Original Article

CRISPR/Cas9 ribonucleoprotein (RNP) complex enables higher viability of transfected cells in genome editing of acute myeloid cells

Qinquan Cheng[1#], Jing Xia[1#], Kaimin Wang[1#], Yue Zhang[2], Yan Chen[1], Qi Zhong[1], Xue Wang[3], Qi Wu[1,4,5]

[1]Department of Clinical Laboratory, Shanghai Tenth People's Hospital of Tongji University, Shanghai, China; [2]Department of Central Laboratory, Shanghai Tenth People's Hospital of Tongji University, Shanghai, China; [3]Department of Dermatology, Shanghai Ninth People's Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China; [4]Key Laboratory of Endemic and Ethnic Diseases, Ministry of Education, Guizhou Medical University, Guiyang, China; [5]Department of Clinical Laboratory, Shanghai Tenth People's Hospital Chong Ming Branch, Shanghai, China

Contributions: (I) Conception and design: Q Wu; (II) Administrative support: Q Wu; (III) Provision of study materials or patients: Q Zhong, X Wang; (IV) Collection and assembly of data: Q Cheng; (V) Data analysis and interpretation: Q Cheng, Y Chen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work and should be considered as co-first authors.

Correspondence to: Qi Zhong. Department of Clinical Laboratory, Shanghai Tenth People's Hospital of Tongji University, Shanghai, China. Email: Zhongqi0607@163.com; Xue Wang. Department of Dermatology, Shanghai Ninth People's Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China. Email: belongtogether@126.com; Qi Wu. Department of Clinical Laboratory, Shanghai Tenth People's Hospital of Tongji University, Shanghai, China. Email: wuqi496@163.com.

Background: Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) has become an increasingly vital tool for modifying gene expression in a variety of cell types. Lentiviral transduction and electroporation are the two main approaches used to deliver CRISPR/Cas9 into cells. However, the application of CRISPR/Cas9 in primary hematopoietic cells has been limited due to either low transduction efficiency in terms of viral-based delivery or difficult selection and enrichment of transfected and edited cells with respect to electroporation of CRISPR/Cas9 ribonucleoprotein (RNP).

Methods: In this study *in vitro* transcription was used to synthesize the guide RNA (gRNA), and plasmid pL-CRISPR.EFS.GFP was used as its DNA template. Then the *in vitro* transcribed gRNA was labeled with pCp-Cy5 via T4 ligase before incubating with Cas9 protein. Furthermore, CRISPR/Cas9 RNP was electroporated into primary $CD34^+$ cells isolated from cord blood, and cell survival rate and transfection efficiency were calculated and compared to that of lentiviral transduction.

Results: Here, we show that electroporation of CRISPR/Cas9 RNP resulted in higher cell viability compared to electroporation of CRISPR/Cas9 all-in-one plasmid, providing important findings for further studies in hematology via CRISPR/Cas9 technology. Moreover, we established a method for labeling *in vitro*-transcribed gRNA with fluorophore and the sorted fluorescent cells displayed higher knockout efficiency than nonsorted transfected cells.

Conclusions: Electroporation of fluorescence labeled CRISPR/Cas9 RNP is a perspective approach of gene editing. Our study provides an efficient and time-saving approach for genome-editing in hematopoietic cells.

Keywords: CRISPR/Cas9; ribonucleoprotein (RNP); guide RNA (gRNA); electroporation; acute myeloid cells

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Introduction

Acute myeloid leukemia (AML) is a malignant proliferation of abnormal myeloid cells and has an intermediate risk of recurrence (1). Although remarkable advances have been made in hematologic tumor therapy, the majority of AML patients still suffer from relapse after extensive treatment. AML cells are nonadherent cells and the molecular biological approaches for studying AML are very different from those for solid tumors. AML cells are difficult to transfect or transduce, which is the main limitation of AML research and therapy.

The clustered regularly interspaced short palindromic repeats (CRISPR)/*CRISPR*-associated protein 9 (Cas9) system has become an increasingly vital tool for modifying gene expression in a variety of cell types, showing unprecedented potential for tumor therapy (2). Many studies have applied CRISPR/Cas9 to therapeutically relevant *ex vivo* and *in vivo* experiments (3). However, the application of CRISPR/Cas9 is restricted because of the large size of the Cas9 protein (160 kDa). Efficient and safe delivery of CRISPR/Cas9 into AML cells is urgently needed to explore new therapeutic targets. In recent years, CRISPR/Cas9 ribonucleoprotein (RNP) complex has been directly delivered into tumor cells via electroporation, leading to decreased off-target effects due to the short lifetime of the RNP complex (4-9).

Electroporation is an electro-physical method for gene and protein transfer based on square-wave pulse technology and results in high expression of delivery targets and substantial viability of cells (10). Electroporation is already successfully applied to the manufacture of antitumor lymphocytes (11) and delivery of exogenous drugs and nuclear acids (12). CRISPR/Cas9 with electroporation shows great potential for genome editing and tumor therapy. However, the fundamental mechanism of electroporation-mediated DNA/RNA transfer has not yet been fully elucidated. The electric parameters (pulse amplitude, volts, etc.) need to be optimized for various conditions to reduce cell death and increase transfection efficiency (13).

Lentivirus or nanoparticles has been used to deliver CRISPR/Cas9 system into cells in many research studies, but the cell viability or transfection efficiency was always challenging. In this study, electroporation was used to deliver the CRISPR/Cas9 system, including CRISPR/Cas9 all-in-one plasmid and the RNP complex. Compared to CRISPR/Cas9 all-in-one plasmid, the RNP complex enabled higher viability of transfected cells. A novel fluorescent labeling of RNPs was applied to select genome-edited AML cells, which substantially improved the genome editing efficiency. Furthermore, gene editing of $CD34^+$ cells was also carried out in our study. This electroporating approach to deliver CRISPR/Cas9 RNP is simple and easy to operate, which provides a potential option for cancer research and tumor therapeutics. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3279/rc).

Methods

Cell culture

Human leukemia cell lines MV4-11 and Kasumi-1 were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). MV4-11 and Kasumi-1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% and 20% fetal bovine serum, respectively. Cell density was kept between 1×10^5 and 1×10^6 cells/mL. Cells were cultured at 37 ℃ in a humid incubator supplied with 5% CO_2.

Primary $CD34^+$ cells were isolated from cord blood with donors' consent using the commercial isolation kit (STEMCELL Technologies, EasySep™ Human CD34 Positive Selection Kit II, Canada). $CD34^+$ cells were cultured in StemSpan SFEM II (STEMCELL Technologies, Vancouver, BC, Canada) supplemented with FMS-like tyrosine kinase 3 ligand (FLT3L; 100 ng/mL), thrombopoietin (100 ng/mL), and stem cell factor (100 ng/mL; PeproTech, East Windsor, NJ, USA). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was taken from all the patients. The study was approved by institutional ethics board of Shanghai Tenth People's Hospital of Tongji University (22KN250).

Cloning

Guide DNAs (gDNAs) targeting enhanced green fluorescent protein (*EGFP*; sequence: GGCCACAAGTTCAGCGTGTC) was synthesized by TSINGKE Biotechnology Co., Ltd. (Beijing, China). The double strands DNAs were denatured at 95 ℃ for 5 minutes, and then step-wise annealing was carried out. Simultaneously, the pL-CRISPR.EFS.GFP (57818; Addgene, Waterton, MA, USA) vector was digested via FastDigest Esp3I

(Thermo Fisher Scientific, Waltham, MA, USA) at 37 ℃ for 15 minutes and purified on a 1% agarose gel (Biozyme). T4 DNA ligase (Thermo Fisher Scientific) was used to ligate the annealed double-stranded gDNA and digested vector (5:1 molar ratio). The ligated product was transformed into 50 µL of Stbl3 competent cells. Correct clones were confirmed by Sanger sequencing.

Guide RNA (gRNA) in vitro transcription

Plasmid pL-CRISPR.EFS.GFP was used as a DNA template for gRNA *in vitro* transcription. The forward primer was composed of forward primers containing a T7 promoter sequence (TTAATACGACTCACTATAG), the specific targeting sequence, and the sequence corresponding to the 5' terminal of the gRNA scaffold (GTTTTAGAGCTAGAAATAGC). The reverse primer sequence was: AGCACCGACTCGGTGCCACT. The *EGFP* targeting sequence was: GGAGCGCACCATCTTCTTCA (14). Polymerase chain reaction (PCR) products were purified with Gel DNA Recovery Kit (Thermo Fisher Scientific). HiScribe T7 High Yield RNA Synthesis Kit [New England Biolabs (NEB) GmbH, Frankfurt, Germany] was used to perform the *in vitro* transcription according to the manufacturer instructions. The transcribed gRNA products were purified through DNA electrophoresis and were recovered with RNA Clean & Concentrator Kit (Thermo Fisher Scientific).

Electroporation

Purified *in vitro* transcribed gRNA (1 µg) was incubated with 1 µg Cas9 protein (PNA Bio, Newbury Park, CA, USA) at room temperature for 15 minutes before electroporation. Neon transfection system (Thermo Fisher Scientific) was used and the various electroporation parameters were tested, the electroporating voltage which have been tried in this study changed from 900 to 1,600 v. Electroporation in Buffer R (Invitrogen, Carlsbad, CA, USA) was performed for 3 independent experiments, and 150,000–250,000 cells were used in each replicate. After 96 hours, the genome editing efficiency was validated.

Cell viability assays

The MV4-11 cells, Kasumi-1 cells, and $CD34^+$ cells were electroporated with CRISPR/Cas9-GFP all-in-one plasmid and the RNP complex, respectively, with different electroporation parameters according to the aforementioned method. After 96 hours, cells were stained with trypan blue and counted under microscopy. All experiments were repeated at least 3 times.

Electrophoretic mobility shift assay (EMSA)

After incubation of purified gRNA (1 µg) and Cas9 protein (1 µg) at room temperature for 15 minutes, gRNA/Cas9 RNP was loaded on 1.5% agarose gel to check the combining efficiency and electrophoresis was performed in 1× tris-acetate-EDTA (TAE) buffer with the Bio-Rad Powersupplies (130 v). Agarose gel imager was used to document the results.

gRNA 3' end labeling

pCp-Cy5 (30 µM; Sangon Biotechnology, Shanghai, China) was ligated to 1 µg gRNA with T4 RNA ligase (NEB) supplied with 10 mM adenosine triphosphate (ATP) at 16 ℃ overnight. The labeled RNA was purified with RNA Clean & Concentrator-5 (cat. No. R1013; Zymo Research, Irvine, CA, USA). For each experiment, fresh fluorescent labeled gRNA should be prepared in this study.

Flow cytometry analysis

Transfected Cy5-positive cells were sorted by fluorescence-activated cell sorting (FACS; BD FACSAria II; BD Biosciences, Franklin Lakes, NJ, USA) and 1× PBS with 2% FBS were used to collect the sorted cells. The genome editing efficiency was then tested through *GFP* expression under flow cytometry (BD LSR Flow Cytometer, BD Sciences).

Statistical analyses

The data were analyzed with SPSS version 22 (IBM Corp., Armonk, NY, USA). Student's *t*-test was used to confirm the statistical significance, and $P<0.05$ was considered statistically significant.

Results

CRISPR/Cas9 RNP reveals higher viability

To test the efficiency of electroporation on cell viability, we performed electroporation of leukemia cells with CRISPR/

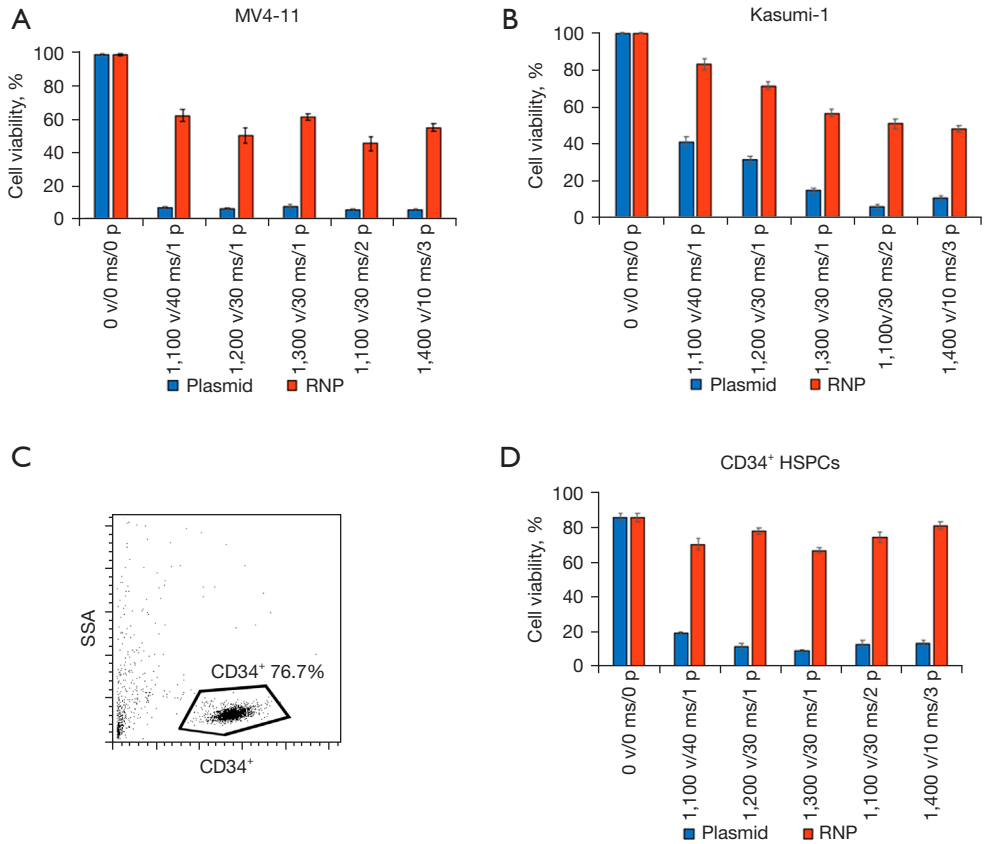

Figure 1 CRISPR/Cas9 RNP reveals higher viability. (A,B) Electroporated MV4-11 and Kasumi-1 cells were stained with trypan blue and counted under microscopy. Viability was calculated by the formula (viability = living cells of electroporated samples/living cells of nonelectroporated control × 100). (C) $CD34^+$ cells were isolated and sorted. (D) Electroporated $CD34^+$ cells were stained with trypan blue and counted under microscopy using the formula in (A,B), above. RNP, ribonucleoprotein; SSA, side scatter area; HSPCs, hematopoietic stem and progenitor cells; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9.

Cas9 all-in-one plasmid and gRNA/Cas9 RNPs. When plasmids were transfected into MV4-11 cells, cell viability was only around 5%, while it was up to 10 times higher (45–61%) when RNPs were delivered (*Figure 1A*). For Kasumi-1 cells, when plasmids were electroporated, a relatively higher number of living cells (compared to MV4-11 cells) were observed. Notably, when gRNA/Cas9 RNPs were used, viability was also significantly increased (*Figure 1B*). These results indicated that RNPs were much less toxic to cells, and electroporation with gRNA/Cas9 RNPs was a more viable and acceptable approach.

It is well known that hematopoietic stem and progenitor cells (HSPCs) are difficult to transfect with conventional viral and nonviral methods. Herein, we isolated $CD34^+$ HSPCs from cord blood (*Figure 1C*) and electroporated these cells with CRISPR/Cas9 all-in-one plasmid and gRNA/Cas9 RNPs, respectively. Consistent with the aforementioned results, the cells electroporated with gRNA/Cas9 RNPs showed much higher viability than those with CRISPR/Cas9 all-in-one plasmid (*Figure 1D*). These results collectively identified the higher viability of nonadherent cells after electroporating gRNA/Cas9 RNPs and the potential high delivery efficiency of gRNA/Cas9 RNPs.

Fluorescent labeling CRISPR/Cas9 RNPs enable effective genome editing

As selecting cells transfected with gRNA/Cas9 RNPs is difficult, we designed an easy and effective technique for isolating cells with introduced RNPs. We first labeled *in vitro* transcribed gRNA with pCp-Cy5 via T4 ligase before incubating with Cas9 protein, as shown in the gRNA/

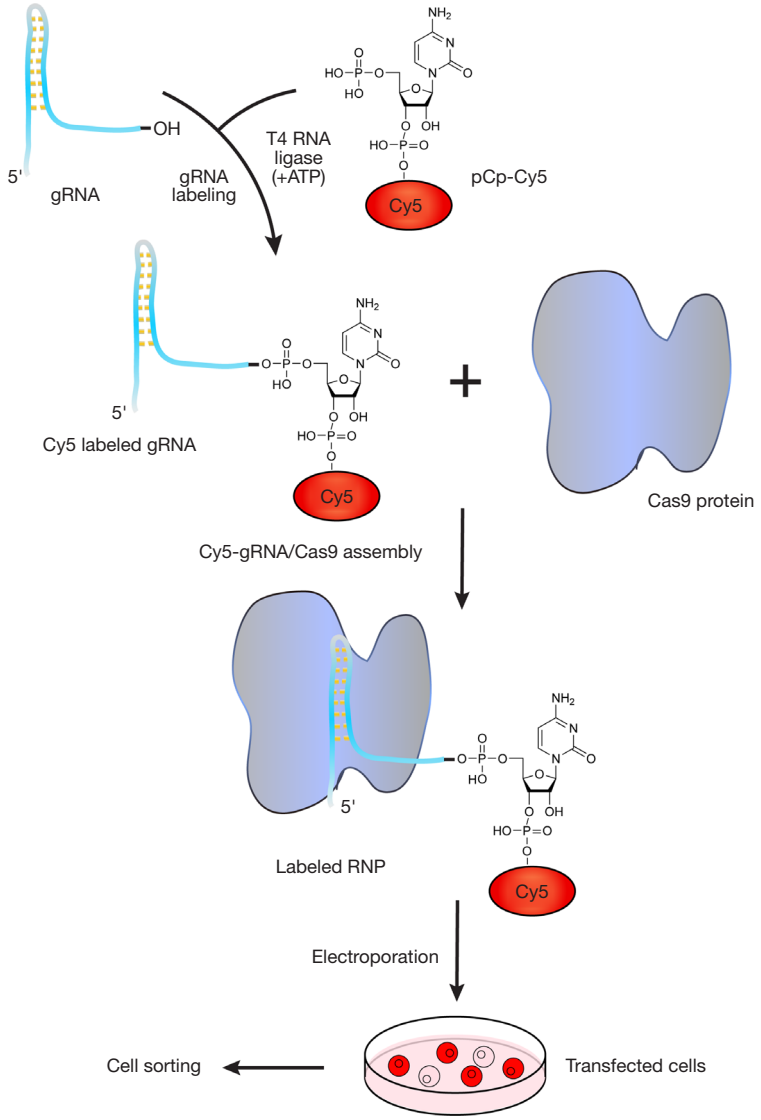

Figure 2 Schematic design of gRNA/Cas9 labeling, assembly, and electroporation. gRNAs were labeled by fluorophore Cy5 for selection. gRNA, guide RNA; ATP, adenosine triphosphate; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; RNP, ribonucleoprotein.

Cas9 RNP labeling schematic (*Figure 2*). Cy5 labeling of gRNA was verified using Imager 600 with red fluorescence (*Figure 3*). Neither gRNA alone (lane 2') nor pCp-Cy5 plus gRNA without T4 RNA ligase (lane 3') displayed a detectable band, while pCp-Cy5 plus gRNA with T4 RNA ligase (lane 4') showed a clear band, indicating the labeling was successful and specific. Further, the *in vitro* transcribed gRNA could effectively bind Cas9 protein in a proportion-dependent manner (*Figure 4A*). In order to test the possible effect of Cy5 labeling on the binding of gRNA to Cas9 protein, EMSA was performed. The results indicated that Cy5 labeling had no obvious effect on the binding of gRNA to Cas9 protein (*Figure 4B*). The above results confirmed the successful production of labeled gRNA/Cas9 RNPs *in vitro*.

To further explore the efficiency of CRISPR/Cas9 RNPs on genome editing, Cy5-labeled gRNA/Cas9 RNPs were electroporated into the MV4-11 cells with *EGFP* under the electric parameters of 1,600 v, 10 ms, and 3 pulses. The expression of targeted gene (*GFP*) was significantly downregulated in the Cy5-sorted cells (44.8%) compared

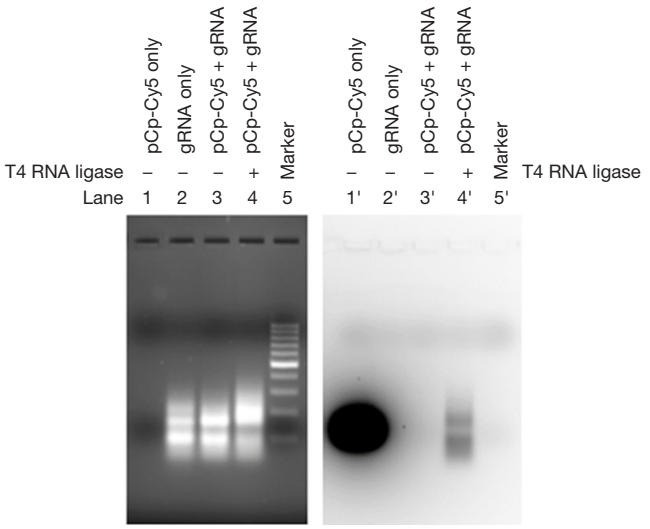

Figure 3 gRNA labeling and verification. gRNAs could be effectively labeled. Left figure was taken with Gel documenter (UV light), while right figure was taken with Amersham imager 600 RGB (Cy5). gRNA, guide RNA.

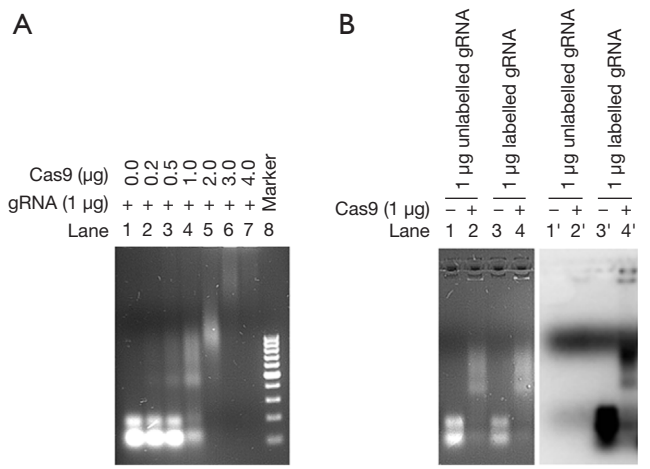

Figure 4 EMSA of gRNA binding to Cas9. Cy5-labeled gRNA did not affect the binding to Cas9 protein (A) and the left figure of (B) were taken with Gel documenter (UV light), while right figure of (B) was taken with Amersham imager 600 RGB (Cy5). Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; gRNA, guide RNA; EMSA, electrophoretic mobility shift assay.

to the unsorted cells (21.5%) (*Figure 5*). In sum, our results identified the substantial benefits of fluorescent labeling CRISPR/Cas9 RNPs on genome editing of nonadherent cells.

Discussion

Recently, nonviral delivery systems for DNA-molecule transport have become increasingly important due to the risk of insertional mutagenesis and probable immunogenicity of viral systems (15). Electroporation has been widely used in gene delivery for vaccine production, transgene expression, and therapy for various cancers (16). As nonadherent cells, AML cells are much more difficult to transfect compared to adherent cells, even with a viral delivery system. The applied range of electroporation is broad and flexible, depending on the transported molecule (mRNA, DNA, protein, etc.) and the various target cell types (adherent and nonadherent cells) (17). Herein, electroporation, which has been used for the past 30 years, was chosen as the delivery method for CRISPR/Cas9 RNPs. The transport efficiency of CRISPR/Cas9 RNPs into AML cells with electroporation is high and cell death is low, confirming the feasibility of electroporation in AML cells.

Traditionally, gene editing has been used to investigate the function of targeted genes and develop novel ways for treating diseases, especially malignancies. A number of gene editing approaches have been used over the past decades, including zinc-finger nucleases, transcription activator-like effector nucleases, and so on (18). Recently, gene editing using CRISPR/Cas9 has quickly risen in popularity due to its simplicity and speed. CRISPR/Cas9, working through RNA-guided Cas9 nucleases, is an efficient tool for gene editing. However, viral-delivered CRISPR/Cas9 plasmids normally result in prolonged expression of the gene editing system, which often increases the frequency of off-target effects and promotes oncogenesis in transduced cells (5). As a result, CRISPR/Cas9 RNPs, with their short lifespan, have been synthesized, and various approaches for delivering CRISPR/Cas9 RNPs have been developed to overcome the drawbacks of viral delivery (19-21). Consistent with previous reports, our results indicated that electroporated CRISPR/Cas9 plasmids into AML cells induced high cytotoxicity. Conversely, CRISPR/Cas9 RNPs delivered with electroporation into AML cells led to more cell viability. Furthermore, Cy5 labeling of gRNA/Cas9 RNPs was used to select positive cells, which could significantly enhance gene editing efficiency. Previous studies have used nanoparticles to deliver CRISPR/Cas9 RNPs, and approximate 30% indel efficiency of target gene have been identified (7), which is significantly lower than that via electroporation (44.8%). Our results indicated that the use of Cy5-labeled CRISPR/Cas9 RNPs could be beneficial

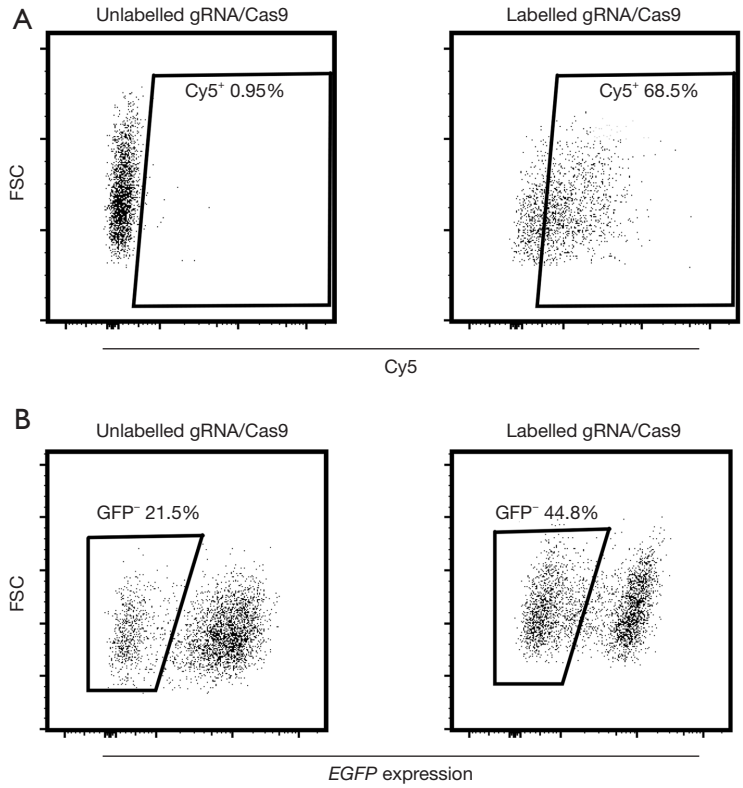

Figure 5 Transfection efficiency and genome-editing efficiency of labeled gRNA/Cas9 RNP. (A) Cy5 labeled and unlabeled RNPs were electroporated into MV MV4-11 cells and sorted with FACS. (B) Selected transfected cells showed higher *EGFP* genome-editing efficiency (44.8% *vs.* 21.5%). FSC, forward scatter; gRNA, guide RNA; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; GFP, green fluorescent protein; *EGFP*, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; RNP, ribonucleoprotein.

for gene editing in nonadherent cells. Of note, the specific mutations generated by CRISPR/Cas9 was mediated by both NHEJ- and HDR-based genome modifications (22). The off-target mutations is normally serious concern about CRISPR/Cas9, which mainly depends on the specificity of targeting regions. To minimize the off-target of gene editing, the tools (http://www.xn--genome-engineering-rl9j.org/crispr/?page_id=41) has been used to design the sgRNA and normally more than one sgRNAs will be recommended in research study. So far, the application of CRISPR/Cas9 RNPs for clinical gene editing is still challenging.

Over the last few years, molecularly targeted inhibitors and chemotherapy drugs have been used to treat malignancies, improving the quality of life for patients (3). However, drug resistance often leads to recurrence. Gene editing, especially CRISPR/Cas9-mediated gene editing, has paved the way for new approaches to cancer therapy. Nanoparticle delivery of CRIPR/Cas9 has been used to rescue fragile X syndrome in mouse model (8), and lentivirus-delivered CRIPR/Cas9 has been confirmed the positive therapeutic role on malignancy (23-26). However, due to the off-target effects of CRISPR/Cas9 and the delivery vehicle, its application has been limited. As CRISPR/Cas9 RNPs degrade in cells, excessive cleavage of untargeted genes is avoided, and thus electroporated CRISPR/Cas9 RNPs may provide a new option for cancer therapy.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3279/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3279/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3279/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was taken from all the patients. The study was approved by institutional ethics board of Shanghai Tenth People's Hospital of Tongji University (22KN250).

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