Tohoku J. Exp. Med., 2023, **260**, 51-61

A New Method for Programmable RNA Editing Using CRISPR Effector Cas13X.1

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Type VI CRISPR-Cas13 is the only CRISPR system that can bind and cleave RNA without DNase activity. We used the newly discovered, smaller Cas13X.1 protein to construct an editing system in mammalian cells, aiming to break the delivery restrictions of CRISPR-Cas13 system *in vivo* and promote the application of Cas13X system in clinical therapy. We employed exogenous fluorescence reporter gene mCherry and endogenous gene transketolase (TKT) closely related to cancer cell metabolism as target genes to evaluate the Cas13X.1 system. The recombinant plasmids targeting exogenous gene mCherry and endogenous gene TKT were constructed based on Cas13X.1 backbone plasmid. The editing efficiency, protein expression level, downstream gene transcript level and safety of Cas13X.1 system were evaluated. Both TKT transcripts of endogenous genes and mCherry transcripts of exogenous genes were significantly degraded by Cas13X.1 system with a knockdown efficiency up to 50%. At the same time, Cas13X.1 down-regulated the expression of the corresponding protein level in the editing of transcripts. In addition, the transcripts of key metabolic enzymes related to TKT were also down-regulated synchronously, suggesting that the degradation of TKT transcripts by Cas13X.1 system affected the main metabolic pathways related to TKT. The morphology, RNA integrity and apoptosis of cells loaded with Cas13X.1 system were not affected. The Cas13X.1 system we constructed had strong RNA knockdown ability in mammalian cells with low cellular toxicity. Compared with other CRISPR-Cas13 systems, Cas13X.1 system with smaller molecular weight has more advantages *in vivo* delivery. The Cas13X.1 system targeting TKT transcripts also provides an alternative method for the study of anti-cancer therapy.

Keywords: CRISPR-Cas13X; gene knockdown; programmable RNA targeting; RNA editing; RNA methods; type VI CRISPR-Cas systems
Tohoku J. Exp. Med., 2023 May, **260** (1), 51-61.
doi: 10.1620/tjem.2023.J011

Introduction

The programming techniques of accurately editing specific DNA or RNA sequences in target cells have changed modern life science and have also become the key technology of gene therapy. Among them, the CRISPR editing system, which is only composed of Cas nuclease effector and guide RNA, can be applied to almost all biological genomes (Jinek et al. 2012; Chang et al. 2020). Compared with the early zinc-finger nuclease (ZFN) and transcription activator-like effector nucleases (TALENS), CRIPSR technology with high versatility, high editing efficiency, simple design and low cost quickly occupies a dominant position in the field of gene editing (Cong et al. 2013; Jinek et al. 2013; Knott and Doudna 2018).

Currently, known CRISPR-CAS systems are classified

Received January 12, 2023; revised and accepted February 8, 2023; J-STAGE Advance online publication February 23, 2023
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into two classes (1 and 2) (Makarova et al. 2015; Agarwal and Gupta 2021). The class 2 CRISPR-Cas system includes type II (Cas9), type V (Cas12), and type VI (Cas13). Cas9 and Cas12 systems can accurately identify and cut specific DNA sequences, which is especially suitable for editing specific genes to treat durable diseases (Wang et al. 2020). However, relying on DNA editing to treat diseases may lead to ineffective treatment and even initiate other risks. For example, it has been repeatedly reported that CRISPR effector-induced DNA double-strand breaks may cause chromosome instability (Rayner et al. 2019), decreased adaptability (Chen et al. 2020), cell cycle disorders (van den Berg et al. 2018) and even activation of tumor suppressor protein TP53 (Haapaniemi et al. 2018). Type VI CRISPR system (Cas13) may avoid these potential risks. The Cas13 system initiates RNA enzyme activity to degrade specific RNA sequences through guided RNA (gRNA) recognition of specific RNA sequences. From a therapeutic point of view, it is almost impossible for Cas13 to damage DNA to cause the potential risk triggered by double-strand breaks because of its lack of DNase activity (Konermann et al. 2018). In view of these advantages, Cas13 has been used for various disease treatments and has shown definite efficacy. In addition, using Cas13d system to target editing human immunodeficiency virus (HIV)-1 RNA is a new strategy which can effectively fight against HIV. The results show that Cas13d system can effectively inhibit HIV-1 in primary CD4 cells (Nguyen et al. 2021). Myosin VI p.C442Y mutation leads to dominant hereditary deafness in humans. The RNA base editor combinates with Cas13 and RNA editing enzyme adenosine deaminase acting on RNA 2 deaminase domain variant (ADAR2dd^{E488Q}) can correct the myosin VI p.C442Y mutation of mouse model and save the hearing of mice (Xiao et al. 2022).

CRISPR-Cas13 editing system for clinical treatment faces the following difficulties. At present, CRISPR system mainly depends on virus delivery for clinical treatment. Due to the limitation of adeno-associated virus (AAV) delivery *in vivo*, only miniature CRISPR effectors are suitable for producing CRISPR-based treatments (Wang et al. 2020). The previous research had found verified that amino acid sequences of Cas13a (1152 aa), Cas13b (1092 aa), Cas13c (1110 aa), and Cas13d (967 aa) protein were too long to be delivered *in vivo* by AVV (Abudayyeh et al. 2017; Smargon et al. 2017; Konermann et al. 2018). In addition to the delivery obstacle, the sustained expression of Cas13 protein may produce a harmful immune response (Ferdosi et al. 2019). Therefore, solving the problems of delivery and immune response of CRISPR-Cas13 system is vital to more accurate, efficient and safe application of CRISPR-Cas13 editing technology in the use of clinical genetic diseases. Our editing system based on the recently discovered Cas13X.1 protein may solve some of the appeal problems. The amino acid sequence of Cas13X.1 protein is only 775 aa, which is beneficial to the transmission of editing system *in vivo* utilizing virus delivery. In addition, the homologous genes of Cas13X.1 originated from microorganisms that live in high-salt habitats and are not closely related to humans, reducing the risk of preexisting immunity found in Cas9 and Cas12 (Xu et al. 2021).

We established the Cas13X.1 editing system in 293T cells, evaluating the editing efficiency and cytotoxicity of this system, which can be used as the basis for follow-up clinical research. The system we constructed can edit the endogenous and exogenous genes of cells and obtain high editing efficiency. Cell proliferation and apoptosis analysis confirmed that the Cas13X.1 system had no detectable cytotoxicity to cells. The Cas13X.1 system we constructed provides a new method for subsequent gene therapy.

Methods

Cas13X.1 gRNA design

Query the sequence information of target genes such as mCherry and TKT from NCBI database. We designed the gRNA targeting mRNA of mCherry and TKT on the gRNA online design evaluation platform (https://zlab.bio/guide-design-resources) (Supplementary Table S1). Then, the pre-insertion sequence corresponding to gRNA on the vector is synthesized (Beijing Genomics institute, Shenzhen, China).

Plasmid constructions and sequencing analysis

RNase Free water, NEbuffer, Cas13X.1 backbone plasmid (171379, Addgene, Shanghai, China) (Supplementary Table S2) and BbsI-HF enzyme (R3539, New England Biolabs, Ipswich, MA, USA) were successively added to the enzyme-free ep tube. Components were mixed by pipetting the reaction mixture up and down, followed with a quick spin-down in a microcentrifuge. After 3 h of reaction at 37°C, BbsI-HF enzyme completed the cleavage of the BbsI restriction site at the skeleton plasmid. The enzyme-cutting products were detected by electrophoresis in 1% agarose gels (agarose G-10, Baygene, Shanghai, China) stained with goldview (DH392-5, DINGGUO CHANGSHENG, Beijing, China). The larger band of the cutting product (around 7,800 bp) was excised and extracted by Gel Extraction Kit (D2500, Omega Bio-tek, Omega, GA, USA) to be carrier segment in the next step. The pre-inserted sequence was connected with the carrier segment by T4 DNA Ligase (M0202, New England Biolabs) following the manufacturer manual. The successfully linked recombinant plasmid was transferred into E.coli DH5α competent cells (9057, Takara, Dalian, China) for amplification. The monoclonal colonies with ampicillin resistance were selected and incubated in LB liquid medium overnight at 37°C. The obtained bacterial solution was sent for sanger sequencing (Beijing Genomics institution).

Cell culture, transfection, fluorescence imaging and flow cytometry analysis

Mammalian experiments were performed using HEK293T cell lines (American Type Culture Collection,

Manassas, VA, USA). Cell medium was prepared by high glucose DMEM with sodium pyruvate and glutamine (C11995500BT, Gibco, Grand Island, NY, USA), additionally supplemented with 1% penicillin-streptomycin (SV30010, Hyclone, Logan, UT, USA) and 10% fetal bovine serum (A3161001C, Gibco). Transfection of HEK293T was conducted with Lipofectamine 3000 (L3000015, Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's manual. Cells were sorted by MoFlo Astrios EQ at 48 h after transfection. Flow cytometry results were analyzed with kaluza analysis. Moreover, fluorescent photographs were taken under a Nikon Eclipse Ti2-A microscope and analyzed by ImageJ software.

Quantitative PCR with reverse transcription and analysis

To quantify RNA knockdown efficiency of Cas13X.1 effectors, RNAs were extracted from successfully transfected cells with Eastep® Super Total RNA Extraction Kit (LS1040, Promega, Madison, WI, USA) and reverse-transcribed to cDNAs with GoScript™ Reverse Transcription Mix (A2800, Promega). Quantitative PCR (qPCR) was performed with the cDNA for each sample on a CFX Connect Real-Time system (BIO-RAD, Hercules, CA, USA), using GoTaq® qPCR Master Mix (A600A, Promega). qPCR results were analyzed with the $-\Delta\Delta$CT method, in which differences between average CT values of target genes and reference gene GAPDH for three biological replicates were used to calculate the relative expression level of the target gene and normalized by that of control groups.

Apoptosis and necrosis assays

For apoptosis and necrosis analysis, live cells of the gRNA-targeting group and control group were freshly harvested and stained with Annexin V-EGFP (C1062, Beyotime, Shanghai, China) and DAPI (D4054, US Everbright, San Ramon, CA, USA). Stained cells were sorted by CYTOFLEX Lx at 48 h after transfection. Flow cytometry results were analyzed with CytExpert analysis.

Ribosomal RNA integrity analysis

To assess the ribosomal RNA integrity analysis, total RNA was extracted from 293T cells transfected with Cas13X.1 recombinant plasmid using an Eastep® Super Total RNA Extraction Kit (LS1040, Promega). Then, 1 μg of total RNA was subjected to denaturing gel electrophoresis and stained with Goldview (DH392-5, DINGGUO CHANGSHENG) for RNA-integrity analysis. RNA-integrity analysis was performed using ImageJ software.

Protein extraction and analysis

About 1×10^6 293T cells were lysed using RIPA (P0013B, Beyotime) with complete proteinase inhibitor (bb-3321, BestBio, Shanghai, China) on ice for 15 mins. After centrifugation, the cellular total protein quantification was performed using a BCA kit (P0012, Beyotime). Then, the protein was denatured by heating and subjected to SurePAGE^{TM} (M00655, Nanjing, China). Proteins were transferred to 0.2 mm PVDF membranes (BioRad Laboratories), which were blocked using 5% non-fat milk in phosphate-buffered saline (PBS) solution for 1 h at room temperature. The membranes were incubated overnight using primary antibodies such as anti-mCherry (ab167453, Abcam, Cambridge, England), anti-TKT (64414S, CST), GAPDH (10494-1-AP, Proteintech, Rosemont, IL, USA) and β-Actin (20536-1-AP, Proteintech). Anti-mCherry and anti-TKT antibodies were used at 1:1,000 dilution in 1% Tris buffered saline with Tween 20 (TBS-T) buffer. Internal reference antibodies were used at 1:5,000 dilution in TBS-T buffer. Membranes were washed in TBS-T and incubated with secondary antibodies (926-68073, LI-COR Biosciences, Lincoln, NE, USA) at 1:10,000 dilution. Protein bands were visualized with Odyssey® CLx Infrared Imaging System (Li-COR Biosciences) and quantified by densitometric analysis using ImageJ software.

Statistical analysis

All statistical values were presented as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) with least significant difference (LSD) (equal variances assumed) or Tamhane's T2 (equal variances not assumed) was used for comparisons and $p < 0.05$ was considered to be statistically significant.

Material availability

All materials are available upon reasonable request.

Results

Establishment of CRISPR-Cas13X.1 knock-down mCherry system

Most CRISPR CAS proteins have too large molecular weight, which is not conducive to delivery through viral vectors, plasmid transfection and other delivery methods *in vivo*, affecting their wide application in the field of life sciences. The amino acid length of recently discovered Cas13X.1 protein is lower than that of the previous Cas13 series protein (Cas13a, Cas13b, Cas13c, and Cas13d) and has similar RNase activity (Fig. 1A) (Xu et al. 2021). Therefore, it is easier for Cas13X.1 protein to transfect cells through viral vectors and play a full editing role. The gRNA in the Cas13X.1-protein complex recognizes and specifically binds the target RNA to activate the specific digestion activity of the protein effector, which leads to the degradation of the target RNA (Fig. 1B) (Abudayyeh et al. 2017). In order to establish a CRISPR-Cas13X.1 editing system, we constructed a mCherry knocking-down recombinant plasmid on the basis of the Cas13X.1 backbone plasmid (Fig. 1C). This plasmid was expected to degrade the mRNA of mCherry and down-regulate the expression of exogenous mCherry (Fig. 2A). We designed the gRNA sequence targeting mRNA of mCherry on the gRNA online

design evaluation platform (Guo et al. 2021). There are two BbsI restriction sites at the 5' end of the the direct repeat (DR) sequence of the circular Cas13X.1 backbone plasmid. After cleavage with BbsI enzyme, the pre-inserted spacer sequence (expressing gRNA after transcription) is ligated with the digested and extracted backbone plasmid to obtain the recombinant plasmid (Fig. 2B). The electrophoretic results of the BbsI enzyme products of the backbone plasmid showed that the backbone plasmid was cut and could connect with the spacer sequence (Fig. 2C). The recombinant plasmid obtained after inserted spacer sequence was sequenced, and the sequencing results showed that the plasmid was constructed accurately.

Efficient interference activity of Cas13X.1 against transcripts in HEK293T cells

To identify the editing effect of Cas13X.1 recombinant plasmid, we used a mCherry reporting system based on HEK293T cells to detect the RNA targeted interference activity of Cas13X.1. At 2 days after transfection with the mCherry-targeting recombinant plasmids and backbone plasmid (non-gRNA), we observed that the fluorescence intensity of mCherry protein decreased significantly in the mCherry-targeting group (Fig. 3A). At the same time, there was no detectable fluorescence in the blank control group (non-transfection), indicating that the fluorescence originated from exogenous gene expression, suggesting the knockdown effect of Cas13X.1 system on exogenous transcripts. The number of mCherry fluorescence positive cells

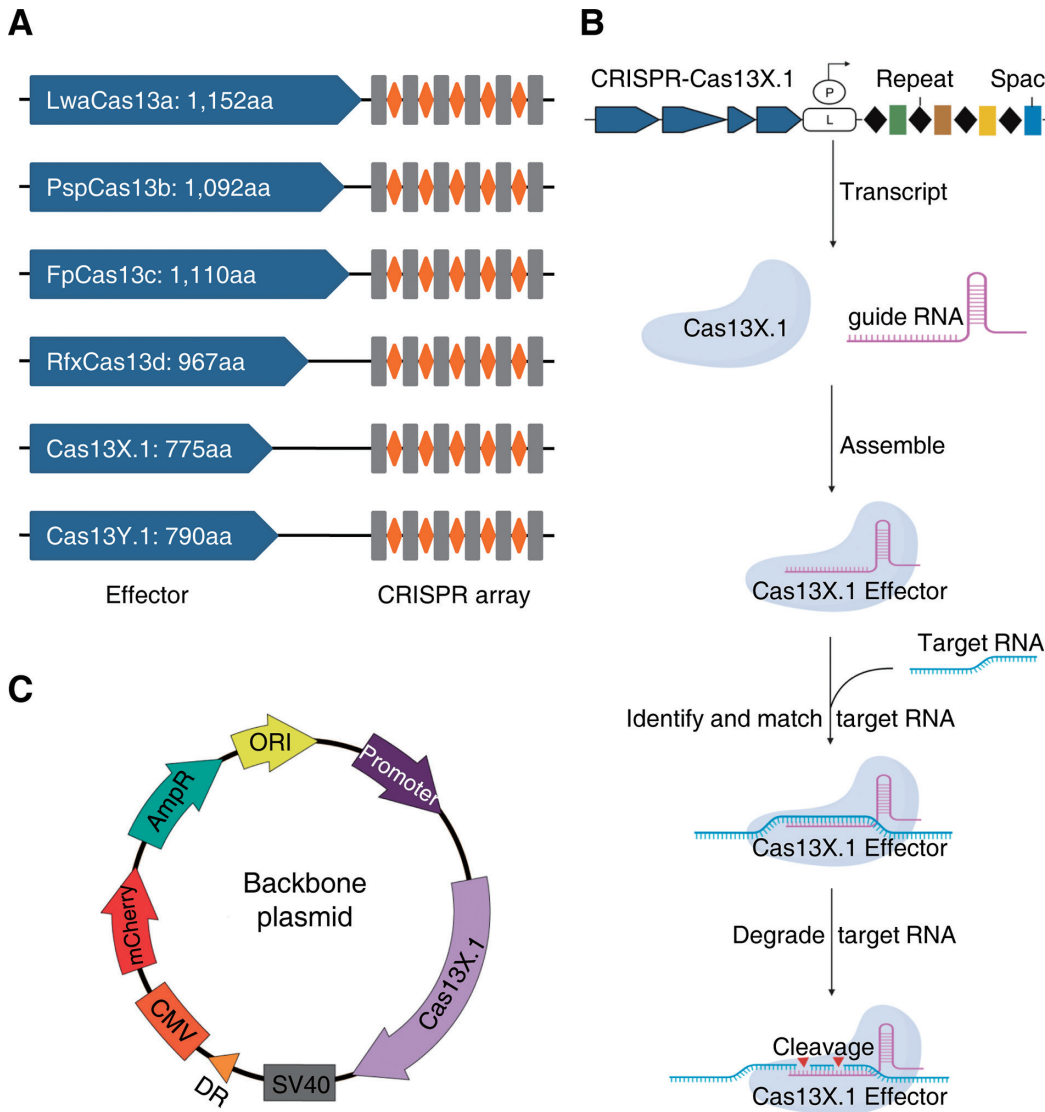

Fig. 1. Characterization of CRISPR-Cas13X.1 systems.
A. Family members and protein sizes of Cas13 protein. B. Schematic of mRNA knockdown by Cas13X.1. The plasmid was transfected into cells to express Cas13X.1 protein and guided RNA (gRNA) to assemble a complex with specific RNA nucleases. The Cas13X.1/gRNA complex recognizes and degrades the target RNA sequence. C. Characteristic backbone plasmid schematics. The inset shows multiple Cas13X.1 elements including Cas13X.1, mCherry and direct repeat (DR) sequence.

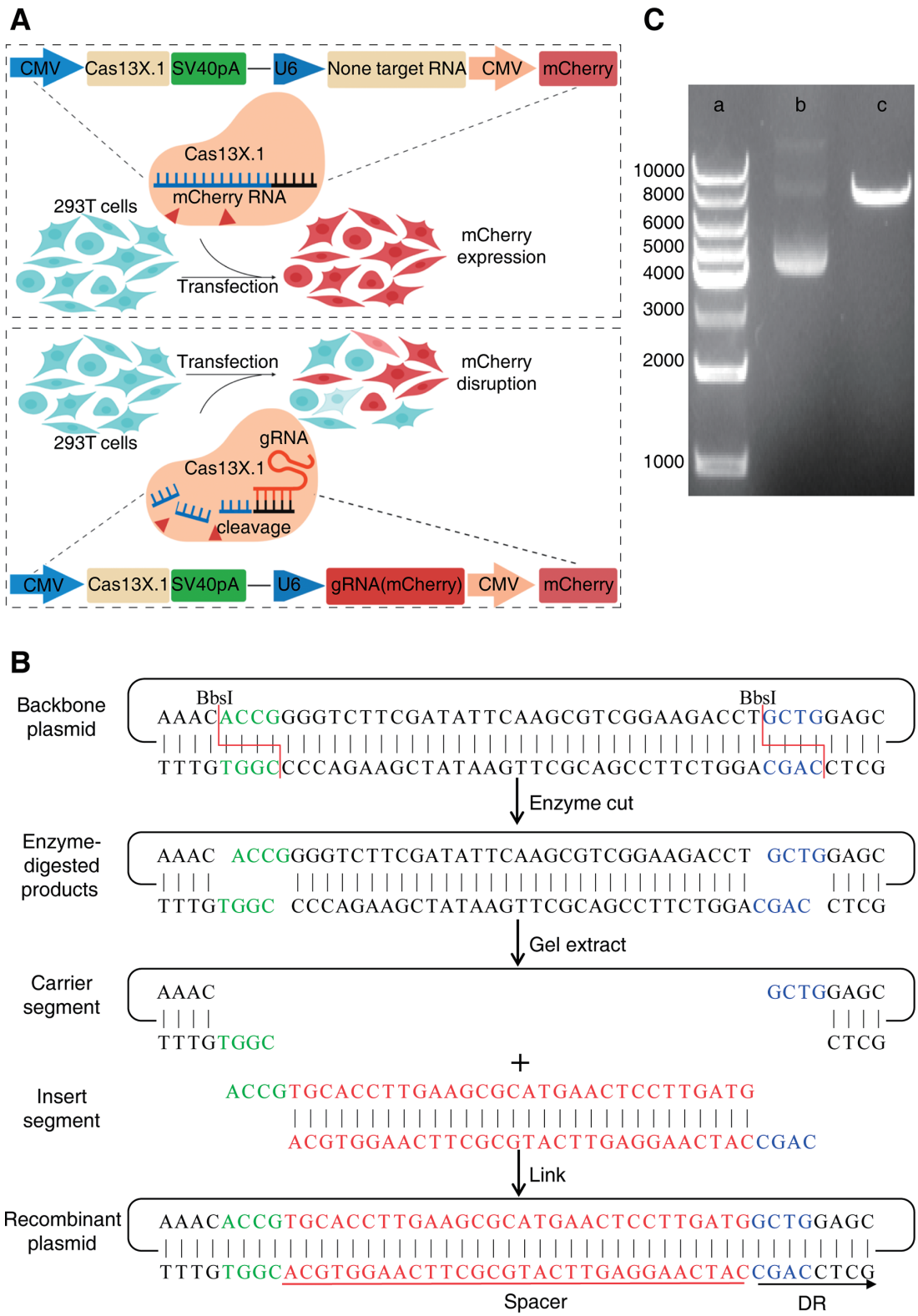

Fig. 2. Construction of Cas13X.1 targeting mCherry knockdown system.
A. Schematic of targeting mCherry knockdown by Cas13X.1 system in cells. The Cas13X.1 system with guided RNA (gRNA) can degrade mCherry mRNA. The Cas13X.1 system without gRNA cannot degrade mCherry mRNA. B. Schematic of CRISPR-Cas13X.1 knockdown mCherry plasmid modification. The designed spacer was inserted into the backbone plasmid by BbsI-HF endonuclease to construct a recombinant plasmid with mCherry-targeting knockdown ability. C. Gel electrophoresis of cleaved Cas13X.1 backbone plasmid. The complete backbone plasmids (b band) were isolated between 4,000 bp and 5,000 bp position. The cleaved backbone plasmids (c band) were isolated at the location of between 6,000 bp and 8,000 bp due to the destruction of the superhelix structure.

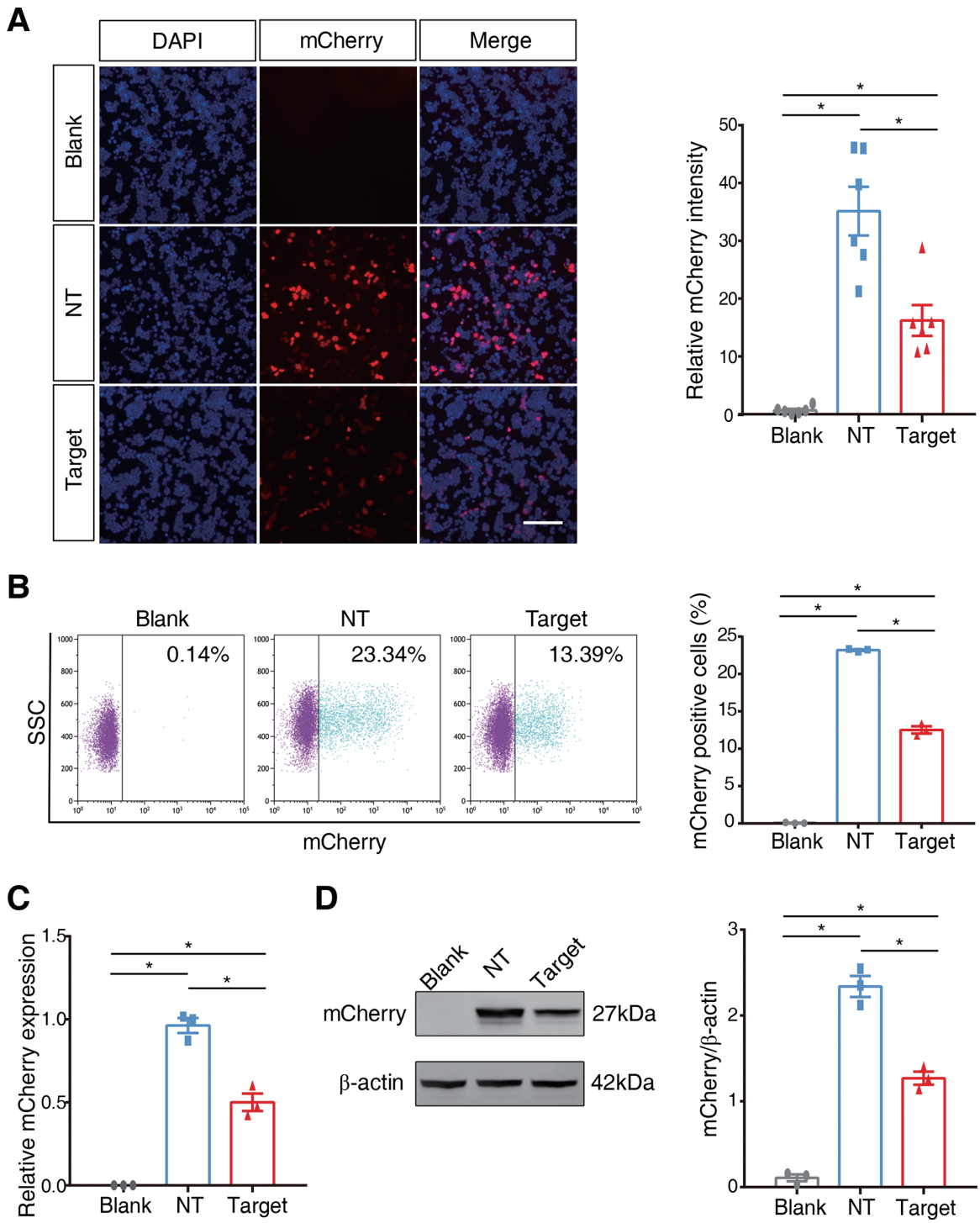

Fig. 3. Efficient interference activity of Cas13X.1 against transcripts in HEK293T cells.
A. Fluorescence imaging of mCherry-targeting group (Target), non-target group (NT) and blank control (Blank). The mCherry reporter inhibition assay results of comparing relative fluorescence intensity among mCherry-targeting group (Target), non-target group (NT) and blank control (Blank). Scale bar = 500 μm. Data are presented as mean ± SEM. n = 6, *p < 0.05. B. Knockdown efficiency was quantified as the percentage of mCherry + cells compared with NT. Left is the flow cytometry analysis result and right is the quantitative analysis bar chart. SSC, side scatter. Data are shown as mean ± SEM. n = 3, *p < 0.05. C. Evaluation of Cas13X.1-mediated knockdown on mCherry. Expression levels of mCherry mRNA were detected by qRT-PCR in 293T cells. Data are shown as mean ± SEM. n = 3, *p < 0.05. D. Western blot analysis of the expression of mCherry protein in blank control group (Blank), NT and mCherry-targeting group (Target). The relative levels of target proteins were quantified using ImageJ software. Data are shown as mean ± SEM. n = 3, *p < 0.05. One-way analysis of variance (ANOVA) with LSD (equal variances assumed) or Tamhane's T2 (equal variances not assumed) was used for comparisons, and p < 0.05 was considered to be statistically significant.

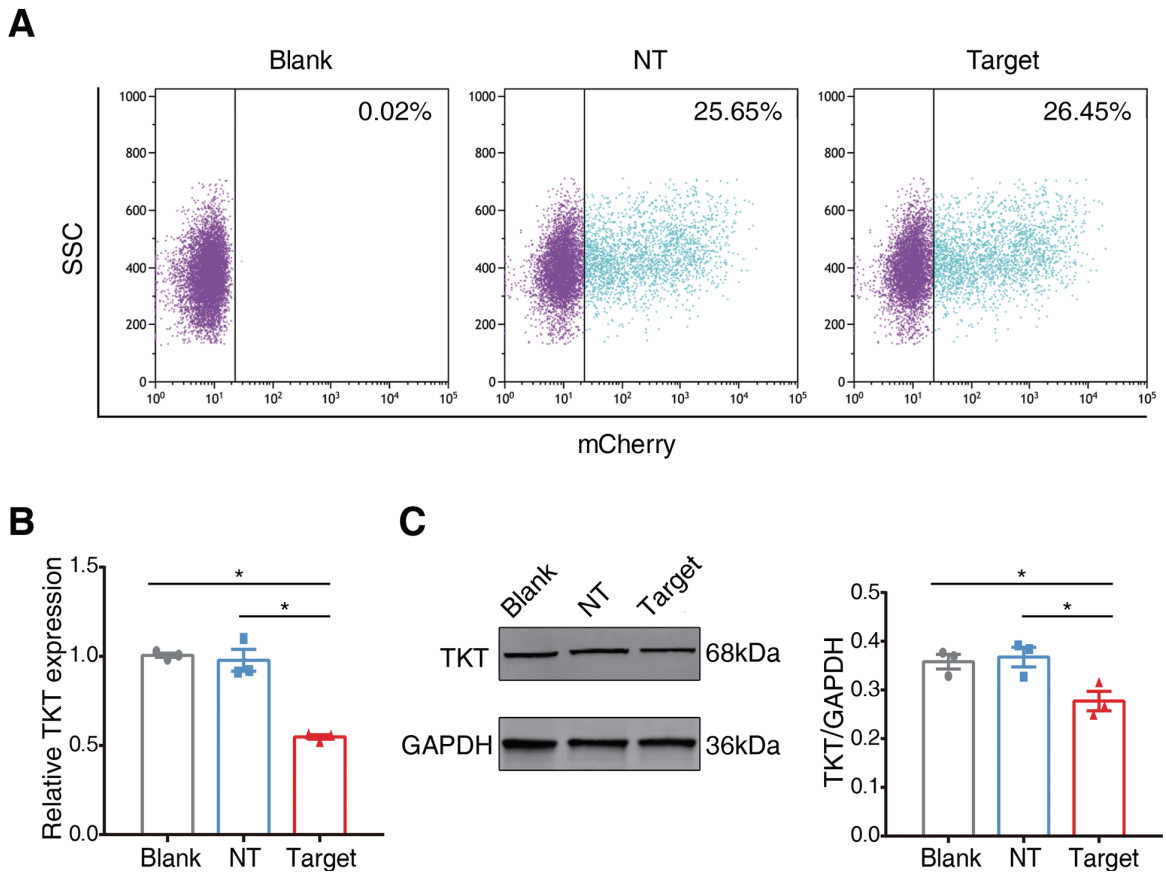

Fig. 4. Efficient knockdown of endogenous gene transketolase (TKT) with Cas13X.1.
A. The mCherry positive cells in non-target group (NT) and TKT-target group (Target) were sorted by flow cytometry. Blank, blank control; SSC, side scatter. B. Evaluation of Cas13X.1 knockdown efficiency against TKT in HEK293T cells. Expression levels of TKT mRNA were detected by qRT-PCR in 293T cells. Data are shown as mean ± SEM. n = 3, *p < 0.05. C. Western blot analysis of the expression of TKT protein in blank control group (Blank), non-target group (NT) and TKT-targeting group (Target). Data are shown as mean ± SE. n = 3, *p < 0.05. One-way analysis of variance (ANOVA) with LSD (equal variances assumed) or Tamhane's T2 (equal variances not assumed) was used for comparisons and p < 0.05 was considered to be statistically significant.

analyzed by flow cytometry sorting in both groups also supported this result. The positive rate of mCherry fluorescence was lower in mCherry-targeting group (Fig. 3B). We then evaluated the ability of mCherry-target recombinant plasmid to knock-down mCherry reporting level in HEK293T cells at the transcriptional level and translational level. The transcriptional level of mCherry in the mCherry-targeting group was about 50% lower than that in non-target group (Fig. 3C). The significant decrease of mCherry protein level in mCherry-targeting group suggested that the recombination plasmid could down-regulate the expression of exogenous genes through editing at the RNA level (Fig. 3D).

Moreover, we also constructed a recombinant plasmid targeting cellular endogenous gene transketolase (TKT), assessing the versatility of Cas13X.1 system for RNA interference. 48 h after transfection, the positive cells in TKT-targeting group and non-target group were obtained by flow sorting for follow-up analysis (Fig. 4A). We observed a 50% knock-down efficiency of TKT transcripts by quantitative PCR with reverse transcription (qRT-PCR), which is equivalent to the knock-down effect of RNAi (Fig. 4B) (Lu and Zhu 2017). There was no significant difference in the level of TKT transcription between the blank control group and the non-targeting group, which suggested that the backbone plasmid and transfection reagent did not affect the transcription of endogenous TKT. The results of lower protein levels in TKT-targeting group suggest that the Cas13X.1 knockdown system can down-regulate expression level of endogenous genes through RNA editing (Fig. 4C). The expression level of TKT protein was also not affected by the backbone plasmid.

Cas13X.1 has low cytotoxicity

The Cas13X.1 knockdown system showed no detectable toxicity in HEK293T cells. There was no difference in cell integrity and intracellular granules between the representative microimages of mCherry targeting and non-targeting in HEK293T cells after transfection, suggesting that the expression of Cas13X.1 system had little effect on cell morphology (Fig 5A). The potential collateral activity of Cas13X.1 system can cause cytotoxicity (Abudayyeh et al.

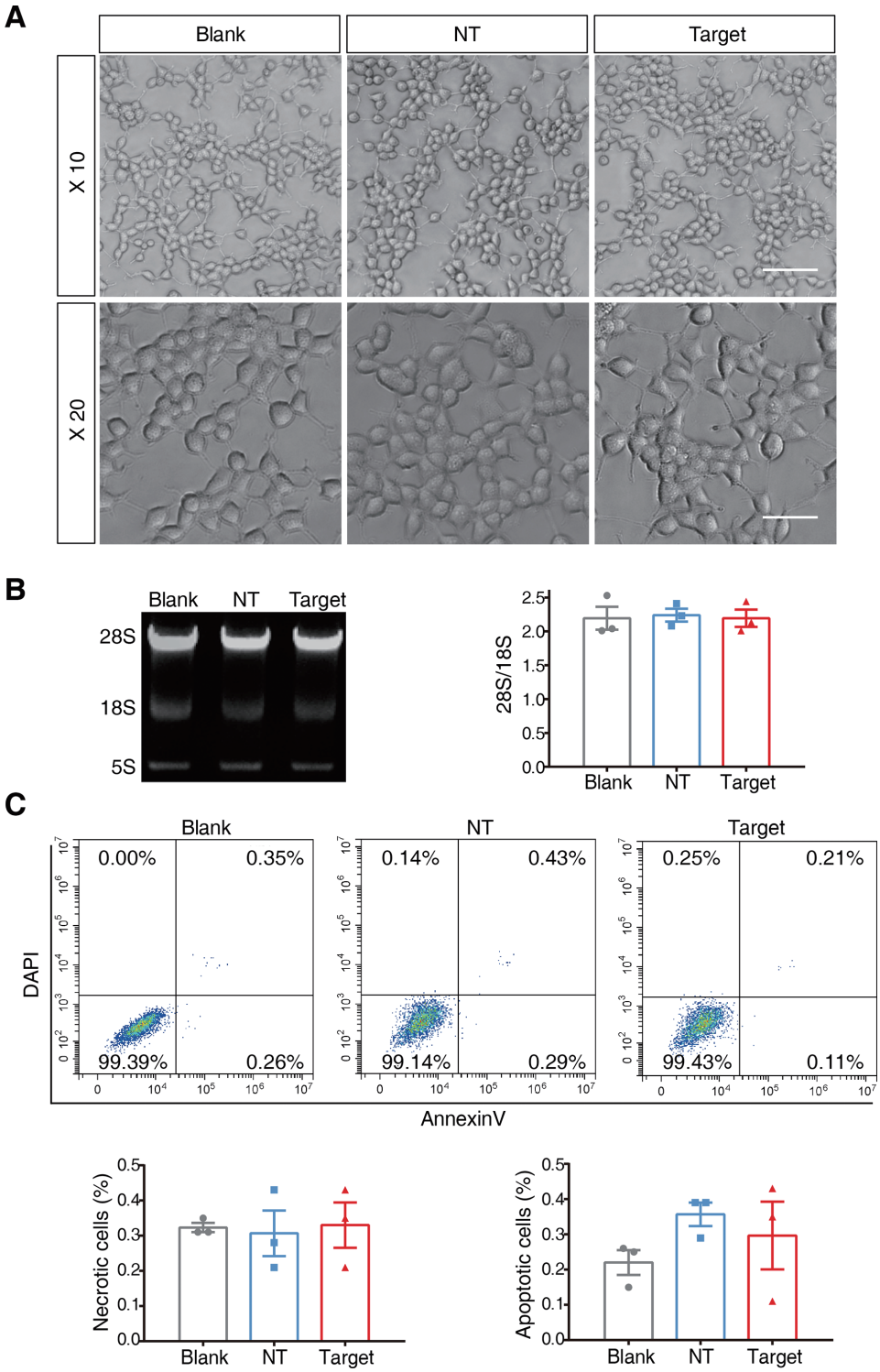

Fig. 5. Cas13X.1 lacks cytotoxicity in mammalian cells.

A. Representative microimages of 293T cells in blank control group (Blank), non-target group (NT) and mCherry-targeting group (Target) at 48 h after transfection. Scale bar of ×10 group = 100 μm, scale bar of × 20 group = 50 μm. B. Ribosomal RNA integrity, measured by ratio of intensity between 28S and 18S ribosomal RNA band, in 293T cells treated with Cas13X.1 for mCherry knockdown. Left is the electrophoretic map of blank control group (Blank), non-target group (NT) and mCherry-targeting group (Target). Right is the intensity ratio of 28S and 18S ribosomal RNA bands in each group. Data are shown as mean ± SEM. n = 3. One-Way ANOVA analysis shows no significant difference in the RNA integrity among three groups. C. Annexin V/DAPI staining of apoptotic or necrotic 293T cell in blank control group (Blank), non-target group (NT) and mCherry-targeting group (Target). Necrosis, AnnexinV+/DAPI+; Apoptosis, AnnexinV+/DAPI−. Data are shown as mean ± SEM. n = 3. One-way analysis of variance (ANOVA) shows no significant difference for apoptosis and necrosis among three groups.

2016). Collateral activity means that the Cas13 effector will activated non-specific RNA nucleases during targeted cleavage of the target sequence, resulting in intracellular non-target RNA degradation (Abudayyeh et al. 2017). Activation of non-specific RNA nucleases of Cas13 effector in mammalian cells will result in detectable changes in RNA size distribution (Rath et al. 2015). Therefore, we used RNA integrity test to evaluate whether the constructed Cas13X.1 system has detectable collateral activity. The results showed no difference in the RNA integrity between targeting and non-targeting conditions from Cas13X.1 knockdown system (Fig. 5B). Apoptosis and proliferation analysis also showed that the Cas13X.1 system had little cytotoxicity (Fig. 5C).

Discussion

The emerging CRISPR-CAS system has accelerated the development of gene editing technology. The optimized CRISPR-Cas effector can accurately edit genes, regulate gene expression and modify epigenetics (Modell et al. 2022). This excellent tool is widely used in anti-cancer treatment and genetic disease correction. As for the usage, programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in T cells are knocked out by Cas9 technique to improve the anti-tumor ability of T cells (Shi et al. 2017; Lu et al. 2020). CRISPR-Cas9 can safely knock out the mutated β-globin gene and restore the expression of fetal γ-globin in human erythrocytes, thus treating sickle cell disease and β-thalassemia (Ye et al. 2016; Antoniani et al. 2018). Other than the Cas9 gene editing technology widely used in clinical therapy, the CRISPR-Cas13 system targeting RNA, as one of the safest and most effective RNA editing systems, also brings new ideas to gene therapy. Type VI CRISPR-Cas effector Cas13 protein can effectively knock down transcripts in mammalian cells under the guidance of gRNA (Abudayyeh et al. 2017). The knockdown efficiency of Cas13 is similar to that of traditional RNAi knocking efficiency, but the off-target efficiency is significantly reduced (Konermann et al. 2018). The Cas13-RNA knockdown system can be used to interfere with RNA in a variety of biological processes (Zeballos and Gaj 2021). For instance, the lentiviral library constructed based on Cas13-RNA system can easily screen the circular RNA which has a significant impact on cell fate (Li et al. 2021). Before this, there was a lack of simple and effective tools to identify functional circular RNA because the circular RNA was basically the same as the linear mRNA sequence except at back-splicing junction (BSJ) sites (Li et al. 2018). In addition to editing circular RNA, Cas13-mediated RNA knockdown system can also inhibit cancer cell proliferation and metastasis by regulating the level of lncRNA-GACAT3 (Zhang et al. 2020). The Cas13-assisted restriction of viral expression and readout (CARVER) established based on the RNA cleavage activity of Cas13 can be used to target the edit of many ssRNA viruses' RNA. This system can effectively reduce the titer of lymphocytic choroid plexus meningitis virus (LCMV), influenza A virus (IAV) and herpetic stomatitis virus (VSV) in human cells. This indicates that Cas13 system has a broad prospect in the field of antivirus (Freije et al. 2019). However, most of the studies on the treatment of cancer with Cas13 are stagnant *in vitro*: Cas13 system down-regulates the target RNA of cancer cell lines to inhibit the proliferation or induce apoptosis of cancer cell lines (Zhao et al. 2018; Che et al. 2020; Fan et al. 2020; Saifullah et al. 2020; Singh and Bhatia 2021). The molecular weight of most Cas13 systems is too large to be transmitted *in vivo* through viral vectors.

We established a target knockdown system based on Cas13X.1 in 293T cells. The constructed targeting knockdown system targets the transcripts of exogenous gene mCherry and cellular endogenous gene TKT, respectively. The result of RNA level detection and protein analysis shows that the constructed Cas13X.1 system has high knockdown efficiency for both endogenous and exogenous genes. TKT is an enzyme closely related to the metabolism of cancer cells, such as liver cancer and lung cancer. Down-regulating the expression of TKT by RNAi can inhibit the proliferation and migration of cancer cells (Zhang et al. 2007; Lu and Zhu 2017). The knockdown efficiency of the recombinant Cas13X.1 plasmid targeting TKT transcripts in cells is approximately 40%, which is equivalent to that of RNAi (Zhang et al. 2007). Moreover, Cas13X.1 system does not affect cell proliferation, apoptosis and RNA integrity. Low cytotoxicity is the basis of subsequent application of Cas13X.1 system in the field of clinical therapy. The Cas13X.1 system we constructed brings new strategies to the gene therapy of cancer.

In conclusion, compared with the CRISPR-based DNA editing strategy, Cas13-based RNA editing events are instantaneous and reversible in nature, which will reduce the risk of long-term editing. Compared with other proteins in the Cas13 system, Cas13X.1 has a smaller molecular weight and a more compact protein structure, which is more suitable for transmission *in vivo* for therapeutic purposes. The Cas13X.1 knockdown system we constructed can be widely used in a variety of tumor target gene therapy and become a powerful tool in clinical treatment. However, the potential collateral activity may hinder the application of the system in clinical treatment. The activation of collateral activity is one of the main reasons for the cytotoxicity of Cas13 system. Although collateral activity was undetectable for Cas13X.1 proteins in 293T cells, a more sensitive method is needed to evaluate whether the editing system activated collateral activity *in vivo* in future therapeutic applications.

Acknowledgments

We are grateful to Dr. Xu Chunlong and Dr. Huo Xiaona, Institute of Neuroscience, State Key Laboratory of Neuroscience, Key Laboratory of Primate Neurobiology, CAS Center for Excellence in Brain Science and

Intelligence Technology, for the gift of backbone plasmid and discussion of the study.

This work was supported by the National Natural Science Foundation of China (82020108021).

Author Contributions

L.L. and W.L. were the primary researchers of this study. J.J. planned the study and wrote the manuscript. L.L., W.L., L.L., J.D. and Q.C. designed and conducted experiments. H.Z. and C.G. performed the statistical analysis. J.S. assisted with the analysis of apoptosis. D.W. assisted with cell experiments. P.L. and M.W. designed experiments and supervised the whole project. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Files

Please find supplementary file(s);
https://doi.org/10.1620/tjem.2023.J011