CRISPR/Cas9-Mediated Three Nucleotide Insertion Corrects a Deletion Mutation in MRP1/ABCC1 and Restores Its Proper Folding and Function

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INTRODUCTION

Many genetic disorders, such as color blindness, cystic fibrosis, hemochromatosis, hemophilia, phenylketonuria, polycystic kidney disease, and sickle cell disease, can be corrected using gene editing technologies. CRISPR/Cas9 is a powerful tool for genome editing that can correct missense and nonsense mutations by inserting or deleting nucleotides.

A three-nucleotide deletion in cystic fibrosis transmembrane conductance regulator (CFTR)/ABCC7 (CFTR/ABCC7) resulting in the absence of phenylalanine at position 508 leads to mis-fold of the mutated protein and causes cystic fibrosis. Presynaptic single-strand nick or DSB is critical for the repair of the CRISPR/Cas9-mediated single-strand nick or DSB repair in mammalian cells. HDR is a complex processing of orchestrated reactions involving multiple factors. In addition, presynaptic single-strand DNA invasion (searching for homologous sequences) plays a crucial role for initiation of the HDR.

However, HDR is a complex processing of orchestrated reactions involving multiple factors. In addition, presynaptic single-strand DNA invasion (searching for homologous sequences) plays a crucial role for initiation of the HDR. The greatest challenge in HDR-mediated gene correction is the creation of recombinogenic DNA ends near the mutation site. Development of the CRISPR/Cas9 system provides a mean to cut the DNA (making either a nick or double-strand DNA break [DSB]) near the mutation site. Unfortunately, non-homologous end-joining (NHEJ), albeit without ensuring restoration of the DNA sequence around the break site, plays a dominant role over HDR for any DSB repair in mammalian cells.

To evaluate the relative contribution of NHEJ and HDR specifically to the repair of the CRISPR/Cas9-gRNA-mediated single-strand nick or DSB, we have used another ABCC family member, the multidrug resistance-associated protein (MRP1/ABCC1), as a model system in which we had shown previously that a single phenylalanine deletion (ΔF728) at a comparable position in nucleotide binding domain 1 (NBD1) as F508 in CFTR/ABCC7 causes similar mis-folding and loss of drug transport function.

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molecular weight (MW) of ~190 kDa, which can be efficiently detected with our mAbs 42.4 and 897.2.\textsuperscript{36} In contrast, the expression of MRP1/ABCC1-\textsuperscript{D728} mainly forms an immature protein with an apparent MW of ~170 kDa that can be efficiently detected with 897.2, but not by 42.4, because F728 is part of this mAb’s epitope.\textsuperscript{36} Thus, if MRP1/ABCC1-\textsuperscript{D728} mutation is corrected, a mature band at ~190 kDa can be efficiently detected with our 42.4 mAb.

We have found that the rate of HDR mediated by gRNA complementary to \textsuperscript{AF728} mutated DNA is significantly higher than the one mediated by gRNA complementary to the WT donor DNA, whereas the rate of gene correction mediated by gRNA complementary to the WT donor DNA is significantly higher than those gRNAs complementary to either the 5’ side or 3’ side of MRP1/ABCC1-\textsuperscript{AF728}. However, the mutation rate introduced by gRNA complementary to \textsuperscript{AF728} mutated DNA is significantly higher than the one mediated by gRNA complementary to the WT donor DNA. Thus, the data presented here provide a gRNA designing guidance for genome editing.

RESULTS

MRP1/ABCC1-\textsuperscript{AF728} Is Used as a Model System for Genome Editing

Deletion of phenylalanine 508 in CFTR/ABCC7, which is present in most cystic fibrosis patients, results in mis-processing of the ion channel protein within epithelial cells.\textsuperscript{20,21} Phenylalanine at position 728 in MRP1/ABCC1 also results in a similar thermolability and mis-folding of the drug transporter so that only the endoplasmic reticulum (ER)-retained immature form of the protein (~170 kDa) is detected by our MRP1/ABCC1 mAb 897.2 (Figure S1). In addition, this thermolabile mutant can form both mature (190 kDa) and immature protein (170 kDa) at 27°C,\textsuperscript{35} detected with 897.2.\textsuperscript{36} However, regardless of whether this mutant forms mature or immature protein, they cannot be detected with our MRP1/ABCC1 mAb 42.4 (Figure S1) mainly because F728 is part of this mAb’s epitope.\textsuperscript{36} In other words, once this deletion is corrected, the corrected version can be detected with either 897.2 or 42.4. Thus, our mAbs and \textsuperscript{AF728} mutated MRP1/ABCC1 provide an excellent model system for optimization of genome editing with the CRISPR/Cas9 system.

Effects of gRNAs Complementary to MRP1/ABCC1-\textsuperscript{AF728} Deletion Mutant on Knockin of the Three Deleted Nucleotides

The first set of gRNA was designed to cover the MRP1/ABCC1-\textsuperscript{AF728} deletion region, but artificially inserted a G at the beginning of the shorter gRNA1 (Figure 1A; Table 1). The second set of gRNA was designed to cover the MRP1/ABCC1-\textsuperscript{AF728} deletion region with a longer version of gRNA2 without any modification (Figure 1A; Table 1). These two sets of gRNAs were cloned into either pSpCas9(BB)-2A-GFP or pSpCas9n(BB)-2A-GFP (Table 1) and used to transfect MRP1/ABCC1-\textsuperscript{AF728} expressing baby hamster kidney (BHK) cells (Table 2, groups 1–12). The results in Figure 1B and Table 2 indicated that the gRNA alone (Table 2, groups 1, 4, 7, and 10), regardless of whether these gRNAs are the shorter or longer version and regardless of whether they were cloned in Cas9 or Cas9n vector, or donor DNA along with GFP-expressing Cas9 or Cas9n
from the host, i.e., BHK cell (type 2 in Figure 1D); 12 of them had a one nucleotide insertion (types 6 and 7 in Figure 1D); and 3 used total RNA isolated from colony 2-12 (Figure 1C) to do reverse transcription, PCR amplification, and cloning. Sequence analysis of 23 clones showed that 2 of them had perfect insertion of the deleted 3 nt in ∆F728 (type 1 in Figure 1D); 3 of them had perfect sequence from the host, i.e., BHK cell (type 2 in Figure 1D); 12 of them had deletion mutations (types 3, 4, 5, and 9 in Figure 1D); 3 of them had a one nucleotide insertion (types 6 and 7 in Figure 1D); and 3 of them had deleted 16 nt and inserted 75 nt in the deleted region (type 8 in Figure 1D), reflecting the domination of HDR by NHEJ of the modified DNA ends.

| Table 1. gRNA Constructs |
|--------------------------|
| Name          | Vector                | Sequence            |
| Cas9.gRNA1   | pSpCas9(BB)-2A-GFP   | 5’-GAAACATCTCTT-3’ |
| Cas9n.gRNA1 | pSpCas9n(BB)-2A-GFP | 5’-GAAACATCTCTT-3’ |
| Cas9.gRNA2   | pSpCas9(BB)-2A-GFP   | 5’-GAAACATCTCTT-3’ |
| Cas9n.gRNA2 | pSpCas9n(BB)-2A-GFP | 5’-GAAACATCTCTT-3’ |
| Cas9.gRNA3   | pSpCas9(BB)-2A-GFP   | 5’-GCACTCTTTTGGATGTCAG-3’ |
| Cas9n.gRNA3 | pSpCas9n(BB)-2A-GFP | 5’-GCACTCTTTTGGATGTCAG-3’ |
| Cas9.gRNA4   | pSpCas9(BB)-2A-GFP   | 5’-GAAACATCTTGGATGTCAG-3’ |
| Cas9n.gRNA4 | pSpCas9n(BB)-2A-GFP | 5’-GAAACATCTTGGATGTCAG-3’ |
| Cas9.gRNA5   | pSpCas9(BB)-2A-GFP   | 5’-GCACTCTTGGATGTCAG-3’ |
| Cas9n.gRNA5 | pSpCas9n(BB)-2A-GFP | 5’-GCACTCTTGGATGTCAG-3’ |
| Cas9.gRNA6   | pSpCas9(BB)-2A-GFP   | 5’-GCACTCTTGGATGTCAG-3’ |
| Cas9n.gRNA6 | pSpCas9n(BB)-2A-GFP | 5’-GCACTCTTGGATGTCAG-3’ |

The individual colonies isolated from group 2 were screened with MRPI/ABCC1 mAb 42.4 (Figure 1C), and 29.2% of the colonies were positive (Table 2, group 2). In order to further confirm that the three deleted nucleotides were knocked in by this treatment, we used total RNA isolated from colony 2-12 (Figure 1C) to do reverse transcription, PCR amplification, and cloning. Sequence analysis of 107 clones showed that 4 of them had perfect insertion of the deleted 3 nt in ∆F728 (type 2 in Figure 2D) and 103 of them had perfect ∆F728 sequence (type 1 in Figure 2D). Interestingly, we have not found any mutation in the amplified fragment (from 2,224 to 2,590) covering the F728 region, suggesting that the Cas9.gRNA3-mediated cutting mainly occurred at the WT donor DNA. These results also suggested that the rate of HDR induced by Cas9.gRNA3 is low, but this treatment significantly reduced the possibility of introducing new mutations into our interested target gene.

Effects of gRNAs Complementary to WT MRPI/ABCC1 on Knockin of the Three Deleted Nucleotides

The results described above indicated that the NHEJ of the ends generated by Cas9.gRNA1 that perfectly matched with the ∆F728 deletion target dominates the HDR to correct the three-nucleotide deletion, suggesting that this kind of gRNA is not ideal for gene correction. Therefore, new sets of gRNAs were designed (Table 1; Figure 2A). The first set of gRNA was designed to cover the F728 region, but artificially inserted a G at the beginning of the shorter gRNA (Figure 2A, gRNA3; Table 1, Cas9.gRNA3 and Cas9n.gRNA3). The second set of gRNA was designed to cover the F728 region with longer version without any artificial modification (Figure 2A, gRNA4; Table 1, Cas9.gRNA4 and Cas9n.gRNA4). Upon transfection of MRPI/ABCC1-∆F728-expressing BHK cells with these gRNAs, whole cell lysates were analyzed by western blot probed with mAb 42.4. The results in Figure 2B and Table 2 indicated that gRNA alone (Figure 2B and Table 2, groups 13, 16, 19, and 22) or donor DNA plus Cas9 vector (Figure 2B and Table 2, groups 15, 18, 21, and 24) did not yield a protein detected by mAb 42.4, suggesting that gRNA alone or donor DNA along with vector cannot efficiently induce the HDR reaction between the WT donor DNA and the ∆F728 mutated target. In contrast, co-transfection of Cas9.gRNA3 with WT donor DNA yielded a clear band at the position of mature MRPI/ABCC1 (Figure 2B and Table 2, group 14), whereas co-transfection of Cas9n.gRNA3 (Table 2, group 17) with WT donor DNA did not yield a detectable amount of mature MRPI/ABCC1 (Figure 2B and Table 2, groups 15, 18, 21, and 24) which also did not yield a protein detected by mAb 42.4, suggesting that gRNA alone or donor DNA along with vector cannot efficiently induce the HDR reaction between the WT donor DNA and the ∆F728 mutated target. In addition, co-transfection of Cas9n.gRNA3 or Cas9n.gRNA2 with WT donor DNA also did not yield a detectable amount of mature MRPI/ABCC1 (Figure 1B, Table 2, groups 5 and 11), suggesting that a Cas9n-mediated nick may not be sufficient to efficiently induce the HDR reaction between the MRPI/ABCC1-∆F728 deletion target DNA and the WT donor DNA.

The individual colonies isolated from group 14 were screened with MRPI/ABCC1 mAb 42.4 (Figure 1C), and 6.5% of the colonies screened were positive (Table 2, group 14). These results were further verified by using mAb 897.2 to detect both the mature form, i.e., the corrected form, and the immature form, i.e., the original ∆F728 (Figure S2). In order to further confirm the three deleted nucleotides were knocked in by this treatment, we used total RNA isolated from colony 14-41 (Figure 2C) to do reverse transcription, PCR amplification, and cloning. Sequence analyses of 107 clones showed that 4 of them had perfect insertion of the deleted 3 nt in AF728 (type 2 in Figure 2D) and 103 of them had perfect ∆F728 sequence (type 1 in Figure 2D). Interestingly, we have not found any mutation in the amplified fragment (from 2,224 to 2,590) covering the F728 region, suggesting that the Cas9.gRNA3-mediated cutting mainly occurred at the WT donor DNA. These results also suggested that the rate of HDR induced by Cas9.gRNA3 is low, but this treatment significantly reduced the possibility of introducing new mutations into our interested target gene.
Effects of gRNAs Complementary to the 5’ Side or 3’ Side of the MRP1/ABCC1-ΔF728 Deletion Target Site on Knockin of the Three Deleted Nucleotides

The results in the previous section indicated that Cas9.gRNA3 complementary to WT donor DNA resulted in extremely low frequency of mutation in the region of interest. In this section, we test the effects of gRNAs complementary to both target DNA and WT donor DNA on knocking in of the three deleted nucleotides. We have designed gRNA5, 36 bp upstream of the ΔF728 deletion mutant, and gRNA6, 34 bp downstream of the ΔF728 target site (Figure 3A). In the meantime, we have generated mutated donor DNAs so that gRNA5 or gRNA6 will only recognize the target DNA, but not the donor DNA (Figure 3A). Upon transfection of MRP1/ABCC1-ΔF728-expressing BHK cells with these gRNAs, whole cell lysates were analyzed by western blot probed with mAb 42.4. The results in Figure 3B and Table 2 indicated that gRNA alone (Table 2, groups 25 and 29) or donor DNA

| Transfection Group No. | Cas and gRNA | Donor           | WT MRP1 in Mixed Population | Total Colonies Analyzed | MRP1 Positive Colonies |
|------------------------|--------------|-----------------|-----------------------------|-------------------------|------------------------|
| 1                      | Cas9.gRNA1   | N/A             | N/A                         | N/A                     | N/A                    |
| 2                      | Cas9.gRNA1   | WT donor        | Yes                         | 24                      | 7                      |
| 3                      | Cas9         | WT donor        | N/A                         | N/A                     | N/A                    |
| 4                      | Cas9n.gRNA1  | N/A             | N/A                         | N/A                     | N/A                    |
| 5                      | Cas9n.gRNA1  | WT donor        | N/A                         | N/A                     | N/A                    |
| 6                      | Cas9n        | WT donor        | N/A                         | N/A                     | N/A                    |
| 7                      | Cas9.gRNA2   | N/A             | N/A                         | N/A                     | N/A                    |
| 8                      | Cas9.gRNA2   | WT donor        | N/A                         | N/A                     | N/A                    |
| 9                      | Cas9         | WT donor        | N/A                         | N/A                     | N/A                    |
| 10                     | Cas9n.gRNA2  | N/A             | N/A                         | N/A                     | N/A                    |
| 11                     | Cas9n.gRNA2  | WT donor        | N/A                         | N/A                     | N/A                    |
| 12                     | Cas9n        | WT donor        | N/A                         | N/A                     | N/A                    |
| 13                     | Cas9.gRNA3   | N/A             | N/A                         | N/A                     | N/A                    |
| 14                     | Cas9         | WT donor        | Yes                         | 46                      | 3                      |
| 15                     | Cas9         | WT donor        | N/A                         | N/A                     | N/A                    |
| 16                     | Cas9n.gRNA3  | N/A             | N/A                         | N/A                     | N/A                    |
| 17                     | Cas9n.gRNA3  | WT donor        | N/A                         | N/A                     | N/A                    |
| 18                     | Cas9n        | WT donor        | N/A                         | N/A                     | N/A                    |
| 19                     | Cas9.gRNA4   | N/A             | N/A                         | N/A                     | N/A                    |
| 20                     | Cas9.gRNA4   | WT donor        | N/A                         | N/A                     | N/A                    |
| 21                     | Cas9         | WT donor        | N/A                         | N/A                     | N/A                    |
| 22                     | Cas9n.gRNA4  | N/A             | N/A                         | N/A                     | N/A                    |
| 23                     | Cas9n.gRNA4  | WT donor        | N/A                         | N/A                     | N/A                    |
| 24                     | Cas9n        | WT donor        | N/A                         | N/A                     | N/A                    |
| 25                     | Cas9.gRNA5   | N/A             | N/A                         | N/A                     | N/A                    |
| 26                     | Cas9.gRNA5   | WT donor        | N/A                         | N/A                     | N/A                    |
| 27                     | Cas9.gRNA5   | 5’ mut donor    | N/A                         | N/A                     | N/A                    |
| 28                     | Cas9         | WT donor + 5’ mut donor | N/A | N/A | N/A |
| 29                     | Cas9.gRNA6   | N/A             | N/A                         | N/A                     | N/A                    |
| 30                     | Cas9.gRNA6   | WT donor        | N/A                         | N/A                     | N/A                    |
| 31                     | Cas9.gRNA6   | 3’ mut donor    | N/A                         | N/A                     | N/A                    |
| 32                     | Cas9         | WT donor + 3’ mut donor | N/A | N/A | N/A |
| 33                     | Cas9.gRNA1+Cas9.gRNA3 | WT donor      | Yes                         | 24                      | 1                      |
| 34                     | Cas9.gRNA1+Cas9.gRNA5 | WT donor      | N/A                         | N/A                     | N/A                    |
| 35                     | Cas9.gRNA1+Cas9.gRNA6 | WT donor      | N/A                         | N/A                     | N/A                    |

mut, mutation.
plus Cas9 vector (Table 2, groups 28 and 32) did not yield an amount of mature MRP1/ABCC1 protein detectable by mAb 42.4, suggesting that gRNA alone or donor DNA along with vector cannot efficiently induce the HDR reaction between the donor DNA and the ΔF728 mutated target DNA. In addition, co-transfection of Cas9.gRNA5 with WT donor DNA (Figure 3B and Table 2, group 26) or with mutated donor DNA (Figure 3B and Table 2, group 27) did not yield an amount of mature MRP1/ABCC1 protein detectable by mAb 42.4, suggesting that gRNA5 along with WT donor DNA or along with mutated donor DNA cannot efficiently induce the HDR reaction between the donor DNA and the ΔF728 mutated target DNA. Further-
more, co-transfection of Cas9.gRNA6 with WT donor DNA (Figure 3B and Table 2, group 30) or with mutated donor DNA (Figure 3B and Table 2, group 31) did not yield an amount of mature MRP1/ABCC1 protein detectable by mAb 42.4, suggesting that gRNA6 along with WT donor DNA or along with mutated donor DNA cannot efficiently induce the HDR reaction between the donor DNA and the ΔF728 mutated target DNA.

Because none of these treatments yielded an amount of mature MRP1/ABCC1 protein detectable by mAb 42.4, individual colonies were not isolated from these GFP-positive cells. In order to test whether these treatments generate mutations or correction of the ΔF728 deletion, we used total RNA isolated from mixtures of groups of 26, 27, 30, or 31 to do reverse transcription, PCR amplification, and cloning. Sequence analyses of 54 clones from group 26 showed that 49 of them had perfect ΔF728 sequence (type 2 in Figure 3C), 1 of them had a 1 nt deletion at the cutting site (type 4 in Figure 3C), and 1 of them had a 94-nt deletion (type 3 in Figure 3C), suggesting that cutting at the 5’ side of ΔF728 caused mutations. Sequence analyses of 50 clones from group 27 (type 5 in Figure 3C) or 56 clones from group 30 (type 1 in Figure 3D) showed no gene correction and no mutation. However, sequence analyses of 44 clones from group 31 showed that 40 of them had perfect ΔF728 sequence (type 2 in Figure 3D); 1 of them had perfect insertion of the deleted 3 nt in ΔF728 (type 3 in Figure 3D); 2 of them had perfect sequence from the host, i.e., BHK cell (type 4 in Figure 3D); and 1 of them had a single mutation at the 5’ side of ΔF728 (type 5 in Figure 3D), suggesting that cutting at the 3’ side of ΔF728 in target DNA caused gene correction and gene mutations.

Effects of Combination of gRNA1 with gRNA3, gRNA5, or gRNA6 on Knocking in of the Three Deleted Nucleotides

The findings described above indicated that gRNA5 or gRNA6 recognizing both target DNA and donor DNA at the 5’ side or 3’ side of the ΔF728 deletion mutant did not efficiently induce HDR between the ΔF728 mutated target DNA and the WT donor DNA. In contrast, gRNA1 recognizing the ΔF728 mutated target DNA or gRNA3 recognizing the WT donor DNA covering F728 did induce the HDR reaction between the ΔF728 mutated target DNA and the WT donor DNA. In this section, we test the effects of the combination of gRNA1 with gRNA3, gRNA5, or gRNA6 (Table 2) on knocking in of the three deleted nucleotides. The treatment with combination of gRNA1 and gRNA3 yielded a band at the position of mature MRP1/ABCC1 (Figure 4A), whereas the combination of gRNA1 with gRNA5 or gRNA6 did not yield any detectable amount of mature MRP1/ABCC1 (Figure 4A), suggesting that the rate of HDR induced by gRNA1 and gRNA3 is significantly higher than the combination of gRNA1 with gRNA5 or gRNA6.

The individual colonies isolated from group 33 were screened with MRP1/ABCC1 mAb 42.4 (Figure 4B), and 4.2% of the colonies had a 1 nt deletion at the cutting site (type 4 in Figure 3C), and 1 of them had a 94-nt deletion (type 3 in Figure 3C), suggesting that cutting at the 5’ side of ΔF728 caused mutations. Sequence analyses of 50 clones from group 27 (type 5 in Figure 3C) or 56 clones from group 30 (type 1 in Figure 3D) showed no gene correction and no mutation. However, sequence analyses of 44 clones from group 31 showed that 40 of them had perfect ΔF728 sequence (type 2 in Figure 3D); 1 of them had perfect insertion of the deleted 3 nt in ΔF728 (type 3 in Figure 3D); 2 of them had perfect sequence from the host, i.e., BHK cell (type 4 in Figure 3D); and 1 of them had a single mutation at the 5’ side of ΔF728 (type 5 in Figure 3D), suggesting that cutting at the 3’ side of ΔF728 in target DNA caused gene correction and gene mutations.

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The individual colonies isolated from group 33 were screened with MRP1/ABCC1 mAb 42.4 (Figure 4B), and 4.2% of the colonies
were positive (Table 2, group 33). In order to further confirm that the three deleted nucleotides were knocked in by this treatment, we used total RNA isolated from colony 33-10 (Figure 4B) to do reverse transcription, PCR amplification, and cloning. Sequence analyses of 91 clones showed that 12 of them had the original 3 nt deletion in DF728 (type 1 in Figure 4C), 11 had perfect insertion of the deleted 3 nt in DF728 (type 2 in Figure 4C), 50 had deletion mutations (types 3–9 in Figure 4C), 3 had 1 nt insertion (types 10 and 11 in Figure 4C), and 15 had 3 nt deletion and 9 nt insertion at the deletion site (type 12 in Figure 4C). Thus, although the treatment with gRNA1 and gRNA3 induced HDR reaction between the target DNA and the donor DNA, the NHEJ of the modified DNA ends dominates the HDR in a cloned cell line.

As shown in Figure 4, insertion of the 3 nt into the ΔF728 deletion site promoted the conversion of the immature ΔF728 variant into mature WT MRP1/ABCC1, suggesting that correction of the deletion restored its proper folding. To test whether the folded protein trafficked to the cell surface and restored its drug transport function, we assayed the ability of the cells to resist the cytotoxic action of daunorubicin. Indeed, the daunorubicin killing profile of MRP1/ABCC1-ΔF728-expressing cells is similar to BHK cells (Figure 4D), whereas the IC50 value of 33-10 is approximately 7.5-fold higher than that of BHK or MRP1/ABCC1-ΔF728-expressing cells (Figure 4E), suggesting that correction of these three-nucleotide deletions by CRISPR/Cas9-gRNA restored its drug transport function.

DISCUSSION

Development of the CRISPR/Cas9 system provides an opportunity to generate recombinogenic DNA ends near the mutation sites. However, the design of gRNAs is restricted by the available protospacer adjacent motif (PAM) and a guanine residue approximately 20 bp upstream of the PAM sequence near the mutation sites. We have found that our MRP1/ABCC1-ΔF728 deletion mutant, along with our MRP1/ABCC1 mAbs, provides an excellent model system to test the HDR-mediated repair of the three-nucleotide deletion mutation. We had found earlier that NHEJ efficiently occurred
for any DSB repair in mammalian cells. In addition, we also had found that the modifications at the break sites could cause deleterious mutations. Using our model system, we attempt to clarify the following three important issues: (1) whether gRNAs can be designed to increase the efficiency of the HDR-mediated repair of the three-nucleotide deletion mutation, (2) whether gRNAs can be designed to increase the safety of the CRISPR/Cas9-gRNA mediated HDR, and (3) whether the CRISPR/Cas9-gRNA-donor DNA system can be designed to create a PAM sequence near the site of interest.

The result presented in Table 2 indicated that the rate of HDR mediated by gRNA complementary to target DNA (Table 2, group 2) is approximately 29%, which is comparable with the experiments done in other cell systems. This rate is significantly higher than that using gRNAs complementary to the donor DNA (Figure S3; Table 2, group 2 versus group 14), suggesting that the efficiency of HDR mediated by DSB is directly associated with the distance of mutation from the DSB site. This conclusion is consistent with that of another study. However, it has also been recently reported that gRNA located 87 bp upstream of the deletion mutant CFTR-D508 induced HDR. Our sequence analysis also confirmed that the gRNA located 34 bp downstream of the deletion mutant D728 induced HDR (Figure 3D, type 3).

The correction of three nucleotide deletion in CFTR-D508 has been achieved in either induced pluripotent stem cells or cystic fibrosis patient-derived organoids. However, modification of the ends generated by DSB and the NHEJ-mediated mutation is still a big issue for genome editing. Our sequencing data strongly support this conclusion. For example, the ratio of the precise knockin of the three deleted nucleotide and mutations, including deletion and insertion, that resulted from NHEJ is 1:10.5 if the gRNA complementary to the target DNA is used (Figure 1D), and this ratio is slightly lower (1:6.2) when gRNAs complementary to target DNA and donor DNA are used (Figure 4C). In contrast, only precisely modified product was found when gRNA complementary to WT donor DNA was used (Figure 2D), meaning that this gRNA

| Figure 4. Effects of Combination of gRNA1 with gRNA3, gRNA5, or gRNA6 on Knockin of the Three Deleted Nucleotides |
| (A) A representative western blot, probed with MRP1/ABCC1 mAb 42.4, showing the WT MRP1/ABCC1 protein generated by HDR. (B) A representative western blot, probed with MRP1/ABCC1 mAb 42.4, showing the WT MRP1/ABCC1 protein generated by HDR in isolated individual colonies from transfection group 33. (C) DNA sequence analyses of the clones derived from the reverse transcription products of total RNA isolated from 33-10. The minus sign (−) indicates the deleted nucleotide. The small red letters indicate the inserted nucleotides. (D) Chemosensitivity assay of the BHK cells expressing the WT MRP1, ΔF728, and a recombinant clone (33-10) expressing WT MRP1 protein generated by CRISPR/Cas9 mediated HDR. (E) IC50 values (nM) of daunorubicin are: 6.03 ± 0.56 (BHK), 6.95 ± 1.38 (ΔF728), 114.45 ± 23.99 (MRP1), and 51.82 ± 3.37 (33-10). *p = 0.011; ***p < 0.0001. |
preferentially binds to the donor DNA and makes a DSB at the donor DNA. This result was also interpreted as that the mutations introduced by the DSB occurred at the donor DNA, but not at the target DNA. Off-target site searching from human genome for this gRNA4 revealed that there is only one nucleotide sequence (5’-CATCCCTTTTGGATTCAGC-3’) located on human chromosome 3 that has three mismatches with this gRNA (with 575 score considering the score of gRNA as 1,000). Previous studies indicated that off-target effects are limited to sites with only one mismatch,28 suggesting that gRNA3 complementary to this gRNA revealed that there is only one nucleotide sequence (5’-CATCCCTTTTGGATTCAGC-3’) located on human chromosome 3 that has three mismatches with this gRNA (with 575 score considering the score of gRNA as 1,000). Previous studies indicated that off-target effects are limited to sites with only one mismatch,28 suggesting that gRNA3 complementary to this gRNA (Table 3). The amplified fragments were cloned into pB.MRP1.WT-donor5 and pB.MRP1.WT-donor6 (without changing amino acid sequences) by using the corresponding forward/reverse primers listed in Table 3 and the QuikChange site-directed mutagenesis kit from Stratagene.36 Regions containing these mutations were sequenced to confirm that the correct clones were obtained.

RNA Extraction, Reverse Transcription, PCR Amplification, Cloning, and Sequence Analysis
Total RNA was isolated from BHK cells with RNaseasy Mini kit from QIAGEN, according to the protocol provided by the manufacturer. Reverse transcription, with MRP1-6A (Table 3) as primer, was performed with RT first strand kit from QIAGEN, according to the protocol provided by QIAGEN. The reverse transcription products were used as a template to amplify the 367 bp (from 2,224 to 2,590) fragment with MRP1.C682A-forward and MRP1.D792A-reverse primers (Table 3). The amplified fragments were cloned into pBluescript and sequenced with T3 primer (Table 3).

The data presented here provide important guidelines for gRNA and donor DNA design for genome editing.

MATERIALS AND METHODS

Materials
Most of the chemicals, including daunorubicin and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), were purchased from Sigma. DMEM/F-12 medium and fetal bovine serum were derived from Thermo Scientific. QuikChange site-directed mutagenesis kit was from Stratagene. Anti-mouse Ig conjugated with horseradish peroxidase was from Amersham Biosciences. Chemiluminescent substrates for western blotting were from Pierce. pSpCas9(2B)-2A-GFP (PX458) and pSpCas9n(2B)-2A-GFP (PX461) were derived from Addgene.

Cell Lines, Cell Culture, and Transfection
WT MRP1/ABCC145 and MRP1/ABCC1ΔF728-expressing BHK cells were grown in DMEM/F-12 medium containing 5% fetal bovine serum at 37°C in 5% CO₂. Subconfluent cells were transfected with plasmid DNAs listed in Table 1 in the presence of 20 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM dextrose, and 125 mM NaCl. 65, 66 GFP-positive cells, 14 days post-transfection, were sorted out in our Flow Cytometry Core with BD FACSDiva software. Individual colonies were picked and analyzed once the WT MRP1/ABCC1 protein was detected with 42.4 μM from a whole mixture of GFP-positive cell lysates.

gRNAs and Donor Plasmid Construction
The annealed gRNAs (Table 3), such as gRNA1-forward and gRNA1-reverse, were cloned into the BbsI digested pSpCas9n(2B)-2A-GFP (Table 1), and the inserted gRNAs were confirmed by DNA sequencing. The WT donor DNA was generated by insertion of the BamHI (1036)-EcoRI (4076) MRP1/ABCC1 cDNA fragment into pBluescript (named as p.B.MRP1.WT-donor5). In order to generate mutated donor DNAs so that gRNA5 or gRNA6 will not efficiently bind to them, we mutated the five residues within the gRNA5 or gRNA6 (Figure 3A) region in pB.MRP1.WT-donor5 (without changing amino acid sequences) by using the corresponding forward/reverse primers listed in Table 3 and the QuikChange site-directed mutagenesis kit from Stratagene.36 Regions containing these mutations were sequenced to confirm that the correct clones were obtained.

Table 3. List of Oligonucleotides

| Number | Name     | Sequence                                      |
|--------|----------|-----------------------------------------------|
| 1      | gRNA1-forward | 5'-CACCGAAAACATCCTTGGAATTCAGC-3'             |
| 2      | gRNA1-reverse | 5'-AAACCTGTACATCCAGAGTGTTC-3'               |
| 3      | gRNA2-forward | 5'-CACCGAAAACATCCTTGGAATTCAGC-3'             |
| 4      | gRNA2-reverse | 5'-AAACCTGTACATCCAGAGTGTTC-3'               |
| 5      | gRNA3-forward | 5'-CACCGCATCCTTTTGGATTCAGC-3'               |
| 6      | gRNA3-reverse | 5'-AAACCTGTACATCCAGAGTGTTC-3'               |
| 7      | gRNA4-forward | 5'-CACCGAAAACATCCTTTTGGATTCAGC-3'             |
| 8      | gRNA4-reverse | 5'-AAACCTGTACATCCAGAGTGTTC-3'               |
| 9      | gRNA5-forward | 5'-CACCGCATCCTTTTGGATTCAGC-3'               |
| 10     | gRNA5-reverse | 5'-AAACCTGTACATCCAGAGTGTTC-3'               |
| 11     | gRNA6-forward | 5'-CACCGCATCCTTTTGGATTCAGC-3'               |
| 12     | gRNA6-reverse | 5'-AAACCTGTACATCCAGAGTGTTC-3'               |
| 13     | gRNA5-mut-forward | 5'-CAACAGGCTTGCCTCGATTTCCCTCAAAAGGATGTCCTGC-3'            |
| 14     | gRNA5-mut-reverse | 5'-CAACAGGCTTGCCTCGATTTCCCTCAAAAGGATGTCCTGC-3'            |
| 15     | gRNA6-mut-forward | 5'-CAACAGGCTTGCCTCGATTTCCCTCAAAAGGATGTCCTGC-3'            |
| 16     | gRNA6-mut-reverse | 5'-CAACAGGCTTGCCTCGATTTCCCTCAAAAGGATGTCCTGC-3'            |
| 17     | MRP1.C682A-forward | 5'-GGTGGGCAAGGCTGATTTCCCTCAAAAGGATGTCCTGC-3'            |
| 18     | MRP1.D792A-reverse | 5'-GGTGGGCAAGGCTGATTTCCCTCAAAAGGATGTCCTGC-3'            |
| 19     | MRP1.6A-forward | 5'-GGAATTTACACCTCCTCAAAAGGATGTCCTGC-3'            |
| 20     | T3 primer | 5'-GATTATACACCTCCTCAAAAGGATGTCCTGC-3'            |
Identification of the MRP1/ABCC1 Protein

Western blot was performed according to the method described previously. 36 42.4 mAb was used to identify the WT MRP1/ABCC1 protein, whereas 897.2 mAb was used to detect the WT MRP1/ABCC1 protein and the MRP1/ABCC1-ΔF728 mutated protein. The secondary antibody used was anti-mouse Ig conjugated with horseradish peroxidase. Chemiluminescent film detection was performed according to the manufacturer’s recommendations (Pierce).

Chemosensitivity Assay

The daunorubicin sensitivities of BHK cells expressing variant MRP1/ABCC1 proteins were determined by employing the CCK-8 cytotoxicity assay. In brief, cells were plated in a volume of 90 μL at 3,000 cells per well in 96-well plates. After incubation at 37°C overnight, 10 μL of the media containing varying concentrations of daunorubicin was added to the wells and incubated for an additional 4 days at 37°C. At the end of drug exposure, 10 μL of CCK-8 solution was added to each well and incubated for 1–4 hr. The absorbance at 450 nm was determined by using Universal Microplate Spectrophotometer derived from BioTek.

Statistical Analysis

The results in Figure 4E were presented as means ± SD from the triplicate experiments. The two-tailed p values were calculated based on the unpaired t test from GraphPad Software Quick Calcs. By conventional criteria, if p < 0.05, the difference between two samples is considered to be statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2017.05.005.

AUTHOR CONTRIBUTIONS

X.C. contributed to conception and design of the study. Q.X., Y.H., and X.C. contributed to acquisition of the data. X.C. and Q.X. wrote the manuscript. All of the authors contributed to interpretation of the data and provided input for the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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