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Original Article

Accelerated Apoptosis and Down-Regulated FMRP in Human Neuroblastoma Cells with CRISPR/*Cas9* Genome Editing

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Abstract

Background: Fragile X syndrome (FXS) is a genetic disease with intellectual disabilities. FXS is often caused by the CGG-repeat expansion mutation in the *FMR1* gene with suppressed *FMR1* transcription and decreased protein levels in the brain of the patients. The RNA-guided CRISPR/*Cas9* system is a promising targeted genomic editing tool in gene therapy of FXS. In order to evaluate its feasibility, the present study used CRISPR/*Cas9* system to target the *FMR1* 5'-UTR sites in cultured human neuroblastoma cells.

Methods: PCR and DNA clone were used to construct plasmids. CRISPR function was tested by Western blot and flow cytometry. Data were analyzed by a two-tailed unpaired Student's *t*-test using GraphPad software. This research was conducted from 2020 to 2022 in the Second Affiliated Hospital of Soochow University, Suzhou, China.

Results: Cell cycle analysis showed significant differences in G1, S and G2/M phases between the two groups ($P<0.05$). In the knockout cells, apoptosis was accelerated ($P<0.05$) with a significantly down-regulated ($P<0.05$) expression of FMRP as compared with the control group.

Conclusion: This study provides further understanding about the FMRP function and molecular mechanism of *FMR1* gene in nerve cells, and suggests the feasibility of gene therapy in FXS by CRISPR/*Cas9* gene editing system.

Keywords: Fragile X syndromes; CRISPR associated protein 9; Gene editing

Introduction

Fragile X syndrome (FXS) is usually characterized by developmental delay and intellectual disability along with various behavioral issues. *FMR1* full mutation (>200 CGG repeats in the 5'-UTR of *FMR1*) or other loss-of-function variants can cause FXS. The presence of a premutation-sized repeat (55-200 CGG repeats) may cause fragile X-associated tremor/ataxia syndrome (FXTAS) and fragile X-associated primary ovarian insufficiency (FXPOI) (1). The (CGG) n with high polymorphism can be divided into four types: 1) normal repeat range (n=6-40), 2) the middle scope of repeat (n = 41-60), 3) pre-mutation (n = 61-200), (4) full mutation (n > 200). The larger the value of n (CGG) n, the more unstable is the offspring. (CGG) n in the process of human batches will expand. When pre-mutation carried by the mother is passed to the offspring, most

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(CGG) n extended to full mutations and increased the chances of FXS in offsprings (2). The prevalence of the pre-mutation alleles was 117:100,0000 in males and 344:100,000 in females, but in a meta-analysis, the incidence of FXS was 14:100,000 (1). The close relationship between *FMR1* gene expression and neural activity was suggested as early as 2000 (3). Mental retardation caused by *FMR1* expansion mutations is caused by the lack of expression of the fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein and the function of brain can be affected in the absence of the protein. Many reports focus on the mechanism by which the deficiency of FMRP influences the disease. FMRP is critical for neural differentiation in mouse adult neural stem cell experiment (4). Various molecular signaling pathways involved in abnormal neuronal development were identified in the *FMR1* knockout mouse (5). The function of FMRP in the human nervous system has also been confirmed (6-8).

No specific treatment is available for FXS, only psychopharmacological treatment and therapeutic services are administered to improve symptoms. For FXTAS, symptomatic and supportive treatment is individualized for clinical interventions. For individuals with FXPOI, gynecological or reproductive endocrinological evaluation can facilitate appropriate treatment. In the research of FXS therapy, some strategies such as modeling *FMR1* knockdown and CGG repeat expansion in FXS hPSCs, have been shown to affect synaptic plasticity or dendritic spine morphology in FXS animal models, targeting protein synthesis (9). However, such strategies have been performed on animal models, and are difficult to apply in clinical practice. As *FMR1* expansion mutations is a high risk factor for diseases with no effective pharmacological treatment, alternative therapies such as gene therapy are urgently required. Recently, gene-editing using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is considered as a promising gene therapy tool for many genetic disorders, either in inherited disorders or in tumors (10, 11). The CRISPR/*Cas9* system consists of a *Cas9* protein and a guide RNA (gRNA), which binds to the target DNA site on the genome and works as a nuclease to induce double-strand breaks (11). Considering the therapeutic requirement and gene editing advances, the *FMR1* expansion mutations of CGG is a suitable target using *Cas9* system-mediated gene therapy. The reactivation could be observed in human FXS iPS cells by CRISPR/*Cas9* genome editing (12). Demethylation of the CGG expansion is sufficient for *FMR1* reactivation, suggesting potential therapeutic strategies for FXS (13). One study showed direct editing in differentiated cells, and further investigation is needed to validate the editing capabilities and observe morphological effects on non-dividing neurons (14).

The present study was designed to address the feasibility of gene therapy in FXS by CRISPR/*Cas9* gene editing system in differentiated nerve cells.

Methods

Design and construction of CRISPR plasmids

This study was conducted in the Second Affiliated Hospital of Soochow University in 2020. Lentivirus vectors were used in this study to transfect human neuroblastoma cells. Lenti-*FMR1*-sgRNA vector was constructed based on GV371 vector. First single-stranded DNA oligos were synthesized for sgRNA sequence (5'-TGACGGAGGCGCCGCTGCCA-3'). Then double-stranded DNA was formed by annealing the two single-stranded DNA oligos. The GV371 vector and double-stranded DNA fragments were ligated and transformed into TOP10 competent cells. Positive clones were confirmed by colony PCR and sent for sequencing to verify the correct Lenti-*FMR1*-sgRNA vector (Fig. 1). GV371 vector was purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). Oligos were synthesized by Genewiz, Inc. (Suzhou, China). Competent cells were bought from Tiangen Biotech Beijing Co., Ltd. (Beijing, China).

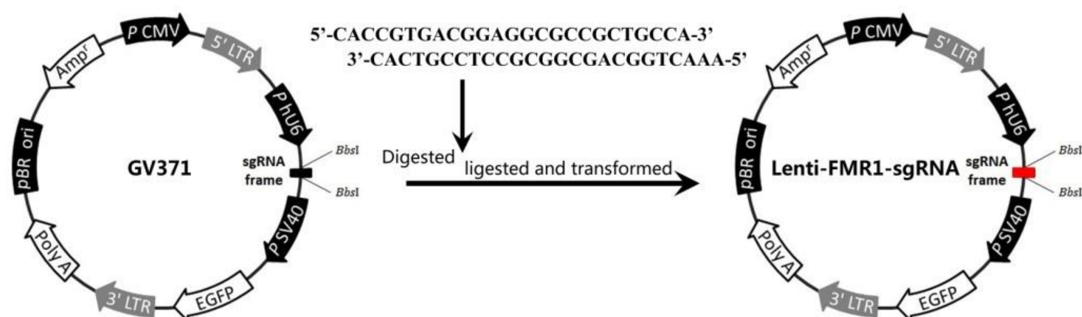

Fig. 1: Construction diagram of *FMR1*-KO-sgRNA lentivirus vector

Cell transfection and culture

Human neuroblastoma cells (SH-SY5Y, Cellular Library of the Chinese Academy of Sciences, SCSP-5014) were cultured in advanced DMEM (Cellular Library of the Chinese Academy of Sciences, SCSP-666) supplemented with 10% FBS, 2 mM Gluta Max (Life Technologies) and penicillin/streptomycin at 37 °C with 5% CO2. Lentivirus transfection was used in this study. Human neuroblastoma cells in logarithmic growth phase were infected by *Cas9* lentivirus. Three days after transfection, puromycin selection was performed to achieve the stable expression of cloned cell line. The cloned cell line was then divided into two groups: the KO group used for knockout test and the NC group used for negative control. These two groups were infected by *FMR1*-KO-sgRNA lentivirus and *FMR1*-NC-sgRNA lentivirus, respectively. Overall, 12.5 μl virus at a titer of 8E+8 TU/ml was used for each group.

Gene editing assay

The expression of green fluorescent protein (GFP) was observed using fluorescence microscope. The cloned cell line was collected to perform the next experiment after the GFP-positive rate was higher than 80%. We collected the edited cells and sequenced their genomic DNA (DP304, Tiangen Biotech Beijing Co., Ltd.) to evaluate the gene editing effect. It has been divided into KO group (KO), tool cell control group (293T) and blank control group (NC).

Two-directional primer extension was setup by Primer-F: 5'-TCAGGCGCTCAGCTCCGTTTCGG -3' and Primer-R: 5'- TGCCTCCCGCCGACACCAA-GAAG -3'. Following denaturation at 95 °C for 5 min, primer extension was performed for 30 cycles with denature at 95 °C for 20 sec, annealing at 60 °C for 20 sec and extension at 72 °C for 30 sec. After the 30 cycles, an extra extension of 5 min was done before the reaction was cooled down to 4 °C. Purified and recycled PCR products and then cloned by TA cloning kit.

About 30 monoclones for each target were randomly sent for sequencing by sequence primers F: 5'-GTGGTTTCAGTGTTTACACCCGC-3'and R: 5'-CCCTAGAGCCAAGTACCTTGTAG -3'), and the sequencing results were analyzed by software to compare the target effective mutation rate.

Western blot analysis

Western blot analysis was used to detect the expression levels of fragile X mental retardation protein (FMRP) in the KO and NC groups. Total protein was extracted from the cells in RIPA lysis buffer (Beyotime, Shanghai, China). The protein concentration was measured by BCA method. Target protein were separated by SDSPAGE and transferred to PVDF membranes. After blocking with 5% BSA, the membrane was incubated with primary antibodies at 4 °C overnight, followed by incubation with the corresponding secondary antibodies for 1 h at room temperature. The membranes were washed three times for 10 min each, incubated with chemiluminescent substrate and imaged by ChemiDoc XRS+ Imaging System

(Bio-Rad). The rabbit anti-FMRP antibody with expected size 71 kDa (ab17722) was bought from Abcam Trading (Shanghai) company Ltd. The mouse anti-GAPDH antibody (sc-32233) was purchased from Santa Cruz Biotechnology. Both the mouse and rabbit IgG antibodies (#7076 and #7074) were from Cell Signaling Technology, Inc.

Cell cycle assay

Cell cycle experiment was performed using Propidium Iodide (PI, Sigma P4864-10ML) staining and analyzed by flow cytometry. Briefly, ≥80% confluent cells were seeded in six-well plates and harvested by trypsin digestion, and then fixed using ice-cold 75% ethanol for one hour on ice. Next, the cells were washed two times with ice-cold D-Hanks, and 0.6-1.0 ml staining solution containing PI (1 mg/ml) and RNase (10 mg/ml) was added to stain the genomic DNA of cells for 30 minutes. The cells were resuspended and the different phases of cell cycle were determined by flow cytometry.

Apoptosis analysis

All cells in the dish, including adherent cells and those in the supernatant, were collected. After washing with ice-cold PBS and 1× binding buffer, the cells were resuspended in 200 μl 1× binding buffer. Then 10 μl of Annexin V-APC was added into the cell suspension and kept at room temperature in the dark for 10-15 min. Flow cytometry was performed on a BD Aria-IIu flow cytometer. The flow data was analyzed and the figures were generated with Guava inCyte.

Statistical analysis

The data were presented as mean ± SD. Data were derived from at least three independent experiments. Data were analyzed by a two-tailed unpaired Student's t-test using GraphPad software v.5.0 (La Jolla, CA, USA). *P-values < 0.05; **P-values < 0.01; ***P-values < 0.001.

Results

Construction and expression of two lentivirus vectors

Figure 2 shows a schematic view of CRISPR/*Cas9* binding to the *FMR1* 5'-UTR. The PAM sequence GGG is shown in red in Fig. 2a. The single guide RNA (sgRNA) oligos were designed to target the upstream of the CGG repeat located in the 5'-UTR of *FMR1*. Fig. 2b shows the sequencing data of selected clones, indicating the successful construction of Lenti-*FMR1*-sgRNA vectors. The two candidate transformed lentivirus vectors were further confirmed by puromycin selection and observation of the expression level of GFP. As shown in Fig. 3a, the expression rate of GFP was higher than 60% under field of view with fluorescence microscope.

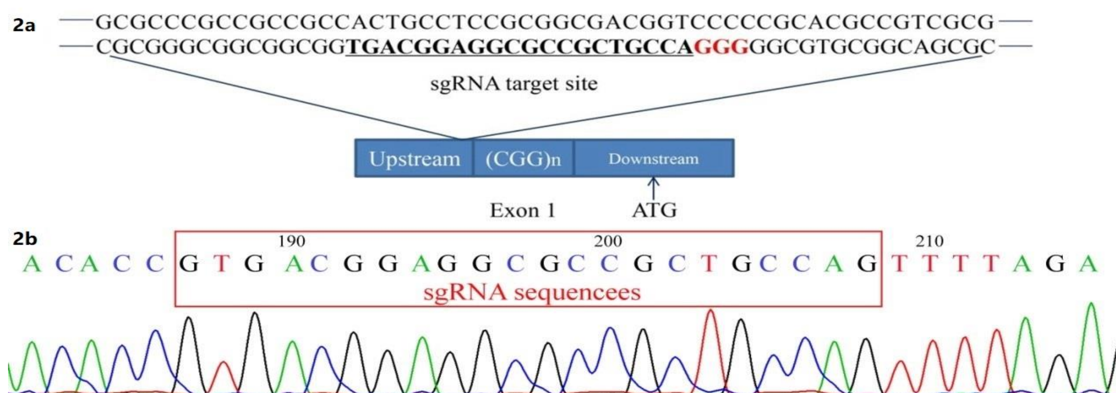

Fig. 2: Sequence result diagram of correct construction of lentivirus vector and its confirmed sequence
2a. The RGEN-binding site in a schematic view of the *FMR1* 5'-UTR. The PAM sequence is shown in red.
2b. The Sanger sequencing chromatographs of the correct constructed Lenti-*FMR1*-sgRNA vector

Sequencing validation of CRISPR/Cas9 mutation efficiency

As shown in Fig. 3b, A 381bp target-specific PCR fragment was detected in the control group and tool cell 293T group. For the target bands of KO group were diffuse, the mismatch enzyme assay could not be carried out, CRISPR/*Cas*9 mutation efficiency was verified by TA cloning and sequencing.

Thirty clones were tested by KO, of which 26 had deletion mutations of different degrees, three had double peaks, and one was empty. Among them, T-3, T-21, and T-29 had three double peaks; T-30 is empty. Moreover, T-8, T-9, T-10, T-14, T-24 had five deletion positions, which were inconsistent with other deletion positions, and were analyzed separately. The remaining 21 clones were all deletion mutations as shown (Fig. 4). From the above sequencing results, the mutation rate of this target was more than 80%.

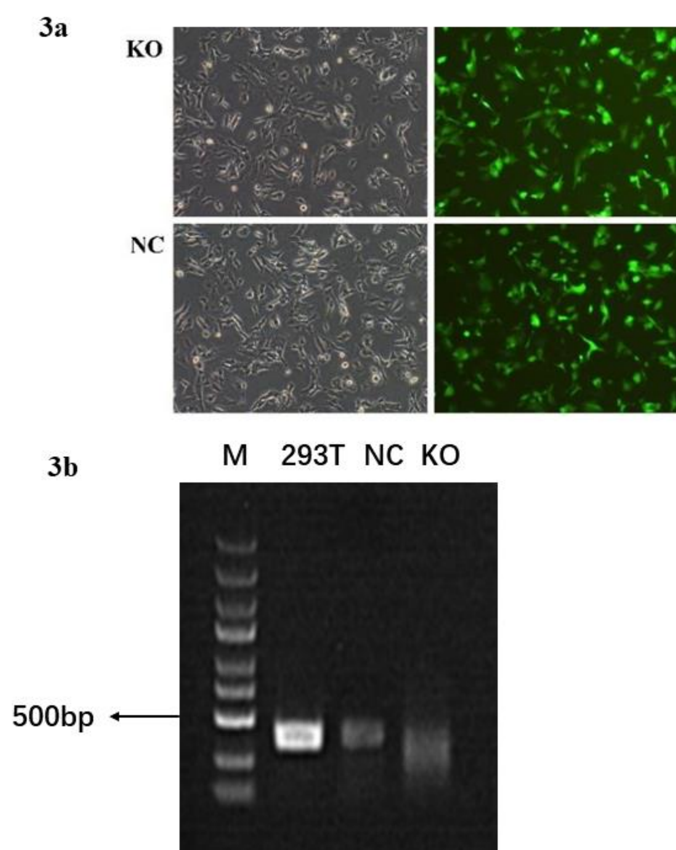

Fig. 3: Fluorescence image of cells transfected with lentivirus vector and PCR amplification of agarose gel image after collecting DNA

3a. The fluorescence images of cells with lentivirus vectors were successfully transferred in KO and NC groups. Both photos are displayed at 100x magnification.

3b. Agarose electrophoresis of 381 bp target bands amplified by PCR after extracting genomic DNA from KO group and NC group, and set up tool cell control group

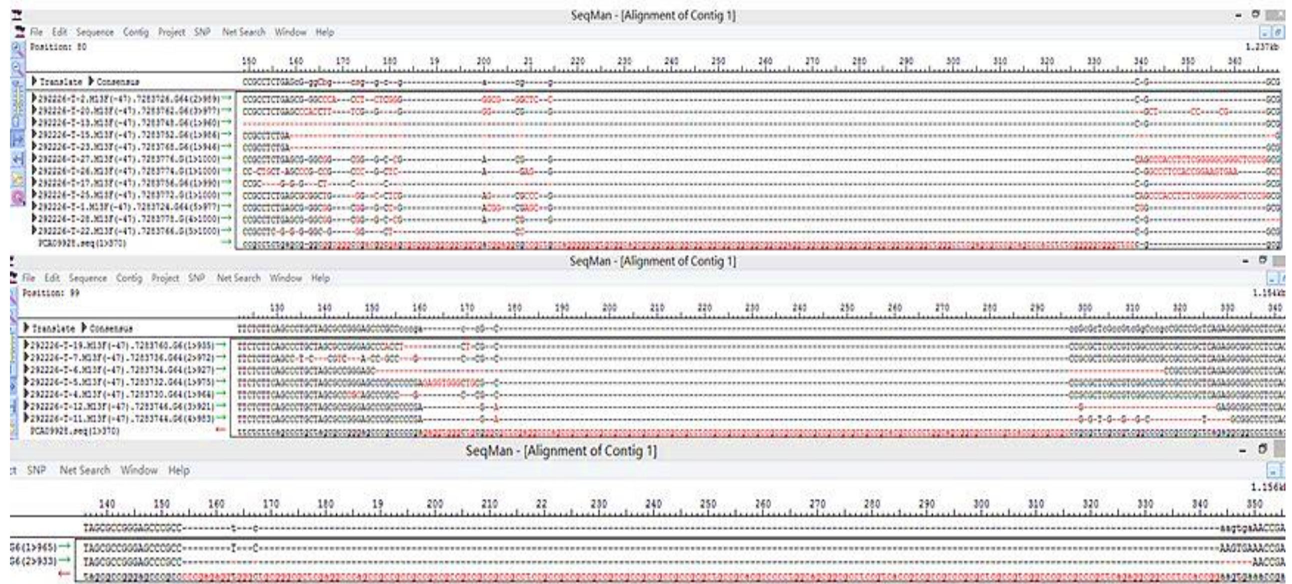

Fig. 4: Sequence alignment of deletion and mutation of 21 clones after sequencing

Expression of fragile X mental retardation protein

We detected the expression levels of fragile X mental retardation protein in the *FMR1* knockout group and the negative control group. The protein gel electrophoresis is shown in Fig. 5, and the FMRP level was normalized to the NC group. The gel analysis revealed that the expression of fragile X mental retardation protein in the KO group was significantly down regulated compared with the NC group ($P<0.05$). After *FMR1* gene was partially knocked-out, the fragile X mental retardation protein was down regulated; indicating that the gene editing in nerve cells was successful and further suggesting that fragile X mental retardation protein played an important role in nerve cells.

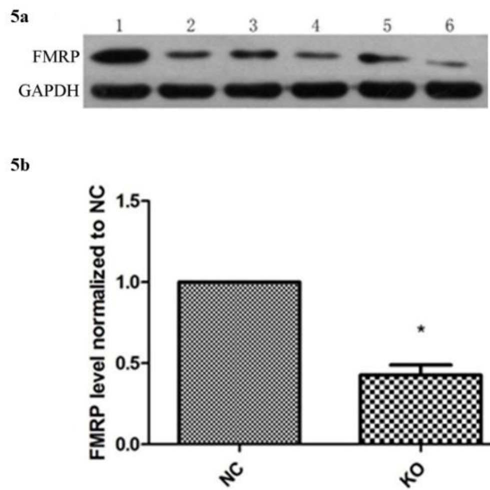

Fig. 5: Western blot results of FMRP expression level

5a. Line 1,3,5 showed the WB of NC group, Line 2,4,6 showed the WB of KO group. The molecular weight of FMRP is about 71 kDa.

5b. Statistical results of protein gray value of KO group and NC group

Knockout of FMR1 altered the cell cycle

In order to investigate whether the cell cycle was altered after editing by CRISPR/*Cas9*/sgRNA system, we assayed the cell cycle spectra between the *FMR1* knockout group and the negative control group. As shown in Fig. 6 and Table 1, there was significant difference between the KO group and the NC group in G1, S and G2/M phases (*t*-test, *P*<0.05). There were more cells in the S phase in the control group and more cells in the G2/M phase in the KO group.

Table 1: Cell cycle in different stage between KO and NC group

Cell Cycle	*NC*	*KO*	*P-value*
G1	53.88	56.03	0.036
S	34.42	28.84	0.002
G2/M	11.7	15.13	0.003

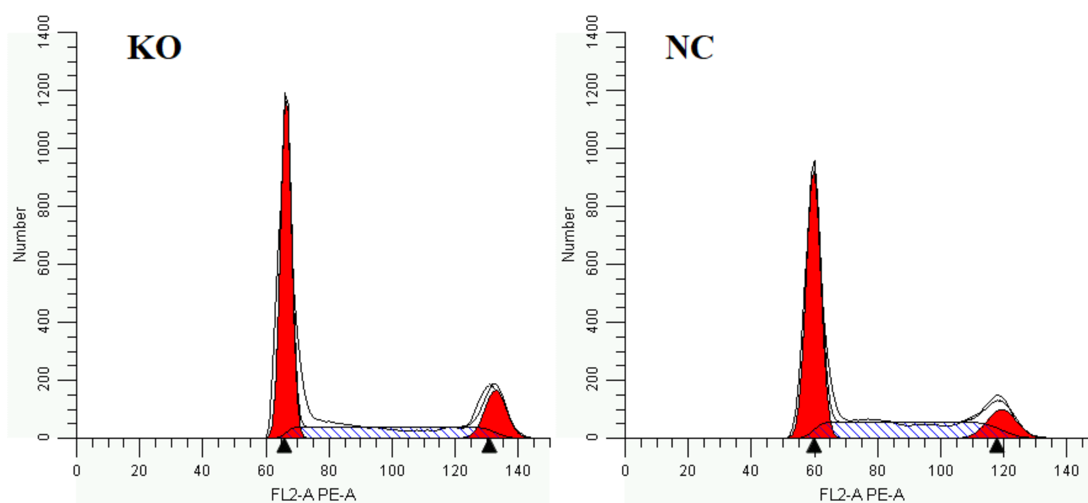

Fig. 6: Results of cell cycles in KO group and NC group

Knockout FMR1 will accelerate cell apoptosis

To explore the impact of *FMR1* on cell apoptosis, we also tested the cell apoptosis between the *FMR1* knockout cells and the negative control group. We observed that the cell apoptosis was accelerated in the KO group, compared with the NC group (t-test, *P*<0.05), as shown in Fig. 7 and Table 2.

Table 2: Cell apoptosis between KO and NC group

Cell apoptosis	*NC*	*KO*	*P-value*
AVERAGE	3.83	2.85	
STDEV	0.159	0.2346	
t-test Analysis			0.0038

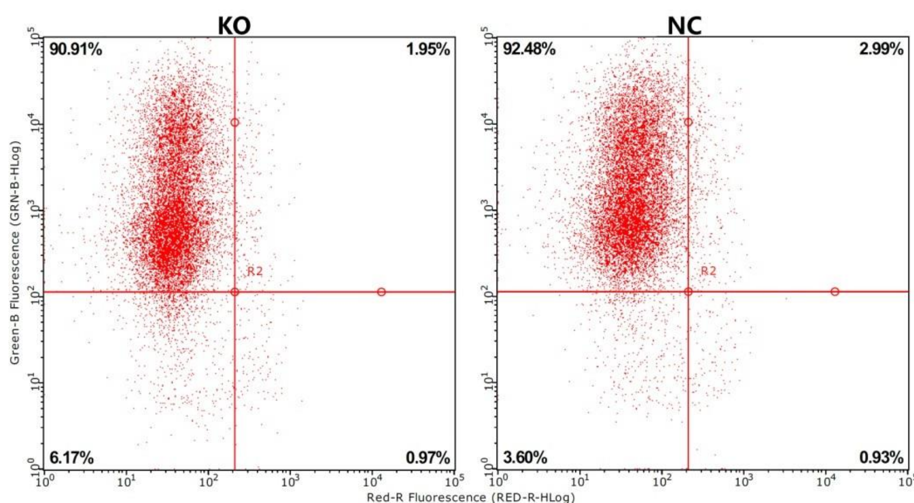

Fig. 7: Results of apoptosis in KO group and NC group

Discussion

Finding an effective treatment is a critical and extremely urgent requirement for genetic diseases. Gene therapy provides a promising option, especially for those genetic diseases with severe phenotype, such as Fragile X syndrome. The preliminary research based on cells and animals are essential steps for exploring a feasible treatment option. Since functional and morphological damages in nerve cells are the main pathological changes of Fragile X syndrome, research focused on neural cell level is important. In the present study, we investigated the feasibility of gene therapy for FXS on human neuroblastoma cells using CRISPR/*Cas9* gene editing system.

The first study employing CRISPR to edit *FMR1* was published in 2015 with human induced pluripotent stem cells (iPSCs), but the *FMR1* expression levels in edited iPSCs were shown to be similar to the control cells with normal CGG repeat alleles (15). In addition, CRISPR system was used to target *FMR1* in HEK 293 cells and iPSCs, and found that very limited clonal lines were reactivated following CRISPR editing (16). Therefore, whether differentiated nerve cells could be edited and lead to the change of *FMR1* expression level remains unknown. In this study, the majority of human neuroblastoma cells, approximately 60%, could be edited successfully by CRISPR/*Cas9* system, and the *FMR1* expression level in edited human neuroblastoma cells was significantly lower than that in the negative control group. The trend of FMRP expression change was in accordance with previous studies (17, 18). Furthermore, compared with the control group, there were differences in cell cycle in the edited human neuroblastoma cells. There were more cells in the G1 and G2/M phases in the edited human neuroblastoma cells and less cells in the S phase. The cell cycle progression was delayed and the maintenance of proliferating neural progenitors into postnatal stages was extended in *FMR1* KO mice (19). Furthermore, apoptosis was accelerated in the edited groups. This finding may be because numerous apoptosis-associated factors were considered to influence the effects of *FMR1*, such as miR-410-3p, PI3K, Akt and FoxO3a (17, 20). Previous reports showed the involvement of FMRP in the DNA damage response, a cellular response that triggers activation of the p53 pathway (21, 22). *FMR1* overexpression could alleviate oxidative stress and apoptosis via regulating oxidative stress and apoptosis associated factors in differentiated cardiomyocytes (17). The change of cell apoptosis might be caused by programmed cell death (PCD), while FMRP participates in PCD in neurons (23). The difference in different phases of cell cycle between the KO group and the NC

group of nerve cells is a novel observation in the present study. This article first detected the change of whole cell cycle through comparing the FMRP knockout cell line and normal cell line.

Researchers excised the expanded CGG-repeat in cells using the CRISPR/*Cas9* genome editing, and observed transcriptional reactivation in the CRISPR-edited cells. The excision of the expanded CGG-repeat from the fragile X chromosome can result in *FMR1* reactivation (16). Case reports also showed deletions of *FMR1* in human cells. Both large deletions (all CGG repeats and some of the flanking sequences) and small deletions (19 repeats remaining) were found in patients whose mothers harbored a full mutation (24, 25). Case reports supported that CRISPR/*Cas9* can be used to correct mutations in *FMR1* to cure FXS.

The present study was designed to explore the strategy of gene therapy for Fragile X syndrome, using CRISPR/*Cas9* system to target the *FMR1* 5'-UTR sites in human neuroblastoma cells. We used gene editing in differentiated nerve cells and found that the differentiated nerve cells could be edited successfully with the corresponding expression level of fragile X mental retardation protein. Meanwhile, functional consequences following gene editing, cell cycle changes and accelerated apoptosis were observed. Gene therapy of fragile X syndrome by CRISPR/*Cas9* system is thought to be very promising. The present data added important experimental evidence to support the gene therapy for genetic diseases.

Conclusion

This study provides further understanding about the FMRP function and molecular mechanism of *FMR1* gene in nerve cells, and suggests the feasibility of gene therapy in FXS by CRISPR/*Cas9* gene editing system.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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