Highly efficient and safe genome editing by CRISPR-Cas12a using CRISPR RNA with a ribosyl-2′-O-methylated uridinylate-rich 3′-overhang in mouse zygotes

Dae-In Ha1,2, Jeong Mi Lee1,2, Nan-Ee Lee1,2, Daesik Kim1, Jeong-Heon Ko1,2 and Yong-Sam Kim1,2,3

Abstract

The CRISPR-Cas12a system has been developed to harness highly specific genome editing in eukaryotic cells. Given the relatively small sizes of Cas12a genes, the system has been suggested to be most applicable to gene therapy using AAV vector delivery. Previously, we reported that a U-rich crRNA enabled highly efficient genome editing by the CRISPR-Cas12a system in eukaryotic cells. In this study, we introduced methylx modifications at C2 in riboses in the U-rich 3′-overhang of crRNA. When mixed with Cas12a effector proteins, the ribosyl-2′-O-methylated (2-OM) U-rich crRNA enabled improvement of dsDNA digestibility. Moreover, the chemically modified U-rich crRNA achieved very safe and highly specific genome editing in murine zygotes. The engineered CRISPR-Cas12a system is expected to facilitate the generation of various animal models. Moreover, the engineered crRNA was evaluated to further improve a CRISPR genome editing toolset.

Introduction

The clustered regulatory interspaced palindromic repeat (CRISPR) system has “democratized” genome editing technology because of its easy construction, high performance, and versatile applications in eukaryotic systems1–4. Since the development of the CRISPR-Cas9 system, various CRISPR systems have been identified in bacteria and archaea, including CRISPR from Prevotella and Francisella 1 (Cpf1, also known as Cas12a), Cas13, and Cas14 (recently classified as Cas12f); these systems constitute a diverse genome editing toolbox in which each tool has unique utility5–7. The CRISPR genome-editing tool consists of a gene-targeting guide RNA and a Cas endonuclease8. These two components form a ribonucleoprotein (RNP) complex that recognizes target sequences accompanying a protospacer-adjacent motif (PAM), subsequently inducing a double-stranded break (DSB) either inside or outside the protospacer region8.

Although the CRISPR system per se has shown surprisingly high genome editing performance, efforts to further improve the CRISPR system have been made by engineering both Cas nucleases and guide RNAs. Catalytically modified Cas97 and Cas12a8 have been determined to have expanded utility for base editing9–12, epigenetic regulation13,14, transcriptional inhibition (CRISPRi)/activation (CRISPRa)15,16, library screening17, and, recently, prime editing systems9. In addition, sophisticated engineering of Cas variants has enabled high-fidelity18–21 and unrestricted gene targeting10,11. In addition, efforts to engineer guide RNAs have been rigorously undertaken to ameliorate technical and clinical limitations in research and gene therapy.

Plasmid DNA is the preferred material for genome editing in cell lines due to its high stability and
transfection efficiency and to the ease with which its production can be scaled. However, plasmid DNA delivery is undesirable for engineering the genomes of plants and zygotes; rather, RNP is the preferred form of genome editor because of the lack of concern regarding chromosomal integration, its controlled expression and the possibility of specific gene editing\(^{12}\). Moreover, RNP delivery has an additional advantage because chemically modified guide RNAs can be used for efficient genome editing with improved efficiency and target specificity; in addition, regulation of biological toxicity, sensitive and specific molecular imaging, multiplexing, and genome editing flexibility is possible with modified guide RNAs\(^{13}\). Because guide RNAs are amenable to a variety of chemical modifications and mass production, chemically modified guide RNAs can be simply mixed with Cas proteins at determined stoichiometries prior to injection into zygotes\(^{14\sim16}\) or protoplasts\(^{17\sim18}\).

Previously, we reported that a uridylate-rich 3′-overhang in crRNA significantly improved the genome editing efficiency of CRISPR-Cas12a in eukaryotic cells without compromising off-target activity\(^{19}\). The engineered U-rich Cas12a system was explored exclusively in eukaryotic cell lines, and crRNA was transcriptionally produced from DNA templates, indicating that a chemically unmodified, natural form of crRNA was used for all experiments. In this study, we tested several types of chemical modifications in the polyuridylated 3′-overhang of crRNA and found that 2′-O-methylation (2-OM) in the ribose ring enabled genome editing of mouse zygotes with further improved genome editing efficiency and specificity. Furthermore, the 2-OM U-rich crRNA showed significantly reduced cellular toxicity when injected into zygotes with a microinjector. The optimized form of crRNA is expected to facilitate manipulation of zygotes and, subsequently, generation of animal models in a highly safe and efficient fashion.

Materials and methods
Preparation of vector constructs for in vitro cleavage assay
The human DNMT1 gene was ampliﬁed through polymerase chain reaction (PCR) using Pfu polymerase (Biofact, PD302-50h). Primers were screened with Primer3web version 4.1.0 and synthesized by Bioneer Corporation (Korea). The amplicon and primer sequences for DNMT1 are noted in Fig. S1. The PCR products were puriﬁed with a HiGene Gel & PCR Puriﬁcation system (Biofact, GP104–100), and cloned into an All in One PCR Cloning vector (Biofact, VT201-020). The cloned vector constructs were extracted using an NucleoBond Xtra Midi kit (Machery-Nagel, 740410.50).

Preparation of gRNA and Cpf1 protein
Each gRNA was designed to target the DNMT1 or Trp 53 gene with a TTTV PAM (Fig. S1). Each gRNA was synthesized by Integrated DNA Technologies, Inc. For an in vitro cleavage assay, recombinant Acidaminococcus sp. Cpf1 (AsCpf1) was prepared. Codon-optimized Cpf1 CDS from Acidaminococcus sp. was ligated into a pET-28a(+) plasmid vector (Addgene, 69864-3), and the vector was cloned to transform BL21(DE3) E.coli cells (Thermo Fisher, EC0114). The transformed colony was cultured at 37 °C in LB broth in the presence of 50 µg/ml kanamycin following preculture for 4 h in the same medium. When the optical density reached ca. 0.6, isopropyl β-D-thiogalactopyranoside (IPTG) was added at 30 mM, and culture proceeded at 30 °C overnight. After induction, E.coli cells were harvested by centrifugation at 3000 rpm for 30 min. The pellets were suspended in phosphate-buffered saline (PBS) with an EDTA-free protease inhibitor cocktail and lysed in a sonicator for 30 min at 30% pulse power with 2-s intervals (Sonic Materials, VCX-500). The lysates were cleared by centrifugation at 13,000 rpm for 30 min. After being filtered through a 0.22 µm filter unit, the supernatant was loaded onto a Ni\(^{2+}\)-affinity column using an FPLC Purification System (ÄKTAA Purifier, GE Healthcare). The concentrations of the purified proteins were determined from Coomassie blue staining of the bands on SDS-PAGE gels using bovine serum albumin (BSA) as a calibrator.

In vitro digestion assay
Target-carrying plasmid vectors were digested in the presence of AsCpf1 protein (2.5–5 ng/µl) and 10 pM gRNA in 1X NEB 3.1 buffer. After digestion at 37 °C for 1 h, each sample was loaded onto a 1% agarose gel containing 5% ethidium bromide (EtBr). The band intensities were measured by illumination with ultraviolet (UV) light to calculate the levels of DNA cleavage.

In vivo experiment
C57BL/6J mice were cared for and used for preparation of fertilized eggs. The procedures for the use and care of mice were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), KIRIB. All mice were bred in an isolated facility that was specific pathogen-free with a constant temperature of 24°C, a humidity of 40%, and light cycles of 12 h. Forty-eight hours before human chronic gonadotropin (hCG) (Sigma-Aldrich, CG100) injection, 5 IU of pregnant mare serum gonadotropin (PMSG) (Daesung Microbiological Labs, A71001) was intraperitoneally injected into 5-week-old C57BL/6J female mice. Two days after mating, zygotes were collected from the ampullae of the oviducts, and the cumulus cells surrounding the zygotes were degraded by incubation in M2 medium (Sigma-Aldrich, M7167) containing 3 mg/ml hyaluronidase (Sigma-Aldrich, H3506) at room temperature for 5 min. After several washes in M2 and KSOM media (Merck, MR-121-D), the zygotes were
cultured in KSOM medium for 5–6 h to select fertilized eggs. Fertilized eggs that formed polar bodies were used for RNP delivery.

**RNP delivery via electroporation**

Fertilized eggs were suspended in Opti-MEM (Gibco, 31985-070) in the presence of RNP and placed on microscope slides with platinum-plated electrodes (NEPA GENE, CUY505P5). RNP was delivered into zygotes using a NEPA 21 Super Electroporator (NEPA GENE) according to the recommended protocol. Four days after incubation of the eggs in KSOM medium at 37 °C, cells at the morula stage were harvested for preparation of genomic DNA.

**Deep sequencing analysis**

Morula cells were suspended in PCR tubes with blastocyst lysis buffer (BLB) containing 60 µg/ml yeast tRNA (Invitrogen, AM7119) and 125 µg/ml proteinase K (Qiagen, 1114886). To prepare crude genomic DNA, the PCR tubes were placed in a thermal cycler, incubated at 95 °C for 10 min and 56 °C for 20 min and then stored at 4 °C. Using a REPLI-g Mini Kit (Qiagen, 150025), the crude genomic DNA was PCR-amplified using KAPA HiFi HotStart DNA Polymerase (Roche) according to the manufacturer’s instructions. The resulting PCR amplicons carrying Illumina TruSeq HT dual indexes were subjected to 150-bp paired-end sequencing using an Illumina iSeq 100. The indel frequencies were calculated by MAUND, which is available at [https://github.com/ibs-cge/maund](https://github.com/ibs-cge/maund).

**Selection of candidate off-target sites**

To compare the specificity of each gRNA, potential off-target sites associated with a Trp53 on-target sequence were screened with the Cas-Offinder program ([http://www.rgenome.net/cas-offinder](http://www.rgenome.net/cas-offinder)) with criteria of fewer than two bulges and three mismatches.

**Statistical analysis**

Statistical tests of indel efficiency were performed in SigmaPlot software using a two-tailed Student’s t test. P-values < 0.05 were considered to indicate significance and are presented in the Legends section.

**Results**

** Improvement of DNA cleavage activity of Cas12a via U-rich CRISPR RNA with 2′-O-methyl ribose**

A variety of chemical modifications have been extensively explored for Cas9 and Cas12a in the directed repeat and spacer regions of the guide RNAs. However, no experimental efforts have been made to investigate the effects of chemical modifications in the U-rich 3′-overhang region in crRNA on the functionality of the Cas12a system. Based on previous reports that modifications in bases or ribose sugars of guide RNAs improve stability and editing efficiency, we focused on chemical modifications of bases and ribose sugars in the U-rich regions (Fig. 1a). The base modifications into nitrogen (N5) were made at C5 in the pyrimidine ring to render a pseudouridine base (ψ). The hydrogen atom attached to C5 was also replaced with a methoxyl group, thereby producing 5′-methoxyuridine (moU). Alternatively, the

---

**Fig. 1** Scheme for the controllable CRISPR-Cas12a system with a 3′-polyuridinylated (U) crRNA and chemical modifications at the U-rich 3′-overhang. **a** The U-rich crRNA Cas12a system was compared to a tuner system in which the polyuridinylated 3′-overhang acts as an indel knob. By modulating the number of Us, we can regulate the indel efficiency of the CRISPR-Cas12a system in eukaryotic cells. We aim to further enhance the Cas12a system by introducing chemical modifications in the U-rich 3′-overhang. **b** Chemical modifications in crRNA were implemented in both bases and ribose sugars. The uracil base was substituted with pseudouridine (ψ) or 5′-methoxyuridine (moU). Ribose was chemically modified at C2 with a fluoro or a methyl group. The modified atom is noted in red letters.
hydroxyl group at C2 in ribose was modified with a fluorine or methyl group, hereafter designated F or OM, respectively (Fig. 1b).

We employed an in vitro digestion assay to assess guide RNA efficiency, in which plasmid DNA vectors harboring a 20-nt DNMT1 protospacer sequence were used as digestion substrates for the Cas12a-crRNA RNP complex (Fig. 2a). Under limited-digestion conditions, a subset of supercoiled plasmid vectors was transformed through an open circular state into linearized DNA. The ratio of linear to supercoiled forms and/or linear to open circular forms was regarded as a measure of crRNA efficiency. The incubation time and concentrations of RNP and substrate plasmid DNA were predetermined in this experiment. In line with the findings of the previous study, the U-rich crRNA significantly increased the DNA digestion activity in vitro (lane 4 vs lane 5). However, base modification of the crRNAs (ψ and moU) did not yield any increased cleavage activity (Fig. 2b). Rather, it compromised the modulatory effects of the U-rich 3′-overhangs. The F U-rich crRNA was also ineffective in modulating crRNA efficiency. In contrast, the OM U-rich crRNA and the unmodified U-rich crRNA showed comparable levels of digestion efficiency. Thus, we decided to test OM-crRNA under altered conditions. We increased the input of AsCas12a in the presence of equal amounts of crRNA and plasmid DNA but used a shortened incubation time (Fig. 2c). As the input of AsCas12a increased, a difference in cleavage activity became evident between unmodified and OM U-rich crRNA (p < 0.05). Notably, OM U-rich crRNA showed markedly higher efficiency than canonical crRNA (Fig. 2d). Taken together, these findings indicate that ribosyl-2′-O-methylation in the 3′-overhang of crRNA further potentiated the efficiency of Cas12a in vitro.

Low toxicity and improved efficiency of OM U-rich crRNA for genome editing in zygotes

In addition to efficiency, toxicity is a critical issue in genome editing in zygotes because the number of zygotes available is limited. Although modification of the 5′-terminal triphosphates of guide RNAs into hydroxyl groups has been shown to improve cellular viability26, less is known about the effects of OM modifications, particularly in the U-rich 3′-overhang, on the viability of zygotes. To investigate the cellular toxicity of OM U-rich crRNA, we injected AsCas12a with either canonical or modified crRNAs into zygotes and monitored viability at the morula stage 4 days after injection (Fig. 3a). For this, we tested the toxicity of RNP preparations in zygotes in

Fig. 2 Determination of the highly efficient form of a chemical modification in guide RNA through an in vitro digestion assay. a Scheme for an experimental procedure to assess the efficiency of various guide RNAs. A pUC19 vector harboring the DNMT1 target sequence was digested with RNP. The crRNA was varied with sequence and/or various chemical modifications. Digested samples were run on agarose gels, on which supercoiled, linear, and open circular forms of digested vectors were separated with different mobilities. The efficiency of the crRNAs was assessed by comparing the band intensities. b crRNAs with different chemical modifications (F, OM, omU, and ψ) were compared with respect to dsDNA digestibility. The guide RNA efficiency can be calculated from the band intensity ratio of an open-circular over a linear form. M refers to a 1-kb molecular ladder (lane 1). c–d The increase in Cas12a concentration caused by OM U-rich crRNA improved DNA digestibility. Increased concentrations of Cas12a promoted the digestion rate of supercoiled plasmid vectors through open-circular to linear forms. d In the presence of Cas12a (5.0 ng/µl) and 10 pM crRNA, compared to canonical and unmodified U-rich crRNA, OM U-rich crRNA mediated significantly better digestion efficiency.
five independent experiments. The first three experiments (experiment 1 to experiment 3) yielded, overall, low survival rates for all the types of crRNAs. The overall low survival rates likely stemmed from the long durations of zygote manipulation due to the large number of zygotes tested ($n = 50$, 66, 62 for each type crRNA). Nonetheless, OM crRNA enabled high-viability genome editing in zygotes (Fig. 3b). To shorten the duration of zygote manipulation, we decreased the number of zygotes to 22 and 24 for experiments 4 and 5, respectively. As a result, the overall survival rate increased significantly to more than 60%. In these two experimental batches, OM U-rich crRNA was responsible for the improved viability of zygotes (Fig. 3b, Table S1). To investigate whether OM U-rich crRNA shows improved genome editing efficiency in zygotes as measured by a DNA cleavage assay in vitro. Because experiments 4 and 5 showed convincingly high cell viability, we investigated indel efficiency in morula cells of the two experiments. The viable morula-stage cells were pooled, and genomic DNA was prepared. Deep sequencing analysis was performed with PCR-amplified DNA products, with Trp53 as the target. As compiled in Table 1, the U-rich crRNA significantly improved indel efficiency ($0.296$ to $0.783$ and $0.173$ to $0.979$ for experiments 4 and 5, respectively). OM crRNA either retained or further improved the indel efficiency of the U-rich crRNA-AsCas12a RNP complex. Notably, in experiment 4, all 18 surviving morula cells showed biallelic indel mutations (Table 2, Fig. S2). In experiment 5, OM U-rich crRNA also showed a better indel efficiency than a canonical crRNA. Although the OM crRNA was slightly less efficient than an unmodified U-rich crRNA, the overall yield of mutations was higher than that of the
over, the mismatch resided in an area with a high motif, few indel mutations were found for the three sequencing data and found that, apart from the poly-G in a targeted manner. We searched for potential off-target investigate this possibility, we measured off-target effects in silico sequence similarities should be avoided through analysis. Then, highly specific and efficient genome engineering can be achieved via 3′-U-rich crRNA with ribosyl-2′-O-methylation in the CRISPR-Cas12a system.

Discussions

It has been more than four years since Zhang and colleagues first introduced the CRISPR-Cas12a system; since then Cas12a has been proposed to provide multiple advantages over Cas9 for genome editing purposes. First, Cas12a has a smaller gene size than most Cas9 analogs, which is a critical element for viral transduction of genes. Moreover, the guide RNA for Cas12a (crRNA) is less than half the size of the sgRNA for Cas9, which makes it much easier to synthesize the crRNA with high purity. Additionally, credible research articles suggest that Cpf1 has higher specificity than Cas9. These traits are critical for clinical applications of programmable nucleases. Nonetheless, the adoption of Cas12a has been less enthusiastic than expected. The paucity of research articles searchable in PubMed reflects this notion; indeed, almost 12,000 CRISPR-Cas9-related research articles were deposited in PubMed in the 4 years after the first publication in Science in January 2013. In contrast, only 400 or so papers concerning Cas12a were published during the same period of time. Moreover, many more studies on Cas9 than on Cas12a have been published during the same period (October 2015–present). We propose that this startlingly low adoption of Cas12a stems mainly, if not exclusively,

Table 1 Increased indel efficiency of AsCas12a mediated by U-rich crRNA with OM modification in zygotes.

| Experiment | Trp53 (Experiment #4) | Trp53 (Experiment #5) | Wnk1 | Ahrr |
|------------|------------------------|------------------------|------|------|
|            | Viability | Indel (%) | Viability | Indel (%) | Viability | Indel (%) | Viability | Indel (%) |
| Canonical crRNA | 16       | 72.73 | 29.24 | 15       | 62.50 | 17.26 | 5 | 30.00 | 77.15 |
| U-rich crRNA   | 14       | 63.64 | 77.96 | 17       | 70.83 | 97.88 | 5 | 79.67 | 14 |
| OM U-rich crRNA | 18       | 81.82 | 99.62 | 22       | 91.67 | 81.64 | 10 | 50.00 | 84.38 |

unmodified U-rich crRNA considering the higher viability. Because the increased indel efficiency of OM U-rich crRNA was similarly observed for the Wnk1 and Ahrr genes, we conclude that OM U-rich crRNA facilitates the generation of genome-engineered mouse models with increased safety and efficiency. Further study is needed to determine whether the improved functionality of the engineered crRNA is applicable to zygotes of animals other than mice.

Preserved high specificity of Cas12a by OM U-rich crRNA

Despite the improved indel efficiency and mitigated cellular toxicity of OM U-rich crRNA, there remained a concern that the engineered guide RNA might impair the innately high specificity of Cas12a, thereby compromising the utility of the engineered CRISPR–Cas12a system. To investigate this possibility, we measured off-target effects in a targeted manner. We searched for potential off-target sites for Trp53 using the Cas-OFFinder program with criteria of fewer than three mismatches and two bulges. We screened five potential sites (Table S4) and performed deep sequencing analysis using genomic DNA prepared from the morula-stage cells of the previous experiments 4 and 5.

As shown in Table 2, OM U-rich crRNA did not significantly affect the off-target activity for four (OF1, OF2, OF3, and OF5) of the five potential off-target sites (Table 2). It appeared that OF1 showed a high indel frequency independent of the type of crRNA. However, we found that it originated from sequencing errors because OF1 has a polyguanylated sequence immediately downstream of the protospacer sequence. We manually investigated the sequencing data and found that, apart from the poly-G motif, few indel mutations were found for the three crRNAs tested. One intriguing result was observed for OF4, which carried a sequence almost identical to that of the target site with a single nucleotide mismatch. Moreover, the mismatch resided in an area with a high mismatch tolerance in the CRISPR-Cas12a system. For OF4, compared to canonical and unmodified U-rich crRNA, OM crRNA increased the level of indel mutations in one experimental batch (experiment 5). We hypothesize that the increased indel frequency may have originated from the improved efficiency of the modified crRNA. It is important to mention that a more careful identification of potential off-target sites is necessary, and accordingly, targets with off-target sites showing high sequence similarities should be avoided through in silico analysis. Then, highly specific and efficient genome engineering can be achieved via 3′-U-rich crRNA with ribosyl-2′-O-methylation in the CRISPR-Cas12a system.

Discussion

It has been more than four years since Zhang and colleagues first introduced the CRISPR-Cas12a system; since then Cas12a has been proposed to provide multiple advantages over Cas9 for genome editing purposes. First, Cas12a has a smaller gene size than most Cas9 analogs, which is a critical element for viral transduction of genes. Moreover, the guide RNA for Cas12a (crRNA) is less than half the size of the sgRNA for Cas9, which makes it much easier to synthesize the crRNA with high purity. Additionally, credible research articles suggest that Cpf1 has higher specificity than Cas9. These traits are critical for clinical applications of programmable nucleases. Nonetheless, the adoption of Cas12a has been less enthusiastic than expected. The paucity of research articles searchable in PubMed reflects this notion; indeed, almost 12,000 CRISPR-Cas9-related research articles were deposited in PubMed in the 4 years after the first publication in Science in January 2013. In contrast, only 400 or so papers concerning Cas12a were published during the same period of time. Moreover, many more studies on Cas9 than on Cas12a have been published during the same period (October 2015–present). We propose that this startlingly low adoption of Cas12a stems mainly, if not exclusively,
from its low efficiency compared with that of Cas9. Thus, improving the efficiency of Cas12a is a critical factor for making the best use of the inherent advantages of Cas12a over Cas9. Unlike the guide RNA for Cas9, the crRNA for Cpf1 has a 5′-oriented direct repeat sequence and a target-complementary sequence located in the 3′-region. These features suggest that there is room for engineering of the crRNA at the 3′-end. Previously, we observed that 3′-terminal addition of multiple uridine bases to the crRNA significantly improved Cas12a indel efficiency. In this study, we further engineered Cas12a crRNA by modifying the U-rich 3′-overhang with 2′-O-methylation. This modification improved the safety and efficiency of genome engineering with the CRISPR-Cas12a system and is expected to facilitate the generation of various animal models. Moreover, the engineered crRNA was evaluated to add a powerful tool to the genome editing toolbox.

Ribonucleoprotein (RNP) complexes have been suggested to increase the specificity of gene editing26, and guide RNA modifications can have synergistic effects on specificity. 3′-Phosphorothioate linkage, either alone or together with 2′-O-ribose modifications, confers Cas9 with significantly improved target specificity21. It is interesting to note that an RNA-DNA hybrid guide exhibits enhanced specificity37. In particular, highly specific editing has been achieved by bridged nucleic acids (BNAs) in which the 2′-O and 4′-C atoms of the ribose are joined through a methylene bridge. These BNAs include N-methyl-substituted 2′,4′-bridged

| Table 2 Preserved high specificity of AsCas12a with OM U-rich crRNA. |
|---------------------------------------------------------------|
| Site | crRNA Type | Read Counts | Indel Frequency (%) | Read Counts | Indel Frequency (%) |
|      |            | #4          | #5             |            | #4                  |
|      |            | WT | Indel | Total | Indel | Total | WT | Indel | Total |
| On Target | none | 9522 | 24 | 9546 | 0.25 | 9440 | 30 | 9470 | 0.32 |
|          | canonical | 6516 | 2692 | 9208 | 29.24 | 7852 | 1638 | 9490 | 17.26 |
|          | U-rich | 2073 | 7334 | 9407 | 77.96 | 180 | 8303 | 8483 | 97.88 |
|          | OM U-rich | 37 | 9772 | 9809 | 99.62 | 1493 | 6640 | 8133 | 81.64 |
| OF1 | none | 541 | 276 | 817 | 33.78 | 256 | 77 | 333 | 23.12 |
|      | canonical | 555 | 270 | 825 | 32.73 | 593 | 299 | 892 | 33.52 |
|      | U-rich | 657 | 266 | 923 | 28.82 | 421 | 168 | 589 | 28.52 |
|      | OM U-rich | 344 | 186 | 530 | 35.09 | 146 | 55 | 201 | 27.36 |
| OF2 | none | 10503 | 0 | 10503 | 0 | 9337 | 0 | 9337 | 0 |
|      | canonical | 10562 | 0 | 10562 | 0 | 10433 | 2 | 10435 | 0.02 |
|      | U-rich | 11181 | 0 | 11181 | 0 | 9467 | 3 | 9470 | 0.03 |
|      | OM U-rich | 9104 | 3 | 9107 | 0.03 | 8945 | 0 | 8945 | 0 |
| OF3 | none | 11337 | 7 | 11144 | 0.06 | 9224 | 0 | 9224 | 0 |
|      | canonical | 11173 | 0 | 11173 | 0 | 10844 | 0 | 10844 | 0 |
|      | U-rich | 11337 | 14 | 11351 | 0.12 | 10818 | 0 | 10818 | 0 |
|      | OM U-rich | 11041 | 0 | 11041 | 0 | 8487 | 0 | 8487 | 0 |
| OF4 | none | 9676 | 0 | 9676 | 0 | 10360 | 6 | 10366 | 0.06 |
|      | canonical | 5148 | 5456 | 10604 | 51.45 | 6595 | 3482 | 10077 | 34.55 |
|      | U-rich | 5304 | 5517 | 10821 | 50.98 | 6022 | 3571 | 9593 | 37.23 |
|      | OM U-rich | 5758 | 3748 | 9506 | 39.43 | 3070 | 5695 | 8765 | 64.97 |
| OF5 | none | 11740 | 0 | 11740 | 0 | 10100 | 8 | 10108 | 0.08 |
|      | canonical | 7659 | 0 | 7659 | 0 | 11346 | 0 | 11346 | 0 |
|      | U-rich | 11762 | 0 | 11762 | 0 | 10793 | 0 | 10793 | 0 |
|      | OM U-rich | 10577 | 0 | 10577 | 0 | 8702 | 0 | 8702 | 0 |

*OF1 carries a polyguanidylated sequence, and most reads had sequencing errors in the poly-G region. Manual reading confirmed the absence of reads with indel mutations outside the polyguanidylated region. The OF1 sequence is "TTTCCCTCATGCAGATAAGATGGGGGGGGGGG."
ribose (BNA<sup>NC</sup>[N-Me]) and S-constrained ethyl ribose<sup>22</sup>. OM U-rich crRNA was responsible for further improvement of the genome editing efficiency of Cas12a, but the improved efficiency led to undesirable off-target effects at targets with high sequence similarity that were tolerant of single mismatches (Table 2). If this drawback is addressed, the engineered Cas12a system will yield unsurpassed high genome editing performance, as is required in clinical settings. Further applications of chemical modifications that are validated to improve specificity, including BNAs, will help achieve more advanced genome editing in terms of specificity and safety.

Acknowledgements

This work was supported by grants through the KRIBB Research Initiative Program and the Bio & Medical Technology Development Program of the Ministry of Science and ICT (NRF-2016M3A9B6K03343) and through the R&D Convergence Program of the National Research Council of Science & Technology (CAP-15-03-KRIBB).

Author details

1 Genome Editing Research Center, KRIBB, Daejeon 34141, Republic of Korea. 2 KRIBB School of Bioscience, Korea University of Science and Technology (UST), 34141 Daejeon, Republic of Korea. 3 CenKore, Daejeon 34141, Republic of Korea.

Author contributions

Y.-S.K. and J.-H. conceived the study and designed the experiments. D.-H., J.-M.L., and N.-E.L. performed most of the experiments. D.K. performed the genome-wide off-target analysis. Y.-S.K. wrote the manuscript. All authors approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Publisher's note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary information

accompanies this paper at https://doi.org/10.1038/s12276-020-00521-7.

Received: 13 May 2020 Revised: 4 September 2020 Accepted: 15 September 2020.

Published online: 9 November 2020

References

1. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823 (2013).
2. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816–821 (2012).
3. Jinek, M. et al. RNA-programmed genome editing in human cells. Elife 2, e00471 (2013).
4. Mali, P. et al. RNA-guided human genome engineering via Cas9. Science 339, 823–826 (2013).
5. Zetsche, B. et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas systems. Cell 163, 759–771 (2015).
6. Shmakov, S. et al. Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. Mol. Cell 60, 385–397 (2015).
7. Abdulayev, O. O. et al. C2c2 is a single-component, programmable RNA-guided RNA-targeting CRISPR effector. Science 360, 385–397 (2018).
8. Adli, M. The CRISPR tool kit for genome editing and beyond. Nat. Commun. 9, 1911 (2018).
9. Anzalone, A. V. et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576, 149–157 (2019).
10. Hu, J. H. et al. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature 556, 57–63 (2018).
11. Topf, E. et al. Improved LbCas12a variants with altered PAM specificities further broaden the genome targeting range of Cas12a nucleases. Nucleic Acids Res. 48, 3732–3733 (2020).
12. Moon, S. B. & Kim, D. Y. Koo, J. H. & Kim, Y. S. Recent advances in the CRISPR genome editing tool set. Exp. Mol. Med. 51, 1–11 (2019).
13. Moon, S. B., Kim, D. Y., Koo, J. H., Kim, J. S. & Kim, Y. S. Improving CRISPR genome editing by engineering guide RNAs. Trends Biotechnol. 37, 870–881 (2019).
14. Chen, S., Lee, B., Lee, A. Y., Modzelewski, A. J. & He, L. Highly efficient mouse genome editing by CRISPR ribonuclease electroporation of yoygotes. J. Biol. Chem. 291, 14457–14467 (2016).
15. Modzelewski, A. J. et al. Efficient mouse genome engineering by CRISPR-Cas9 system. Nat. Protoc. 13, 1253–1274 (2018).
16. Dumeau, C. E. et al. Introducing gene deletions by mouse zygote electro-poration of Cas12a/Cpf1. Transgenic Res. 28, 525–533 (2019).
17. Liu, X. W. et al. Lipid-mediated delivery of DNA-free CRISPR-Cas9 ribonuclease proteins into plant cells. Plant Cell Rep. 39, 245–257 (2020).
18. Park, J. & Choe, S. DNA-free genome editing with preassembled CRISPR/Cas9 ribonuclease proteins in plants. Transgenic Res. 28, 61–64 (2019).
19. Bin Moon, S. et al. Highly efficient genome editing by CRISPR-Cpf1 using CRISPR RNA with a uridylate-rich 3’-overhang. Nat. Commun. 9, 3651 (2018).
20. Andries, O. et al. N(1)-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. J. Control Release 217, 337–344 (2015).
21. Basila, M., Kelley, M. L. & Smith, A. V. Minimal 2’-O-methyl phosphorothioate linkage modification pattern of synthetic guide RNAs for increased stability and efficient CRISPR-Cas9 gene editing avoiding cellular toxicity. Plos ONE 12, e0188593 (2017).
22. Cromwell, C. R. et al. Incorporation of bridged nucleic acids into CRISPR RNAs improves Cas9 endonuclease specificity. Nat. Commun. 9, 1448 (2018).
23. Hendel, A. et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nat. Biotechnol. 33, 985–989 (2015).
24. Kariko, K., Muramatsu, H., Keller, J. M. & Weissman, D. Increased erythropoiesis in human cells with chemical modification of synthetic guide RNAs for increased stability and efficient CRISPR-Cas9 gene editing avoiding cellular toxicity. Plos ONE 12, e0188593 (2017).
25. Kaya, E. et al. A bacterial Argonaut with noncanonical guide RNA specificity. Proc. Natl Acad. Sci. USA 113, 4057–4062 (2016).
26. Kim, S. et al. CRISPR RNAs trigger innate immune responses in human cells. Genome Res. https://doi.org/10.1101/gr.231963.118 (2017).
27. Li, B., Luo, X. & Dong, Y. Effects of chemically modified messenger RNA on protein expression. Bioconjug Chem. 27, 849–853 (2016).
28. Li, B. et al. Synthetic oligonucleotides inhibit CRISPR-Cpf1-mediated genome editing. Cell Rep. 23, 3262–3272 e3263 (2018).
29. Li, B. et al. Engineering CRISPR-Cpf1 CRNAs and mRNAs to maximize genome editing efficiency. Nat. Biomed. Eng. 1, https://doi.org/10.1038/s41551-017-0066 (2017).
30. Mir, A. et al. Heavily and fully modified RNAs guide efficient SpyCas9-mediated genome editing. Nat. Commun. 9, 2641 (2018).
31. Rahdar, M. et al. Synthetic CRISPR RNA-Cas9-guided genome editing in human cells. Proc. Natl Acad. Sci. USA 112, 6710–6715 (2015).
32. Ryan, D. E. et al. Improving CRISPR-Cas specificity with chemical modifications in single-guide RNAs. Nucleic Acids Res. 46, 792–803 (2018).
33. Yin, H. et al. Structure-guided chemical modification of guide RNA enables potent non-viral in vivo genome editing. Nat. Biotechnol. 35, 1179–1187 (2017).
34. Kim, D. et al. Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. Nat. Biotechnol. 34, 863–868 (2016).
35. Kleinester, B. P. et al. Genome-wide specificities of CRISPR-Cas Cpf1 nucleases in human cells. Nat. Biotechnol. 34, 869–874 (2016).
36. Wang, Y. et al. Specificity profiling of CRISPR system reveals greatly enhanced off-target gene editing. Sci. Rep. 10, 2269 (2020).
37. Yin, H. et al. Partial DNA-guided Csa9 enables genome editing with reduced off-target activity. Nat Chem Biol 14, 311–316, https://doi.org/10.1016/j.chembi.2559 (2018).