPH oxidase activity as wild type (WT) cells. In contrast, we observed increased DNA methylation of the MRP8 promoter compared with the miR223 and CatG/cFes promoters in differentiated cells, which correlated with the reduced expression from the MRP8 promoter. In summary, we demonstrated that the myeloid-specific miR223 and CatG/cFes promoters allow stable transgene expression in iPSC-derived myeloid cells and further confirmed that some cell type-specific promoters remain methylated in the *AAVS1* safe harbor locus during differentiation. Thus, evaluation of different promoters might be necessary to avoid unexpected transgene silencing in the *AAVS1* locus.

EXPERIMENTAL PROCEDURES

Plasmids

The AAVS1-specific donor plasmid used for genetic correction was described elsewhere.²⁵ In brief, the donor plasmid consisted of two 750 bp homology arms including a splice acceptor site following a puromycin resistance cassette for selection of targeted clones and a bovine growth hormone polyadenylation signal for termination. The therapeutic codon-optimized cDNA for *NCF1* (synthesized by GeneArt/Thermo Fisher, Regensburg, Germany) was inserted into the donor plasmid through *SaII* and *PacI* restriction sites and was terminated by a herpes simplex virus thymidine kinase polyadenylation signal. The miR223 promoter was exchanged by the CatG/cFes and MRP8 promoter using *NotI* and *SaII* restriction sites (all restriction enzymes: New England Biolabs (NEB), Ipswich, MA).

The CRISPR-Cas9 plasmid carried a human U6 promoter to drive expression of the sgRNA. The 20 nucleotide long sgRNA target sequence for the AAVS1 locus was cloned after phosphorylation and annealing of 100 pmol oligodeoxynucleotides 5'-AAVS1 (5'-CACCGTCACCAA TCCTGTCCCTAG) and 100 pmol 3'-AAVS1 (5'-AAACC TAGGGACAGGATTGGTGAC) through two *BsmBI* restriction sites. Phosphorylation was performed at 37°C for 45 min using the T4 polynucleotide kinase (NEB) and annealing was achieved by incubation at 98°C for 150 s followed by a cool down to 22°C at a rate of $-0.1^{\circ}\text{C}/\text{min}$. Behind the sgRNA cassette, an SFFV promoter was cloned to drive expression of spCas9 and a dTomato fluorescence protein.

Cell culture

Human-iPSCs were cultivated on irradiated mouse embryonic fibroblasts C3H (MEFs) as described before.²⁶ The iPSC medium contained F12/Dulbecco's modified Eagle's medium (DMEM) (Gibco/Thermo Fisher, Waltham, MA) supplemented with 20% knockout serum replacement (Gibco/Thermo Fisher), 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (PanBiotech, Aidenach, Germany), 2 mM L-glutamine (Biochrom, Berlin, Germany), 1% nonessential amino acids (Gibco/Thermo Fisher), 0.1 mM β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 20 ng/mL β -FGF (kindly provided by the Department of Technical Chemistry, Leibniz University Hannover). Once per week, colonies were picked onto new MEFs in the presence of 10 μM ROCK inhibitor Y-27632 (Tocris, Bristol, UK). MEFs were seeded 1 day in advance in low-glucose DMEM (PanBiotech) supplemented with 15% fetal bovine serum (FBS Standard; PanBiotech), 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin, 2 mM L-glutamine, 1% nonessential amino acids, and 0.1 mM β -mercaptoethanol onto 0.1% bovine gelatin-coated plates (Sigma-Aldrich).

Gene editing of iPSCs

Two different p47^{phox}-deficient iPSC lines, p47- Δ GT (generated by CRISPR-Cas9 modification of healthy WT iPSCs derived from CD34⁺ cells) and p47-CGD (generated through reprogramming of peripheral blood from a CGD patient), were used in this study.²⁷ For genetic correction, 5×10^4 p47^{phox}-deficient iPSCs were seeded in a 24-well plate on Geltrex (Thermo Fisher) in the presence of 10 μM ROCK inhibitor Y-27632. After 24 h, 250 ng of the CRISPR-Cas9 plasmid and 250 ng of the donor plasmid were transfected using Lipofectamine Stem Reagent (Thermo Fisher). In brief, 25 μL F12/DMEM supplemented with 1 μL Lipofectamine Stem Reagent was added to 25 μL F12/DMEM mixed with 250 ng CRISPR-Cas9 plasmid and 250 ng donor plasmid. The mixture was incubated for 10 min at room temperature and added to the iPSCs. After 24 h, the iPSCs were detached using trypsin (PanBiotech) and plated on puromycin-resistant DR4 MEFs for selection. Single iPSC colonies were selected in iPSC medium supplemented with 0.2 $\mu\text{g}/\text{mL}$ puromycin (Invivogen, San Diego, CA) and analyzed for correct targeting.

Genetic analysis

Genomic DNA was isolated from all iPSC clones using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). All PCRs were performed using the Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher). The 5'-junction of the inserted donor construct was amplified with primers 5F (5'-GACCTGCATTCTCTCCCCTGG) and 5R (5'-GGGCTTGTA CTCTCGGTCATCTCG) (930 bp amplicon). The 3' junction was amplified using primers 3F (5'-CCAAGTTCGGGTGAAGGCC) and 3R (5'-AAGCCT GAGCGCCTCTCCTG) (846 bp amplicon). Random insertion of the donor plasmid was addressed by amplifying the 5'- and 3'-termini of the plasmid with primers D5F (5'-GGGTGTCGGGGCTGGCTTAAC) and 5R (961 bp amplicon) and with primers D3F (5'-GTGAGAATGGTGCG TCCTAGG) and D3R (5'-TATAGTCCTGTCGGGTTT CGCC) (684 bp amplicon), respectively. Monoallelic or biallelic targeting was assessed by amplification of WT sequence around the Cas9 cleavage site using primers WF (5'-GACAGCATGTTTGCTGCCTCC) and WR (5'-GGA TCCTCTCTGGCTCCATCG) (320 bp amplicon). All PCR products were analyzed by electrophoresis on 1% agarose gels (Invitrogen/Thermo Fisher).

Myeloid differentiation of iPSCs

All iPSC clones were differentiated into the myeloid/granulocytic lineage using an embryoid body (EB)-based protocol as previously described.²⁸ In brief, iPSC colonies were grown to high density and detached using dispase (Roche, Basel, Switzerland). The colonies were transferred into EB medium (KO/DMEM (Gibco/Thermo Fisher) supplemented with 20% knockout serum replacement, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin, 2 mM

L-glutamine, 1% nonessential amino acids, 0.1 mM β -mercaptoethanol, and 10 μ M ROCK inhibitor Y-27632. After 5 days on an orbital shaker at 80 rpm, EBs were picked into APEL2 medium (StemCell Technologies, Vancouver, Canada) supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin, 50 ng/mL human granulocyte colony-stimulating factor (G-CSF; Peprotech, Rocky Hill, NJ), and 25 ng/mL human interleukin-3 (IL-3; Peprotech). Differentiated cells were harvested once per week and cultured in RPMI 1640 medium (PanBio-tech) supplemented with 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin, 2 mM L-glutamine, and 100 ng/mL G-CSF.

Myeloid cell morphology was analyzed by Pappenheim staining. About 5×10^4 differentiated cells were spun onto microscope glass slides using a Cytospin 4 (Thermo Scientific). After May-Grünwald and Giemsa staining according to manufacturer's instructions (Pappenheim staining; Sigma-Aldrich), cells were analyzed with an Olympus BX51 microscope (software Cell^F version 3.4; Olympus, Tokyo, Japan).

Differentiated cells were analyzed for surface marker expression by flow cytometry (CytoFlex S; Beckman Coulter, Brea, CA) using the following antibodies: CD45-FITC (Clone: 5B1; Miltenyi, Bergisch-Gladbach, Germany), CD11b-PE (Clone: M1/70.15.11.5; Miltenyi), CD66b-APC (Clone: G10F5; Biolegend, San Diego, CA), and the viability dye DAPI (Sigma-Aldrich). Before antibody staining, cells were blocked with human FcR blocking reagent (Miltenyi).

Intracellular p47^{phox} staining

Myeloid cells were fixed and permeabilized using the FOXP3 intracellular staining kit (Biolegend). In brief, fixation was performed for 20 min in Fix/Perm solution. After washing once with PBS and once with Perm buffer, the cells were incubated in Perm buffer for 15 min. Primary p47^{phox} antibody (BD, Franklin Lakes, NJ) staining was done for 30 min followed by 30 min incubation with a goat antimouse DyLight488 secondary antibody (Thermo Fisher). Data were acquired using a CytoFlex S flow cytometer.

Dihydrorhodamine assay

The dihydrorhodamine (DHR) assay was performed as previously described,²⁵ with minor modifications. In brief, 10^5 cells were stimulated with 40 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) in the presence of 1,000 U catalase (Sigma-Aldrich) in 500 μ L Hank's buffered salt solution containing Mg/Ca (Gibco/Thermo Fisher). After stimulation for 5 min, 250 ng DHR (Sigma-Aldrich) was added to the cells for 15 min. The reaction was stopped by placing the cells on ice. Data were recorded by flow cytometry (CytoFlex S).

Bisulfite sequencing

Genomic DNA from iPSCs and myeloid cells was isolated using the QIAamp DNA Blood Mini Kit. Bisulfite conversion was performed using the EpiTect Bisulfite Kit (Qiagen) according to manufacturer's instructions. For the bisulfite conversion, 20 μ L genomic DNA was mixed with 85 μ L bisulfite mix and 35 μ L DNA Protect Buffer and incubated for 5 min at 95°C, 25 min at 60°C, followed by another 5 min at 95°C, 85 min at 60°C, 5 min at 95°C, and finally 175 min at 60°C. After cleanup of the bisulfite-converted DNA using spin columns, the promoter sequences for miR223, CatG/cFes, and MRP8 were amplified by seminested PCR using a Taq polymerase (Qiagen). The respective primers are listed in Table 1. All PCR products were isolated from a 1% agarose gel and cloned into pCR2.1 plasmid through TA cloning. Five colonies were picked from each plate for plasmid isolation. The plasmid was Sanger sequenced using the M13 primer (5'-TGTAACGACGCGCCAGT).

Statistical analysis

Statistical significance was determined using one-way analysis of variance (ANOVA) with GraphPad Prism 7. All bar graphs represent the mean $\pm$ SD. *** $p \leq 0.001$.

Table 1. Primer sequences used for bisulfite sequencing

Primer	Sequence (5'-3')
miR223_1_fw	TGATTGTATAGTTTATAGGGTTTATGTTTAGAAG
miR223_1_rev_1	TAAAAAACCCRTAATCAAAATTAACAAACCACTC
miR223_1_rev_2	CCCATAACTCCAAATCACATATCTTCTCACTC
miR223_2_fw	GAGTGAAGAATATGTGATTGGAGTTTATGGGG
miR223_2_rev_1	CAAACTACAAAAAATAAAAAACACCTAAC
miR223_2_rev_2	AAACACCTAACTACCTAACTACCTATAATC
miR223_3_fw	GGTTTGTATTATGTTGTAGTAGTAGATTTTTTAT
miR223_3_rev_1	AAACCAACAAACAAATATATCTAATAAAAAATA
miR223_3_rev_2	TACCTTAATCATAAAAAATACTAATTCCTCC
CatG/cFes_1_fw_1	TTTTTGTGTTTGGAGTATTTTGGAAATTTG
CatG/cFes_1_fw_2	TTTTTTTTTGTGTTGGGTTTTATTTTTG
CatG/cFes_1_rev	CACAATCCATTCTCTACTACCATAAAAAAT
CatG/cFes_2_fw_1	AATTTTTATGTTAGTAGGAGAAATGGGATTGTG
CatG/cFes_2_fw_2	TGAGGAGGAGGAGTATAGTAGTAATTGATTGGG
CatG/cFes_2_rev	AAACCCCAACAACTACTAAACCCCAAAACCC
CatG/cFes_3_fw_1	TGGGATTAGGGTTTTTTTTTTTTTTTTT
CatG/cFes_3_fw_2	GGGTTTTTGGGTTTAGTATTTGTTGGGGTTT
CatG/cFes_3_rev	AAACCCAACAAACAAATATCTAATAAAAAATA
MRP8_1_fw	TAAGGGGGAGGATTGGGAAGATAATAGTAGGTATG
MRP8_1_rev_1	CCAAAAATATAAAAAATCCCTACAACCAACAACA
MRP8_1_rev_2	CCAATCAAAAATTAACAAAAAATACTCAATAAACACT
MRP8_2_fw_1	GTGGGGAGAGGATTGTTTTTTTTGAAATTTGGGG
MRP8_2_fw_2	TGGGGAATTGGTTATTTTTTTTTTTTTTAGGTATG
MRP8_2_rev	CCTATTCTATAAACTAAAAATAAACCATACCTAA
MRP8_3_fw_1	TGAGGTATGGTTATTTTATGTTTATAGAATAGG
MRP8_3_fw_2	TAGGGTAGGAATGGATATAGTTTTTGG
MRP8_3_rev	TCATTTTAAATACATACACTCAATAAAAAACATTCCTCC
MRP8_4_fw_1	TGGAGGAATGTTTTATTGAGTGTATG
MRP8_4_fw_2	TGATGTATTTAATTTAATTTAGTTTTAGGGATGTATG
MRP8_4_rev	TCAAAAACAACTCTCCCTACCAAAATAC
MRP8_5_fw_1	TGGTTGAGAAATTAGAGATTGTAGTAATTTGG
MRP8_5_fw_2	GTAATTTTGGTAGGGAGAAGTTGTTTTGA
MRP8_5_rev	CCACCTCTCGCTCAATCTACCACTTAC

**** $p \leq 0.0001$. Additional supplemental experimental procedures can be found in Supplementary Data.

RESULTS

Targeting the AAVS1 safe harbor locus for correction of p47-CGD

The overall aim of this study was to correct p47^{phox} deficiency by insertion of a therapeutic codon-optimized *NCF1* cDNA into the *AAVS1* safe harbor locus using CRISPR-Cas9. To maintain myeloid-specific expression of p47^{phox}, we tested three different myeloid-specific promoters: miR223, CatG/cFes, and MRP8 (Fig. 1A). Before targeting the *AAVS1* locus in iPSCs, we used a fluorescence reporter assay in HT1080 cells to determine the on-target activity of the applied sgRNA. The reporter construct consisted of a super-folder green fluorescent protein (sfGFP), which contained the sgRNA target sequence behind the ATG start codon of sfGFP (Supplementary Fig. S1A). We used lentiviral vectors for the delivery of the CRISPR-Cas9 system to mediate efficient gene transfer and to track genetically modified cells through the dTomato fluorescent protein on the CRISPR-Cas9 construct (Supplementary Fig. S1A).

After delivery of the CRISPR-Cas9 components and cleavage of the target sequence, the induced DSBs were repaired by nonhomologous end joining. In ~66% of

cleaved alleles, the repair would result in a frameshift and a subsequent loss of sfGFP fluorescence, which can be detected through flow cytometry. We measured an on-target activity of 43–47% on average for the *AAVS1*-specific sgRNA at different multiplicities of infection (Supplementary Fig. S1B–D). After validation of the sgRNA, two different p47^{phox}-deficient iPSC lines (p47-ΔGT generated by CRISPR-Cas9 and p47-CGD generated by reprogramming²⁷) were corrected by applying the different donor constructs and the CRISPR-Cas9 plasmid through lipofection. We identified a biallelic corrected clone for each promoter construct and each iPSC clone (p47-ΔGT: miR223 no. 4, CatG/cFes no. 5, MRP8 no. 16, p47-CGD: miR223 no. 4, CatG/cFes no. 12, MRP8 no. 12) through PCR screening for the 5'- and 3'-junction site and the WT sequence around the Cas9 cleavage site (Fig. 1B, C). Random insertion of the donor plasmid in selected clones was excluded by two additional PCR amplifications for the 5'- and 3'-side of the donor plasmid. The screening results of all analyzed iPSC clones are shown in Fig. 1D.

In most cases, we achieved more monoallelic corrected clones than biallelic corrected clones most likely due to the low frequency of HDR. We observed between 40% and 80% random insertion of the donor plasmid, which might be due to an excessive amount of donor plasmid in the transfection reaction. All gene-edited iPSC clones maintained

Figure 1. Targeting the *AAVS1* locus for genetic correction of p47-ΔGT and p47-CGD iPSCs. **(A)** Schematic of the *AAVS1* safe harbor locus and the gene correction strategy for p47^{phox} deficiency. The Cas9 is guided to intron 1 of *PPP1R12C* (*AAVS1* locus) and introduces a DNA double-strand break, which induces homology-directed repair through the donor template. The arrows indicate primer binding sites used for PCR-based screening of corrected iPSC clones. **(B)** PCR-based screening to identify corrected p47-ΔGT clones. The primers were used as indicated in **(A)**. **(C)** PCR-based screening to identify corrected p47-CGD clones. **(D)** Quantitative analysis of gene correction and random integration summarized from all analyzed p47-ΔGT and p47-CGD iPSC clones. 2A, peptide cleavage site; F, forward primer; HAL, homology arm left; HAR, homology arm right; iPSC, induced pluripotent stem cell; NCF1co, codon-optimized cDNA encoding *NCF1* gene; pA, polyadenylation signal; Puro, puromycin resistance gene; R, reverse primer; SA, splice acceptor site.

their typical iPSC morphology (Supplementary Fig. S2A). Moreover, surface staining revealed expression of the pluripotency markers SSEA-4 and TRA-1-60 in all iPSC clones, which confirmed that they remained pluripotent after gene editing (Supplementary Fig. S2B). Finally, quantitative PCR demonstrated similar expression of the pluripotency genes *OCT4*, *NANOG*, and *DNMT3B* compared with embryonic stem cells (Supplementary Fig. S2C). In summary, we genetically corrected two different $p47^{\text{phox}}$ -deficient iPSC lines by insertion of a codon-optimized *NCF1* minigene into the *AAVS1* safe harbor locus.

Differentiated myeloid cells had restored $p47^{\text{phox}}$ expression and functional NADPH oxidase activity

To analyze $p47^{\text{phox}}$ expression, all iPSC clones were differentiated into myeloid cells using an EB-based differentiation protocol.²⁸ To drive the differentiation into the granulocytic lineage, we added G-CSF and IL-3 to the hematopoietic differentiation medium. Staining of differentiated cells with May–Grünwald and Giemsa revealed segmented neutrophils as well as myeloid progenitors (Fig. 2A). Macrophages were also observed in many differentiations, but there was great variation regarding the amount of macrophages produced among different experiments. Further characterization of the differentiated myeloid cells by staining for the surface markers CD45, CD11b, and CD66b revealed 91–99% CD45-positive cells (Fig. 2B). These cells were 80–94% CD11b positive and 26–46% double positive for CD11b and CD66b (Fig. 2C).

Restored $p47^{\text{phox}}$ expression was assessed through intracellular staining in the parental iPSCs and the differentiated CD45⁺ myeloid cells. As expected in iPSCs, the WT and corrected clones lacked $p47^{\text{phox}}$ expression as did the $p47^{\text{phox}}$ -deficient iPSC lines (Fig. 3A). In differentiated myeloid cells, $p47^{\text{phox}}$ expression from the miR223 promoter was similar to WT cells ($66.3 \pm 7.4\%$ $p47^{\text{phox}}$ -positive cells) and in the range of $57.3 \pm 21.6\%$ $p47^{\text{phox}}$ -positive cells (Fig. 3B, C). The $p47^{\text{phox}}$ expression from the CatG/cFes promoter ranged from $19.5 \pm 13.6\%$ $p47^{\text{phox}}$ -positive cells, whereas the MRP8 promoter resulted in only $6.9 \pm 6.5\%$ $p47^{\text{phox}}$ -positive myeloid cells. These findings indicated strong differences in the activities of various promoters and that expression from the myeloid-specific promoters was restricted to differentiated myeloid cells with no leakiness observed in iPSCs. We also compared the $p47^{\text{phox}}$ expression in some clones with a monoallelic correction to the clones with the biallelic correction and observed similar expression levels (Fig. 3C).

Functional NADPH oxidase activity was assessed in a DHR assay, which detected ROS production by the conversion of DHR into green-fluorescent rhodamine (Rho) 123. As expected, all iPSC clones failed to produce ROS in this assay due to the fact that the components of the NADPH oxidase are not expressed in iPSCs (Fig. 4A).

Upon differentiation, cells corrected with the miR223 promoter yielded $73.9 \pm 18.3\%$ Rho123-positive cells, which was slightly below WT levels ($93.5 \pm 2.8\%$ Rho123-positive cells) (Fig. 4B, C). The CatG/cFes promoter yielded $18.6 \pm 13.0\%$ Rho123-positive cells, which was again inferior to the miR223 promoter but higher than the MRP8 promoter ($6.3 \pm 4.3\%$ Rho123-positive cells). Taken together, differentiated myeloid cells derived from corrected iPSCs revealed restored NADPH oxidase activity, which reflected the $p47^{\text{phox}}$ expression levels (Fig. 3C). In contrast to the MRP8 promoter, cells corrected with the miR223 and CatG/cFes promoter expressed therapeutically relevant levels of $p47^{\text{phox}}$ and restored NADPH oxidase activity. The miR223 promoter achieved the highest levels of $p47^{\text{phox}}$ expression and was comparable with WT cells.

Myeloid-specific promoters were differentially silenced in the *AAVS1* safe harbor locus

To analyze whether promoter silencing in the *AAVS1* locus had an effect on the transgene expression, we performed bisulfite sequencing of the myeloid-specific promoters in iPSCs and in the differentiated myeloid cells to assess the DNA methylation status, which is a good predictor of gene expression.²⁹ Based solely on promoter sequence, all three myeloid-specific promoters contain different amounts of CpGs that could be potentially methylated. Although the miR223 promoter contained only four CpGs, the CatG/cFes promoter had 52 CpGs and the MRP8 promoter had 13 CpGs. Owing to a high CpG density and a CpG island in the 3'-part of the CatG/cFes promoter, we only analyzed the first 12 CpGs of the CatG/cFes promoter, which covered the first half of the promoter. We observed extensive (85–98%) CpG methylation of all promoters in iPSCs (Fig. 5A–C). Upon myeloid differentiation, the promoter methylation status decreased to 20–25% for the miR223 promoter, 8–40% for the CatG/cFes promoter, and 64–80% for the MRP8 promoter. In conclusion, we observed that the analyzed myeloid-specific promoters were mainly methylated in the *AAVS1* safe harbor locus of iPSCs and demethylated to different extents upon myeloid differentiation.

DISCUSSION

The *AAVS1* locus has been described as a genomic safe harbor with open and active chromatin that allows higher expression of an inserted transgene compared with the *CCR5* gene.⁸ However, we and others showed that certain cell type-specific promoters, when integrated into the *AAVS1* locus, become methylated and mediate low transgene expression in differentiated cells.²¹ In our study, we tested three different myeloid-specific promoters (miR223, CatG/cFes, and MRP8) to drive expression of a therapeutic *NCF1* cassette in the *AAVS1* locus. In the context of iPSC-derived myeloid cells, we demonstrated that the

Figure 2. Myeloid differentiation of corrected iPSC clones. **(A)** All iPSC clones were differentiated into myeloid cells. Cytopsin from corrected iPSC-derived myeloid cells were stained with Pappenheim staining solutions to assess the cell morphology of differentiated cells (scale bar = 20 μ m). **(B)** Differentiated iPSC-derived myeloid cells were stained for the hematopoietic marker CD45. The expression was analyzed in viable cells by flow cytometry. *Black line/unfilled* = isotype control, *gray* = stained cells. **(C)** Upon surface marker staining, the expression of the myeloid marker CD11b and the granulocytic marker CD66b was measured in CD45⁺ iPSC-derived myeloid cells by flow cytometry. Representative flow cytometry dot plots of three independent experiments/differentiations are shown.

Figure 3. Differentiated myeloid cells had restored p47^{phox} expression. **(A)** All iPSC clones were stained intracellularly for p47^{phox}. The expression of p47^{phox} was analyzed in viable iPSCs by flow cytometry. Data represent one independent experiment. As negative control, the parental p47^{phox}-deficient iPSCs were used, which received the whole antibody staining. The gating is based on the respective parental p47^{phox}-deficient p47-ΔGT (also for WT) or p47-CGD clone. *Black line/unfilled*=stained negative control, *gray*=stained samples. **(B)** After myeloid differentiation all clones were stained for p47^{phox}. Upon intracellular antibody staining, p47^{phox} expression was measured in CD45⁺ iPSC-derived myeloid cells through flow cytometry. Representative histograms of three independent experiments/differentiations are shown. As negative control, the parental p47^{phox}-deficient iPSC-derived CD45⁺ myeloid cells were used, which received complete antibody staining. The gating is based on the respective parental p47^{phox}-deficient p47-ΔGT (also for WT) or p47-CGD clone. *Black line/unfilled*=stained negative control, *gray*=stained samples. **(C)** The bar graph summarizes p47^{phox} expression in corrected p47-ΔGT (*black symbols*) and p47-CGD clones (*white symbols*) from three independent differentiations. Diamonds represent monoallelic corrected clones ($n=3-7$, mean $\pm$ SD, statistics: one-way ANOVA, *** $p<0.001$, **** $p<0.0001$). ANOVA, analysis of variance; SD, standard deviation; WT, wild type.

miR223 and CatG/cFes promoter achieved therapeutically relevant transgene expression levels and NADPH oxidase activity. In a study by Merling *et al.*, the authors targeted the AAVS1 locus with a constitutively expressing CAG promoter to drive expression of p47^{phox} in patient-derived iPSCs.³⁰ Upon differentiation, only 26% Rho123-positive cells were generated from p47^{phox}-corrected granulocytes in the earlier study. The authors claimed that this low rate was caused by variable differentiation rates and probably a low yield of myeloid cells in that specific assay. However, for other CGD lines, the authors measured up to 90% Rho123-positive cells using the CAG promoter, which is comparable with our data with the miR223 promoter.

Brendel *et al.* compared the expression strength of the miR223 promoter with that of other myeloid-specific pro-

motors, including the cFes and MRP8 promoter.²² In the former comparison, they observed superior performance of the miR223 promoter compared with the other promoters with regard to p47^{phox} induction and Rho123-positive cells in differentiated murine granulocytes. Although the degree of epigenetic modifications might differ between murine hematopoietic stem and progenitor cells and iPSCs, the expression strength of the miR223 promoter in comparison with the MRP8 promoter in murine granulocytes was quite similar to our data in human iPSC-derived myeloid cells.

To maintain pluripotency, pluripotent stem cells possess a unique DNA methylation profile.^{29,31} In general, DNA methylation plays an important role in establishing and maintaining tissue-specific gene expression and correlates inversely with gene expression.³² We observed that

Figure 4. Corrected myeloid cells had functional nicotinamide adenine dinucleotide phosphate oxidase activity. **(A)** The DHR assay was performed on undifferentiated iPSCs. Upon PMA stimulation, DHR is converted into green fluorescent Rho123 in the presence of ROS. The frequency of Rho123⁺ cells was analyzed in viable iPSCs by flow cytometry. Unstimulated cells were used as a negative control. *Black line/unfilled*=unstimulated cells, *gray*=stimulated cells. Histograms represent one independent experiment. **(B)** The DHR assay was performed on iPSC-derived myeloid cells. The frequency of Rho123⁺ cells was analyzed in CD45⁺ myeloid cells by flow cytometry. As negative control, stimulated p47^{phox}-deficient myeloid cells (p47-ΔGT or p47-CGD) were used. The gating is based on the respective parental p47^{phox}-deficient p47-ΔGT (also for WT) or p47-CGD clone. *Black line/unfilled*=stimulated negative control, *gray*=stimulated WT and corrected cells. Representative histograms of three independent experiments/differentiations are depicted. **(C)** The bar graph represents the results of all DHR assays performed on corrected p47-ΔGT (*black symbols*) and p47-CGD clones (*white symbols*) from three independent differentiations. Diamonds represent monoallelic corrected clones ($n=3-8$, mean $\pm$ SD, statistics: one-way ANOVA, **** $p<0.0001$). DHR, dihydrorhodamine; PMA, phorbol 12-myristate 13-acetate.

all three myeloid-specific promoters were highly methylated in the iPSC state, in which the promoters are inactive. The differentiation of pluripotent stem cells is associated with extensive epigenetic reprogramming that involves changes in DNA methylation, binding of transcription factors, and modification of DNA-binding proteins, which changes the chromatin structure and makes it accessible for the transcription machinery.³³

Upon myeloid differentiation, we measured a different degree of demethylation for the promoters tested in this study, which, in turn, partly correlated with the observed transgene expression. We observed quite heterogeneous results for the CatG/cFes promoter with regard to p47^{phox} expression levels and the methylation status in the two different iPSC lines. Despite 40% CpG methylation in the 5'-part of the promoter in p47-CGD CatG/cFes clone no. 12,

we observed up to 40% p47^{phox} expression and NADPH oxidase activity. In contrast, p47-ΔGT CatG/cFes clone no. 5 that had 8% methylated CpGs yielded only 15% p47^{phox} expression and NADPH oxidase activity. Moreover, the demethylation of the myeloid-specific promoters could be completely different when targeting primary HSC.

So far, methylation of the CatG/cFes promoter has not been observed in neutrophils of CGD patients who were treated with gene therapy (unpublished data). Although it has been described that the MRP8 promoter relies on methylation of certain CpGs to bind specific transcription factors and to drive expression, it performed very poorly in our assays despite high DNA methylation.³⁴ Also in the context of lentiviral vectors, the MRP8 promoter shows a high abundance of repressive histone marks and low transgene expression in iPSC-derived myeloid cells.³⁴

Figure 5. DNA methylation analysis of different myeloid promoters in iPSCs and iPSC-derived myeloid cells. CpG methylation analysis of the miR223 promoter (A), CatG/cFes promoter (B), and MRP8 promoter (C) through bisulfite sequencing in iPSCs and iPSC-derived myeloid cells (open circle, nonmethylated CpG; filled circle, methylated CpG; mean percentage of CpG methylation is indicated).

The inclusion of insulators or ubiquitous chromatin opening elements (UCOE), such as the 1.8 kb element or a shortened CBX3 version, could help to overcome transgene silencing.^{21,34–36} Insulators are DNA sequences that block the spread of heterochromatin and thus protect against silencing and DNA methylation.³⁷ Ordovàs *et al.* observed that the OCT4 promoter, which was inactive in the *AAVS1* locus without insulators, had 100% transgene expression when flanked by insulator sequences.²¹ However, others demonstrated that mosaicism can occur in a safe harbor locus even with insulators, for example, the murine *Rosa26* and *ColA1* genes.^{38,39} Insertion of a CBX3 element upstream of the internal promoter can also protect against epigenetic transgene silencing and stabilize expression of transgenes that are integrated into the genome. Müller-Kuller *et al.* showed that the addition of CBX3 next to the MRP8 promoter increased transgene expression by

two-fold in iPSC-derived myeloid cells and the expression intensity by five-fold in a myelomonocytic cell line.³⁴

We conclude that the targeted integration of a therapeutic transgene into a genomic safe harbor locus can reduce the risk of insertional mutagenesis compared with semirandomly integrating vectors. However, the criteria of predictable transgene function do not apply without restrictions as sequence-dependent transgene silencing can occur in the *AAVS1* safe harbor locus in human pluripotent stem cells.²¹ In this study, we tested three different myeloid-specific promoters that were integrated into the *AAVS1* locus and observed that the miR223 promoter performed highly reliably, with corrected cells producing similar NADPH oxidase activity compared with WT cells. The CatG/cFes promoter also allowed stable and therapeutically relevant p47^{phox} expression levels in iPSC-derived myeloid cells, whereas the MRP8 promoter was prone to silencing. Thus,

the correct promoter choice is essential to achieve sufficient gene expression, for example, in a therapeutic setting. Moreover, the inclusion of insulators and UCOEs could help to overcome transgene silencing for promoters that are prone to epigenetic silencing through DNA methylation or addition of repressive histone marks.

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AUTHOR DISCLOSURE

No competing financial interests exist.

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SUPPLEMENTARY MATERIAL

Supplementary Data

Supplementary Figure S1

Supplementary Figure S2

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