

Construction and optimization of a base editor based on the MS2 system

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Abstract

Background: Catalytic defect Cas9-cytosine deaminase fusion is widely used in base editing. The Multiple copy numbers of the MS2 binding site (MBS) can recruit multiple MS2 coat proteins (MCPs), which are usually applied to amplify signals. Our study aimed to apply the MS2 signal amplification system to the base editing system in order to achieve simultaneous mutations of multiple bases at the target genome site.

Methods: Multiple copy numbers of the MS2 were ligated to the 3'-end of sgRNA, and MCP was fused to the 5'-end of cytosine deaminases. The MS2 was recognized by MCP to recruit cytosine deaminase for base substitutions (C-T) at the target site. Different Cas9 variants, different cytosine deaminases and different copy numbers of MS2 were tested in this system, and the different versions of base editors were compared by editing efficiency and window.

Results: In this study, dCas9, nCas9 (D10A) and nCas9 (H840A) were used. Among these 3 Cas9 variants, dCas9 exhibited higher base mutation efficiency. Two cytosine deaminases were then applied and the efficiency of rAPOBEC1 deaminase was found to be higher than AID. We also increased the copy numbers of MS2 linked to sgRNA from 2 to 12. Disappointingly, the sgRNA-12x MS2 did not improve the editing efficiency or increase the editing window.

Conclusion: An optimal version of base editor based on the MS2 system, MS2-BE-rAPOBEC1 (sgRNA-2x MS2, MCP-rAPOBEC1 and dCas9), was obtained. This tool can simultaneously mutate multiple bases at the target site, providing a new approach for the study of genome functions.

KEYWORDS

base editing, functional screening, MS2-MCP, simultaneously multiple mutagenesis

1 | INTRODUCTION

When CRISPR/Cas9 is used for genome manipulation, a DNA double strand break (DSB) can result, which causes DNA damage. In order to reduce the damage, a CRISPR-based editing strategy, base editor (BE), has been developed that generates programmed base changes

without forming DSBs.^{1,2} BE mainly consists of a Cas9 variant and a deaminase. With cytidine deaminase, the target C will be converted to U and the base pair mismatch of uracil-guanine can be misread, resulting in a C-to-T or G-to-A conversion.³ The primary base editor BE1 consists of rat deaminase APOBEC1 (rAPOBEC1) fused to inactivated Cas9 (dCas9).¹ An optimized version, BE2, results from

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a fusion of uracil DNA glycosylase inhibitor (UGI) to the C-terminus of BE1. The UGI can increase the base editing efficiency by inhibiting uracil N-glycosylase (UNG) to decrease the uridine excision and the base excision repair (BER) pathway.¹ A further improved version, BE3, which uses nickase Cas9 (D10A) instead of dCas9, has significantly improved base editing efficiency, giving fewer indels and by-products.^{1,4} Subsequently, a series of BEs with different deaminases have been developed to increasing fidelity, efficiency or editing scope and decrease indels and byproducts.⁴ However, most of these BEs do not have a wide enough window to use for studying regulatory elements.

The MS2 system (MS2-MCP) is usually applied to molecular imaging in cells to amplify signals. The coat protein of the MS2 bacteriophage (MCP) specifically binds to the MS2 binding site (MBS) which contains 19 nucleotides.^{5,6} Taking advantage of this feature, fusion of multiple copy numbers of MS2 to a particular RNA molecule can result in the recruitment of MCP coexpressed with fluorescence proteins, and the RNA molecule can be detected by fluorescence microscopy in living cells.^{7,8} This approach has been successfully applied to studying transcription and translation of single mRNAs in mammalian cells.⁹⁻¹² The MS2-MCP system has also been used in the CRISPR system to image DNA molecules,^{13,14} and the MS2 system fused with a truncated activation-induced cytidine deaminase (AID) (CRISPR-X) without UGI has been shown to cause a large variety of transitions from cytidine with low efficiency.¹⁵ However, the low efficiency of CRISPR-X and the variety of mutations makes the editing results uncontrollable, restricting their applications.

Due to the low efficiency and narrow editing window, mostly within the protospacer, of many editing tools, they are of limited use in functional studies. In this study, we applied the MS2 system to a base editing system and obtained an optimal version of base editor MS2-BE-rAPOBEC1. This system can efficiently achieve simultaneous mutations of multiple bases between -34 and -2 nt relative to PAM at the target genome site and provides a new approach to the study of genome functions.

2 | MATERIALS AND METHODS

2.1 | Plasmid construction

To construct pST1374-MCP-rAPOBEC1-UGI-GB1 (MCP-rAPOBEC1) and pST1374-MCP-AID-UGI-GB1 (MCP-AID), the sequences of UGI-GB1 and AID were synthesized (Genewiz) and cloned into the pUC57 vector. The rAPOBEC1 was amplified by PCR from BE3 (Addgene, 73021), as reported,¹ and the MCP was amplified by PCR from lenti MS2-P65-HSF1_Hygro (Addgene, 61426).¹⁶ Two mutations (D10A and H840A) were introduced into pST1374-NLS-flag-linker-Cas9 (Addgene, 44758). We then fused MCP to the 5'-end of rAPOBEC1/AID in the pST1374 vector. We amplified 2x MS2 and 12x MS2 from pUC57-12x MS2 synthesized in Genewiz and assembled them into U6 vectors to obtain U6-sgRNA-2x MS2-puro or U6-sgRNA-12x MS2-puro backbones.

Then oligoes of sgRNAs were synthesized, annealed and cloned into the backbones. All plasmid sequences were confirmed by Sanger sequencing.

2.2 | Cell culture and transfection

An HEK293FT cell line (from ATCC) was confirmed as free of mycoplasma contamination and cultured with 10% FBS and 1% penicillin and streptomycin in DMEM (Gibco) in a cell incubator with 5% CO₂. Cells were seeded in 24-well plates at an appropriate density per well and grown to 70%-80% confluence. The plasmids consisting of 0.3 µg sgRNA (sgRNA-2x MS2 or sgRNA-12x MS2), 0.6 µg cytosine deaminase (MCP-rAPOBEC1 or MCP-AID) and 0.4 µg Cas9 (dCas9, nCas9 (D10A) or nCas9 (H840A)) in 100 µL OPTI-MEM containing 2.6 µL LipofectamineTM 2000 (Thermo Fisher Scientific, 11668019) were transfected into cells. Twenty-four hours after transfection, puromycin (InvivoGen, nt-pr-1) at a final concentration of 2 µg/mL was added to the culture media. Forty-eight hours later, genomic DNA from the transfected cells was extracted using the phenol-chloroform method.

2.3 | The blue/white colony assay

SgRNAs were designed to target the *supF* tRNA gene on the shuttle vector pSP189. pSP189 with three components of the MS2-BE system (sgRNA: sgRNA-2x MS2 or sgRNA-12x MS2; cytosine deaminase: MCP-rAPOBEC1 or MCP-AID; and Cas9 variants: dCas9, nCas9 (D10A) or nCas9 (H840A)) was transfected into cells and 24 hours later puromycin was added for resistance selection. After 48 hours the mutant pSP189 was extracted following a standard DNA extraction protocol. The mutant pSP189 was then transduced into *lacZ^{amb} Escherichia coli*, which resulted in the formation of white instead of blue colonies.¹⁷ The efficiency of MS2-BEs was analyzed by calculating the ratio of white/blue colonies. After calculating the ratio of white/blue colonies, the white colonies were collected and analyzed for mutations by performing Sanger sequencing. The base editing frequency of each site was calculated as the number of colonies that contained mutations/the total number of sequenced white colonies.

2.4 | Data statistics and analysis

All results were repeated three times independently. Where possible, error bars indicate SD in the figures. Significance was determined by analysis of variance (one-way ANOVA): *****P* < 0.0001, ****P* < 0.001, ***P* < 0.01, **P* < 0.05, ns (not significant).

3 | RESULTS

3.1 | MS2-based base editor system (MS2-BE)

Inspired by the findings that nicking the DNA strand can stimulate genomic DNA repair to convert C-T^{1,2} and that UGI can inhibit the

function of UNG,⁴ we constructed a new BE system using nickase Cas9 (D10A) and UGI, which is used in BE3, combined with the MS2 system. We constructed the MS2-BE using three plasmids: (a) MCP fused to the 5'-end of the cytosine deaminase AID fused with UGI (MCP-AID); (b) two copy numbers of MS2 fused to the 3'-end of sgRNA (sgRNA-2x MS2) (Figure 1A); and (c) nickase Cas9, nCas9 (D10A).

To sensitively observe the efficiency of this system, we applied the episomal shuttle vector pSP189 as a reporter system.¹⁸ With this reporter, once the *supF* region is mutated, the tRNA becomes inactivated and the color of the transduced *E coli* colonies changes from blue to white. Three sgRNAs (F1, F2, and F3) (Table 1) were designed to target the *supF* region of pSP189. Using these three sgRNAs, we analyzed the editing window and efficiency of the newly constructed MS2-BE by sequencing the white colonies. The data showed that the C-to-T conversions occur over a wide window, from positions -32 to 28 (counting from PAM), around the target site (Figure 1B) and the average editing efficiency of each site was 0.0519 (Figure 1C), which means that the MS2-BE can successfully introduce programmable base substitutions across a wide window but with low efficiency.

TABLE 1 The sgRNA sequences of pSP189

sgRNA name	sgRNA sequence (5'-3')
F1	TCTGCCGTCATCGACTTCGA
F2	CGAACCTTCGAAGTCGATGA
F3	GAATCCTTCCCCTCTAACCA

3.2 | MS2-BEs with different Cas9 variants

To optimize the constructed MS2-BE system, we attempted to change the components. First, regarding Cas9 variants, we wondered whether replacing nCas9 (D10A) with another nickase Cas9 (nCas9 (H840A), which produces a nick on the non-complementary strand) or the catalytically inactivated Cas9 (dCas9) would improve the mutation effect of the MS2-BE system. We compared the mutation efficiency of the MS2-BE systems with the different Cas9 variants using the pSP189 reporter system and blue/white colony assay. The results showed that, among the three Cas9 variants, dCas9 produced a higher ratio of white colonies (12.4%

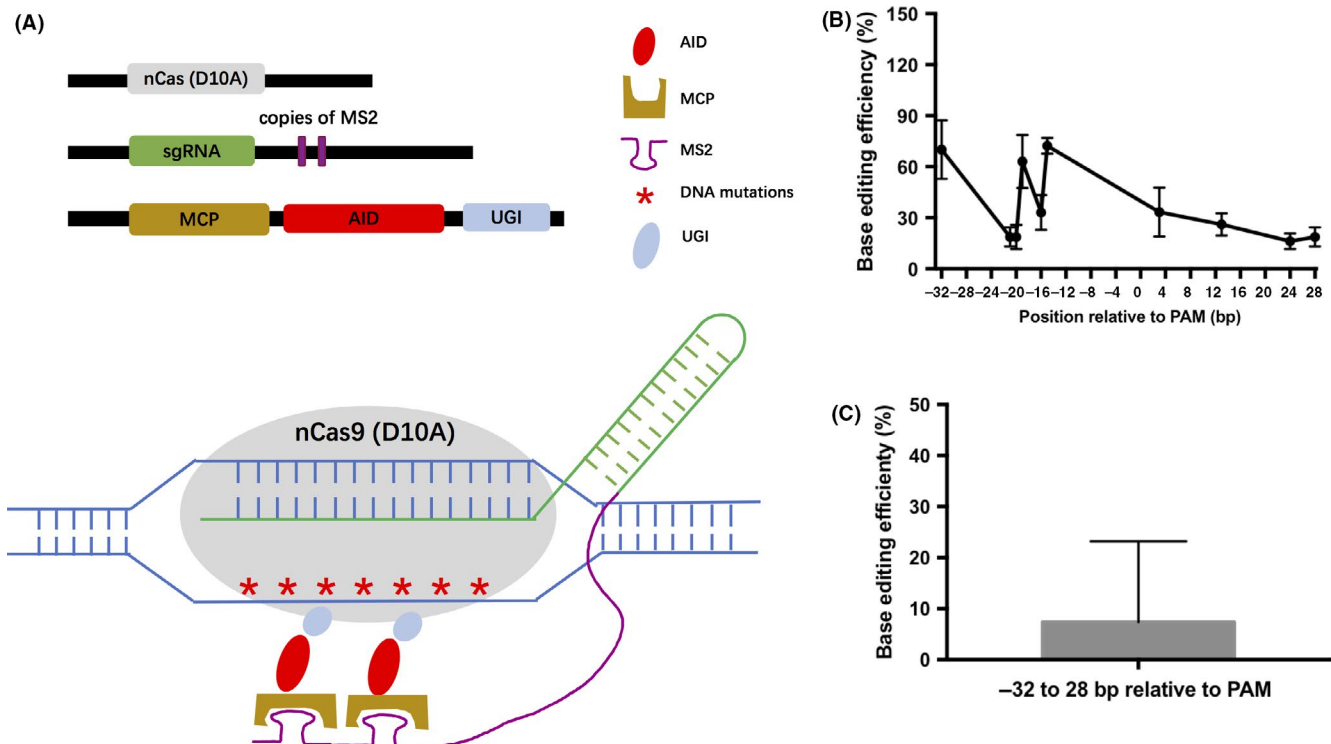


FIGURE 1 The generation of MS2-BE. A, A scheme of the MS2-BE system, which consists of nCas9 (D10A), sgRNA-2x MS2 and MCP-AID-UGI. Two copies of MS2 were fused to the 3'-end of sgRNA and MCP was fused to the 5'-end of AID-UGI. The two plasmids are co-transfected with nCas9 (D10A), which is guided by sgRNA to the binding site. MCP-AID-UGI is then recruited by sgRNA-2x MS2 around the target site to induce C-to-T conversions. B, Analysis of the base editing efficiency of MS2-BE. SgRNA-2x MS2 (F1, F2 and F3), MCP-AID-UGI and nCas9 (D10A), together with the pSP189 reporter vector, were cotransfected into HEK293FT cells and the mutant reporter vectors were extracted and transduced to *E coli*. The resulting white colonies were selected for Sanger sequencing. Analysis of the sequencing results showed that the C-to-T conversions occurred over a wide window, from positions -32 to 28 (50 nt) counting from PAM. Error bars (\pm) indicate the SD of three independent replicates. C, The average base editing efficiency of MS2-BE over 50 nt. We calculated the efficiency from the data in (B) and the average editing efficiency of each mutation site is shown. Error bars (+) indicate the SD of three independent replicates

vs 5.5% vs 1.63%, $P < 0.05$; Figure 2), demonstrating that, of the three Cas9 variants in MS2-BE system, dCas9 performed most efficiently.

3.3 | MS2-BEs with different cytosine deaminases

The second component of MS2-BE is the deaminase. In addition to cytosine deaminase AID, we created a second deaminase by fusing rAPOBEC1-UGI to the 3'-end of MCP to construct MCP-rAPOBEC1. Then we compared the editing efficiency of the MS2-BE systems with different deaminases using our reporter system and blue/white colony assay. The results showed that, using dCas9, MCP-rAPOBEC1 resulted in a much higher ratio of white/blue colonies than MCP-AID (12.4% vs 4.47%, $P < 0.0001$; Figure 3). Moreover, the same results were obtained using nCas9 (D10A) (4.9% vs 1.2%, $P < 0.01$; Figure 3), but no significant differences were seen using nCas9 (H840A) (1.63% vs 0.98%, ns; Figure 3). These results indicated that the rAPOBEC1 deaminase acted more efficiently than AID when combined with dCas9 or nCas9 (D10A).

3.4 | MS2-BEs with different copy numbers of MS2

Since increasing the copy numbers of MS2 could recruit more MCP conjugated to deaminases,¹⁵ we hypothesized that increasing the copy number of MS2 in our system would enhance the mutation effect. Therefore, we created a second sgRNA complex by fusing 12 copies of MS2 to the 3'-end of sgRNA (sgRNA-12x MS2) and calculated the efficiency of the adapted MS2-BEs (dCas9, MCP-rAPOBEC1

or MCP-AID and sgRNA-2x MS2 or sgRNA-12x MS2) using our reporter and blue/white colony assay. The data show no significant difference in editing efficiency using sgRNA with different copy numbers of MS2 (MCP-rAPOBEC1: 2x MS2, 12.57% vs 12x MS2, 7.1%, $P < 0.01$; MCP-AID: 2x MS2, 4.47% vs 12x MS2, 5.3%, ns; Figure 4A), but the MCP-rAPOBEC1 MS2-BE was more efficient than the MCP-AID MS2-BE (sgRNA-2x MS2: MCP-rAPOBEC1, 12.57% vs MCP-AID 4.47%, $P < 0.001$; sgRNA-2x MS2: MCP-rAPOBEC1, 7.1% vs MCP-AID 5.3%, ns; Figure 4A). Therefore, these results showed that increasing the copy number of MS2 from 2 to 12 did not improve the efficiency of mutation and provided further proof that MCP-rAPOBEC1 performs more efficiently in the MS2-BE system than MCP-AID.

In order to confirm the mutation scope of the MS2-BE system, the white colonies of both groups (2x MS2 and 12x MS2) were subjected to Sanger sequencing. The efficiency of editing C to T on each site from positions -50 to 50 (counting from PAM) were analyzed (Figure 4B). The data showed that the mutation hotspot region of MS2-BE was between positions -32 to -2. The mutation hotspot region was defined as the region in which the editing efficiency was higher than the average efficiency at each site between positions -50 and 50. The average mutation frequencies at each site within each hotspot region of sgRNA were 0.173 (2x MS2) and 0.0925 (12x MS2) ($P < 0.05$; Figure 4C). And the average base editing efficiencies of MS2-BEs with MCP-AID (data in Figure 1B) and MCP-rAPOBEC1 using sgRNA-2x MS2 in the hotspot region were 8.32% and 17.26% ($P < 0.05$; Figure 4C). In addition, the average counts of C-to-T conversions in every white colony selected for Sanger sequencing were calculated. The results showed that MS2-BE with

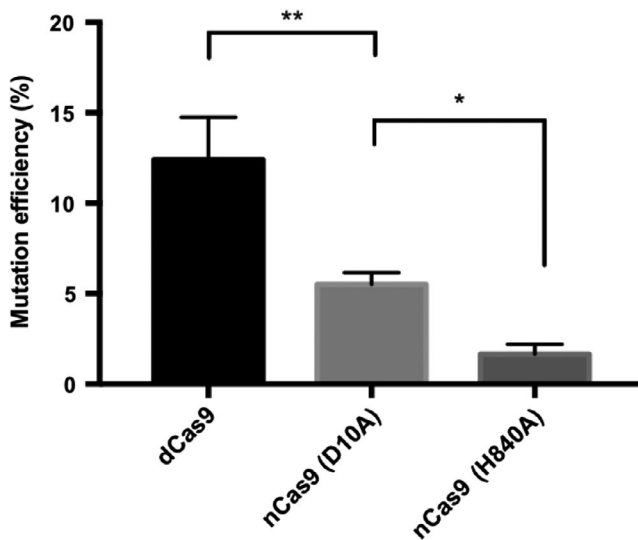


FIGURE 2 The mutation editing efficiency of MS2-BEs with different Cas9 variants. The mutation efficiency of MS2-BEs with different Cas9 variants (dCas9, nCas9 (D10A) and nCas9 (H840A)) is shown. MS2-BEs consisting of sgRNA-2x MS2, MCP-AID-UGI and dCas9/nCas9 (D10A)/nCas9 (H840A) were applied to target the pSP189 reporter system and the percentage of white/blue colonies was calculated. The data were analysed using one-way ANOVA. Error bars (+) indicate the SD of three independent replicates. * $P < 0.05$; ** $P < 0.01$

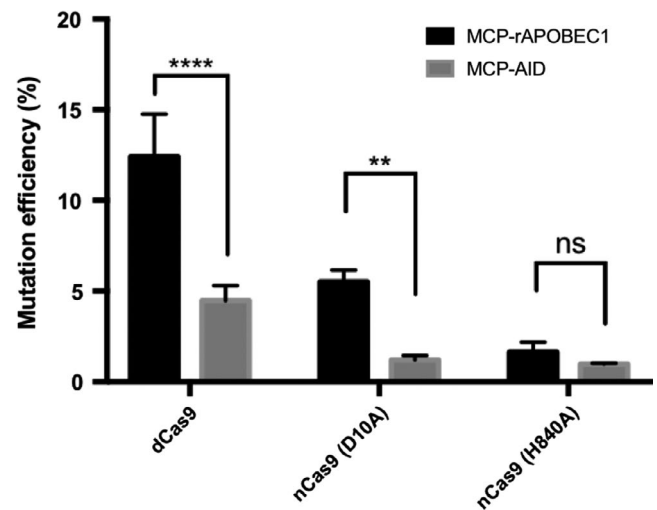


FIGURE 3 The mutation efficiency of MS2-BEs with different cytosine deaminases. Analysis of the mutation efficiency of MS2-BEs with different cytosine deaminases (MCP-rAPOBEC1 and MCP-AID) is shown. MS2-BEs consisting of sgRNA-2x MS2, MCP-rAPOBEC1/MCP-AID and dCas9/nCas9 (D10A)/nCas9 (H840A) were applied to target the pSP189 reporter system and the percentage of white/blue colonies was calculated. The data were analyzed using two-way ANOVA. Error bars (+) indicate the SD of three independent replicates. ns, not significant; $P > 0.05$; ** $P < 0.01$; **** $P < 0.0001$

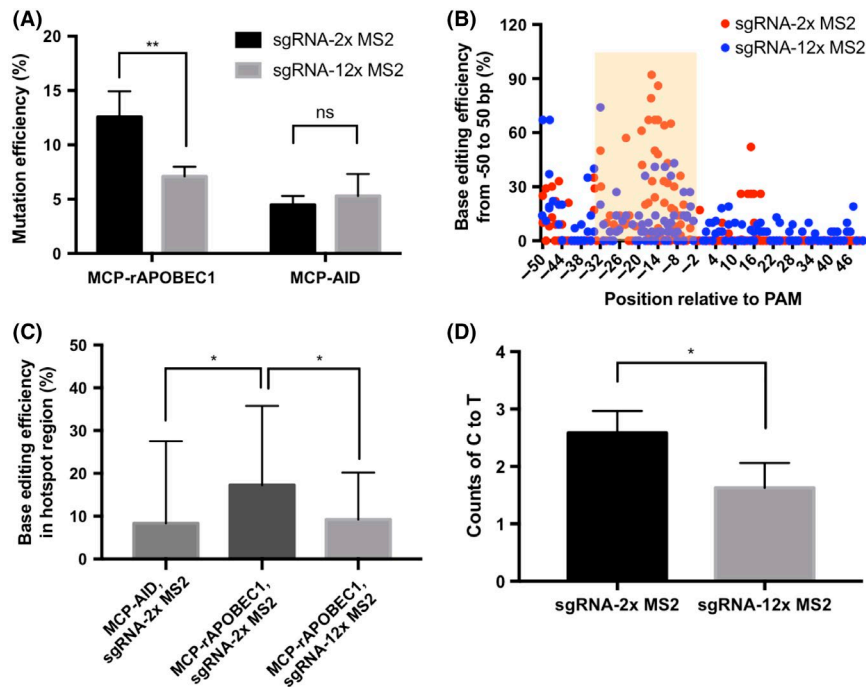


FIGURE 4 The base editing efficiency and window of MS2-BEs with different copy numbers of MS2. A, Analysis of the mutation efficiency of MS2-BEs with different copy numbers of MS2. MS2-BEs, consisting of sgRNA-2x MS2/sgRNA-12x MS2, MCP-rAPOBEC1/MCP-AID and dCas9 were applied to target the pSP189 reporter system and the percentage of white/blue colonies was calculated. The data were analyzed using two-way ANOVA. Error bars (+) indicate the SD of three independent replicates. ns, not significant; $P > 0.05$; $**P < 0.01$. B, The base editing window of MS2-BEs with different copy numbers of MS2. MS2-BEs consisting of sgRNA-2x MS2/sgRNA-12x MS2, MCP-rAPOBEC1 and dCas9 were applied to target the pSP189 reporter system, and the mutated reporter vector were transduced into *E. coli*. The white colonies were subjected to Sanger sequencing and the C-to-T conversions from positions -50 to 50 were shown as spots ($n = 3$ for each sgRNA). The mutation hotspot regions (positions -32 to -2 relative to PAM) of both sgRNA-2x MS2 and sgRNA-12x MS2 (shaded rectangle) were defined as the region in which the editing efficiency was higher than the average efficiency of each site from positions -50 to 50 . C, The average base editing efficiency of MS2-BEs with different copy numbers of MS2 in the hotspot region. The average base editing efficiency of MS2-BEs with different copy numbers of MS2 (data in (B)) and the average base editing efficiency of MS2-BEs with MCP-AID (data in Figure 1B) or MCP-rAPOBEC1 using sgRNA-2x MS2 in the hotspot region (positions -32 to -2) was calculated. The data were analyzed using a one-tailed Student's *t* test. Error bars (+) indicate the SD of 31 mutation sites of 3 sgRNA. $*P < 0.05$. D, Analysis of simultaneously occurring mutations. MS2-BEs consisting of sgRNA-2x MS2/sgRNA-12x MS2, MCP-rAPOBEC1 and dCas9 were applied to target the pSP189 reporter system, and the mutated reporter vectors were transduced into *E. coli*. The white colonies were subjected to Sanger sequencing. The average C-to-T mutation counts in each white colony were calculated and shown. The data were analyzed using a two-tailed Student's *t* test. Error bars (+) indicate the SD of three independent replicates. $*P < 0.05$

sgRNA-2x MS2 could simultaneously introduce more transitions of C to T than MS2-BE with sgRNA-12x MS2 (2.59 vs 1.63, $P < 0.05$; Figure 4D). Neither the efficiency nor the editing window of the MS2-BE system was improved by using more copy numbers of MS2. Taken together, the results show that the optimal version of MS2-BE consists of dCas9, sgRNA-2x MS2 and MCP-rAPOBEC1 (termed MS2-BE-rAPOBEC1), providing a system that can simultaneously produce multiple substitutions over a wide window (-32 to -2).

4 | DISCUSSION

The CRISPR/Cas9 base editor has been developed by adding the MS2 system to provide genome editing. In this study we compared different Cas9 variants, deaminases and different copy numbers of MS2 combined as a MS2-BE and acquired an optimal MS2-BE

system, consisting of sgRNA-2x MS2, MCP-rAPOBEC1 and dCas9, which we named MS2-BE-rAPOBEC1. We showed that this system could simultaneously mutate multiple bases at the target site within a hotspot region between positions -32 and -2 and avoided the DSBs caused by Cas9. Given these advantages, we suggest that the system is suitable for screening for functional elements.

Surprisingly, in our study, the efficiency of C-to-T mutation is not improved by increasing the copy number of MS2. Since Ma et al reported that CRISPR-Sirius was constructed by inserting eight copies of MS2 into the tetraloop of sgRNA (sgRNA-In-MS2),¹⁸ we speculate that where MS2 is inserted into sgRNA is a key factor, and fusing 12x MS2 to the 3'-end of sgRNA (sgRNA-12x MS2) may decrease the stability of the entire RNA secondary structure and so interfere with the editing efficiency. Strategies to alter the relative position of MS2 within sgRNA and need to be explored to further optimize the MS2-BE system.

5 | CONCLUSION

We have designed an editing tool based on the MS2 system, MS2-BE-rAPOBEC1, which can achieve simultaneous mutations of multiple bases at the target genome site without introducing DSBs. This system provides new opportunities for studying directed evolution, activation or repression of transcription and translation and screening of regulatory elements.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

JC designed the study. SF and WJ performed the experiments. SZ and WJ worked on the statistical analysis of the data and wrote the paper. All authors read and approved the final manuscript.

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