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ORIGINAL ARTICLE

Evaluation of the CRISPR/Cas9 system evaluation as genome editing platform for the Mucopolysaccharidosis IVA using a strategy for induction of higher homologous recombination frequency

Diego A. Suarez[1,2], **Andrés Felipe Leal**[2], **Carlos Javier Alméciga-Díaz**[2*]

Edited by
Angela Johana Espejo Mojica
editorus@javeriana.edu.co

1. Facultad de Medicina, Universidad Nacional de Colombia, Bogotá D.C.
2. Institute for the Study of Inborn Errors of Metabolism, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá D.C.

*cjalmeciga@javeriana.edu.co

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Abstract

Mucopolysaccharidosis IVA (MPS IVA) is a lysosomal storage disease caused by mutations in the gene encoding for the hydrolase N-acetylgalactose-6-sulfate sulfatase (GALNS). GALNS deficiency leads to a progressive buildup of partially degraded chondroitin-6-sulfate and keratan-6-sulfate within the lysosomes. Several therapeutic strategies are under evaluation. Recently, we validated the use of CRISPR/Cas9-based gene therapy for MPS IVA, recovering up to 40 % of normal GALNS activity, leading to a positive outcome on classical MPS IVA biomarkers. In this study, we extended our previous findings by performing the co-delivery of the CRISPR/Cas9 system with the proteins E4orf6 or E1B, which are inhibitors of the non-homologous end-joining repair pathway that may favor an increase in the homology-directed repair mechanism. The CRISPR/Cas9 vectors were transfected in GM00593 and GM01361 human MPS IVA fibroblasts using a lipofection-based delivery. After 30 days post-transfection with CRISPR/Cas9 systems in the absence of the E4orf6 or E1B, we found an increase in the GALNS activity up to 4.17 and 11.2-fold when compared to untreated GM01361 and GM00593 cells, respectively. Partial recovery of the lysosomal mass was achieved in human MPS IVA fibroblasts after treatment, while a modest reduction in oxidative stress was observed only in treated GM01361 cells. None of these cells transfected with CRISPR/Cas9 plasmids expressing E4orf6 or E1B showed improvement for the test variables. Together, our results support using CRISPR/Cas9 as a potential GT-based strategy for treating MPS IVA and highlight the importance of seeking approaches to improve the impact of gene editing as a therapeutic approach.

Keywords: AAVS1 *locus*; CRISPR/Cas9; Mucopolysaccharidosis IV A; homologous recombination

1. Introduction

Mucopolysaccharidosis IV A (MPS IVA, Morquio A syndrome; OMIM # 253000) is an autosomal recessive disease caused by variants in the gene encoding for the lysosomal enzyme N-acetylgalactosamine-6-sulfate sulfatase (GALNS, EC: 3.1.6.4) (1). GALNS is responsible for the first step in the catabolism of glycosaminoglycans (GAGs), chondroitin-6 sulfate (C6S), and keratan sulfate (KS) [1, 2]. A deficient GALNS activity leads to the lysosomal buildup of partially degraded KS and C6S in several tissues, such as connective tissue, bone, and cornea [2, 3]. MPS IV A most common symptoms include generalized skeletal dysplasia, spinal cord compression, tracheal obstruction, ligamentous laxity, hearing loss, respiratory disturbances, and heart valve disease without impairment of the central nervous system [4, 5]. Disease prevalence varies

from 1:71,000 to 1:179,000 across multiple countries [6]. In Colombia, the estimated combined frequency for both MPS IVA and MPS IVB was 0.68 per 100,000 newborns, being the most frequent MPS in the country [7]. A recent study revealed that MPS IV A is the most prevalent form of MPS in Colombia, with an estimated share of 58.4% of diagnosed MPS patients within 20 years [8]. According to the epidemiological report of rare diseases in Colombia, 199 patients were reported between 2016 and 2022, representing 0.45% of all Colombian rare disease cases [9]. A more recent report indicated an estimated proportion of 0.11% of total rare diseases in Colombia in 2022 [10].

The only approved treatment for MPS IV A is enzyme replacement therapy (ERT), involving the intravenous administration of the recombinant elosulfase alfa [11–13]. Clinical trials showed 41% and 59.4% reductions in urine KS [13, 14]. Moreover, there was a boost in the six-minute walk and cardiopulmonary exercise tests but not in the three-minute stair climb test [11, 13]. Despite better outcomes on clinical tests, there is no improvement in cervical compression, joint hypermobility, and bone growth, suggesting a limited effect on bone abnormalities [15, 16]. There is also no evidence of oxidative stress reduction in patients under ERT [17]. Hematopoietic stem cell transplantation (HSCT) has been conducted on MPS IV A patients with considerable recovery of bone density, decreased apnea and snoring, and recovery of voluntary gait and airway integrity [18]. Additional trials showed improvement in joint hypermobility and reduction in airway obstruction [19, 20]. Increased GALNS activity levels comparable to normal levels have been reported in all cases [18–21]. Even with the positive effects reported using this approach, more studies are necessary to assess the effectiveness of this strategy in correcting bone abnormalities [22]. Pharmacological chaperones [23, 24] and substrate degradation therapy [25] have also been explored as potential therapeutic alternatives for MPS IVA.

Gene therapy (GT) potentially allows for correcting the genetic mutation causing the disease with only one intervention [26]. Gene therapy studies for MPS IV A have used lentiviral, adeno-associated, and γ-retroviral vectors, showing a significant increase in GALNS activity in several cell types [27–32]. Preclinical evaluation in a murine model for MPS IV A using adeno-associated virus (AAV) showed increased GALNS activity and reduced levels of KS in plasma, leading to reduced storage in articular cartilage, growth plate region, meniscus surrounding the articular cartilage, ligaments, heart valves, and heart muscle [33, 34]. In addition, using a modified capsid viral vector with increased hydroxyapatite affinity induced higher tropism for bone and a significant increase in GALNS activity in this tissue [35]. More recently, it was possible to increase GALNS activity in bone, cartilage, and peripheral organs and reduce KS levels on the whole body of a rat model for MPS IV A by using AAV9 vectors carrying GALNS cDNA, preventing abnormal development on bones, teeth, joints, trachea, and heart [36]. Thus, GT represents a promising therapeutic alternative for treating MPS IV A.

Genome editing offers new alternatives in the design of gene therapy strategies for different diseases. Among the different genome editing strategies, CRISPR/Cas9 has several advantages over other approaches, such as zinc finger nucleases or transcription activator-like effector nuclease [37–39]. CRISPR/Cas9 consists of an endonuclease complex (Cas9) naturally occurring in several bacteria. Cas9 is associated with a small RNA sequence known as gRNA, which guides the Cas9 toward a specific *locus*, allowing for precise double-strand break (DSB) and insertion of a donor sequence through homologous recombination (HR) [38]. One considerable limitation of the CRISPR/Cas9 is a reduced HR frequency [40]. In this regard, an increase in HR frequency is possible by inhibiting the non-homologous end-joining (NHEJ) pathway [41]. Chu *et al*. proved that AAV-derived proteins may inhibit the NHEJ pathway, leading to a higher HR frequency when using CRISPR/Cas9 on HEK 293 cells and murine embryonic fibroblasts [42].

We recently reported that the use of CRISPR/nCas9 leads to an increase in GALNS activity, significantly recovering GAG levels, lysosomal mass, and reactive oxidative species (ROS) without detectable off-target events in human MPS IVA fibroblasts [43, 44]. In this study, we expanded our CRISPR/Cas9 approach for MPS IVA by assessing the utilization of E4orf6 and E1B proteins in a gene therapy context. Our objective was to improve HR frequency in HEK293FT cells and skin fibroblasts derived from MPS IVA patients, thereby appraising the therapeutic efficacy of this strategy.

2. Materials and Methods

2.1. Cell cultures

Human embryonic kidney cells (HEK 293FT) (ThermoFisher ScientificTM, USA) were cultivated on Dulbecco's Modified Eagle Medium (DMEM, Biowest, USA) supplemented with 10 % fetal bovine serum (FBS, Biowest, USA) and 1X penicillin-streptomycin (Biowest, USA). Human MPS IV A skin fibroblasts GM00593 and GM01361 were obtained from the Coriell Institute and maintained in DMEM plus 15 % FBS. All cells were incubated at 37°C and 5 % CO_2, and experiments were conducted on cells between 9 and 18 passages.

2.2. CRISPR/Cas9 plasmids

CRISPR/Cas9 and donor plasmids were purchased from the Addgene repository and included a CRISPR/Cas9 plasmid containing the sequence of *Streptococcus pyogenes* Cas9, a cloning site for the sgRNA, and the mCherry as a fluorescent reporter (Addgene: 64324, hereinafter CRISPR/Cas9). Two additional plasmids, bearing Addgene 64324 backbones, were also used (**Fig. 1B** and **Fig. 1C**): Addgene 64221 with the sequence encoding for E4orf6 (hereinafter CRISPR/Cas9-E4orf6) and Addgene 64222 with the sequence encoding for E1B (hereinafter CRISPR/Cas9-E1B). The co-expression of E4orf6 and E1B increases the HR ratio through NHEJ blocking [42]. The sgRNA sequence was obtained from Chu *et al*. [42] to target the AAVS1 *locus*. The sgRNA was cloned into CRISPR/Cas9 plasmids by enzymatic digestion using the BbsI enzyme following the protocol described by Chu *et al*. [42]. The sgRNA sequence insertion into CRISPR/Cas9 plasmid was confirmed by Sanger sequencing.

2.3. CMV-GALNS Donor plasmid

The donor plasmid (CMV-GALNS Donor) was constructed by PCR amplification of the CMV-GALNS fragment from a pIRES-neo-GALNS plasmid [27] using primers with restriction sites for *ClaI* (forward) and *MluI* (reverse) (**Table 1**). The PCR product was purified using the GeneJET PCR Purification Kit (ThermoFisher Scientific, USA) and digested with *ClaI* (NEB# R0197S, USA) and *MluI*-HF (NEB # R3198S, USA) according to the manufacturer's instructions. The final product was ligated into the Addgene 64215 vector previously digested with *ClaI* (NEB# R0197S, USA) and *MluI*-HF (NEB # R3198S, USA). Ligation occurred with a T4 ligase (NEB # M0202S, USA) according to the manufacturer's instructions. The resulting CMV-GALNS donor plasmid, containing the human *GALNS* cDNA under the control of a cytomegalovirus (CMV) promoter, is a commonly used promoter for the strong expression of proteins in mammalian cells [45]. Two homologous arms flanked the CMV-GALNS expression cassette, which mediates its recombination on the AAVS1 *locus*. This *locus* also has a puromycin resistance gene for selecting the edited cells (Fig. 1). The correct assembly of the plasmid was confirmed by enzymatic digestion, PCR, and Sanger sequencing (**Fig. 2**).

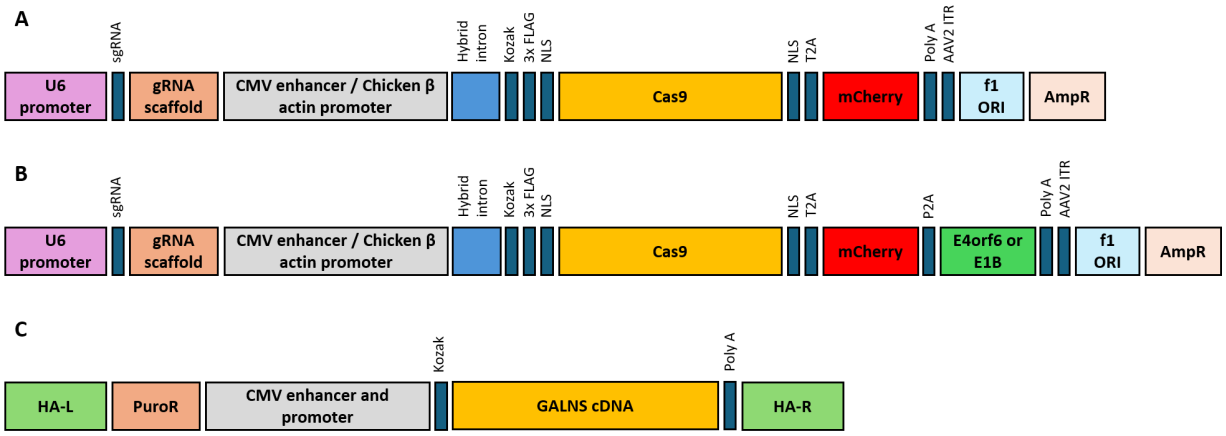

Figure 1. CRISPR-Cas9 and CMV-GALNS-Donor vectors. **(A)** CRISPR/Cas9 encodes for the spCas9 and mCherry fluorescence (red) proteins. **(B)** CRISPR/Cas9-E4Orf6 and CRISPR/Cas9-E1B vectors are like CRISPR/Cas9 but they also encode for the E4orf6 and E1B proteins, respectively. CMV: cytomegalovirus, Cas9: *Streptococcus pyogenes* Cas9, NLS: nuclear localization signal, T2A, and P2A: self-cleaving peptides, AmpR: ampicillin resistance. (C) CMV-GALNS expression cassette harboring a puromycin resistance gene (PuroR) flanked by two homology recombination arms (HA-L and HA-R) for the AAVS1 *locus*.

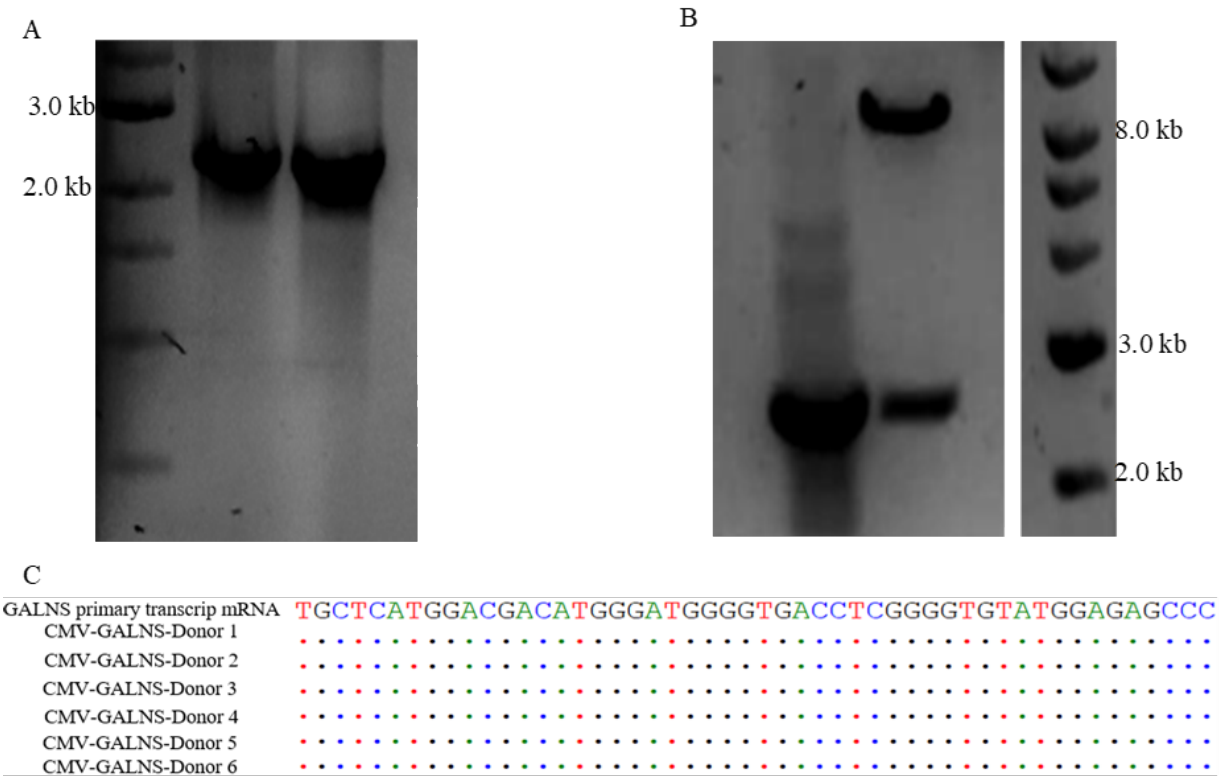

Figure 2. Assembly of CMV-GALNS Donor. **(A)** Agarose gel of PCR products showing amplicons of CMV-GALNS cassette (approximately 2.4 kb) using a pIRESneo-GALNS vector as template (27). ClaI and MluI-1 primers were used. **(B)** Verification by PCR (left) and enzymatic digestion with ClaI and MluI-HF enzymes of the CMV-GALNS-Donor vector (right). The lower band is the CMV-GALNS cassette, and the upper band is the Addgene 64215 (42) backbone. **(C)** Sanger sequencing of PCR products confirming the presence of the GALNS cDNA sequence on the assembled CMV-GALNS Donor. Molecular weight marker: 1 kb DNA ladder (NEB # N3232L).

Table 1. List of primers used in this study for sgRNA cloning into CRISPR-Cas9 vectors, CMV-GALNS Donor assembly, T7 assay, TIDE analysis, and homologous recombination verification. Highlighted letters correspond to the sgRNA sequence.

Primer	Sequence (5' - 3')	Function
ClaI	ACAGCAATCGATATTGATTATTGACTAGT TATTAATAGTAATCAATTACGGGGTCA	Donor assembly
MluI -1	GCGTATACGCGTCTAGTGGGACCAGAGGCACTT	Donor assembly
Forward T7	TCCGACGGATGTCTCCCTTG	T7 assay and TIDE analysis
Reverse T7	TCTCCCTCCCAGGATCCTCT	T7 assay and TIDE analysis
Forward P1	GGCAGCCTGTGCTGACCCATCGAGTC	Homologous recombination confirmation
Reverse R2	CGGGATTCTCCTCCACGTCACCGCA	Homologous recombination confirmation
sgRNA-F	CACCGACCCCACAGTGGGGCCACTA	sgRNA sequence assembly and cloning
sgRNA-R	AAACTAGTGGCCCCACTGTGGGGTC	sgRNA sequence assembly and cloning

2.4. T7 assay

To assess whether the CRISPR/Cas9 system with its respective sgRNA produces DSB at the AAVS1 *locus*, HEK 293FT cells were transfected at 100,000 cells/well in a 12-well plate with 500 ng of CRISPR/Cas9 vector using Lipofectamine 3000 (ThermoFisher ScientificTM, USA). Three days after transfection, genomic DNA was extracted using the Monarch® Genomic DNA Purification Kit (NEB # T3010S, USA), following the manufacturer's instructions. A region of the AAVS1 *locus* was obtained using primers that amplify a 300 bp fragment containing the Cas9 cut site (Table 1). The T7 assay was performed using the EnGen® Mutation Detection Kit (NEB # E3321S, USA), according to the manufacturer's instructions. The Cas9 on-target cut was calculated through densitometry on a 1 % agarose gel using the Gel Analyzer 19.1 software (www.gelanalyzer.com).

2.5. Indel detection by chromatogram decomposition analysis

HEK 293FT cells were transfected with 500 ng of CRISPR/Cas9-sgRNA plasmid. Three days post-transfection, genomic DNA was extracted as described above. A 300 bp region of the AAVS1 *locus* containing the cutting site was amplified using the Q5® High-Fidelity DNA Polymerase (NEB # M0515, USA) with the same primers used for the T7 assay. The PCR fragments were purified using the Monarch® Genomic DNA Purification Kit (NEB # T3010S, USA) and sequenced by the Sanger method (Macrogen, Republic of Korea). The resulting chromatograms were analyzed using the TIDE (Tracking of Indels by Decomposition) bioinformatic tool [46].

2.6. Homologous recombination on AAVS1 *locus* evaluation

HEK 293 FT cells were transfected with 500 ng of CRISPR/Cas9 and 500 ng of CMV-GALNS-Donor vector. Two days post-transfection, positive mCherry fluorescence cells were selected by fluorescence-activated cell sorting (FACS) using a BD FACSAria™ II cell sorter at 477 nm/678 nm excitation/emission. Edited cells were selected with puromycin (3 μ g/mL), following previous reports [42]. Only edited cells were expected to survive upon puromycin addition. After 15 days, genomic DNA was extracted as previously described. A 1.3 kb fragment was amplified by PCR using the Q5® High-Fidelity DNA Polymerase (NEB # M0515, USA)

kit and the forward F1 primer, annealing to a genomic region near the recombination site upstream of the left homology arm, and the Reverse R2 primer (Table 1). PCR amplicons were Sanger-sequenced for homologous recombination confirmation.

2.7. MPS IVA skin fibroblast transfection

MPS IVA fibroblasts were transfected with different plasmid combinations: 1) 500 ng of CRISPR/Cas9 and 500 ng of CMV-GALNS-Donor; 2) 250 ng of CRISPR/Cas9-E4orf6, 250 ng CRISPR/Cas9-E1B and 500 ng of CMV-GALNS-Donor; and 3) 500 ng of CMV-GALNS-Donor alone. Transfections occurred using Lipofectamine 3000 (Thermo Fisher Scientific). Briefly, 500 ng of CRISPR/Cas9 vectors and/or 500 ng of CMV-GALNS Donor vector were transfected on 12-well plates containing 30.000 cells/wells according to previous studies [43, 47]. Fibroblasts from a healthy male donor were obtained under informed consent and used as normal controls. Transfection efficiencies were evaluated with flow cytometry using a BD FACSAria II cell sorter at 477 nm/678 nm excitation/emission for mCherry-positive cells. Cells were labeled with propidium iodide (1 µg/mL) (Fisher Scientific, US) at 566 nm/574 nm excitation/emission to exclude non-viable cells, having recorded about 10.000 events. Transfection by epifluorescence was also confirmed using a Zeiss AXIO observer Z1 microscope (Zeiss Jena, Germany) at 560 nm / 630 nm excitation/emission for mCherry positive cells. Images were processed using the ImageJ 1.54m software [48].

2.8. GALNS activity assay

GALNS activity was evaluated following a previously reported protocol [43, 49]. 4-methylumbelliferyl-β-D-galactopyranoside-6-sulfate reagent (Toronto Chemicals Research, North York, Canada) served as the substrate for the GALNS enzyme. One unit of enzymatic activity (U) consisted of substrate nanomoles catalyzed per hour, whereas specific activity was reported as U/mg of total protein. Briefly, 20 μL of 2 mM substrate was incubated with 10 μL of total cell lysate. After 17 h of incubation at 37 °C, 2 μL of β-galactosidase from *Aspergillus oryzae* (10 mg/mL) was added, and the reaction was incubated for 2 hours at 37 °C. The reaction was halted with 150 μL of stop buffer (0.5 M Na_2CO_3/$NaHCO_3$, pH 10.7). Fluorescence was measured in a Twinkle LB 970 fluorescence microplate reader at Excitation/Emission: 360 nm/445 nm (Berthold Technologies Microplate, Germany). A standard curve was made using 4-methyl-umbelliferone serial dilutions. A solution of 0.2 M sodium acetate, 1% Triton X-100 with 1 mM PMSF, was used as the lysis buffer. GALNS activity was evaluated on days three, fifteen, and thirty after transfection.

2.9. Lysosome mass quantitation with LysoTracker Deep Red

Lysosomal mass was evaluated by flow cytometry using LysoTracker labeling, as previously described [31, 43], 30 days following MPS IVA fibroblasts transfection with 1) 500 ng of CRISPR/Cas9 and 500 ng of CMV-GALNS-Donor; 2) 250 ng of CRISPR/Cas9-E4orf6, 250 ng CRISPR/Cas9-E1B, and 500 ng of CMV-GALNS-Donor; or 3) 500 ng of CMV-GALNS-Donor alone. MPS IVA fibroblasts were incubated with 50 nM LysoTracker Deep Red (Invitrogen, USA) for 1 hour at 37 °C. Subsequently, the fibroblasts were collected by trypsinization, centrifuged, and washed twice with 1× PBS. The fibroblasts were resuspended with 300 μL of Hanks buffered saline solution (HBSS) at a 1:1000 dilution of propidium iodide (final concentration of 1 μg/mL, Fisher Scientific, US). The mean fluorescence intensity (MFI) of the LysoTracker Deep Red was

analyzed using a BD FACSAria II cell sorter at 477 nm/678 nm excitation/emission, and cell viability was determined with propidium iodide at 560 nm/630 nm excitation/emission. WT fibroblasts served as normal controls.

2.10. Evaluation of reactive oxygen species (ROS)

After transfection, as described above, MPS IVA fibroblasts were resuspended in HBSS buffer with 1 μM 2',7'-dichlorodihydrofluorescein diacetate reagent (H2DCFDA, Invitrogen, USA), following the manufacturer's instructions. Fluorescence was measured at 560 nm / 630 nm excitation/emission. After 30 minutes at 37 °C, the fibroblasts were washed with HBSS buffer and resuspended in the same buffer with propidium iodide (1 μg/mL) to assess viability (560 nm / 630 nm excitation/emission). Cells were analyzed on a BD FACSAria II cell sorter. ROS were measured at thirty days post-transfection. WT fibroblasts served as normal controls.

2.11. Statistical analysis

Results were analyzed with one-way ANOVA, Kruskal-Wallis tests, or two-tailed Student's t-tests. Differences between groups were significant at $p < 0.05$. Analyzes were performed with the GraphPad Prism 8.0.2 package (GraphPad Software, Inc).

3. Results and Discussion

3.1. CRISPR-Cas9 plasmids and sgRNA validation in HEK293FT cells

CRISPR/Cas9 requires a single-guide RNA (i.e., sgRNA) to drive the Cas9 enzyme toward a specific *locus* in the genome [37]. Previously, we demonstrated the utility of the AAVS1 *locus* as a safe harbor for inserting an expression cassette containing GALNS cDNA [43]. In this work, we integrated the CMV-GALNS cassette within the same *locus* (Fig. 2) but with the novel strategy of blocking the NHEJ pathway [42]. A sgRNA sequence against the AAVS1 *locus* [42] (Table 1) was cloned into three CRISPR/Cas9 vectors (CRISPR/Cas9, CRISPR/Cas9-E4orf6, and CRISPR/Cas9-E1B) (Fig. 1A–C). The insertion of the sgRNA sequence was validated by Sanger sequencing (**Fig. 3A**). To confirm that a Cas9-dependent DSB on the AAVS1 *locus* took place, we transfected HEK 293FT cells with the CRISPR/Cas9 vector. After three days post-transfection, we observed a mean transfection efficiency of 17.2 % ($n = 2$, SD = $\pm$0.71 %) via mCherry expression detection with flow cytometry (Fig. 3B). Then, through a T7 assay, we detected a DSB frequency of 17.6%, suggesting that Cas9 was able to promote a DSB towards the AAVS1 *locus* (Fig. 3C). To confirm the T7 findings, we also sequenced the PCR products and analyzed the chromatograms by tracer decomposition. As expected, the results confirmed the presence of indels in sequences obtained from HEK293FT cells transfected with CRISPR/Cas9-sgRNA, suggesting DSB events on the AAVS1 *locus*. Deletions between 2 to 6 nucleotides were the most common indels after DSB by CRISPR-Cas9 (Fig. 3D and E).

The on-target DSB efficiency of 17.6% on the AAVS1 locus (Fig. 3C) correlates with the results of indel detection by chromatogram decomposition, which showed an indel frequency of 18.5% (Fig. 3D and E). Altogether, these results confirm that our employed CRISPR/Cas9 system induced on-target DSB in the desired locus. Under similar conditions, Chu *et al*. obtained 45% DSB on positive-transfected cells. As the on-target cut reported here was estimated from whole cell populations and not solely from positive-transfected cells (*i.e*., fluorescence-activated cell sorting [FACS]), we expected it to be lower than that reported by Chu *et al*. [42].

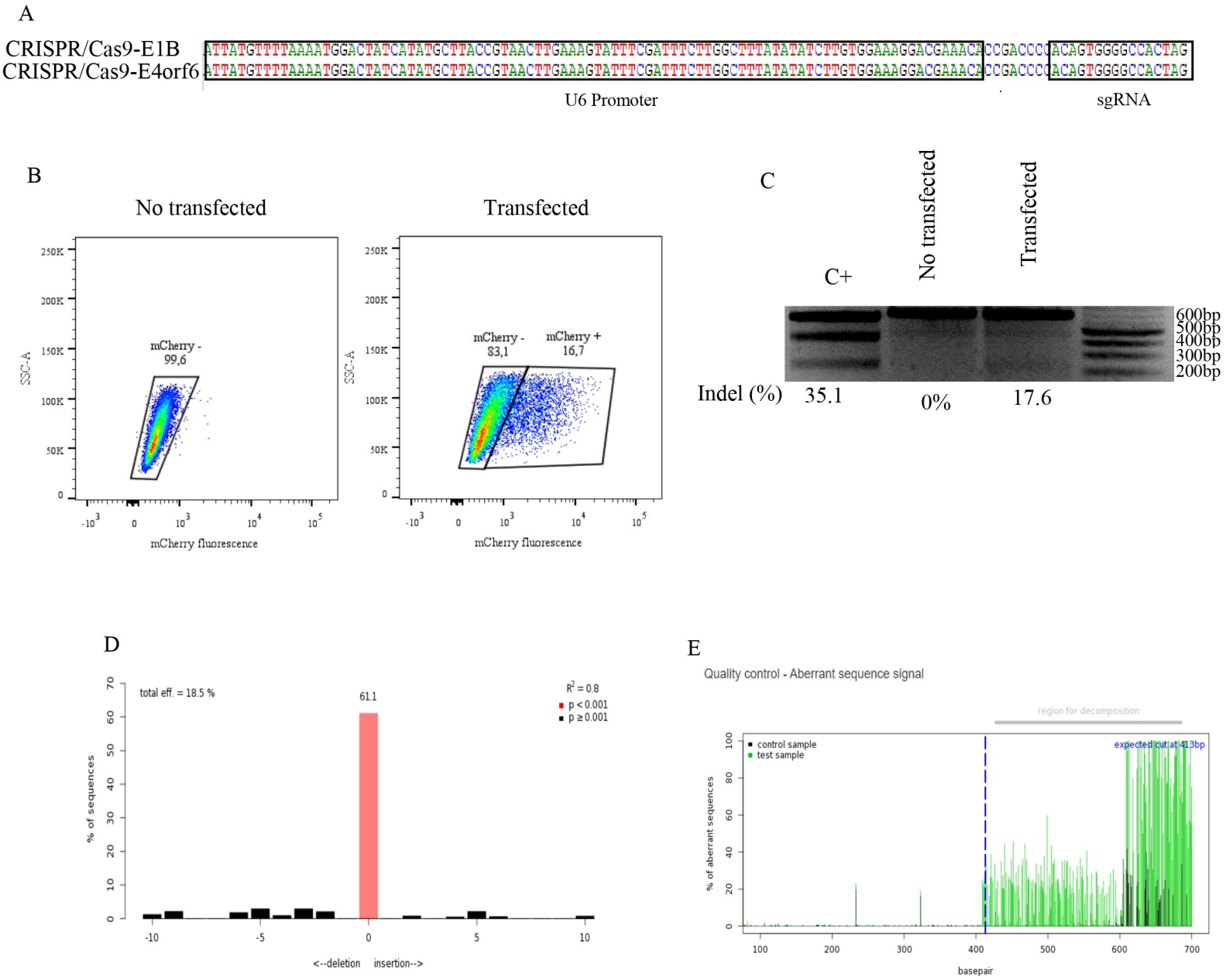

Figure 3. CRISPR/Cas9 vectors validation in HEK293FT cells and indel detection by chromatogram decomposition analysis of genomic DNA from HEK 293FT cells treated with CRISPR/Cas9 vectors. **(A)** Sequence alignment of the U6 promoter region of CRISPR/Cas9-E1B and CRISPR/Cas9-E4orf6 vectors showing the sgRNA fragment sequenced by the Sanger method **(B)**. Transfection efficiency of HEK 293FT cells. Representative flow cytometry showing transfection efficiency after transfection with CRISPR/Cas9 vector. Transfection efficiency was measured 3 days post-transfection. **(C)** T7 endonuclease assay on HEK 293FT cells transfected with CRISPR/Cas9 vector showing cutting efficiency on AAVS1 *locus*. **(D)** Predicted indel proportion of an AAVS1 *locus* region sequence obtained from cells transfected with CRISPR-Cas9 vectors. Predicted Indels with p<0,001 are shown with red bars. **(E)** Chromatogram decomposition of sequences from cells transfected with CRISPR/Cas9 (Green lines) vs. untreated (control) cells (Black bars). The dotted blue line shows the region where the CRISPR/Cas9 was predicted to cut the genomic AAVS1 *locus*. The larger the lines, the more background noise is expected in the genomic region, indicating the presence of Indels.

In previous work, we reported a DSB efficiency of 36.35% on the whole treated cell population and 32% on positive-transfected cells [43]. We hypothesize that the difference between the DSB percentage between our previous study and the present one may rely on transfection efficiencies (present study: 16.7% vs. Leal & Alméciga: 32.8%) and the Cas9 variants evaluated (present study: Cas9 wild type vs. Leal & Alméciga: nCas9).

There are no reports of potential off-target effects by the sgRNA used in the present study [42]. Although we did not confirm experimentally the occurrence of off-target events, we conducted a bioinformatics analysis to reveal the most probable off-target sites leading to unwanted effects (**Table 2**) [50–52]. Mismatches proximal to the protospacer adjacent motif (PAM) are unlikely to induce off-target effects [52]. Therefore, from the ten most probable off-target sites, eight are unlikely to produce off-target effects, and the two remaining targets, located in non-coding regions, are also unlikely to trigger deleterious effects if these events occur.

3.2. Homologous recombination by CRISPR-Cas9 and GALNS donor transfection

Homologous recombination after DSB-induced CRISPR/Cas9 requires a donor sequence flanked by homologous recombination arms [53]. Chu *et al*. designed HR arms to target the homologous recombination of a donor sequence into the AAVS1 *locus* [42], a safe harbor region for the accommodation of foreign genetic material [54]. We used the same HR arms in the present study. To establish the HR events under our experimental conditions, we transfected HEK 293FT cells with CRISPR/Cas9 and GALNS Donor plasmids. We performed FACS two days post-transfection to isolate mCherry-positive cells. From four isolation attempts, we grew the cells and extracted the DNA after fifteen days, from which we performed a PCR using primers flanking the recombination region of the expression cassette (**Fig. 4A**). All samples showed a ∼1.3 kb band, suggesting that the CMV-GALNS cassette was inserted into the AAVS1 *locus* (Fig. 4B). Further confirmation by Sanger sequencing revealed that cells transfected with the CRISPR/Cas9 system (i.e., CRISPR/Cas9 and CMV-GALNS-Donor plasmid) had the CMV-GALNS cassette inserted into the AAVS1 *locus* (Fig. 4C).

We measured GALNS enzyme activity on the HEK 293FT cells transfected with CRISPR/Cas9 and/or donor plasmids seven days post-transfection. We observed a 1.19-, 3.17-, and 0.96-fold change in GALNS activity, compared to untreated cells, after transfection with 1) CMV-GALNS Donor, 2) CMV-GALNS Donor and CRISPR/Cas9, and 3) CMV-GALNS Donor, CRISPR/Cas9-E4orf6, and CRISPR/Cas9-E1B CRISPR/Cas9, respectively (Fig. 4D), suggesting that insertion of the CMV-GALNS cassette induced the expression of the GALNS enzyme.

The evaluation of GALNS activity after transfection of HEK 293FT cells with the CRISPR/Cas9 system showed that co-transfection with CRISPR/Cas9 and CMV-GALNS-Donor induced a 3-fold enzyme activity increase compared to the untransfected control. However, we did not detect a significant increase in GALNS activity in cells simultaneously transfected with CRISPR/Cas9-E1B, CRISPR/Cas9-E4orf6, and CMV-GALNS-Donor. One possible explanation for this lack of enzymatic activity increase may be associated with the co-transfection of three vectors, likely reducing the success of a simultaneous triple transfection in the same cell.

Table 2. List of potential off-target human genome sequences for the sgRNA sequence used in this study. The highlighted nucleotides differ from the sgRNA sequence. The location of the chromosome and whether the sequence is in an intronic or intergenic region is shown. Possible off-target events were estimated using the bioinformatic tool Cas-OFFinder (50). The reference genome Homo sapiens (GRCh38/hg38) – Human was used for the off-target prediction.

Sequence	Chromosome	Location
ACCCCACAGTGGGGCCACTA	19	*AAVS1 locus*
ACCCCACAGTGcaGCCACcA	16	Intergenic
ACagCACAGTGaGGCCACTA	16	Intergenic
ACCCCACAaTGaGGCCACgA	15	Intronic
ACCCCACAGTGGGGCtAaTg	15	Intergenic
AaCCCACAcTGGGGCCACTc	12	Intergenic
ACCCCACAGTGGGGCtACag	18	Intronic
cCCCCAgAGTGcGGCCACTA	19	Intronic
ACCCCACAGTGGGGCggCaA	19	Intronic
AaCCCAgAGTGGGGCCtCTA	1	Intergenic
gCCCCACAGTGGGGaCAgTA	5	Intronic

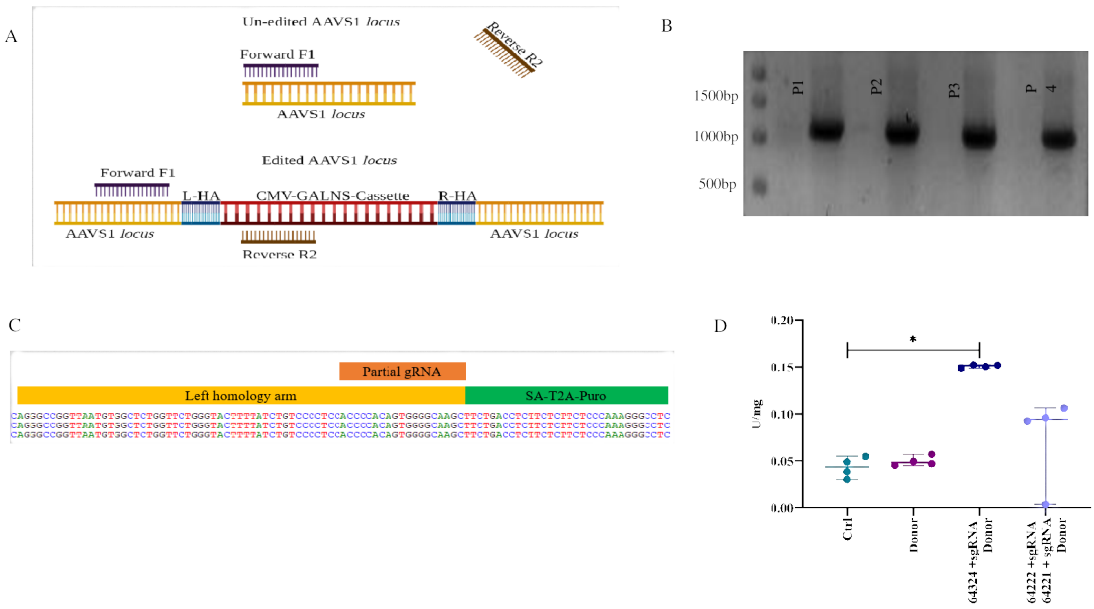

Figure 4. CRISPR/Cas9 and CMV-GALNS-Donor vectors validation in HEK293FT cells. **(A)** Schematic showing how junction PCR works for homologous recombination verification. An un-edited DNA sequence cannot anneal with both primers, while an edited sequence will amplify a sequence of 1.3 kb by annealing with both primers (created with BioRender.com). The forward primer (Forward F1) is upstream of the left homology arm (L-HA), while the reverse primer (Reverse R2) is within the CMV-GALNS cassette upstream of the right homology arm (R-LA). **(B)** Agarose gel electrophoresis of PCR products using a primer forward flanking the AAVS1 *locus* outside the recombination cassette and a primer reverse flanking a region inside the recombination cassette. Four HEK 293FT cell populations were isolated after having sorted the cells transfected with CRISPR/Cas9 and CMV-GALNS-Donor vectors. For each population, DNA was isolated, and a fragment of 1.3 kb was obtained by PCR. **(C)** Representative sequence alignment of PCR products from isolated cells after FACS showing the left homology arm (yellow), which is part of the AAVS1 *locus*; the SA-T2A-Puro fragment (green), which is part of the recombined cassette on AAVS1 *locus*; and the partial sgRNA sequence (orange). **(D)** GALNS specific activity after 7 days post-transfection of HEK 293 FT cells with CRISPR/Cas9 vectors (n= 4); *** p<0,001, **p<0,01, *p<0,5; Kruskal-Wallis test.

3.3. GALNS activity increases in MPS IVA skin fibroblast treated with CRISPR/Cas9

We have shown that gene editing using CRISPR/nCas9 positively impacts GALNS activity levels, GAGs, lysosomal mass, and oxidative stress in MPS IVA fibroblasts [43, 44]. Nevertheless, intracellular and extracellular activity levels were below those observed in wild-type fibroblasts, highlighting the importance of evaluating strategies to increase these levels. Supraphysiological GALNS activity levels have been associated with significant corrections in MPS IVA animal models [36, 55, 56]. Attempting the co-transfection of the CRISPR/Cas9 system along with E1B and E4orf6 proteins may lead to an HR increase [42], resulting in a significant rise in the GALNS activity levels, thus improving the general outcome.

We evaluated the transfection efficiency in MPS IVA fibroblasts three days post-transfection of 500 ng of CRISPR/Cas9 plasmid, observing a higher transfection efficiency in GM01361 fibroblasts than in GM00593 fibroblasts (**Fig. 5A**). In fact, evaluation by flow cytometry showed a transfection efficiency of 35.4 % (n = 4, SD = ± 0.14 %) and 4.91 % (n = 4, SD = ± 0.50 %) for the MPS IVA fibroblasts GM01361 and GM00593, respectively (Fig. 5B). Transfection of E1B or E4orf6 expressing vectors resulted in similar transfection rates compared with cells transfected with only CRISPR/Cas9-mCherry expressing vectors (Fig. 5C). Despite the lack of direct evaluation of the expression of E1B or E4orf6 proteins, the expression of the reporter gene served as an indirect confirmation of the expression of E1B or E4orf6 proteins in the transfected cells. Cas9, mCherry, and E4orf6/E1B cDNAs are within the same ORF and are separated by self-cleaving peptides (Fig. 1).

This study aimed to evaluate whether the expression of E1B or E4orf6 proteins increases gene edition effectiveness in MPS IVA patient fibroblasts as a proof of concept for upcoming *in vivo* studies. Consequently, we decided not to select MPS IVA fibroblast after transfection. Rather, we measured parameters in the mixed population of edited and unedited cells, evaluating then the effect of the transfection of CRISPR/Cas9 and GALNS donor plasmids in GALNS activity in MPS IVA fibroblasts after 3, 15, and 30 days post-transfection. Three days post-transfection, a non-significant increase in enzymatic activity was observed in GM01361 fibroblasts co-transfected with the CRISPR/Cas9-E4orf6, CRISPR/Cas9-E1B, and CMV-GALNS-Donor plasmids (Fig. 5d). After 15 days, GALNS activity in GM01361 fibroblasts showed a tendency to increase with all treatments (but remained not significant) whereas, after 30 days, co-transfection with CRISPR/Cas9 and CMV-GALNS-Donor plasmids showed a significant intracellular GALNS activity increase (p = 0.04), reaching levels 4.17-fold higher than those observed on untreated fibroblasts (untreated = 0.12 U/mg vs. CRISPR/Cas9 and CMV-GALNS-Donor treated cells = 0.81 U/mg) (Fig. 5D).

In the case of GM00593 fibroblasts, intracellular GALNS activity significantly increased after fifteen days post-transfection with CRISPR/Cas9 and CMV-GALNS-Donor cells (p = 0.002). At 30 days post-transfection a 11.2-fold increase (p = 0.014) was observed in fibroblasts transfected with CRISPR/Cas9 and CMV-GALNS-Donor (untreated = 0.048 U/mg vs. CRISPR/Cas9 and CMV-GALNS-Donor treated cells = 0.62 U/mg). Transfection of CRISPR/Cas9-E1B and CRISPR/Cas9-E4orf6 plasmids did not show any significant increase in GALNS activity after thirty days post-transfection, neither in GM00593 nor GM01361 fibroblasts (Fig. 5d), suggesting that this strategy may not result in an HR frequency increase in the MPS IVA fibroblasts. Compared to WT fibroblasts (GALNS activity = 3.98 U/mg), there was an increase in both cell cultures equivalent to a 0.98-fold change in CRISPR/Cas9 and CMV-GALNS-Donor transfected GM01361 cells and a 0.62-fold change in CRISPR/Cas9 and CMV-GALNS-Donor transfected GM00593 cells after 30 days (Fig. 5D).

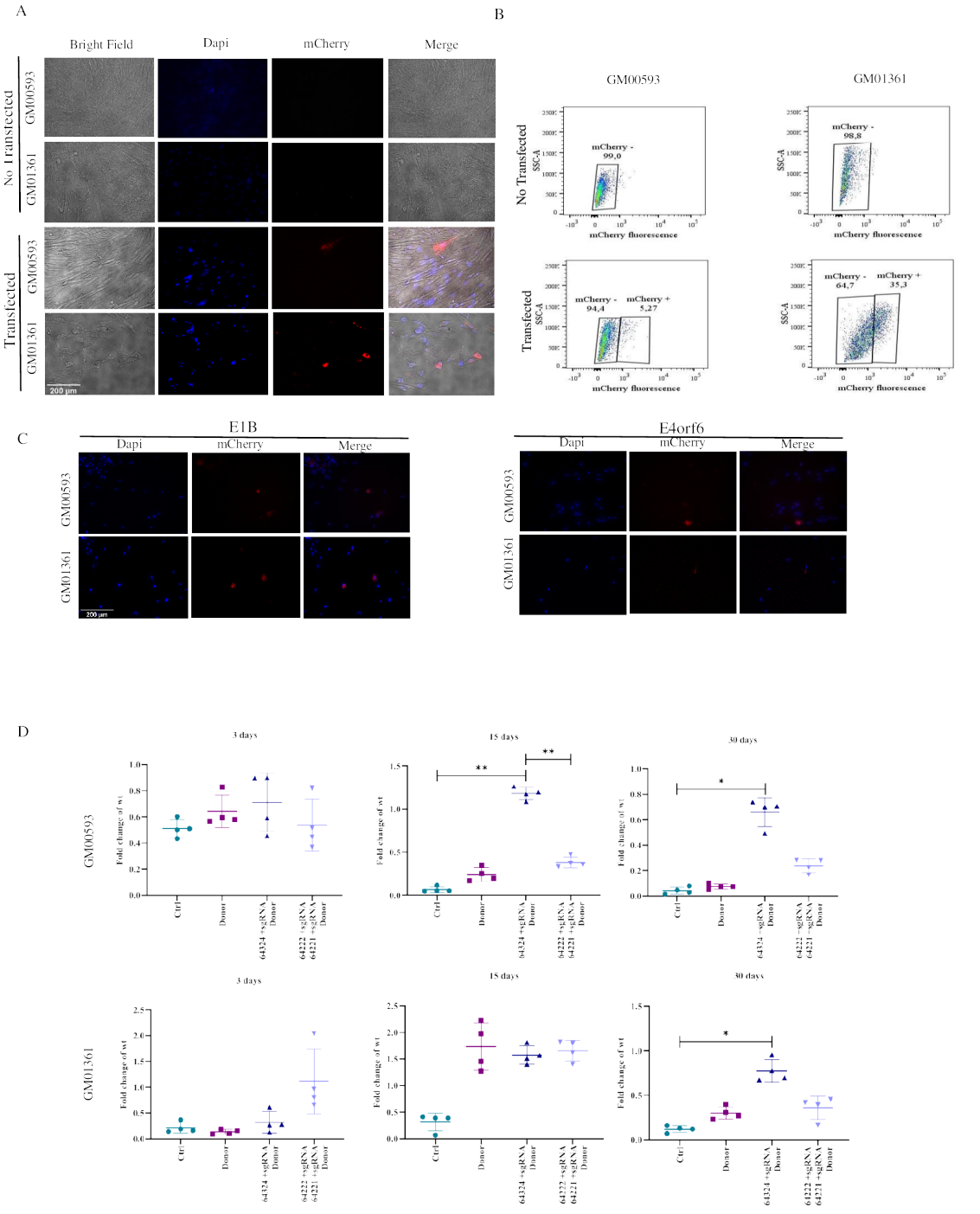

Figure 5. Transfection efficiency and evaluation of the effect of CRISPR/Cas9 system on the GALNS enzyme activity in MPS IVA derived skin fibroblasts. (A) Fluorescence images of GM00593 and GM01361 skin fibroblasts on day three following transfection with CRISPR/Cas9 vectors (20x), control (untransfected) cells are also shown. (B) Transfection efficiencies evaluated by flow cytometry using the mCherry filter. (C) Fluorescence images of MPS IVA fibroblasts with CRISPR/Cas9 vectors with coding sequences for expression of E1B (left) or E4Orf6 proteins (right) assessed on day three post-transfection (20x). (D) Specific GALNS activity. Results are reported as fold changes compared to a WT after 3, 15, and 30 days post-transfection with CRISPR/Cas9 vectors with or without donor vector and donor vector alone in GM00593 and GM01361 skin fibroblasts (n= 4). *** p<0,001, **p<0,01, *p<0,5; One way ANOVA test.

The evaluation of the CRISPR/Cas9 system in two MPS IVA patient-derived fibroblasts (GM00593 and GM01361) showed different transfection efficiencies to those of HEK 293FT cells. GM01361 fibroblasts experienced a higher transfection efficiency than GM00593 cells (35.3 % vs. 5.27 %). After 30 days post-transfection with CRISPR/Cas9 and CMV-GALNS-Donor plasmids, we observed a significant increase in GALNS activity, reaching up to 48.3 % and 54 % of WT levels for the GM01361 and GM00593 fibroblasts, respectively (Fig. 5d). These results agree with those reported by Puentes *et al*. [31] when using lentiviral vectors (LV) to transduce GM01361 and GM00593 fibroblasts. LV led to an increase in GALNS activity between 4- and 12-fold compared to untreated cells. These results also agree with our previous findings using CRISPR/nCas9 in GM00593 fibroblasts, in which a GALNS enzyme activity increased to 40 % of WT levels after 30 days post-transfection [43]. A GALNS intracellular activity increase of about 30 % and 50 % of WT GALNS activity levels was recently reported for GM01361 and GM00593, respectively, when the cells were transfected with CRISPR/nCas9 and GALNS Donor vectors, also showing similar levels of GALNS activity of those observed in the present study [44]. MPS I fibroblasts treated with CRISPR/Cas9 plus an α-iduronidase (IDUA) gene also showed similar results, in which a 4-fold increase in enzyme activity, compared to untreated cells, was observed [47]. Since patients with GALNS activity as low as 40 % to 70 % exhibit a normal phenotype [33], our findings may anticipate a positive therapeutic effect.

3.4. Lysosomal mass and oxidative stress evaluation in gene-edited MPS IVA skin fibroblasts

One common milestone of MPS IVA is the progressive buildup of partially degraded GAGs into the lysosome, increasing its size and number [57]. By delivering a functional copy of the human GALNS gene, we expected to reduce GAG storage and, consequently, lysosome number and size [24, 43, 58]. As previously reported [43], untreated MPS IVA fibroblasts showed increased lysosomal mass compared to WT fibroblasts (**Fig. 6A**). After 30 days post-transfection, only the fibroblasts co-transfected with CRISPR/Cas9 and CMV-GALNS-Donor vectors showed a 13.6 % ($p = 0.02$) and 7.1 % ($p = 0.04$) reduction in the lysosomal mass for the GM01361 and GM00593 fibroblasts, respectively (Fig. 6A).

There is evidence of lysosomal impairment function, alteration of the normal autophagy flux, and oxidative stress on MPS IVA cells [17, 57, 59]. CRISPR/Cas9 gene editing in MPS I and MPS IVA showed a positive outcome in the recovery of lysosomal mass on treated fibroblasts [43, 44, 47]. Under our experimental conditions, MPS IVA fibroblasts treated with CRISPR/Cas9 and CMV-GALNS-Donor vectors significantly reduced the lysosomal mass, which agreed with previous reports [43, 44, 47, 58].

Increased oxidative stress occurs in MPS IVA [17, 43, 44, 57]. As expected, an increase in ROS was observed on MPS IVA fibroblasts compared to WT fibroblasts (Fig. 6B). However, 30 days post-transfection, we did not observe a significant reduction of ROS levels in any of the MPS IVA fibroblasts evaluated. Nevertheless, we observed a non-significant decrease of 14% and 24.6% of the untreated cells signal after treatment with 1) CRISPR/Cas9 and CMV-GALNS-Donor and 2) CRISPR/Cas9-E1B, CRISPR/Cas9-E4orf6, and CMV-GALNS-Donor, respectively in GM01361 fibroblasts (Fig. 6B). This result contrasts with a report by Leal & Alméciga, having employed a CRISPR/Cas9 nickase (nCas9) [43]. This difference can be explained, in part, by the approach used to evaluate oxidative stress profiles. While Leal and Alméciga evaluated mitochondrial-dependent oxidative stress [43], we evaluated global oxidative stress instead.

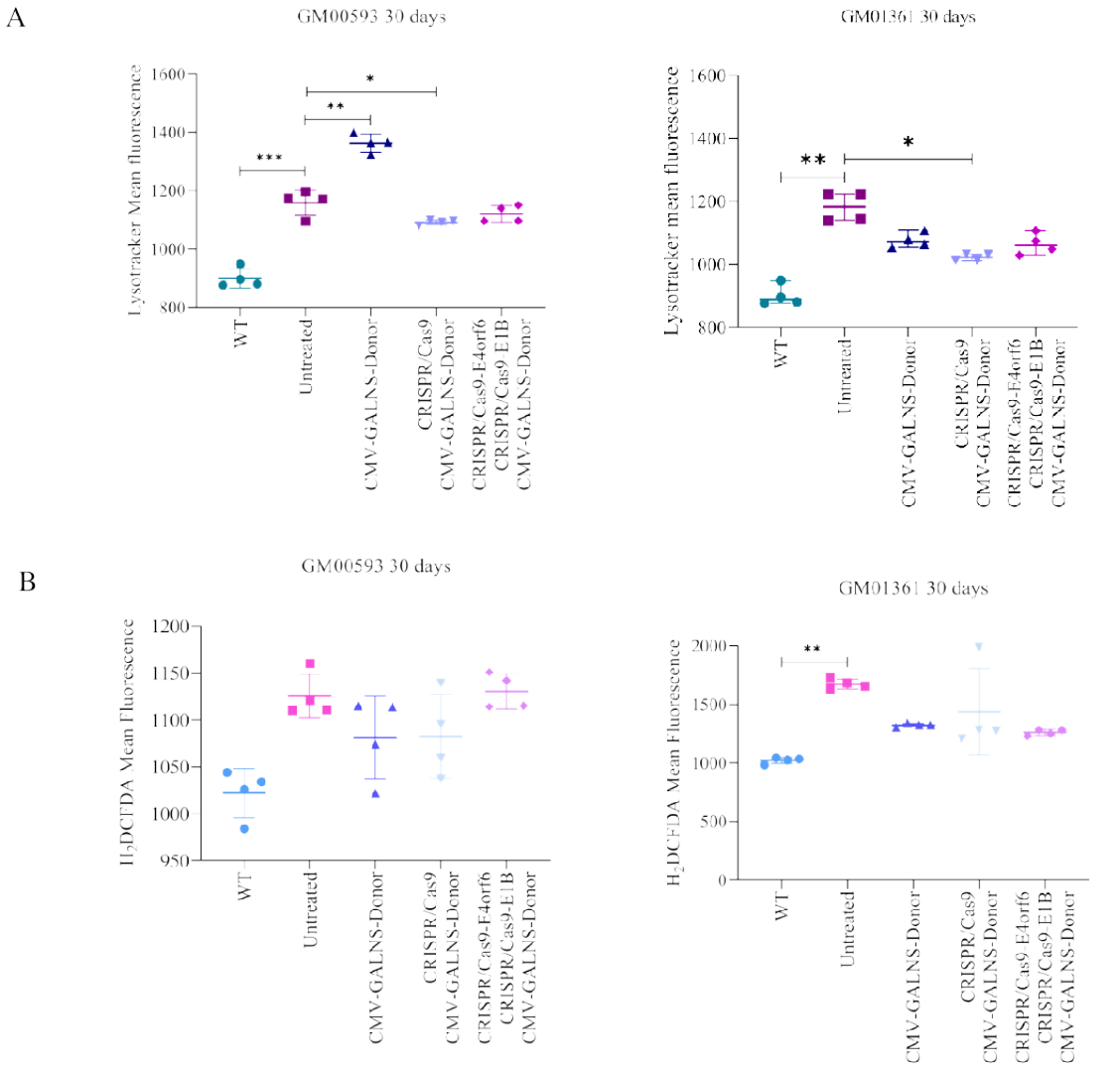

Figure 6. Lysosomal mass and ROS evaluation after 30 days post-transfection with CRISPR/Cas9 vectors in GM00593 and GM01361 skin fibroblasts. **(A)** Lysosomal mass was evaluated using LysotrackerTM Deep Red by flow cytometry. Graphs show Lysotracker mean fluorescence after 30 days post-transfection (n = 2). **(B)** Oxidative stress on MPS IVA fibroblast was evaluated by flow cytometry using the H2DCFDATM probe. Graphs show H2DCFDA mean fluorescence after 30 days post-transfection (n = 4). *** $p < 0{,}001$, ** $p < 0{,}01$, * $p < 0{,}5$; One way ANOVA or Kruskal-Wallis test.

Compared with the results obtained with conventional CRISPR/Cas9 plasmids, we did not detect any significant difference in GALNS activity, lysosomal mass, or oxidative stress with fibroblasts transfected with the plasmids expressing the E4orf6 and E1B proteins [42]. Simultaneous co-transfection of three vectors could affect the expression of each vector in the same cell. A previous study reported that co-transfection could favor the expression of some vectors over others in a vector- and cell-type-dependent manner [60]. Other approaches involving small molecules and Cas9 fusion variants could provide better results [61–63].

4. Conclusions

This study confirmed the suitability of classical CRISPR/Cas9-based gene therapy as a potential treatment strategy for MPS IVA. Our CRISPR/Cas9 system targeting the AAVS1 *locus* offers a suitable platform for inserting a GALNS expression cassette, significantly increasing GALNS activity levels while reducing lysosomal mass. Although no improvement occurred when employing NHEJ inhibitors with the classical CRISPR/Cas9 strategy, novel strategies to increase the frequency of homologous recombination still need to be explored.

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7. Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent arrangements), or non (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript. Potential conflicts of interest related to individual authors' commitments. Potential conflicts of interest related to commitments of editors, journal staff, or reviewers.

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Evaluación del sistema CRISPR/Cas9 como plataforma de edición genómica para la Mucopolisacaridosis IVA utilizando una estrategia para la inducción de una mayor frecuencia de recombinación homóloga

Resumen: La Mucopolisacaridosis IVA (MPS IVA) es una enfermedad de almacenamiento lisosomal causada por mutaciones en el gen que codifica para la hidrolasa N-acetilgalactosamina-6-sulfato sulfatasa (GALNS). La deficiencia de GALNS conduce a una acumulación progresiva de condroitín-6-sulfato y queratán-6-sulfato parcialmente degradados dentro de los lisosomas. Varias estrategias terapéuticas para esta enfermedad están bajo evaluación. Recientemente, validamos el uso de la terapia génica basada en CRISPR/Cas9 para MPS IVA, recuperando hasta un 40 % de la actividad normal de GALNS, lo que condujo a valores positivos en los biomarcadores clásicos de MPS IVA. En este estudio, extendimos nuestros hallazgos previos realizando la co-entrega del sistema CRISPR/Cas9 con las proteínas E4orf6 o E1B, que son inhibidores de la vía de reparación de unión de extremos no homólogos, lo que puede llevar a un aumento en el mecanismo de reparación dirigida por homología. Los vectores CRISPR/Cas9 se transfectaron en fibroblastos humanos MPS IVA GM00593 y GM01361 utilizando un modo de entrega basad en lipofección. Después de 30 días, encontramos que las células transfectadas con los sistemas CRISPR/Cas9 en ausencia de E4orf6 o E1B, mostraban un aumento en la actividad de GALNS en comparación con las células no tratadas, este aumento fue de hasta 4.17 veces en células GM01361 y 11.2 veces en células GM00593. Se logró una recuperación parcial de la masa lisosomal en fibroblastos humanos MPS IVA después del tratamiento. Observamos una modesta reducción del estrés oxidativo únicamente en células GM01361 tratadas. Ninguna de las células transfectadas con plásmidos CRISPR/Cas9 que expresan E4orf6 o E1B mostró mejoría para las variables de prueba. En conjunto, nuestros resultados respaldan el uso de CRISPR/Cas9 como una estrategia potencial basada en terapia génica para el tratamiento de MPS IVA y resaltan la importancia de buscar enfoques para mejorar el impacto de la edición genética como enfoque terapéutico.

Palabras Clave: CRISPR/Cas9; Locus AAVS1; Mucopolisacaridosis IVA; Recombinción homóloga.

Avaliação do sistema CRISPR/Cas9 como plataforma de edição genômica para a Mucopolissacaridose IVA utilizando uma estratégia para indução de maior frequência de recombinação homóloga

Resumo: A mucopolissacaridose IVA (MPS IVA) é uma doença de armazenamento lisossômico causada por mutações no gene que codifica a hidrolase N-acetilglicosamina-6-sulfato sulfatase (GALNS). A deficiência de GALNS leva ao acúmulo progressivo de condroitina-6-sulfato e queratan-6-sulfato parcialmente degradados dentro dos lisossomos. Diversas estratégias terapêuticas para essa doença estão em avaliação. Recentemente, validamos o uso da terapia gênica baseada em CRISPR/Cas9 para MPS IVA, recuperando até 40 % da atividade normal de GALNS, o que resultou em efeitos positivos nos valores de biomarcadores clássicos da MPS IVA. Neste estudo, ampliamos nossos resultados anteriores realizando a co-entrega do sistema CRISPR/Cas9 com as proteínas E4orf6 ou E1B, que são inibidores da via de reparo por junção de extremidades não homólogas, o que pode levar a um aumento no mecanismo de reparo direcionado por homologia. Os vetores CRISPR/Cas9 foram transfectados em fibroblastos humanos MPS IVA GM00593 e GM01361 utilizando um método de entrega baseado em lipofecção. Após 30 dias, observamos que as células transfectadas com os sistemas CRISPR/Cas9 na ausência de E4orf6 ou E1B apresentaram um aumento na atividade de GALNS em comparação com células não tratadas, sendo esse aumento de até 4,17 vezes nas células GM01361 e 11,2 vezes nas células GM00593. Após o tratamento, foi observada uma recuperação parcial da massa lisossômica em fibroblastos humanos com MPS IVA. Observou-se uma modesta redução do estresse oxidativo apenas nas células GM01361 tratadas. Nenhuma das células transfectadas com plasmídeos CRISPR/Cas9 expressando E4orf6 ou E1B mostrou melhora nas variáveis testadas. Em conjunto, nossos resultados apoiam o uso do sistema CRISPR/Cas9 como uma estratégia terapêutica promissora baseada em terapia gênica para o tratamento da MPS IVA e destacam a importância de buscar abordagens que aumentem o impacto da edição genética como estratégia terapêutica.

Palavras-chave: CRISPR/Cas9; Lócus AAVS1; Mucopolissacaridose IV A; Recombinação homóloga.

Dr. Carlos Javier Alméciga-Díaz

Professor at Pontificia Universidad Javeriana, specializing in inborn errors of metabolism. His expertise spans gene therapy, enzyme replacement, glycoengineering, and CRISPR/Cas9 applications. He has pioneered recombinant lysosomal enzyme production and bioinformatics-driven insights into Mucopolysaccharidoses.

ORCID: 0000-0001-6484-1173

Diego A. Suarez

is a biologist with a Master's degree in Biochemistry and extensive experience in developing therapeutic strategies for inborn errors of metabolism. His expertise includes CRISPR/Cas9 gene editing, recombinant enzyme therapies, and pharmacological chaperones. Currently, he is focused on the development and characterization of cellular models for Tay-Sachs disease, advancing research toward innovative treatment approaches.

ORCID: 0000-0001-9519-4706

Dr. Andrés Felipe Leal

is a cell biologist with rich experience in drug development for leukemias, infectious diseases, and inherited metabolism disorders. Dr. Andrés F. Leal's research currently focuses on cell biology and CRISPR/Cas9-based genome editing for lysosomal storage disorders using in vitro, in vivo, and ex vivo approaches at Nemours Children's Health.

ORCID: 0000-0001-5956-1986