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ARTICLE

High rate of HDR in gene editing of p.(Thr158Met) *MECP2* mutational hotspot

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Abstract

Rett syndrome is a progressive neurodevelopmental disorder which affects almost exclusively girls, caused by variants in *MECP2* gene. Effective therapies for this devastating disorder are not yet available and the need for tight regulation of *MECP2* expression for brain to properly function makes gene replacement therapy risky. For this reason, gene editing with CRISPR/Cas9 technology appears as a preferable option for the development of new therapies. To study the disease, we developed and characterized a human neuronal model obtained by genetic reprogramming of patient-derived primary fibroblasts into induced Pluripotent Stem Cells. This cellular model represents an important source for our studies, aiming to correct *MECP2* variants in neurons which represent the primarily affected cell type. We engineered a gene editing toolkit composed by a two-plasmid system to correct a hotspot missense variant in *MECP2*, c.473 C > T (p.(Thr158Met)). The first construct expresses the variant-specific sgRNA and the Donor DNA along with a fluorescent reporter system. The second construct brings Cas9 and targets for auto-cleaving, to avoid long-term Cas9 expression. NGS analysis on sorted cells from four independent patients demonstrated an exceptionally high editing efficiency, with up to 80% of HDR and less than 1% of indels in all patients, outlining the relevant potentiality of the approach for Rett syndrome therapy.

Introduction

Rett syndrome (RTT; OMIM# 312750) is one of the most common genetic causes of intellectual disability in girls with an estimated prevalence of about 1:10.000 [1]. Classic RTT patients present a normal development for the first 6–18 months of life. Subsequently, ability to speak and purposeful hand movements are lost, together with a reduction in interpersonal contact and the appearance of autistic behaviour [2]. Typical features include involuntary movements, with the classic 'hand-washing' stereotypic activities, electroencephalogram abnormalities, postnatal microcephaly, inability to speak and walk. Over the years, patients develop further somatic and neurologic deterioration, resulting in end-stage spastic quadriparesis in part of them. In more than 95% of cases classic RTT is an X-linked disorder resulting from de novo pathogenic variations in the Methyl−CpG-binding Protein 2 (*MECP2*) gene, located on the long arm of chromosome X (Xq28) [2, 3]. Over 100 different pathogenic variations have been identified, including point variants, insertions, duplications and small

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or large deletions. However, eight single nucleotide recurrent changes, namely c.316 C > T (p.(Arg106Trp)), c.397 C > T (p.(Arg133Cys)), c.473 C > T (p.(Thr158Met)), c.502 C > T (p.(Arg168*)), c.763 C > T (p.(Arg255*)), c.810 A > G (p.(Arg270*)), c.880 C > T (p.(Arg294*)) and c.916 C > T (p.(Arg306Cys)), account for ~70% of all identified variations [1, 4, 5]. The most frequent one is the missense change c.473 C > T (p.(Thr158Met)) that accounts for ~10% of classic RTT (https://www.rettdatabasenetwork.org) [5]. This variant is associated with a severe phenotype and increased risk for severe epilepsy, but is reported also in the milder preserved speech variant (PSV or Z-RTT) [6, 7]. The reasons of this wide clinical variability are not yet fully understood but an important role might be played by epigenetic factors and X-chromosome inactivation that influence the degree of *MECP2* expression in the brain [6, 8]. *MECP2* encodes a transcriptional regulator that plays a fundamental role in brain development and functioning and in synaptic maintenance [9, 10]. It has been demonstrated that the reintroduction of wild type (WT) *Mecp2* post-natally in symptomatic KO mice can revert disease phenotype, suggesting that RTT is not irreversible [11]. However, also *MECP2* overexpression causes disease in humans, outlining the fundamental need of a tight regulation of its expression for normal brain functionality [12, 13].

To characterize the biological mechanisms implicated in disease pathogenesis, we established and characterized a human neuronal model based on genetic reprogramming of patient fibroblasts into induced Pluripotent Stem Cells (iPSCs) [14–16]. iPSCs are a valuable source of patient-derived cells like neurons, which represent the most relevant cell type for studying disease mechanisms as well as for testing innovative therapeutic approaches, such as gene editing.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is an adaptive immune system used by bacteria to defend themselves against invading viruses and it is widely used for genome editing [17]. The genome editing CRISPR/Cas9 technology has the ability to correct disease-causing variants and the spur to develop novel molecular therapeutics for genetic diseases [18, 19]. The system requires two components to work: Cas9 nuclease, the molecular scissors able to cut the DNA, inducing double-strand breaks (DSBs); the single guide RNA (sgRNA), a short RNA sequence that leads Cas9 to the target sequence. CRISPR/Cas9 system enables efficient and precise alteration by inducing targeted DNA DSBs that stimulate cellular DNA repair mechanisms, Homology Directed Repair (HDR) and Non-Homologous End-Joining. HDR results either in knock-in events or in correction of point variants by recombination with an exogenous donor DNA used as template. HDR is effective in both dividing and non-dividing cells, like terminally differentiated neurons [20]. It is therefore suitable for the correction of variants in *MECP2*-related disorders. Classical gene therapy for RTT relies on a gene replacement strategy, in which an additive normal copy of *MECP2* is inserted within the genome under an artificial control [21, 22]. However, this strategy is risky for genes like *MECP2* whose over- and under-expression are both deleterious. Therefore, gene editing presently appears as a more appropriate strategy, since this approach allows the edited gene to maintain its native regulation avoiding the risks associated with *MECP2* inappropriate expression. Starting from these premises, we explored the possibility to correct *MECP2* variants with CRISPR/Cas9 technology as a therapeutic tool for RTT. We present here the successful editing of one of the most common *MECP2* variant hotspots, namely c.473 C > T (p.(Thr158Met)), in RTT fibroblasts and iPSC-derived neurons. Our results represent a step forward in the effort to improve the quality of life of patients with rare genetic diseases, finding ready-to-use therapeutic strategies.

Materials and methods

Patients' selection

Four patients with classic Rett syndrome and typical phenotype were recruited by the medical équipe of the Medical Genetics Unit at the University Hospital of Siena, following Informed Consent signature. All patients carry the same causative variant in the *MECP2* gene, namely c.473 C > T (p.(Thr158Met)) (NM_004992.3). The patients have been inserted in the LOVD database http://www.lovd.nl/MECP2 (individual IDs 00274244–00274247). Clinical description of all patients is reported in Table S1.

sgRNA design and plasmid cloning

The Oligonucleotides used for the cloning are listed in Table 1. The variant-specific sgRNA was designed using the MIT CRISPR Design Tool (http://crispr.mit.edu). A WT Donor DNA (#1) was designed accordingly as a 120 bp sequence centred on the mutated nucleotide, where a Protospacer Adjacent Motif (PAM) sequence with a silent variant was added. A variant-specific sgRNA was obtained by annealing and subsequent phosphorylation of single-strand oligonucleotides (#2,3). sgRNA and Donor DNA were cloned into BbsI and AfIII/SalI sites, respectively, in the pAAV2.1_CMV_eGFP3 plasmid backbone [23]. A mCherry/EGFP double reporter system was cloned between NheI/SpeI unique restriction sites replacing the eGFP3 coding sequence of the original plasmid. A target sequence, represented by sgRNA plus PAM, was interposed between mCherry and EGFP coding sequences, using a BsmbI

Table 1 Sequence of oligonucleotides used for plasmids construction.

#1	*MECP2* Donor sequence	GATTGCGTACTTCGAAAAGGTAGGCGACACATCCCTGGACCCTAATGATTTTGACTTCACGGTAA CgGGGAGAGGGAGCCCCTCCCGGCGAGAGCAGAAACCACCTAAGAAGCCCAAATC
#2	*MECP2* sgRNA Fw oligo	CACCGGATTTTGACTTCATGGTAAC
#3	*MECP2* sgRNA Rv oligo	AAACGTTACCATGAAGTCAAAATCC
#4	*MECP2* sgRNA + PAM Wild type FW oligo	GTCGGATTTTGACTTCACGGTAACTGG
#5	*MECP2* sgRNA + PAM Wild type RV oligo	CAGACCAGTTACCGTGAAGTCAAAATCC
#6	Cas9 Fw primer	TAAAGGATCCGATTTTGACTTCATGGTAACTGGATGGACTATAAGGACCACGA
#7	Cas9 Rv primer	TAAACCGCGGGATTTTGACTTCATGGTAACTGGTCAGCGAGCTCTAGGAATTC

restriction site. The same cloning was performed to obtain the mCherry/EGFP reporter harbouring the WT target sequence (#4,5). The px330 Addgene plasmid encoding *Streptococcus pyogenes* Cas9 (SpCas9) was used for Cas9 cloning [24]. Cas9 coding sequence was amplified by PCR using specific primers (#6,7), designed to insert the sgRNA plus PAM sequences to achieve self-cleaving activity, and BamHI/SacII restriction sites at 5′ and 3′ ends of the PCR product, respectively; the amplified product was cloned into pAAV2.1_CMV_eGFP3 backbone, under the control of CMV promoter, between BamHI and SacII restriction sites.

Plasmids were transformed in STBL3 Competent *Escherichia coli* cells and grown in standard Luria-Bentani (LB) medium; [25] plasmids were purified using the EndoFree Plasmid Maxi Kit (Qiagen). All constructs were verified by Sanger sequencing (GA3130 Genetic Analyzer, Thermo Fisher Scientific).

Cell culture and transfection

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (EuroClone) supplemented with 10% Fetal Bovine Serum (FBS) (Carlo Erba), 2 mM L-Glutamine (Carlo Erba), and 1 mM Penicillin/Streptomycin (Carlo Erba) and routinely passed 1:2 with Trypsin-EDTA (Irvine scientific Santa Ana, California, United States). Transient transfection of HEK293 cells was performed using Polyethylenimine transfection reagent 1 ug/ul (Polysciences) according to the manufacturer's instructions. Cells were transfected with 100 ng of targeting plasmid and 400 ng of Cas9 encoding plasmid. FACS analysis was performed 48 h after transfection.

Fibroblasts from mutated patients were obtained from the Telethon Network of Genetic Biobanks. Cells were cultured in DMEM (Biochrom GmbH, Berlin, Germany) supplemented with 10% FBS, 2 mM L-Glutamine and 1 mM Penicillin/Streptomycin (Carlo Erba) according to standard protocols and routinely passed 1:2 with Trypsin-EDTA (Irvine scientific) [25]. Fibroblasts were passed one-two days prior to transfection, in order to have cultures 70–90% confluent on the day of the experiment. Transfection was performed by electroporation using the Neon® Transfection System following manufacturer protocol (Thermo Fisher Scientific®, Waltham, MA, United States). Briefly, fibroblasts were harvested with Trypsin Solution and counted. 1×10^6 cells were transfected with 15 ug of DNA (5 µg Reporter plasmid and 10 µg Cas9 plasmid) and the following instrument parameters: 1 Pulse at 1700 Volts with 20 ms pulse width. EGFP and mCherry positive cells were quantified with a Cytoflex flow (Beckman Coulter) cytometer with blue (488 nm) and a yellow (560 nm) lasers, using a 530/30 (EGFP) and a 585/42 (mCherry) filter.

Neuronal differentiation and neurons transfection

Induced Pluripotent Stem Cells obtained from case three were maintained in feeder-free conditions in mTeSR1 medium (Stem Cell Technologies, Grenoble, France) with 1 mM penicillin/streptomycin (Carlo Erba) and routinely passed by Dispase (Stem Cell Technologies). Cells were differentiated into neurons following the protocol already set up in the laboratory [15]. For transfection, neurons were plated in 12-well plates coated with poly-L-Ornithine and laminin (Sigma-Aldrich, Merck Millipore®, Burlington, Massachusetts, United States) with Terminal differentiation medium (TD) (DMEM:F12 supplemented with 1% N2, 4% B27 with Vitamin A, 55 µM beta-mercaptoethanol and 500 µl penicillin/streptomycin). Neurons were transfected with variant -specific plasmids on day 22 of neuronal differentiation. 2×10^5 cells were transfected using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, California, United States) according to manufacturer's protocol. Transfection efficiency was confirmed with fluorescence microscopy and FACS analysis from 4 to 6 days post-treatment.

Flow cytometry analysis and cell sorting

Cells were analyzed and sorted on a fluorescent-activated cell sorter FACSAria II (Becton Dickinson) using FACSDiva software version 8.0.1 (BD Biosciences-US). To isolate EGFP-positive cells for subsequent analysis, the fibroblasts were detached, resuspended in PBS/EDTA 3 mM/Trypsin 2.5% and placed on ice. The cellular suspension was filtered through a 70 µm filcon filter (BD Biosciences). Fibroblasts were sorted using a 100 µm nozzle and an event rate of 1000/sec.

Ion Torrent S5 sequencing and NGS analysis

DNA was extracted from $EGFP^+$ cells and control cells using the Qiamp DNA micro kit (Qiagen, Hilden, Germany). Ion AmpliSeq 2.0TM Library Kit (Life Technologies™, Carlbad, CA) was used for library preparation. Libraries were purified using Agencourt AMPure XP system, quantified with the Qubit® dsDNA HS Assay Kit reagent (Invitrogen Corporation, Life Technologies™), pooled at an equimolar ratio, annealed to carrier spheres (Ion Sphere™Particles, Life Technologies) and clonally amplified by emulsion PCR (emPCR) using the Ion ChefTM system (Ion Chef™, Life Technologies). Ion 510™, 520™ or 530™ chip were loaded with the spheres carrying single stranded DNA templates and sequenced on the Ion Torrent S5 instrument (Life Technologies™, Carlsbad, CA) using the Ion S5™ Sequencing kit, according to manufacturer's protocol.

For each patient, the FASTQ files of transfected cells and relative controls were downloaded from the sequencing platform (S5 Torrent Server VM) and uploaded to the online analysis tool Cas-Analyzer [26] together with the sgRNA and the Donor in order to obtain the percentage of HDR achieved, considering a comparison range of 15 nucleotides around the cut site. For each sample, the .bam and .bai files were uploaded on IGV Visualization Software (Broad Institute, Cambridge, United States) to precisely visualize both the replacement of the mutated base and the indels events in the neighbouring sites.

Immunoblotting

Proteins from patient-derived fibroblasts were extracted with 10-fold excess of RIPA buffer (Tris-HCl 50 mM, NP-40 1%, Na-Deoxycholate 0.5%, SDS 0.1%, NaCl 150 mM, EDTA 2 mM, pH 7.4). Protease inhibitor cocktail (Sigma, Milano, Italy) was added to all lysates. Lysates were cleared by ultracentrifugation (20.000 × *g* for 30 min at 4 °C) before western blot analysis. Protein concentration was measured with Bradford Assay (BioRad, Hercules, CA, USA). A total of 20 µg of protein from cultured fibroblasts were used in each lane for immunoblotting. Immunosignals were detected by autoradiography using multiple exposures to ensure that signals were in the linear range. Signals were quantified through densitometry using Adobe Photoshop 2020 software (Adobe). The following antibodies were employed for analysis: anti-MeCP2 (Sigma-Aldrich, #M6818) and anti-β-Actin (Santa Cruz, Biotechnology, #sc-47778).

Results

Plasmids design and validation

To validate CRISPR/Cas9 application as therapeutic strategy for the correction of pathogenic *MECP2* variants, we tested its potential for the correction of the most common variant found in RTT patients, the c.473 C > T (p.(Thr158Met)) missense variant. To this aim, we selected a sgRNA that exclusively targets the mutated allele and an appropriate donor DNA harbouring the WT sequence (Fig. 1a). An adjacent spCas9 PAM sequence (NGG) is present and a silent nucleotide substitution was inserted in the PAM sequence; this modification prevents a secondary cut by Cas9 and helps to identify the corrected alleles. We designed a dual-vector approach for contemporary delivery of the correction machinery components to the cells (Fig. 1b): one plasmid expresses SpCas9 under the control of *CMV* promoter, and the second one harbours the variant -specific sgRNA, under the control of U6 promoter, and the Donor DNA along with the mCherry/EGFP reporter system, under

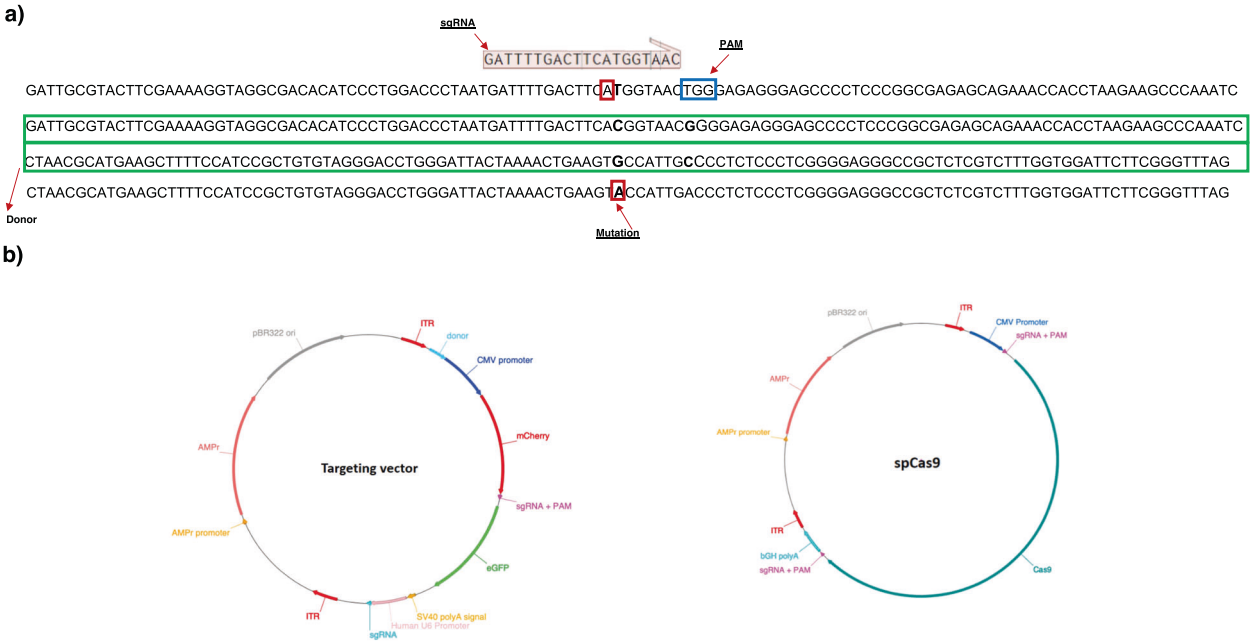

Fig. 1 Design of sgRNA and Donor for *MECP2* variant c.473 C > T (p.(Thr158Met)). a The red box indicates the variant and the grey sequence above is the sgRNA which directs the Cas9 specifically to the target sequence. The PAM sequence, required for Cas9 activity, is outlined by the blue box. After Cas9 activation, the donor sequence, indicated by the green box, is used as template to restore the wild type sequence. **b** Representation of the plasmids structure by ApE tool http://biologylabs.utah.edu/jorgensen/wayned/ape. The relevant components are reported.

the control of *CMV* promoter. The variant-specific sgRNA sequence plus a PAM sequence has been cloned in the Cas9 vector between the promoter and Cas9 coding sequence, allowing Cas9 auto-cleaving and thus avoiding its long-term expression. The double reporter system included into the targeting vector is designed to visualize transfected cells and monitor Cas9 activation. Indeed, mCherry is constitutively expressed, while EGFP expression requires Cas9 protein synthesis and activity. Once expressed, Cas9 is able to recognize and cut the target sequence between mCherry and EGFP, bringing EGFP coding sequence in frame and resulting in the expression of a mCherry/EGFP fusion protein (Fig. 2a, b).

To test the functionality of the reporter system, HEK293 cells were transfected with the targeting vector alone or in combination with the Cas9 vector; mCherry$^+$ and EGFP$^+$ population was investigated by FACS analysis 48 h after transfection (Fig. 3a). About 50% of cells transfected only with the reporter system results as mCherry$^+$ population, but EGFP negative. Conversely, 4.86% of cells co-transfected with reporter and Cas9 plasmid are mCherry$^+$/EGFP$^+$, proving Cas9 activation and plasmids functionality (Fig. 3a).

The specificity of the sgRNA was confirmed in HEK293 cells, by transfection with the Cas9 plasmid and a targeting plasmid in which the target sequence between mCherry and EGFP harbours either the WT or the variant-specific sgRNA. After 48 h green fluorescence was confirmed to be expressed only in cells co-transfected with the Cas9 plasmid and the reporter plasmid with variant-specific sgRNA (Fig. 3b). No EGFP$^+$ cells were observed when the WT sgRNA is interposed between mCherry and EGFP sequence (Fig. 3b), confirming the specificity of the variant-specific sgRNA for the mutated allele.

Correction plasmids were also transfected in mutated iPSC-derived neurons and fluorescence was visualized in vivo 5 days post-transfection. Efficient co-expression of mCherry and EGFP confirmed the correct activation of the vectors in this type of cells (Fig. 3c).

Gene correction in patient's cells

To establish the efficiency of the correction plasmids in patient-derived cells, we transfected fibroblasts obtained from four independent RTT patients harbouring the c.473 C > T (p.(Thr158Met)) variant and quantified fluorescence 48 h post-transfection by FACS analysis. In line with results in HEK293, cell transfected with targeting plasmid alone expressed mCherry but not EGFP. Results from replicate experiments ($n = 3$) in which cells were co-transfected with targeting and Cas9 plasmids showed a consistent cell population which expressed mCherry (~23%), with ~33% of these cells expressing also EGFP. This result indicates proper plasmids activation in patient's cells. Co-transfected mCherry$^+$/EGFP$^+$ fibroblasts were recovered by cell sorting 48 h later and analysed using Next Generation Sequencing

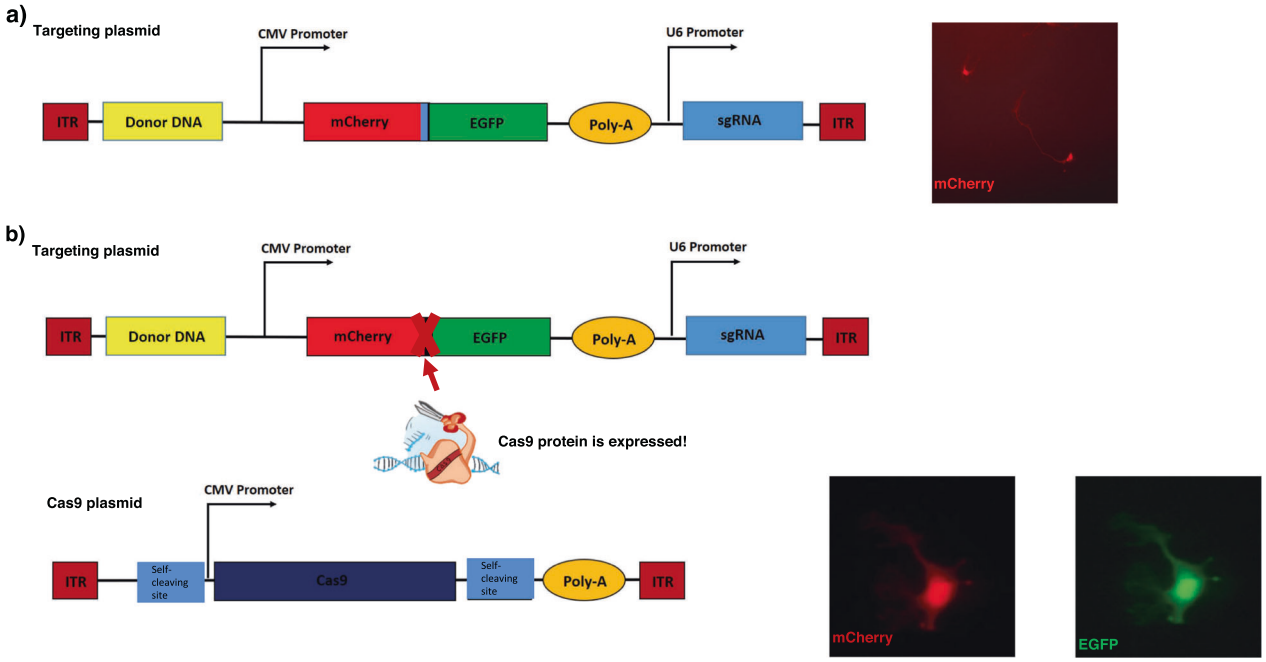

Fig. 2 Plasmids strategy. a The targeting construct brings sgRNA, Donor DNA and the Reporter system composed by mCherry and EGFP. If the targeting plasmid is transfected into the cells, mCherry is expressed constitutively and the cells acquire red fluorescence. **b** The Cas9 construct encodes Cas9 and two self-cleaving targets (light blue boxes) composed by sgRNA + PAM sequence. When a cell is transfected by both targeting and Cas9 plasmids, Cas9 is expressed and cuts the target sequence interposed between mCherry and EGFP (light blue box); this allows EGFP expression resulting in double mCherry^{+}/EGFP^{+} cells. The same specific nuclease activity ensures Cas9 auto-cleaving, avoiding long-term expression.

(NGS) (Fig. 4a). NGS data were uploaded to the CasAnalyzer tool to assess HDR frequency. This analysis demonstrated the efficiency of the correction plasmids, in particular we obtained 64.2% of edited alleles for case 1 (#2204), 50% of edited alleles for case 2 (#304), 83.6% of edited alleles for case 3 (#2271) and 20.4% of edited alleles for case 4 (#2239) (Table 2). For case 3 (#2271), *MECP2*-mutated neurons differentiated from iPSCs were also analysed. Co-transfected mCherry^{+}/EGFP^{+} neurons were recovered by cell sorting 6 days after transfection and analysed using NGS (Fig. 4b). Data analysis in neurons demonstrated that 14% of mutated alleles has been correctly reverted to the WT sequence. Editing experiments in fibroblasts and neurons demonstrated a negligible rate of indels generation. Manual analysis of sequencing reads with IGV for the inclusion of the silent nucleotide change on the PAM sequence (TGG to TAG) demonstrated that it is present in a high proportion of the corrected alleles (about 90%).

In the mouse model, the p.(Thr158Met) variant is associated with ~70% reduction of the levels of Mecp2 protein [27]. We thus asked ourselves whether a similar reduction is observed also in human cells and if it is corrected following gene editing. Western blot analysis was performed on whole protein extracts isolated from WT fibroblasts, patient fibroblasts and co-transfected mCherry^{+}/EGFP^{+} edited fibroblasts. In patient cells we observed a decrease of MeCP2 protein levels (Fig. 4c). Following editing, a statistically significant increase in the expression of MeCP2 was observed compared with native patient cells (Fig. 4c).

TP53 polymorphism characterisation

It is well known that the activation of the DNA repair machinery following Cas9-induced DSBs involves TP53 activation and that TP53 inhibition increases the rate of homologous recombination [28]. The functional TP53 polymorphism c.215 C > G (p.(Pro72Arg)) at amino acid position 72 is known [29]. We reasoned that editing efficiency might be influenced by this TP53 polymorphism. The genotype of our patients at this locus was thus analysed demonstrating homozygosity for the Arg72 allele in 3 patients (Table 3). The fourth patient, displaying the lower editing efficiency, resulted homozygous for the Pro72 allele.

Discussion

RTT is a rare incurable neurodevelopmental disorder due to pathogenic variants in *MECP2* gene. The present study demonstrates that gene editing with CRISPR/Cas9 is an efficient tool for the precise correction of the most common *MECP2* hotspot variant, c.473 C > T (p.(Thr158Met)), in

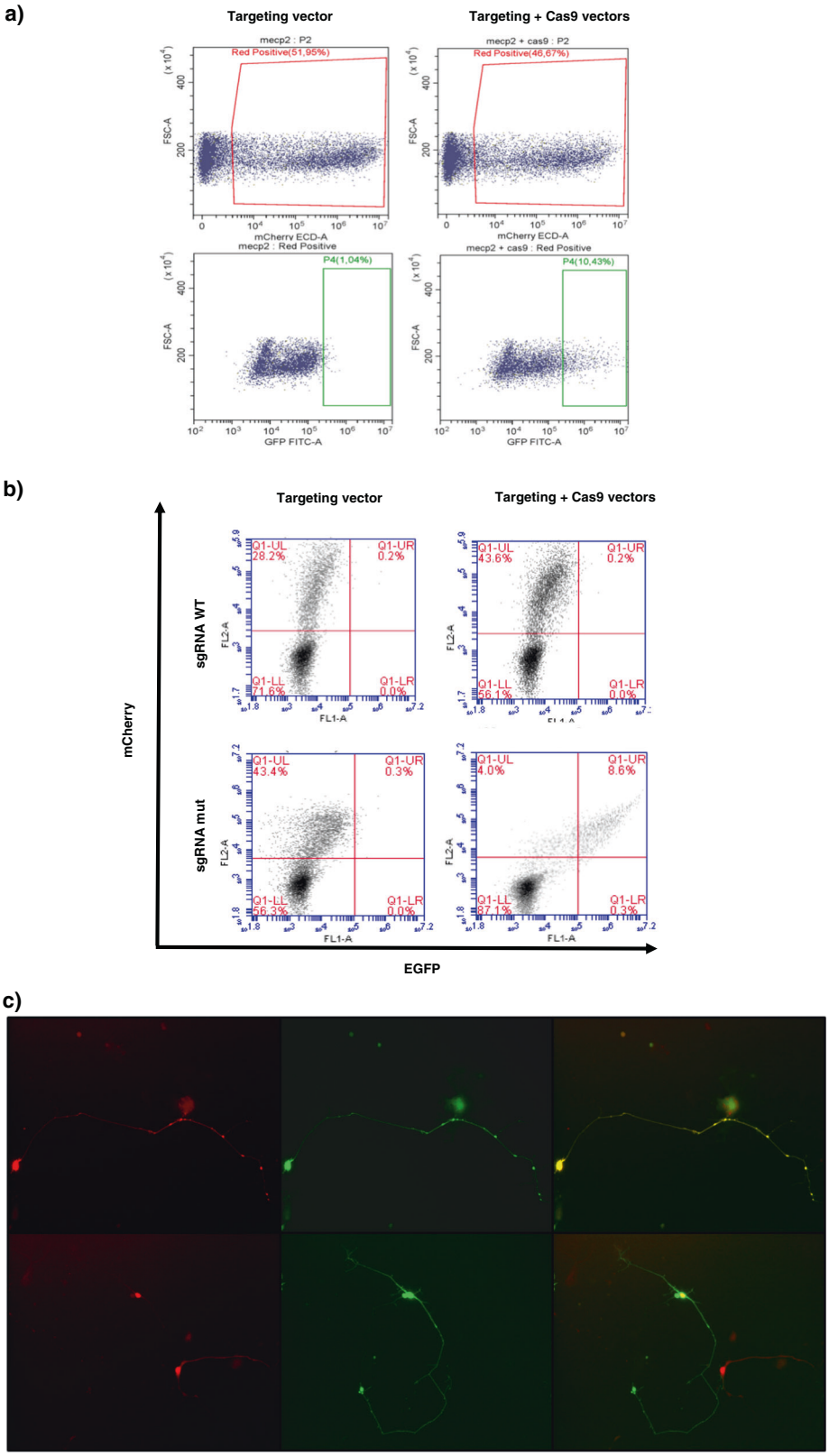

Fig. 3 sgRNA specificity and plasmid functionality in HEK293 cells and *MECP2* neurons. a Representative FACS analysis on HEK293 cells 48 h after transfection with constructs for the c.473 C > T (p.(Thr158Met)) *MECP2* variant. Cells were transfected with the targeting vector alone or in combination with Cas9 vector and fluorescence was quantified. Transfection with the targeting plasmid alone results in 51.95% of mCheery^{+} cells and no EGFP^{+} cells. As expected, following co-transfection 10.43% of mCherry^{+} cells were also EGFP^{+}, confirming the functionality of the system. **b** Specificity of the variant-specific sgRNA was demonstrated by transfecting HEK293 cells with the reporter plasmid expressing the wild type or mutated sequence between mCherry and EGFP. The mCherry^{+}/EGFP^{+} population is gated in the UR quadrant. The double fluorescence (8,6%) expressed by cells co-transfected with targeting vector comprising the variant-specific sgRNA and Cas9 plasmid, but not by those transfected with the vector harbouring the WT sequence, demonstrates the specificity of the selected sgRNA. **c** In vivo fluorescence microscopy images of mutated iPSC-derived neurons 5 days after transfection showing double mCherry and EGFP expression. Images are 20× magnification.

cells deriving from RTT patients, including iPSC-derived neurons. Indeed, our results succeed in demonstrating that HDR can be obtained with an efficiency of up to 80% in fibroblasts and 14% in iPSC-derived neurons. The negligible percentage of indels obtained for all patients indicates the safety of the approach. Brown et al. demonstrated that in

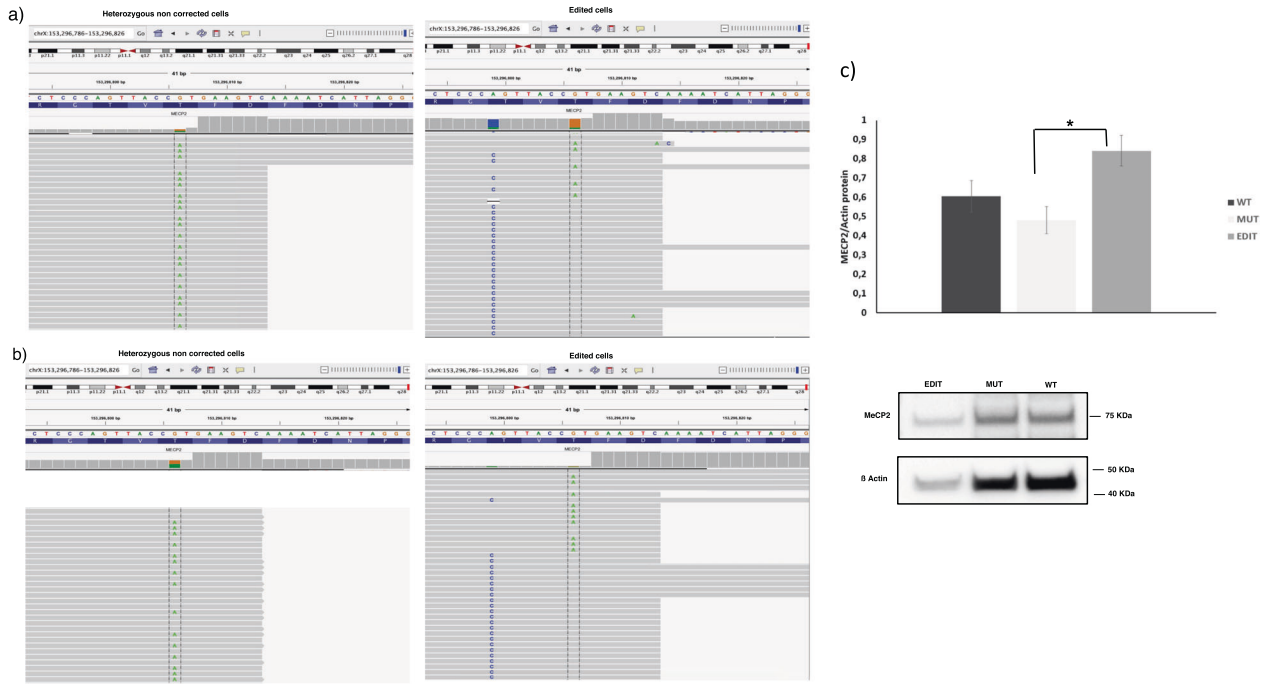

Fig. 4 Efficient editing in *MECP2* mutated cells. a, b Representative NGS results for edited fibroblasts from Case 1 (#2204) (**a**) and iPSC-derived neurons from Case 3 (#2271) **b**. Sequencing results have been visualized by IGV (Integrative Genomic Viewer). The mutated nucleotide is placed between dashed lines. In edited cells, the silent A > C change removing the PAM 7 nucleotides upstream to the variant is present in edited alleles. **c** MeCP2 protein levels were analysed by immunoblotting on extracts obtained from wild type, mutated and edited fibroblasts. Protein levels were quantified with Photoshop 2020 software. MeCP2 levels significantly increase in edited samples compared with mutated not-edited cells. Values on the *y* axis are the averages of percentage of proteins relative to WT normalized to b-Actin expression. $n = 2$. Statistical significance was determined using unpaired student's test ($^*p < 0.05$).

Table 2 Editing efficiency in patients' cells.

Case	Patient Code	Sample	Total reads	HDR efficiency (%)	In/dels frequency (%)	C (WT)	T (M)
1	2204	Fibroblasts	22141	64.2	0.1	83% (18284)	17% (3857)
2	304	Fibroblasts	2675	50	0.2	68.3% (1833)	31.5% (842)
3	2271	Fibroblasts	104	83.6	0	98% (102)	2% (2)
3	2271	iPS-derived Neurons	2935	14	0	56% (1643)	44% (1292)
4	2239	Fibroblasts	1864	20.4	0.5	59.8% (1120)	39.7% (744)

Sample: type of cell analysed; Total reads: number of total reads analysed by NGS; HDR efficiency: percentage of Homology Directed Repair. Indel frequency: percentage of insertions and deletions caused by Cas9 activity; C (WT): wild type nucleotide; T (M): mutated nucleotide

Table 3 TP53 polymorphism characterization in *MECP2* mutated patients.

Case	Patient code	Pro72Arg genotype
1	2204	Arg/Arg
2	304	Arg/Arg
3	2271	Arg/Arg
4	2239	Pro/Pro

T158M mouse models there is ~70% reduction in Mecp2 protein levels compared with WT mice [27]. Our analysis of MeCP2 expression in patient's cells suggests that protein expression is reduced also in patient fibroblasts, although to a lower extent, and that editing results in an increase in protein levels, confirming the correction of protein expression. A few studies have shown that it is possible to apply CRISPR/Cas9-based gene editing in human primary cells, such as fibroblasts, and in iPSCs, including cells from patients with different genetic disorders [30–32]. Interestingly, Le et al. recently applied an approach similar to the one described in this manuscript for the precise manipulation in iPSCs of *MECP2* locus, including the correction of another *MECP2* variant hotspot, c.808 C > T (p.(Arg270*)), obtaining an editing efficiency of 20–30%, confirming that *MECP2* gene can be efficiently modified in different cell types [30].

Gene therapy using a gene replacement approach, as the one recently tested in patients affected by SMA [33], has been extensively tested in RTT mouse models but has resulted in toxicity due to excessive Mecp2 expression [13, 21, 34]. Indeed, this approach relies on the insertion of an additive normal copy of the gene within the genome under an artificial control and it is thus effective when a tight regulation of the target gene is not needed, as in the case of enzymatic defects. A fine native regulation is essential for *MECP2* since both down- and up-regulation lead to brain impairment in humans, outlining the importance of setting up a therapeutic approach that restores native gene expression. Thus, gene replacement therapy approaches might represent a relevant treatment option provided efficient control of *MECP2* expression will be obtained. In this respect, gene editing currently appears a safer therapeutic strategy for RTT, since it allows the edited gene to maintain its native regulation, thus avoiding inappropriate expression. Since the approach is variation-specific, dedicated correction vectors with variation-specific sgRNA and donor DNA need to be designed and validated for each variant. The variant on which we applied gene editing represents only about 10% of RTT cases but this could be however relevant for those patients, considering that there is currently no cure for this syndrome. Moreover, since eight recurrent variants underlie ~70% *MECP2*-mutated RTT cases [1, 4, 9], a relevant portion of patients might be targeted with appropriate validation of a limited number of constructs. Moreover, the results from Le et al. in iPSCs suggest that it might be possible to use a single sgRNA to mediate the insertion of specific sequences into *MECP2* gene by Homologous Recombination [30]; a similar approach could be employed to correct MeCP2 C-terminal deletions, further extending the spectrum of treatable variants.

The Blood-Brain Barrier (BBB) represents one of the major obstacles for the treatment of neurological diseases and for the delivery of genetic cargo to central nervous system (CNS) due to the presence of a physical barrier composed by tight junctions between brain microvascular endothelial cells [35]. With the perspective of employment in clinical trials, we designed a toolkit based on adeno-associated viral vectors (AAVs) which are emerging as a promising tool for in vivo gene delivery [36, 37]. Indeed, AAVs are immune-tolerated and some serotypes are capable of crossing the BBB, an essential feature for in vivo gene therapy of CNS disorders via intravenous injection [38, 39]. However, the packaging of SpCas9, sgRNA, donor DNA template and reporter system in a single AAV vector was not feasible due to the limited packaging capacity of AAVs (4.7 Kb) [40]. Several groups have previously packaged Cas9 and sgRNAs into separate AAV vectors increasing overall packaging capacity [41, 42]. We thus designed an approach based on a correction system that employs two plasmids to be used in combination. In agreement with literature data, our results demonstrate that co-transfection and subsequent gene editing can be efficiently obtained, confirming the feasibility of the proposed strategy. Results from the phase I trial for SMA1 suggest that intravenous delivery of AAV9 particles might have relevant outcomes [33, 43]. However, it will be important to perform correction experiments in mouse models, in order to confirm that efficient correction can be obtained also in vivo. It will be also important to test different administration routes, including intrathecal or intracerebroventricular, to establish the more efficient delivery tool for the CNS; these routes, although more invasive, would have the advantage to allow injecting the correction machinery directly in the CNS without immunity reaction or organ toxicity [44, 45]. Finally, the need of two viral particles for the delivery of the correction machinery likely reduces the effectivity of our toolkit. To overcome this issue, modified AAV particles with increased infectivity for brain cells could be employed [46]. Alternatively, SaCas9, a shorter nuclease derived from Staphylococcus aureus, could be employed to allow packaging in a single AAV particle, provided a specific PAM is available close to the variant. This approach has recently been tested for Leber Congenital Amaurosis, in both cellular and animal models and a phaseI/II trial is currently being performed [47] (ClinicalTrials.gov Identifier: NCT03872479).

The application of CRISPR/Cas9 technology and HDR-mediated repair in the context of gene therapy represents an important innovation in the scientific field. Few papers have attempted to apply gene editing techniques to primary cells, mainly for their short lifespan and extreme sensibility to treatment [31, 48–50]. Moreover, HDR is active primarily during the S phase of cell cycle, limiting its application in post-mitotic cells [51, 52]. However, recent works have demonstrated that HDR is effective also in non-dividing cells, such as terminally differentiated neurons, allowing its application to disorders affecting tissues with limited regeneration/renewal capacity, including the CNS [20, 53]. In particular, Nishiyama et al. compared editing efficiency in mammalian neurons using an approach based on either one or two AAV-based vectors for in vivo delivery to the brain [20]. They demonstrated that editing of neurons can be obtained in both cerebral cortex and organotypic hippocampal slice cultures with an efficiency of 6–15% and that the single and dual-vector systems give comparable results. In line with these data, the results we obtained in iPSC-derived neurons demonstrate that accurate gene editing can be obtained in the primarily affected cells in RTT. The percentage of HDR we obtained in neurons is lower compared with fibroblasts from the same patient, but, interestingly, our results fit with what reported from

Nishiyama et al. in mouse brain, suggesting that we might expect a similar editing efficiency also in vivo [20]. In the perspective of future clinical trials our editing results in patient-derived cells represent a proof of concept demonstrating that the application of HDR-CRISPR/Cas9 gene therapy is possible also in post-mitotic cells with encouraging results. Pre-clinical studies in the mouse models will allow evaluating to which extent the obtained correction efficiency allows correction of disease phenotype.

In spite of the presence of the same *MECP2* variant, editing efficiency in the four patients analysed is variable, ranging from 20% to more than 80%. Based on a recent publication demonstrating that TP53 can affect HDR efficiency [54], we hypothesized that the reported differences in HDR efficiency might be partly explained by TP53 genotype. TP53 maintains genome integrity and prevents cell cycle transition to the S phase in the presence of DNA damage [55]. Cell cycle affects HDR machinery, which is mainly activated in S/G2 phase [56]. Recent publications have shown that TP53 inhibition can increase HDR frequency and precise genome editing efficiency [28, 57, 58]. It is known that c.215 C > G polymorphism in TP53, resulting in the substitution of amino acid Proline with Arginine at position 72, can influence TP53 stability and activity [29]. It is possible that this polymorphism could influence the final correction efficiency in patient cells. Our analysis has shown that the three patients with a higher editing efficiency are homozygous for the less stable Arg72 variant, while the fourth patient having the lowest efficiency is homozygous for Pro72 variant. Although the number of patients is limited, these data suggest a possible correlation between TP53 polymorphism and correction efficiency.

Therapeutic use of CRISPR/Cas9 system for clinical application requires a comprehensive evaluation of the possible off-targets caused by the activity of the Cas9 protein throughout the genome in order to avoid unintended variants. Our results demonstrate that we have a negligible rate of indels in *MECP2* gene and tests in HEK293 cells demonstrate that the selected sgRNA targets only the mutated allele, confirming its high specificity (Fig. 3b); nevertheless, it will be important to confirm the safety and specificity of the system by techniques such as Genome Wide Unbiased Identification of DsBs Enables by Sequencing (Guide-seq), that allow the analysis of the entire genome [59].

Overall, the findings highlighted in the present work represent an important step toward gene therapy based on the application of CRISPR/Cas9 system through HDR in patients with *MECP2* hotspot variants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Informed consent Informed consent was provided to the patients before blood drowning and skin biopsies.

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