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Chemical and biological approaches to improve the efficiency of homologous recombination in human cells mediated by artificial restriction DNA cutter

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ABSTRACT

A chemistry-based artificial restriction DNA cutter (ARCUT) was recently prepared from Ce(IV)/EDTA complex and a pair of pseudo-complementary peptide nucleic acids. This cutter has freely tunable scission-site and site specificity. In this article, homologous recombination (HR) in human cells was promoted by cutting a substrate DNA with ARCUT, and the efficiency of this bioprocess was optimized by various chemical and biological approaches. Of two kinds of terminal structure formed by ARCUT, 3′-overhang termini provided by 1.7-fold higher efficiency than 5′-overhang termini. A longer homology length (e.g. 698 bp) was about 2-fold more favorable than shorter one (e.g. 100 bp). When the cell cycle was synchronized to G2/M phase with nocodazole, the HR was promoted by about 2-fold. Repression of the NHEJ-relevant proteins Ku70 and Ku80 by siRNA increased the efficiency by 2- to 3-fold. It was indicated that appropriate combination of all these chemical and biological approaches should be very effective to promote ARCUT-mediated HR in human cells.

INTRODUCTION

Precise recombination of huge genome has been attracting significant interest of chemists, biochemists and biologists. Homologous recombination (HR) is one of the most powerful ways to manipulate genome in living cells, since a target DNA sequence in genome can be altered to its homologous sequence of choice. In mammalian cells, however, this recombination occurs with only a limited frequency (1). In 1994, it was reported that a double-strand break (DSB) at a specific site in substrate DNA, induced by a rare cutting endonuclease, notably activates the repair machinery in cells and drastically stimulates HR (2). These pioneering works were extended to elegant HR works using zinc finger nucleases, conjugates of a non-specific nuclease domain of FokI restriction enzyme with tandemly assembled zinc finger proteins, which cut genomes at target site (3–8). Furthermore, FokI nuclease was fused with a transcription activator-like effector (TALEN) (9–12), and engineered homing endonucleases have been also developed (13–19). These protein-based DNA cutters have been successfully used for site-directed mutagenesis in many biological and medical applications.

Recently, a completely chemistry-based artificial restriction DNA cutter (ARCUT) has been prepared by combining Ce(IV)/EDTA complex (molecular scissors) and two strands of pseudo-complementary peptide nucleic acid (pcPNA; sequence-recognizing moiety) (20,21). One of the most significant advantages of this chemistry-based tool is that the site of selective scission is a priori determined by Watson–Crick base pairings between the pcPNA strands and the DNA substrate. Thus, ARCUT for aimed scission of genomes can be straightforwardly designed and synthesized without any selection procedure. The site specificity is high enough to cut one site in human genome (22). In a preliminary communication (23), it was shown that DSB introduced by ARCUT is satisfactorily recognized by the repair system in human cells and stimulates HR therein. A strong potential of ARCUT as a new tool for genome manipulation was indicated. In this article, ARCUT-mediated HR in human cells is investigated more in detail. Substrate DNA is cut at a pre-determined site by ARCUT and incubated with donor DNA in human

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cells. Various chemical and biological factors are varied, and their effects on the efficiency of HR are quantitatively analyzed. The chemical factors investigated are the structure of scission terminus (either 5′-overhang or 3′-overhang) and the length of homology region between target DNA and donor DNA. Furthermore, the cell cycle is synchronized to the phases which have been proposed to be preferable for HR. A typical competitive pathway of HR (non-homologous end-joining; NHEJ) is suppressed by using siRNA.

MATERIALS AND METHODS

Preparation of the substrate DNA

The substrate DNA for ARCUT scission (pBFP-N1) was prepared by introducing five amino acid mutations (T65S, Y66H, Q80R, I167T and L231H) into pEGFP-N1 (Clontech) with the use of QuikChange site-directed mutagenesis (Stratagene) according to the manufacturer's protocols. The primers are presented in Supplementary Figure S1 (together with other PCR primers used in this study).

Preparation of donor DNA

The PCR template (pQE30-EGFP) was constructed from pEGFP-N1-mut encoding EGFP with three amino acid substitutions (Q80R, I167T and L231H). The full-length coding region of EGFP mutant was amplified. The product was digested with BamHI and HindIII, and cloned into pQE30Xa plasmid (QIAGEN) which was digested with these restriction enzymes. Using this plasmid as template, the donor EGFP short (119 bp) and the donor EGFP long (717 bp) were prepared by PCR, and purified by QIAquick PCR purification kit (QIAGEN).

Site-selective scission of substrate DNA by ARCUT

The Ce(IV)/EDTA solution was prepared by mixing an aqueous solution of $Ce(NH_4)_2(NO_3)_6$ (20 mM) and EDTA•4Na (20 mM) in HEPES buffer and then adjusting the pH to 7.0 with a small amount of NaOH (20). The synthesis, purification and characterization of pcPNA strands were described elsewhere (24). In pcPNA, pseudo-complementary bases, 2,6-diaminopurine (D) and 2-thiouracil (U_s), are used, in place of conventional nucleobases A and T, to achieve double-duplex invasion efficiently [D/U_s pairs formed in the pcPNA/pcPNA duplex are destabilized by the steric repulsion between amino group of D and the sulphur atom of U_s, whereas D/T and U_s/A pairs in the pcPNA/DNA duplexes are sufficiently stable (25)]. The site-selective scission of DNA by ARCUT was carried out at 37°C and pH 7.0 (5 mM HEPES buffer) for 66 h under the following conditions: [pBFP-N1] = 8 nM, [each of pcPNAs] = 100 nM, [Ce(IV)/EDTA] = 100 μM and [NaCl] = 100 mM. The reactions were stopped by adding ethylenediaminetetramethylenephosphonic acid to a final concentration of 500 μM. Then, the scission fragment (4.7 kb) was purified by agarose gel electrophoresis (1.5%) and extracted from the gel using QIAquick Gel Extraction Kit (QIAGEN).

HR in human cells, and its flow cytometry and sequencing analyses

In DMEM supplemented with 10% fetal bovine serum, 293T cells were maintained at 37°C with 5% CO_2. For the flow cytometric analyses, these cells were seeded at 5×10^4 cells on each well in 24-well dish. On the next day, ARCUT-treated (or non-treated) pBFP-N1 and the donor EGFP gene fragment (in mole ratio 1:7, 800 ng in total) were introduced into these 293T cells using Lipofectamine 2000 (Invitrogen). The ratio of the substrate to the donor DNA was fixed to 1:7, since the ratios 1:7 and 1:15 provided comparable recombination efficiency in our previous report (23). The transfection efficiency was 97.2%, according to a control experiment in which non-treated pEGFP-N1 was introduced into 293T cells under the same conditions (data not shown). When the donor EGFP short was used, a dummy DNA that is irrelevant to either the substrate or the donor was added to adjust the total amount of DNA to 800 ng. After 48 h incubation, the expressions of EGFP and BFP were analyzed by a fluorescence microscope (DMI 6000B, Leica), and the numbers of EGFP positive cells were counted with a Flow Cytometry (Guava EasyCyte Plus, Millipore).

For the sequencing analyses, 293T cells, seeded at 2×10^5 cells on each well in six-well dish, were transfected with ARCUT-scission product and the donor EGFP gene fragment (in mole ratio 1:7, 2 μg in total) using Fugene HD Transfection Reagent (Roche). After 48 h incubation, the plasmids were extracted from the cells and isolated by agarose gel electrophoresis. The plasmids were extracted from the gel using Get pure DNA kit-Agarose (DOJINDO), dissolved in ddH_2O (10 μl), and transformed into 50 μl of *Escherichia coli* DH5α (TOYOBO). After 96 colonies were picked up and cultured in LB media in 96-well deep well plate, the plasmids were purified by Multi Screen Plasmid DNA purification kit (Millipore). The sequences were determined by an ABI PRISM 3130 genetic analyzer using 5′-CAAATGGGCGGTAGGCGTG-3′ as the primer.

HR in the human cells synchronized to G2/M phase

On each well in six-well dish, 293T cells were seeded at 3×10^5 cells. Next day, nocodazole (SIGMA) in DMSO was added to the medium to a final concentration of 0.1 μg/ml, and the cells were cultured for 18 h. In order to confirm that the cells were synchronized to G2/M phase, they were harvested and treated with Guava Cell Cycle Reagent (Millipore) and analyzed with Guava EasyCyte plus. To these synchronized 293T cells, ARCUT-treated pBFP-N1 and the donor EGFP long (in mole ratio 1:7, 2 μg in total) were introduced using Fugene HD Transfection Reagent. The cells were cultured for 48 h, and the recombinant plasmids were extracted and analyzed as described above.

Suppression of Ku70 and Ku80 proteins to promote HR

The siRNAs for Ku70 and Ku80 were purchased from SANTA CRUZ Biotechnology. Non-specific siRNAs are 5′-CGUACGCGGAAUACUUCGAAG-3′ and 5′-UCG AAGUAUUCCGCGUACGAU-3′. To 293T cells, which were seeded at 2×10^5 cells on each well in six-well dish and incubated for 1 day, siRNA (10 nM in 2.5 ml of medium) was introduced using Lipofectamine RNAiMAX (Invitrogen), and the cells were cultured for 96 h. At the time points of 48 and 96 h after the siRNA transfection, the cells were harvested and the total RNA was extracted by using ISOGEN (Nippon Gene) for RT-PCR analysis. The total RNA was treated with DNase (Promega), and used for the reverse transcription with SuperScript III Reverse Transcriptase (Invitrogen). The cDNA was mixed with THUNDERBIRD SYBR qPCR MIX (TOYOBO), and quantitative PCR was performed using a CFX96 Real-Time System (Bio-Rad). The beta-2-microglobulin (B2M) gene was used as internal standard.

For HR experiments, at the time point of 48 h after siRNA treatment, the cells were transfected with ARCUT-treated pBFP-N1 and the donor EGFP long (in mole ratio 1:7, 2 μg in total) using Fugene HD Transfection Reagent. The cells were cultured for 48 h, and the recombinant plasmids were analyzed as described above.

RESULTS AND DISCUSSION

Design of HR experiments

The ARCUT-promoted HR was carried out as shown in Figure 1a. The substrate plasmid pBFP-N1 contains the gene of blue fluorescent protein (BFP), which is under the control of a CMV promoter. On the other hand, the donor DNA codes a part or the whole of enhanced green fluorescent protein (EGFP). Note that these donor DNAs have no initiation codon so that they never express EGFP by themselves. The BFP and the EGFP have the same amino acid sequences except for the amino acids in their chromophore sites (Ser65, His66 and Gly67 for BFP versus Thr65, Tyr66 and Gly67 for EGFP; see Figure 1b). With the use of ARCUT, the chromophore-coding site of the BFP in the substrate DNA was cut, and the scission product was introduced into 293T cells after being purified by gel electrophoresis, together with the donor EGFP fragment. Thus, the amount of the recombinant protein, which is formed in the cells and emits green fluorescence, directly reflects the efficiency of HR reactions in the human cells.

In ARCUT, two pcPNA strands are laterally shifted by several nucleotides and the resultant single-stranded portions (the parts underlined in red in Figure 2a) are cut by the molecular scissors Ce(IV)/EDTA. Thus, in the scission termini, either of the two DNA strands protrudes the other by 10–15 nt, and the overhang structure depends on the direction of the lateral shift of two pcPNA strands (Figure 2b). This feature is in contrast with currently available protein-based cutters, which almost always provide only 5′-overhang termini. In order to investigate the effect of these overhang structures on the efficiency of HR, two sets of ARCUT were designed. In the DNA scission by ARCUT-5′-overhangs (the set of $pcPNA^1$ and $pcPNA^2$), both of the scission ends have 5′-overhang structures (the 5′-end of each DNA strand is protruding). On the other hand, 3′-overhang structures are formed by ARCUT-3′-overhangs (the set of $pcPNA^3$ and $pcPNA^4$). These two ARCUTs cut the substrate DNA at the target site, as was clearly evidenced by the electrophoresis in Supplementary Figure S2.

Effect of the homology length on the efficiency of ARCUT-mediated HR in human cells

In Figure 3a, two donor DNAs of different lengths were used. The donor EGFP long (717 bp) contains a homology sequence of 185 bp in the upstream of the chromophore-coding site and a homology sequence of 513 bp in the downstream. On the other hand, the donor EGFP short (119 bp) contains homology sequences of 50 bp in both the upstream and the downstream. With the use of ARCUT-3′-overhangs (also with ARCUT-5′-overhangs),

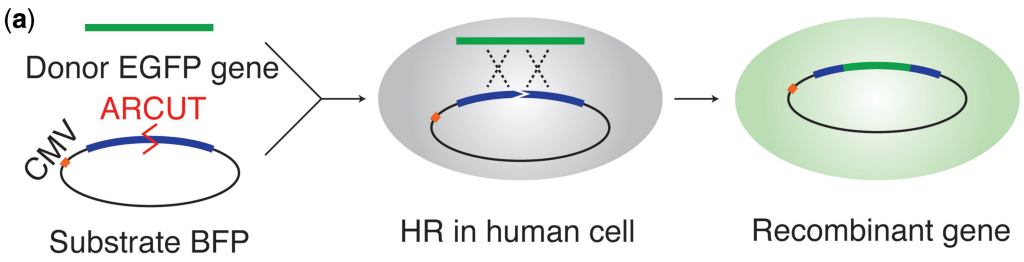

Figure 1. (**a**) HR in human cells to convert the chromophore of BFP to the chromophore of EGFP. (**b**) The sequences of BFP and EGFP near the chromophore-coding regions.

Figure 2. (a) Two sets of ARCUT used in this study to provide different DSB structures (5′-overhangs and 3′-overhangs). Note that the single-stranded portions underlined in red are selectively hydrolyzed by Ce(IV)/EDTA. The terms D and U refer to pseudo-complementary nucleobases 2,6-diaminopurine and 2-thiouracil, respectively. In order to promote the activity of ARCUT, lysines (Lys) and phosphoserines (P) are attached to the termini of pcPNAs (20). (b) Terminal structures of DSB produced by these two types of ARCUT. In order to expose homologous sequences for HR, the overhangs formed by ARCUT must be resected at least down to the borders I and II.

HR was efficient as evidently shown by the emission of green fluorescence from the recombinant protein (Figure 3b). Without the ARCUT scission, however, HR hardly took place. The number of green fluorescence-emitting cells was determined by flow cytometric analysis (Figure 3c). When the donor EGFP long (L) was used, the fraction of green fluorescence-emitting cells in the total cells was 22.5%. With the use of the donor EGFP short (S), the fraction was slightly lower (11.1%). As was previously reported, a longer donor is more favorable for HR (26). However, it is noteworthy that, without the ARCUT treatment, HR is much less efficient with EGFP long and virtually nil with EGFP short. Essential role of ARCUT for efficient HR is reconfirmed.

Effect of DSB structure on the HR efficiency

In order to estimate the efficiency of HR for the 3′-overhang structure and the 5′-overhang structure quantitatively, the recombinant plasmids were extracted from the cells and directly analyzed by sequencing experiments (Table 1). With the use of ARCUT-3′-overhangs, the fraction of the correctly repaired plasmids, which had a chromophore of EGFP, was 10.1% of the total plasmids. With ARCUT-5′-overhangs, the fraction of the repaired plasmids was 6.0%. Apparently, DSB of 3′-overhang structure is 1.7-fold more suitable for the HR than DSB of 5′-overhang structure.

It has been mostly accepted that, in the initial step of HR, the termini of DSB are resected, and the resultant 3′-overhangs invade homologous sequences in the donor DNA (27). With the ARCUT-5′-overhangs, the resection must extend beyond the border I (for the lower DNA strand) or the border II (for the upper DNA strand), since otherwise a 3′-overhang structure is not obtained (Figure 2b). On the other hand, the DSB created by ARCUT-3′-overhangs intrinsically possesses 3′-overhang structures. Thus, the preference of the 3′-overhang structure is consistent with this mechanism. It has been definitely concluded that, in order to achieve HR efficiently in human cells by ARCUT, two pcPNA strands should be laterally shifted toward the C-terminal end of each pcPNA (5′-direction of the complementary DNA strand) to form 3′-overhang termini.

By-products of the ARCUT-mediated HR in human cells

As shown in Table 1, the HR is accompanied by two side reactions, which are categorized into two classes. In the ‘mutation’, several nucleotides were deleted or inserted at the DSB site (a representative sequence is shown in Figure 4a). They cover about 10% of the recombinants. The other one (~4%) is ‘donor integration’ in which the whole of the donor EGFP fragment was ligated into the substrate BFP plasmid (Figure 4b). These by-products could be attributed to NHEJ as an alternative major pathway of DSB repair.

Many other plasmids carried the BFP gene. Apparently, the scission fragment of the BFP, formed by ARCUT, was directly connected each other and returned to the original

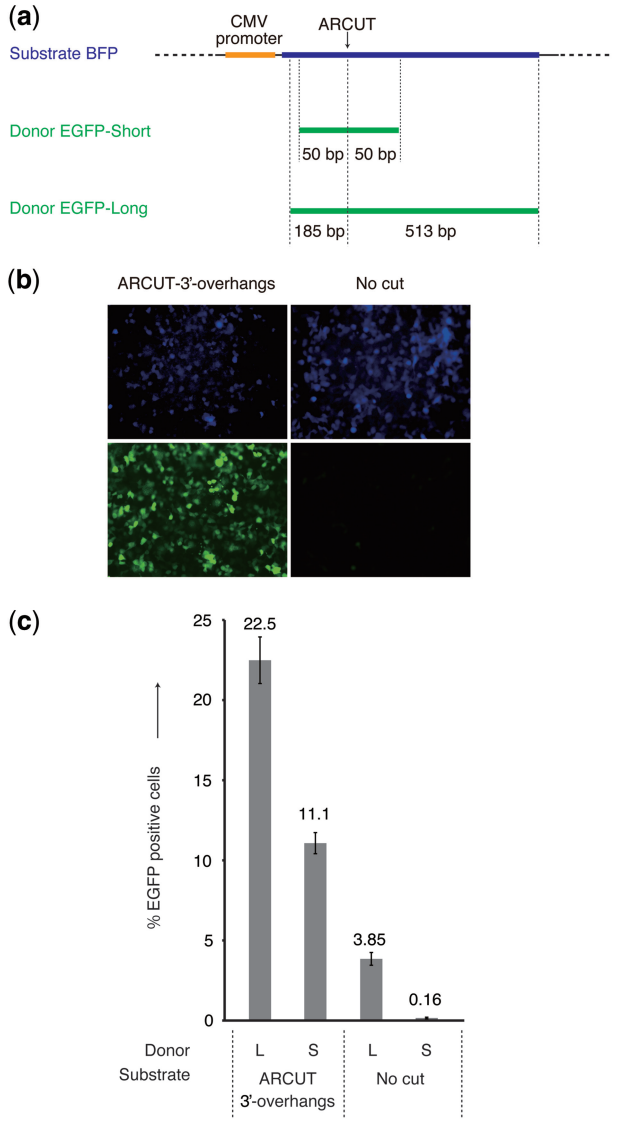

Figure 3. Effects of the homology length on the ARCUT-mediated HR efficiency in human cells. (a) The substrate DNA coding BFP and the donor DNAs having different lengths of homology regions. (b) Typical fluorescence microscopy images of the 293T cells cultured for 48 h after the transfection with the ARCUT-treated substrate DNA and the donor EGFP long (717 bp). The upper and lower panels show blue channel (Ex: 360 nm, Em: 470 nm) and green channel (Ex: 480 nm, Em: 520 nm), respectively. (c) The fraction of green fluorescence-emitting cells in the total cells measured by flow cytometry. The terms L and S refer to the donor EGFP long and the donor EGFP short, respectively.

Table 1. Comprehensive analysis of the plasmids extracted from 293T cells cultured for 48 h after the transfection of ARCUT-scission product together with the donor EGFP long

	Number of plasmids analyzed	EGFP, *n* (%)	Mutation, *n* (%)	Donor integration, *n* (%)	BFP, *n* (%)
5′-overhang	167	10 (6.0)	18 (10.8)	6 (3.6)	133 (79.6)
3′-overhang	158	16 (10.1)	16 (10.1)	7 (4.5)	119 (75.3)

form. This reversal could occur either in human cells or in *E. coli*, which was used for the cloning procedure. Thus, it is not conclusive, which is really the case.

Promotion of the HR by cell cycle synchronization

It has been known that the balance between HR and NHEJ for DSB repair depends on the cell cycle (28,29). HR is up-regulated in later S phase to G2 phase, whereas NHEJ is effective in G1 to early S phase. In Figure 5, 293T cells were synchronized into G2/M phase using nocodazole. The distribution was G0/G1:6.3%, S:31.2% and G2/M:62.4% (the right panel). Compared with asynchronous cells in the left panel (G0/G1:41.8%, S:40.5% and G2/M:17.6%), the population of G2/M is notably increased. Exactly as expected, the HR by ARCUT-3′-overhangs in these nocodazole-treated cells was ~2-fold more effective than in asynchronous cells (Table 2; the entries 1 and 2). The cell cycle synchronization is certainly effective to promote ARCUT-mediated HR in human cells.

Suppression of competitive NHEJ pathway by RNA interference

The efficiency of ARCUT-mediated HR in human cells was also improved by depletion of NHEJ-relevant proteins Ku70 and Ku80. The DSB repair by NHEJ starts by the binding of a heterodimer of these subunits to the DSB ends. Thus, a classical gene targeting through HR was promoted by depletion of Ku70 protein (30,31). In Figure 6, the expressions of Ku70 and Ku80 proteins in 293T cells were repressed with the use of the corresponding siRNA. The levels of the mRNA were decreased to less than 9%. Under these conditions, the efficiency of HR by ARCUT-3′-overhangs was increased by 2.7-fold with Ku70 siRNA and by 2.0-fold with Ku80 siRNA, in comparison with the efficiency with non-specific siRNA (Table 2; see the entries 3–5).

In the Ku80 siRNA-treated cells, the deletions of several nucleotides at the chromophore-coding site slightly prevailed. A similar result was obtained for the treatment with nocodazole (Table 2; the entry 2). Apparently, some of the DSBs formed by the ARCUT were repaired by a pathway other than HR and NHEJ. One of the probable candidates is alternative NHEJ (alt-NHEJ), which involves extensive resection of DSB ends, and is dominant in the cells lacking the Ku proteins (32–34). In order to suppress this side reaction, knock-down of PARP1 as an alt-NHEJ-specific protein could be effective (35,36).

The attempts to combine this approach with the cell synchronization by nocodazole, described in the previous section, and improve the efficiency furthermore were unsuccessful, since the cell viability was severely impaired when the cells were consecutively treated by these two procedures. Further optimization of the experimental conditions should be necessary.

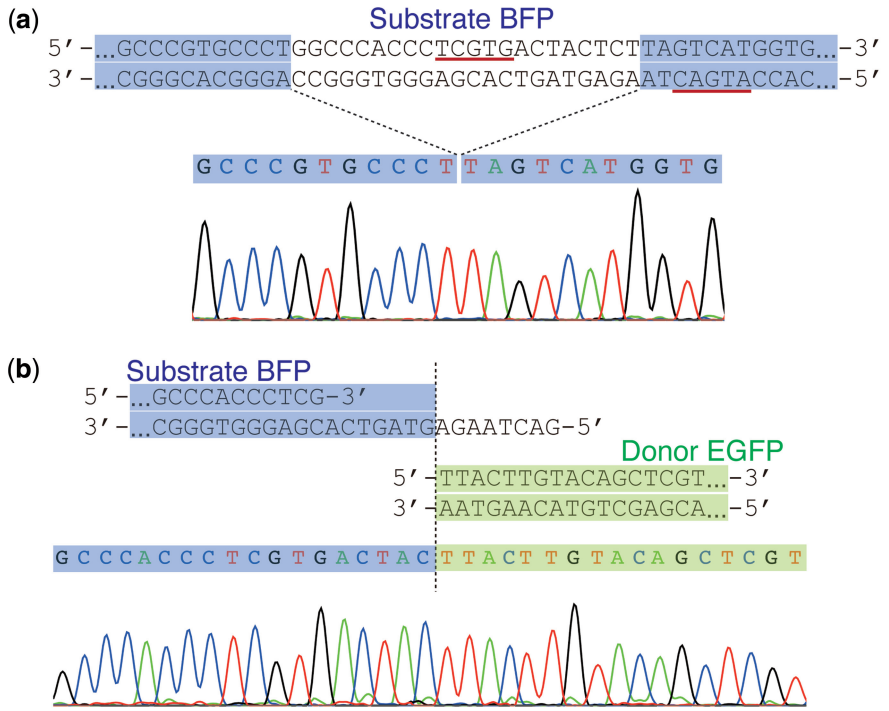

Figure 4. Representative by-products of HR, which are formed through (**a**) 'mutation' and (**b**) 'donor integration'.

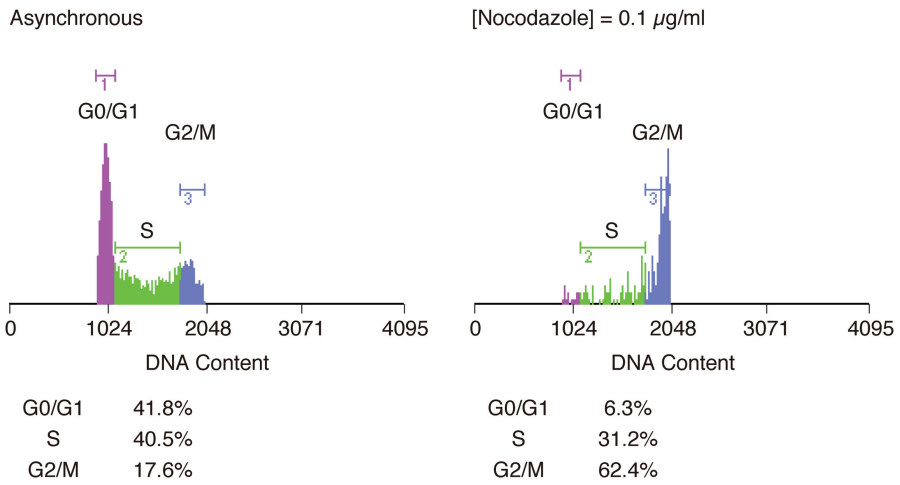

Figure 5. Cell cycle distribution with the treatment of 0.1 μg/ml nocodazole (right) and without the treatment (left). Flow cytometry was carried out at 18 h after the treatment. The results of HR on these cells are presented in Table 2.

Table 2. Effects of cell cycle synchronization and suppression of NHEJ on the product distribution

Entry	Treatment	Number of plasmids analyzed	EGFP, *n* (%)	Mutation, *n* (%)	Donor integration, *n* (%)	BFP, *n* (%)
1	Asynchronous	72	9 (12.5)	7 (9.7)	3 (4.2)	53 (73.6)
2	Nocodazole	83	18 (21.7)	16 (19.3)	3 (3.6)	46 (55.4)
3	Non-specific siRNA	79	10 (12.7)	14 (17.7)	2 (2.5)	53 (67.1)
4	Ku70 siRNA	88	27 (30.7)	13 (14.8)	2 (2.3)	46 (52.3)
5	Ku80 siRNA	84	20 (23.8)	20 (23.8)	4 (4.8)	40 (47.6)

The 293T cells were synchronized to G2/M phase by nocodazole (entries 1 and 2) or treated with siRNAs (entries 3–5). Then, the cells were transfected with ARCUT product together with the donor EGFP long, and cultured for 48 h. The results of the nocodazole treatment and the siRNA treatment are presented in Figures 5 and 6, respectively.

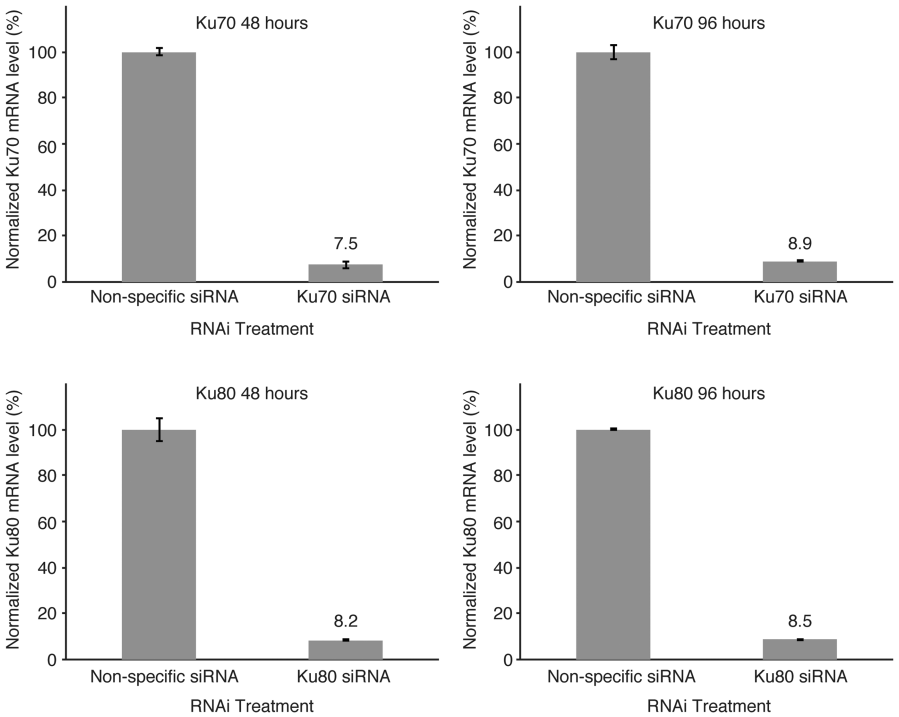

Figure 6. Suppression of Ku70 and Ku80 by siRNA. The mRNA levels in 293T cells were determined by quantitative PCR at 48 h (left) and 96 h (right) after the transfection of the corresponding siRNA. The results of HR on these cells are presented in Table 2.

CONCLUSION

An artificial restriction DNA cutter (ARCUT) is a recently developed chemical tool to cut double-stranded DNA at a pre-determined site, which can be determined simply in terms of Watson–Crick rule. The structure of its scission termini (3′- or 5′-overhang) can be easily and straightforwardly modulated according to our need, although most of protein-based DNA cutters currently available provide only the termini of 5′-overhang structures. In this work, ARCUT was used to form a DSB at target site and stimulate aimed HR in human cells. A substrate DNA, cleaved at a pre-determined site by ARCUT, was introduced into human cells together with a donor DNA, and the efficiency of HR was quantitatively evaluated. Of two kinds of scission ends formed by ARCUT, 3′-overhang terminal structures provide better HR efficiency than 5′-overhang terminal structures. Longer donor DNA is more favorable for ARCUT-mediated HR, although the HR is successful even with very short homology site (e.g. 50 bp in each side). The efficiency of this HR is notably promoted when human cells were synchronized into G2/M phase. The suppression of NHEJ through repression of the relevant proteins Ku70 and Ku80 was also effective to promote ARCUT-mediated HR. All these chemical and biological approaches are, in principle, independent from each other. Accordingly, appropriate combination of them should be very effective to promote ARCUT-mediated HR in human cells. These attempts, as well as the applications of ARCUT to targeting of human genome, are currently under way in our laboratory.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1 and 2.

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Conflict of interest statement. None declared.

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