Chapter 9

Enhancement of Homologous Recombination Efficiency by Homologous Oligonucleotides

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Additional information is available at the end of the chapter

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1. Introduction

Gene targeting is a powerful technology to achieve gene ablation and modification in the study of gene function by phenotypic analysis. This method is widely recognized as being useful; however, its application is not yet versatile. Its main limitation is the small number of cells in which gene replacement occurs efficiently after endogenous homologous recombination. Artificial enhancement of homologous recombination has been hardly successful. Recently, however, several techniques have been reported that can increase the efficiency of homologous recombination. In this chapter, I first summarize the principles and applications of those techniques. Next, I focus on a simple technique, in which the addition of oligonucleotides, homologous to the targeted locus, significantly increases the efficiency of homologous recombination and, subsequently, the number of genetically targeted clones. The greatest benefit of oligonucleotide-aided homologous recombination is its versatility, i.e., its applicability to virtually any cell type. Finally, the presumed molecular mechanisms underlying oligonucleotide-aided homologous recombination are presented.

2. Gene targeting technology

The ultimate goal of genetic molecular biology is to modulate the activity of genes at will. In gene targeting technology, in vivo homologous recombination enables the replacement of a target genomic region with an exogenous DNA fragment that contains a region homologous to the targeted locus (Fig. 1). This technology is indispensable for the analysis of gene function. To acknowledge its importance, the discovery of the principles of gene targeting in mice was awarded the Noble Prize in 2007. Recently, applications of this technology have been expanded to gene therapy and transgenic plants. However, the success of the technique greatly depends on the efficiency of homologous recombination; therefore, it cannot be successfully applied to cells with low homologous recombination efficiency, such

as mammalian somatic cells and higher plant cells. Furthermore, in multiploid somatic cells, simultaneous gene targeting is required, making complete gene replacement extremely difficult. Even in embryonic stem (ES) cells, where homologous recombination occurs with high efficiency, some genes are difficult to target and subject to homologous recombination. To overcome these problems, the development of efficient and versatile methods that can artificially increase the efficiency of homologous recombination is needed.

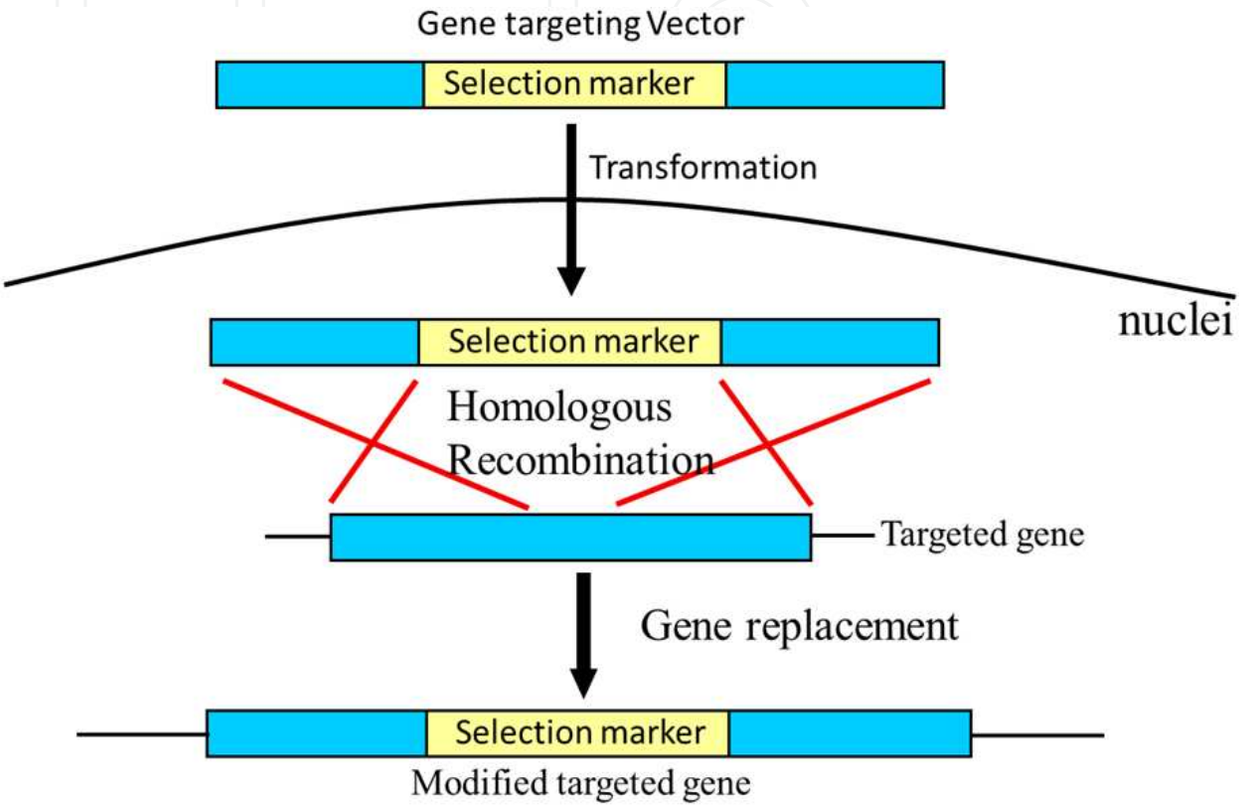

Figure 1. Schematic diagram of principle of Gene Targeting

When extracellular DNA fragment is introduced into cell, gene replacement rarely occurs by homologous recombination. Utilizing this phenomenon, one can 'target' a gene of interest and change its DNA alignment at will.

3. Introduction of vector DNA into cells

Introduction of a targeting vector (DNA fragment) into a cell is the primary step to targeting genes. In general, there are 3 types of strategies to introduce a DNA fragment into a cell: biological, chemical, and physical. The biological method, using a virus vector, yields high transformation efficiencies. However, there remains the risk of insertion of viral vector genes into the host genome with selectivity for the virus. The chemical method, usually utilizing a polycationic polymer, is easy to perform. However, the polymer can be recognized as a foreign substance and become enclosed within endosomes, where it is digested along with the transformation vector. It is possible to make the polymer escape this digesting pathway, but the strategy is generally not very efficient (Colosimo et al., 2003).

Physical methods such as microinjection and electroporation are relatively versatile and most frequently used in gene-targeted cell transformation (Niidome et al., 2002).

4. Construction of gene targeting vectors

In gene targeting, a linear DNA fragment is used as targeting vector. The targeting vector consists of 2 homologous regions to the genome and a drug resistance gene for the selection of transformants (Fig. 1). Drug resistance gene products can degrade drugs such as G418, hygromycin B, puromycin, and blasticidin S, which are toxic to untransformed cells, thus facilitating the survival of the transformed cells in drug-containing medium. The targeting vector is constructed by fusing the 3 DNA fragments in tandem by using basic molecular biological techniques, such as PCR, restriction enzyme digestion, and cloning techniques. Interestingly, Kuwayama et al. have described the construction of a gene targeting vector by PCR only, but unfortunately not in sufficient detail for reproduction (Kuwayama et al., 2002).

Briefly, in the first step, three separate PCR syntheses of a selectable marker cassette and the 5'- and 3'-homologous regions of a target gene. Of the four primers used in amplification of the 5'- and 3'-regions of the target gene, two primers placed proximal to the site of the marker cassette are designed to have sequence tags complementary to the 5'- or 3'-side of the marker cassette. The two primers used in PCR synthesis of the marker cassette are complementary to the tagged primers. By fusion PCR, the 5' and 3' PCR products are connected to the marker cassette via the regions of tagged primers that overlap. And then, a sufficient amount of the disruption construct can be directly amplified with the outermost primers (Fig. 2).

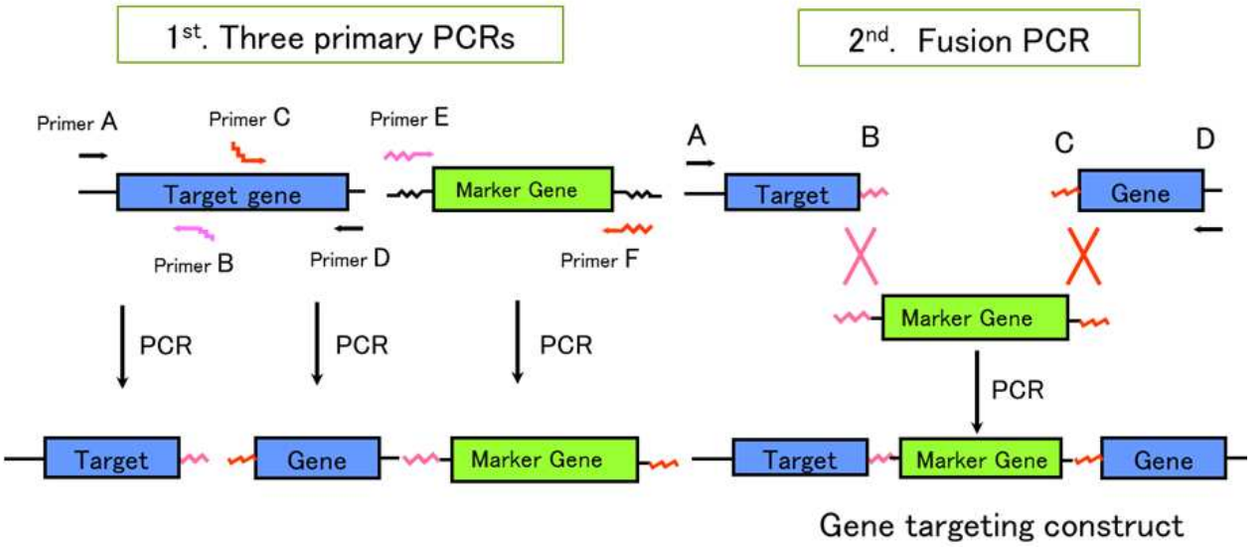

Figure 2. Schematic diagram of PCR-dependent construction of gene targeting vector.

Step 1, the three primary PCR reactions. The 5'- and 3'-flanking regions are amplified with primers (primers A – F) specific for the sequence of the target gene. The primers distal to the selectable marker insertion site are simple primers complementary to the target gene

(primers A and D). The primers directly adjacent to the marker cassette are chimeric (primers B and C). Primers complementary to primers B and C are used to amplify the selection marker gene (primers E and F). Step 2, fusion PCR. The 5´- and 3´-flanking regions are joined to the marker gene and the final PCR product is amplified with the outermost primers A and D. The order in which the flanking sequences are joined to the ends of the selectable marker cassette is discretionary. The final PCR reaction mix containing the targeting vector is subjected to ethanol precipitation and can be directly used to transform cells.

For the transformation, the targeting vector must be linearized by enzymatic digestion at a site outside the insert region; alternatively, the insert region may be amplified by PCR before transformation. One of the critical points in constructing targeting vectors is the length of the homologous regions. Optimum length of the homologous regions for gene targeting varies and depends on the organism, cell type, and targeted locus. In practice, the length of the homologous DNA region, which is cloned or amplified by PCR, is limited. In addition, an excessively long DNA fragment is difficult to introduce into cells. Therefore, the homologous DNA fragment is generally designed to be 5–8 kb in length (Hasty et al., 1993).

5. Principles and applications of gene targeting methods

Introduced DNA fragments containing homologous sequences to genomic DNA rarely induce target gene replacement (Hasty & Bradley, 1993; Rouet et al., 1994; Smih et al., 1995; Jasin, 1996; Mamsour et al, 1988). Notwithstanding its low occurrence, this reaction (so-called homologous recombination) is considered the driving force of evolution and diversity in species. In a test tube, it is possible to manipulate the genomic DNA at will by means of gene targeting in organisms and/or cells with high transformation and endogenous homologous recombination efficiencies. However, even in such model systems, targeting vector transformation results in a high proportion of non-targeted (randomly inserted) transformants. This is because homologous recombination efficiency is generally much lower than genomic insertion efficiency. To overcome this problem, several methods, which eliminate non-targeted transformants, have been proposed.

One such method is positive/negative selection (Mamsour et al., 1988). This method, based on the addition of a negative selection marker gene in one or both ends of the gene targeting vector, aims at the enrichment of the small fraction of cells in which homologous recombination took place. In case of random insertion, the negative selection marker is integrated into the genome along with the targeting vector. The negative marker gene product eliminates non-targeted host cells, and only homologously recombined clones can survive by removal of the negative selection marker gene during homologous recombination. However, the added negative selection marker gene may reduce homologous recombination efficiency. In another method, the "promoterless" method, the promoter region of the selection marker gene is removed and the marker gene can be translated only when homologous recombination occurs. However, when the endogenous promoter activity is low, the marker gene is not expressed at levels high enough to degrade the selection drug.

Any of the 2 abovementioned methods can reduce the number of non-targeted clones, and minimize the time and effort required for selection of the targeted clones. However, none of them achieve the ideal removal of non-targeted clones. In fact, the ratio of targeted clones to non-targeted clones can be as low as 0.1%; in cultured mammalian cells, this percentage can reach up to 20% (Sedivy & Dutriaux, 1999). To increase the proportion of targeted clones, a method has been developed that suppresses non-homologous recombination by mutagenizing host cells. This method targets ku70/ku80 genes, which encode proteins that bind the ends of DNA linear fragments (Kooistra et al., 2004 ; Ninomiya et al., 2004). Without these genes, host cells suppress non-homologous recombination and, as a result, homologous recombination efficiency increases. However, application of this method is restricted to host cells possessing ku70/ku80 orthologous genes. Moreover, the effects of elimination of these genes should be carefully examined in each particular case.

6. Increased homologous recombination efficiency by artificial methods

Some methods have been proposed that artificially increase endogenous homologous recombination efficiency. The common basis of these methods relies on the observation that the occurrence of a DNA double-strand break (DSB) in the targeted region dramatically increases homologous recombination efficiency. Hence, artificial induction of DSB would effectively increase gene targeting efficiency, to (Hasty & Bradley, 1993; Rouet et al., 1994; , Jasin, 1996). At least 3 different DSB-inducing methods have been reported. *I-SceI* is a highly specific restriction enzyme that recognizes an 18-bp-long DNA sequence (Fig. 3). When the recognition site exists within the targeted domain, co-transformation of *I-SceI* with targeting vector results in specific digestion of the genomic DNA at the recognition site, increasing homologous recombination efficiency. The limitations of the method are the necessary pre-existence of a *I-SceI* site in the target region and the absence of that same site from the targeting vector. Moreover, there should be ideally no other *I-SceI* restriction site in the host genome. Therefore, application of the method is restricted to certain organisms and cells.

Another method utilizes a nuclease fused with engineered C2H2 zinc finger protein-based DNA-binding domains, which bind sequences specifically at the targeting region and cause site-directed DSBs (Fig. 4). A limitation of this method is the effort required to accurately and carefully design the zinc finger DNA-binding domain, because binding specificity and affinity are the critical determinants of recombination efficiency. Furthermore, simultaneous expression of zinc finger nucleases (ZFNs) with the gene-targeting construct is also indispensable (Urnov et al., 2005).

Triplex-forming oligonucleotides (TFOs) are known to induce a DNA DSB and repair system and, thus, are expected to increase homologous recombination efficiency *in vivo* (Fig. 5). However, TFOs that bind to double-stranded DNA are restricted to the polypurine or polypyrimidine tract; therefore, this technology is limited to segments with unique target sites (Demidov, 2003).

When the recognition site of I-SceI inserted into the targeted domain, co-transformation of I-SceI enzyme with targeting vector results in specific digestion of the genomic DNA at the recognition site, increasing homologous recombination efficiency.

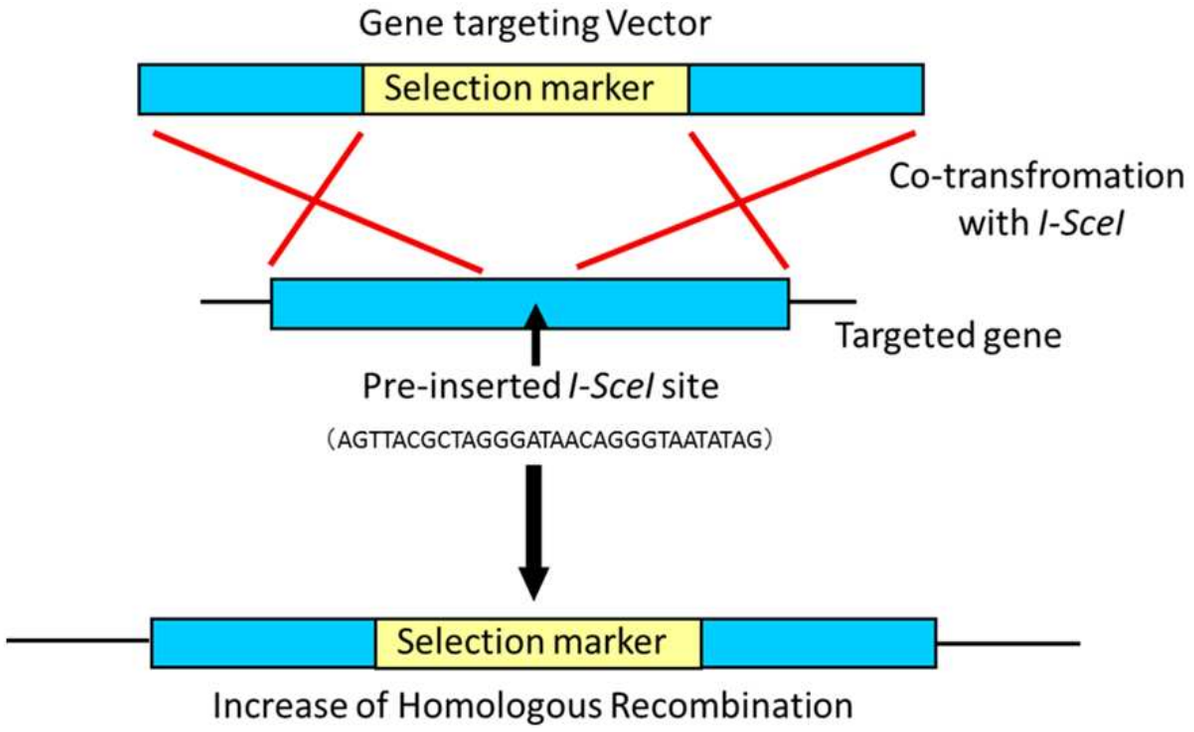

Figure 3. Schematic diagram of Restriction Enzyme (I-SceI) dependent increase of homologous recombination efficiency.

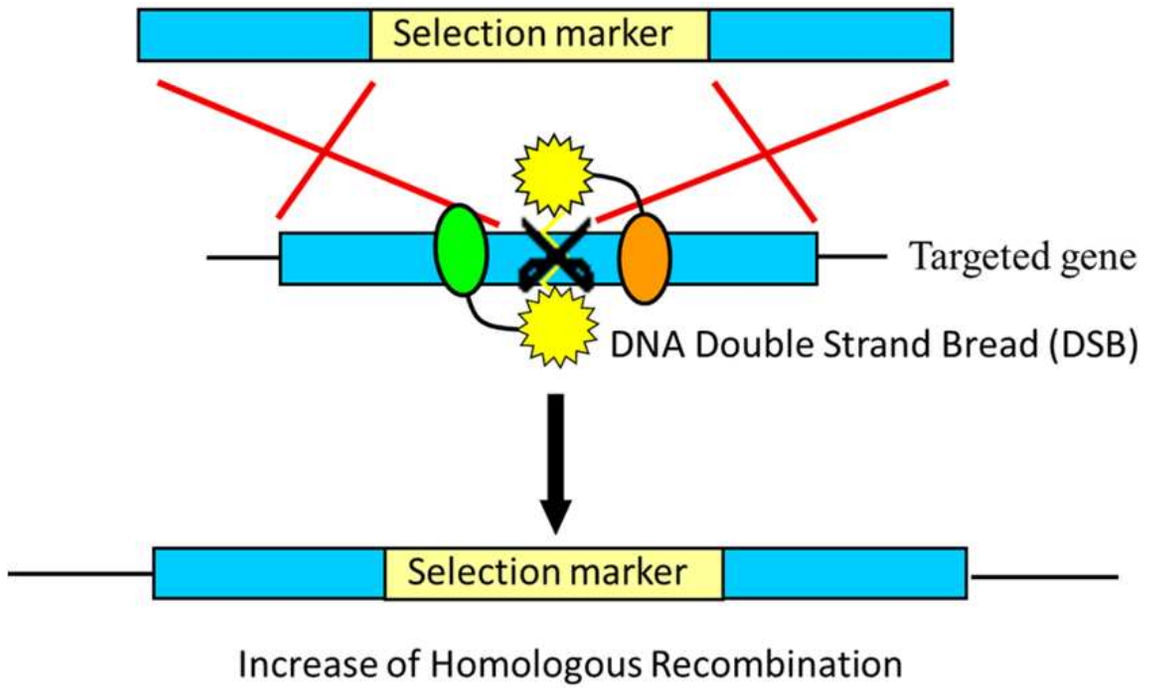

Figure 4. Schematic diagram of zinc finger nucleases (ZFNs) dependent increase of homologous recombination efficiency.

By expressing engineered C2H2 zinc finger protein-based DNA-binding domains which bind sequences specifically at the targeting region, a site-directed DSBs occurs and, thus, increases homologous recombination efficiency.

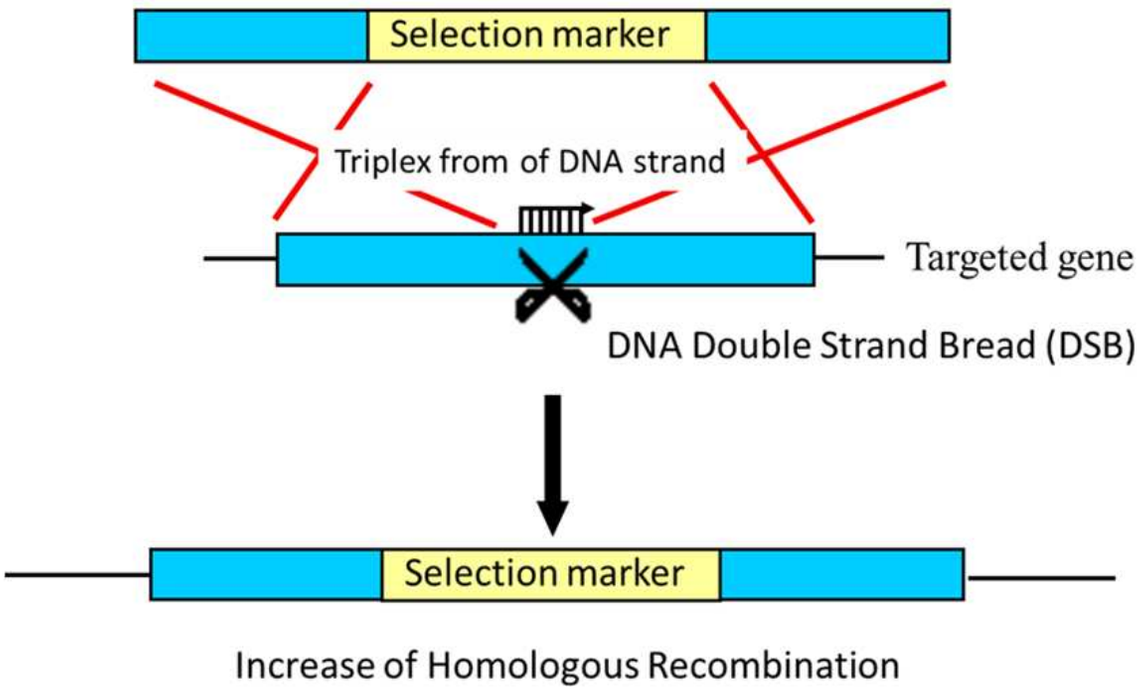

Figure 5. Schematic diagram of Triplex-forming oligonucleotides (TFOs) dependent increase of homologous recombination efficiency.

When a TFO is formed in genome, a DNA DSB is induced at the site and, thus, homologous recombination efficiency increases.

7. Enhancement of homologous recombination efficiency by homologous oligonucleotides

Recently, I described a general gene targeting method in which co-transformation of DNA oligonucleotides (oligomers) could significantly increase homologous recombination frequency and transformation efficiency (Kuwayama et al., 2008). This method is based on the experience that a high concentration of gene-targeting construct generally provides considerably better transformation and homologous recombination efficiencies. However, the amount of gene targeting construct that can be used at each transformation is limited and, furthermore, preparing a large amount of vector DNA is demanding. In order to overcome this limitation, I tested whether addition of only a part of the homologous region of a gene-targeting construct was as effective as the entire construct (Fig. 6).

A single-stranded DNA oligomer is much smaller than the gene targeting vector, and thus, it can be introduced into cells in much larger amounts than the targeting vector. Using the cellular slime mold model organism, *Dictyostelium discoideum*, and mammalian Hela cells, the effect of co-transformation of short homologous DNA oligomers was tested. In D. discoideum, the gbfA gene locus was chosen to be targeted because this gene was reportedly difficult to replace with an endogenous targeting vector. By electroporation, the gene targeting vector and homologous strand of short DNA oligomers were simultaneously transformed into *D. discoideum* cells. The DNA oligomer was about 20 bases in length, and the added concentration was 10 to 100 μM. This concentration was 100 to 10,000 times higher than that of the gene targeting vector. As a result, homologous recombination as well as transformation efficiencies significantly increased. Since this positive effect was also observed with all the genes tested—*pkaC*, *gbfB*, *ctxA*, and *ctxB*—addition of homologous DNA oligomer was considered to be effective in general in *D. discoideum*. The tested oligomers were designed such that they had 20 – 24 monomers, and the sequences at both the ends were identical to those of the flanking regions in the inward direction (Fig. 6). When the wild-type cells were co-transformed with 100 μM of the two inward-directed oligomers, the gene targeting efficiency as well as the transformation efficiency increased in all cases (Fig. 7). These results indicate that the co-transformation of the designed homologous oligomers increases the transformation efficiency.

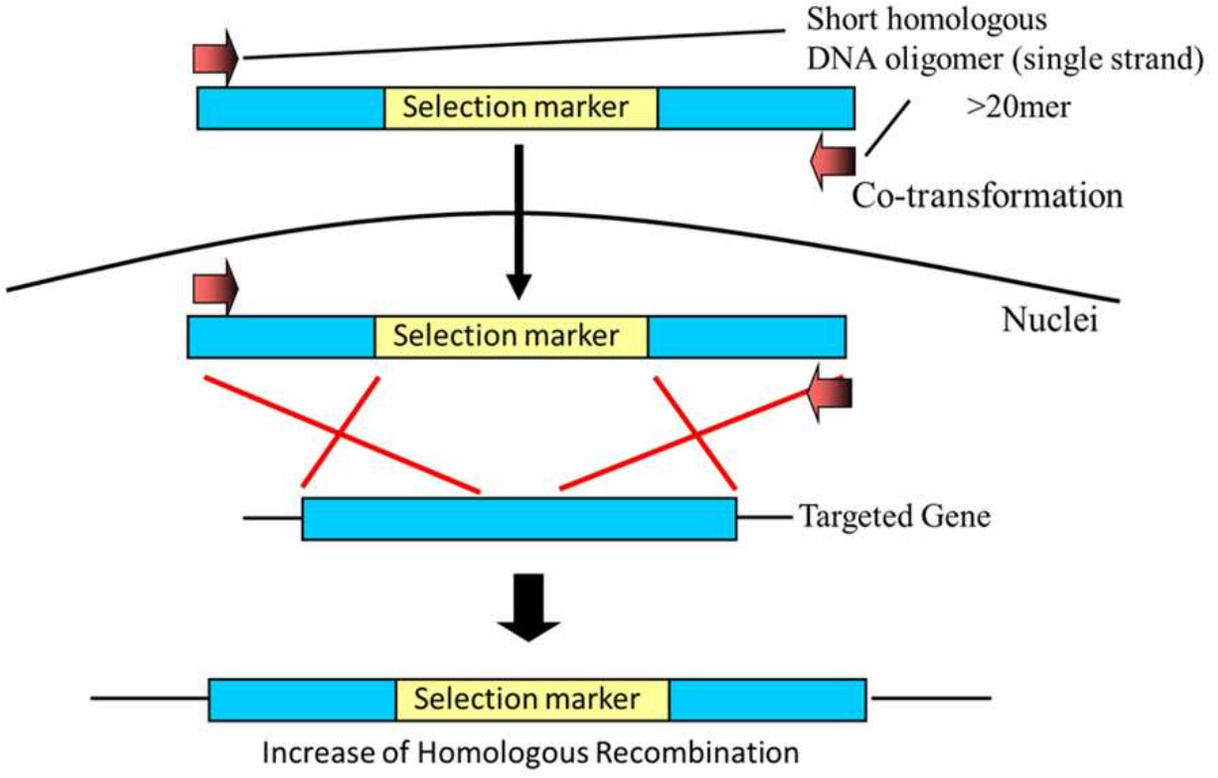

Figure 6. Schematic diagram of increase of homologous recombibation efficiency by short hologous DNA oligomers.

Furthermore, we also observed that, in diploid cells, sister alleles were simultaneously recombined with the targeting vectors. As this effect was also observed in the human cell line, Hela, it was suggested that this effect is not specific to *D. discoideum* cells but is general to all eukaryotic cells.

Why does the simple addition of homologous short DNA oligomers increase specific homologous recombination efficiency? It is not likely that TFOs are responsible because oligomers lacking TFO signature sequences are also effective. Hence, site-specific DSB does not seem to occur. One possibility is that short DNA oligomers can easily be introduced into the nuclei, affecting chromatin structure at the targeting locus and enhancing the interaction between the genomic targeted region and the targeting vector. Another possibility is that the added DNA oligomers contribute to the increase in the number of targeting vector molecules in the cell.

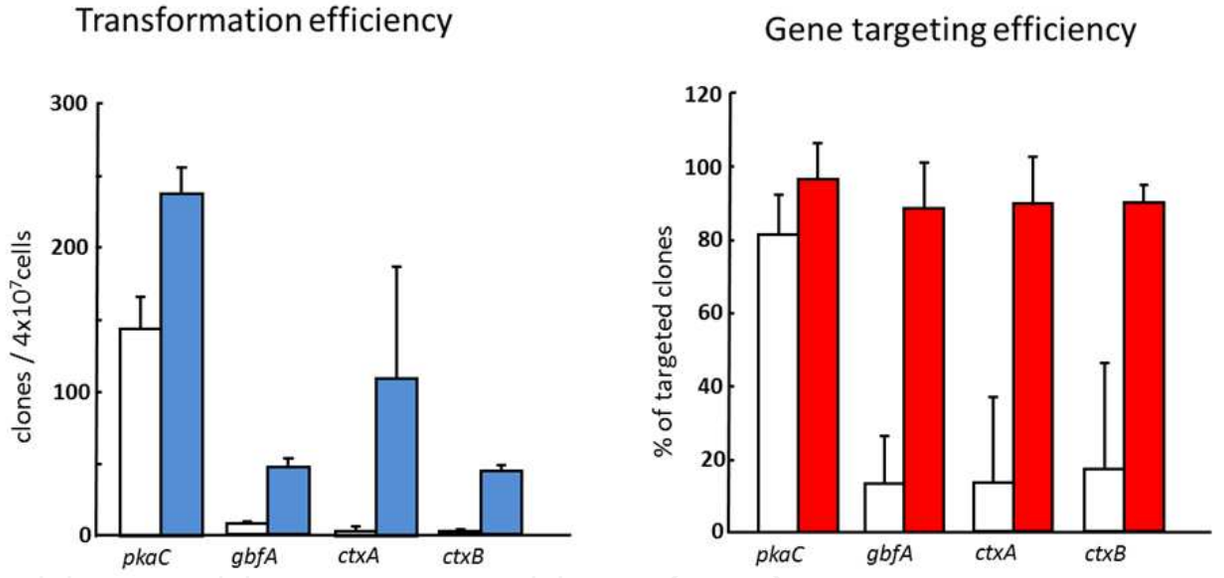

Figure 7. Increase of the homologous oligomers on the transformation and homologous recombination efficiencies.

Co-transformation of high concentration of short homologous DNA oligomers results in significant increase of homologous recombination. This technique also increases transformation efficiency, although the detailed mechanism is not unraveled, yet.

Transformation efficiencies were examined for the pkaC, gbfA, ctxA, and ctxB gene - targeting constructs. The data are represented as the number of primary transformants per transformation (2×10^7 cells). White bars represent transformation without oligomers. Blue bars represent transformation with 100 μM inward oligomers. Homologous recombination efficiencies of the gene- targeting construct without the oligomers (white bar) and with 100 μM oligomers (Red bar) are represented as an average percentage of the gene targeted

transformants to the total number of transformants. Bars represent standard deviation (SD) of 3 independent transformations.

8. Conclusion

The DNA oligomer-aided homologous recombination herein presented can, in principle, be applied to any general transformation method, including microinjection and lipofection, and cell line, including mammalian transformed and primary somatic cells. Furthermore, no cytotoxicity and no random insertion of DNA oligomers into the genome were observed (unpublished data). Although the reason underlying the increase in the homologous recombination efficiency after oligomer addition remains unknown at present, it may become possible in the future to design oligomers to target the most effective position at the locus of interest.

Further improvements in this method are expected to result in practical and clinically safe therapeutic modifications of human cells, in particular, by using artificial nucleic acid analogues such as peptide nucleic acid (PNA) and 2´-O or 4´-C locked nucleic acid (LNA). The use of these analogues is expected to provide higher homologous recombination frequency at low oligomer concentration because PNA and LNA have an increased affinity to native DNA and a high resistance to nucleases, thereby imparting higher biostability (Demidv, 2003). Furthermore, these analogues are low in toxicity (Wahlestedt et al., 2000; Kaihatsu et al., 2004). In the future, it is hoped that this method will contribute to development of genetically engineered high-efficiency yielding transformation methods and to innovation of epochal gene delivery systems.

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