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Chapter

1

General Introduction



Gene targeting by single-stranded oligodeoxyribonucleotides (ssODNs) is a promising technique for introducing site-specific sequence alterations without affecting the genomic organization of the target locus. Over the last five years, significant progress has been made in unravelling the mechanisms and reaction parameters underlying ssODN-mediated gene targeting. Here, we discuss the various mechanistic models that have been proposed and the cellular pathways that may be involved in the targeting process. The DNA mismatch repair (MMR) system imposes a strong barrier to effective application of ssODN-mediated gene targeting. Recent advances that have been made to uncover the role of the MMR system are highlighted. We discuss how the development of new tools such as zinc-finger nucleases (ZFNs) may further promote the efficacy of ssODN-mediated gene targeting.

BACKGROUND

With the advance of human genome sequencing many small DNA sequence variations between individuals have been identified. The consequence of these sequence variations is often difficult to predict: in some cases they may represent neutral polymorphisms, while in other cases they may be deleterious and cause disease. Site-specific gene modification provides a powerful tool to functionally characterize these identified sequence variants and distinguish between polymorphisms and truly pathogenic mutations. Furthermore, permanent correction of a defective gene at its natural chromosomal location may be the ideal treatment for human genetic disease.

Several strategies have been developed for the generation of site-specific sequence alterations. Gene targeting via homologous recombination (HR) requires the design and construction of a targeting vector that is homologous to the target locus [1,2] (Figure 1A). The desired genetic alteration is integrated into the gene of interest together with a selectable marker gene, which is subsequently removed via Cre/lox-mediated recombination [3,4]. Yet, this procedure requires multiple rounds of selection and

clonal purification of the modified cells, which can be laborious and time-consuming.

An alternative approach could be the introduction of subtle gene modifications via short synthetic oligonucleotides that differ from the target locus by one or a few nucleotides [5]. Initial studies focused on the use of chimeric RNA-DNA oligonucleotides (RDOs) to create site-specific sequence alterations [6-9]. RDOs are self-complementary oligonucleotides composed of a contiguous stretch of RNA and DNA residues folded into a double-hairpin configuration (Figure 1B). The RNA residues are modified by 2'-O-methylation of the ribose sugar to make the RDO resistant to ribonuclease activity, whereas the hairpin-capped ends avoid destabilization or destruction of the RDO by helicases or exonucleases. Although the feasibility of RDO-mediated gene targeting was demonstrated in various systems, frequencies varied strongly and results were often irreproducible (see [10] and references therein). Mechanistic studies showed that the RNA-containing strand of the RDO was dispensable and that only the DNA strand was necessary for transfer of the mutation to the target sequence [11,12]. Since

then, research on gene targeting strategies via synthetic oligonucleotides has shifted focus towards the use of single-stranded oligodeoxyribonucleotides (ssODNs) (Figure 1C). Until recently, the addition of chemical modifications to protect the ssODNs against nucleolytic degradation seemed an enormous advance in improving the efficacy of ssODN-mediated gene targeting [13,14]. Now, it is becoming clear that some of the deleterious effects of ssODN-mediated gene targeting on the survival of targeted cells are actually caused by the presence of these protective modifications (Chapter 5).

Here, we describe the various mechanistic models that have been proposed for ssODN-mediated gene targeting and discuss the most important cellular pathways that may be involved in the targeting process. We will focus particularly on the role of the DNA mismatch repair (MMR) system that imposes a strong barrier to effective application of ssODN-mediated gene targeting. Lastly, we discuss how the development of new tools such as zinc-finger nucleases (ZFNs) may further promote the efficacy or could potentially extend the use of ssODN-mediated gene targeting.

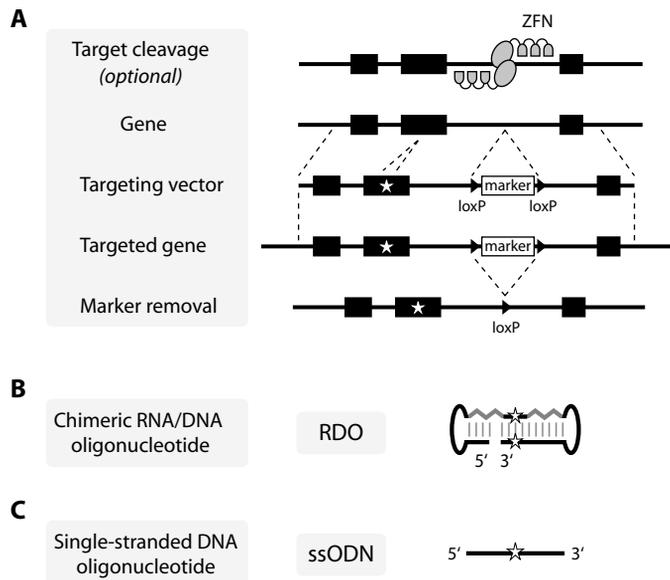


Figure 1 Gene targeting strategies for the introduction of site-specific sequence alterations. (A) Gene targeting via homologous recombination (HR) requires a targeting vector that is homologous to the target locus (~7 kb of total homology) in which the desired genetic alteration is introduced together with a selectable marker gene. The selectable marker gene (typically inserted into an intron) is flanked by loxP sites for subsequent removal by expression of the Cre recombinase. Induction of a DNA double-strand break (DSB) by zinc-finger nucleases (ZFNs) may promote HR between the targeting vector and the chromosomal locus (optional). (B) The chimeric RNA-DNA oligonucleotide (RDO) is composed of a 25-nt DNA stretch (DNA strand) containing the desired sequence alteration and a complementary stretch consisting of both DNA and 2'-O-methyl RNA (chimeric strand) folded into a double-hairpin configuration. Black solid lines indicate DNA residues; grey wavy lines indicate RNA residues. (C) Single-stranded oligodeoxyribonucleotides (ssODNs) of 25- to 120-nt with or without protective end groups are homologous to the target sequence except for one or a few centrally located base alteration(s). The position of the desired sequence alteration is indicated by the asterisk.

MECHANISTIC MODELS FOR ssODN-MEDIATED GENE TARGETING

Repair-dependent model

Several models have been proposed for the mechanism of ssODN-mediated gene targeting. Early models proposed that the initial and rate-limiting step in the targeting process was the formation of a displacement loop (D-loop), caused by pairing between the ssODN and its chromosomal complement (reviewed in [15,16]). After pairing, DNA repair proteins would convert the base alteration to the chromosomal DNA strand using the ssODN as a template (Figure 2, model I). It was postulated that proteins involved in nucleotide excision repair (NER) or mismatch repair (MMR) were responsible for copying the sequence alteration from the ssODN to the chromosomal DNA strand. However, this model is not very plausible when the strand specificity of the MMR system is taken into account: MMR proteins only act on the newly synthesized DNA strand, using the parental DNA strand as a template.

Replication-dependent model

Over the past few years, it has become evident that DNA replication is the predominant cellular process underlying ssODN-mediated gene targeting. In the current model, the ssODN anneals to its chromosomal complement within the context of a replication fork (Figure 2, model II). The single-stranded DNA (ssDNA) regions that are present during DNA synthesis may facilitate ssODN annealing by increasing the accessibility of the target locus. Upon annealing, the replication machinery uses the ssODN as a primer for DNA synthesis, resulting in incorporation of the ssODN into the nascent DNA strand. This model has gained strong support from numerous studies (discussed below). Yamamoto *et al.*

[17] have reported that sense ssODNs (complementary to the transcribed strand) were approximately 20-fold more effective than the complementary anti-sense ssODNs in targeting the *cyc1* gene in *Saccharomyces cerevisiae*. This difference in efficacy was independent of ssODN sequence or length, and was not affected when *cyc1* transcription levels were reduced to approximately 2% of the normal levels. The authors postulated that the strand specificity is caused by the preferential incorporation of the ssODNs into either the leading or lagging strand during DNA replication. Important studies in *Escherichia coli* established a direct link between the direction of replication through the target locus and the strand bias [18-23]. Chromosomal reporter systems were constructed in which the reporter genes had a defined orientation relative to the direction of replication. In these reporter systems, ssODNs corresponding in sequence to the lagging strand consistently performed better than ssODNs corresponding to the leading strand, irrespective of the direction of transcription. In mammalian cells, the direction of the replication fork through the target locus is difficult to determine and may vary due to the firing of different origins in subsequent cell cycles. Therefore, a direct link between the polarity of the ssODN and the direction of replication could not be established [24,25].

Nevertheless, by increasing the number of cells that progressed through S phase during ssODN exposure, many reports have demonstrated that also in mammalian cells ssODN-mediated gene targeting takes place during S phase. Targeting frequencies were elevated when cells were exposed to ssODNs following synchronization at the G₁/S border by mimosine [26,27], aphidicolin [28], or 1-β-D-arabinofuranosylcytosine (Ara-C) [29] or after synchronization in S phase by thymidine or hydroxyurea [28,30].

Within the replication fork the target locus is exposed as a single-stranded region, which may facilitate annealing of the ssODN. Slowing down S phase progression during ssODN exposure, due to the repeated stopping and restarting of replication forks, by thymidine [31,32], hydroxyurea [25,33] or 2',3'-dideoxycytidine (ddC) [28,29], also improved the efficacy of ssODN-mediated gene targeting. Inhibition of the replicative polymerases may lead to functional uncoupling of the replicative helicase and DNA polymerase activities, resulting in the accumulation of larger regions of unwound ssDNA in front of the fork [34,35]. In *S. cerevisiae*, these ssDNA gaps at the replication fork are normally approximately 220 nt in length, while hydroxyurea treatment increases the size of these gaps by ~100 nt [36].

When DNA replication was fully blocked by aphidicolin or Ara-C, targeting frequencies decreased dramatically [24,29,31], indicating that active DNA synthesis is required for effective ssODN-mediated gene correction. Upon annealing to its single-stranded target region, the ssODN may be extended by the replication machinery and form a "pseudo" Okazaki fragment. This implies that the 3' end of the ssODN needs to be accessible for elongation by the replicative polymerases. Radecke *et al.* [37] showed that nuclease-resistant ssODNs with chain-terminating 2',3'-dideoxy residues at their 3' ends were 10-fold less effective than ssODNs with canonical 3'-OH ends, which was confirmed in an episomal targeting system in *E. coli* by Huen *et al.* [22]. In addition, Olsen *et al.* [24] used ssODNs with octyl or HEG (hexaethylene glycol) blocking groups at the ends. The addition of a 5' or a 3' blocking group reduced the targeting frequencies to 77% and 29% of the activity obtained by unblocked ssODNs, respectively. Using ssODNs containing blocking groups at both termini even further reduced the efficacy

to 16%, suggesting that both ssODN ends need to be accessible for efficient ssODN-mediated gene correction. According to the "annealing-integration" model, extension of the annealed ssODN by the replicative polymerases would lead to physical incorporation of the ssODN into the nascent DNA strand. Evidence for this model was provided by Radecke *et al.* [37], who demonstrated that integrated biotin-labelled ssODNs could be recovered from the genome of ssODN-corrected HEK293 cells. After correction of a mutant *EGFP* reporter gene by biotin-labelled ssODNs, genomic DNA was isolated from corrected EGFP-positive cells. Using streptavidin-coated beads, biotin-labelled genomic DNA fragments could be captured and subsequently amplified with *EGFP*-specific primers. This indicates that the ssODN had become physically integrated into the genomic target locus.

Transcription-dependent model

Although the role of replication seems firmly established, replication-independent integration of ssODNs cannot be excluded (Figure 2, model III). Igoucheva *et al.* [38] proposed a model based on the differential accessibility of the DNA strands during transcription to explain the often-observed strand bias in favour of anti-sense ssODNs [14,24,27,31,38-40]. During transcription, ssDNA regions are provided that facilitate binding of the ssODN to its complementary target region. Upon binding of the ssODN in a D-loop structure, endonucleases may excise the chromosomal template DNA followed by integration of the ssODN into the target locus (Figure 2, model III). According to this transcription-coupled model, the increased accessibility of the non-transcribed strand could promote annealing of anti-sense ssODNs, whereas the RNA polymerase activity on the transcribed strand could lead to displacement of sense ssODNs. In inducible episomal reporter systems in *E. coli* [20,23],

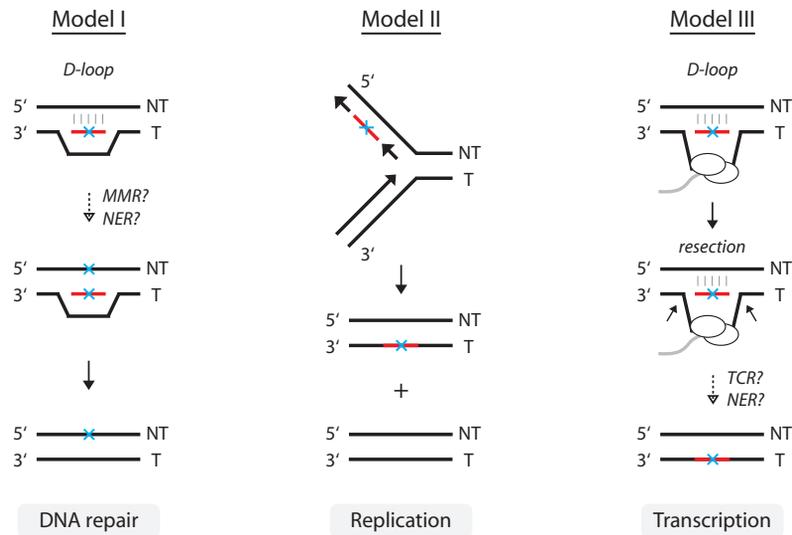


Figure 2 Mechanistic models for ssODN-mediated gene modification.

Hypothetical models for sequence alteration (indicated by blue X) by anti-sense ssODNs (indicated in red) are illustrated. Model I (repair-dependent): the ssODN anneals to its chromosomal complement and serves as a template for repair of this chromosomal DNA strand. Model II (replication-dependent): the ssODN serves as a primer for DNA synthesis during replication and becomes integrated into the newly synthesized DNA strand. Model III (transcription-dependent): annealing of the ssODN in a D-loop structure may impede trafficking of the RNA polymerase complex thereby leading to the recruitment of nucleases. Subsequent D-loop resection and repair activities result in incorporation of the ssODN. NT, non-transcribed strand; T, transcribed strand; MMR, mismatch repair; NER, nucleotide excision repair; TCR, transcription-coupled repair.

S. cerevisiae [41] and mammalian cells [38] increased transcriptional activity improved the overall frequency of ssODN-mediated gene targeting, which has been ascribed to a more open chromatin structure of the target locus. However, the strand bias was not affected by differences in transcriptional activity [20,23,38,41], suggesting that it was largely independent of the differential accessibility of the two DNA strands during transcription. This is further supported by several others who reported a preference for targeting by sense ssODNs rather than anti-sense ssODNs [17,19,25,42-44], or no effect of ssODN polarity on the targeting frequency [45-47]. Moreover, the positive effect of transcriptional activity on the targeting frequency could not be recapitulated in

chromosomally located reporter genes. We have demonstrated that a 20-fold reduction in expression of a tetracycline-regulatable *neo* reporter gene in mouse ES cells had no significant effect on the targeting frequency or the strand bias [25]. Our data are consistent with the results of Yamamoto *et al.* [17], who showed that reduction of *cyc1* expression to 2% of the normal levels by deleting the (potential) TATA elements neither affected the targeting frequency nor the strand bias in *S. cerevisiae*.

It has been suggested that binding of the ssODN to its target locus in a D-loop structure may impede the trafficking RNA polymerase, which could lead to the recruitment of proteins involved in transcription-coupled

DNA repair (TCR), a subpathway of nucleotide excision repair (NER) [48]. Subsequent resection of the D-loop by NER proteins may facilitate ssODN incorporation into the target region. Igoucheva *et al.* [48] reported that episomal gene targeting frequencies were reduced 2- to 3-fold in CHO cell lines deficient for either the ERCC1/XPF or XPG endonucleases. However, the involvement of NER or TCR proteins could again not be confirmed in chromosomal reporter systems. Deletion of the *uvrB* (NER) or *mfd* (TCR) genes only led to minor changes in the targeting frequency and in the strand bias ratio in *E. coli* [20], whereas transient knockdown of the NER proteins XPA, XPF and ERCC1 had no significant effect on the targeting frequency in mouse ES cells [25].

Taken together, these results suggest that transcription may be a determinant of episomal gene targeting, but does not play a role in the targeting of chromosomally located genes. In chromosomal gene targeting, integration of the ssODN seems to occur via mechanisms that are largely independent of transcriptional activity at the target locus.

DNA DAMAGE REPAIR AND RECOMBINATION PATHWAYS

As explained above, it seems unlikely that ssODNs are used as a template for gene repair involving DNA repair proteins. However, it remains possible that DNA repair or recombination proteins facilitate integration of the ssODN into the target region. Treatment with DNA damaging agents such as camptothecin [49] or etoposide [30] has been shown to stimulate ssODN-mediated gene targeting, which was attributed to the induction of DNA double-strand breaks (DSBs). Etoposide directly generates DSBs, while camptothecin

induces single-stranded DNA breaks that can be converted into DSBs when encountered by an advancing replication fork. In S phase, DSBs can be repaired via homologous recombination (HR), a major repair pathway that uses a homologous DNA sequence as template for repair (reviewed in [50,51]). During HR-mediated DSB repair, the broken DNA ends are first processed by exonucleases to generate 3' ssDNA overhangs that are covered by RPA (Figure 3A). Then, the Rad52 protein associates with the RPA-coated ssDNA to accelerate the displacement of RPA by Rad51. The resulting Rad51 nucleoprotein filament searches and invades the homologous double-stranded counterpart, which is supported by the chromatin remodelling protein Rad54. Strand invasion results in the formation of a D-loop, a joint molecule between the broken end and its homologous template. The joint molecule can then serve to prime DNA synthesis, leading to the elongation of the 3' ends. Dissolution of the joint molecules and religation of the strands finally results in error-free repair of the lesion. Based on this model, one can envision that proteins involved in HR may also promote invasion or annealing of the ssODN to its homologous target region in a structure that resembles a D-loop (Figure 3B).

It has been postulated that the increased activity of HR proteins was responsible for the enhanced targeting frequencies in cells treated with camptothecin or etoposide [30,49]. However, besides the recruitment of HR proteins, the DSBs induced by these agents also resulted in replication fork stalling and an accumulation of cells in S phase. Therefore, in these experiments it is unclear whether the targeting frequencies were elevated because the DSBs interfered with replication fork progression or because the DSBs increased the activity of the HR proteins.

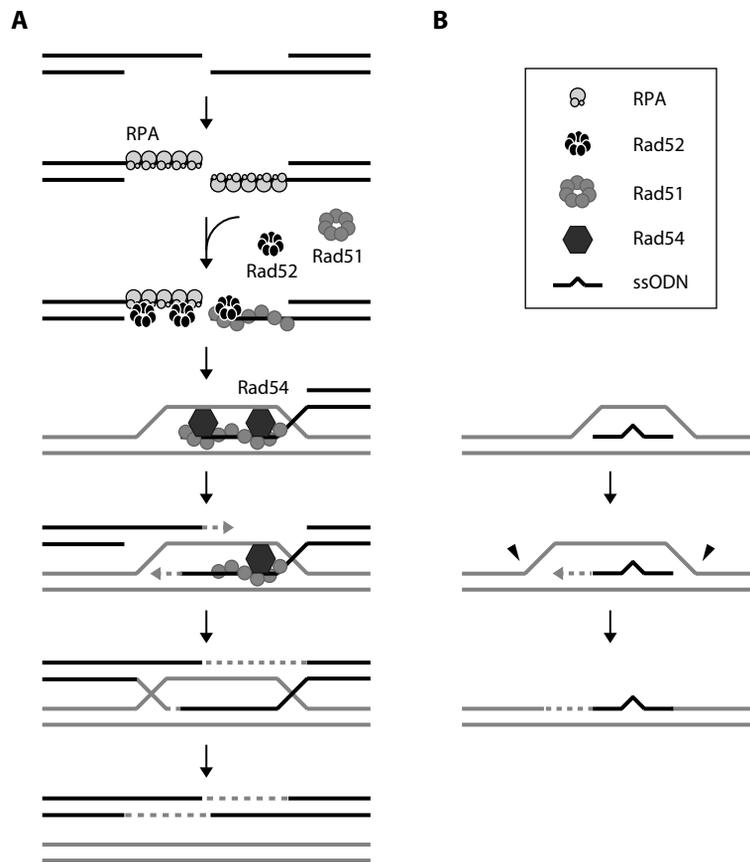


Figure 3 Model for recombinational repair of DNA double-strand breaks in eukaryotes.

(A) Repair is initiated by resection of a DNA double-strand break (DSB) to generate 3' overhangs of single-stranded DNA (ssDNA). The resulting ssDNA overhangs are subsequently coated by replication protein A (RPA). Rad52 recruits Rad51 to the RPA-coated ssDNA, resulting in displacement of RPA and formation of the Rad51 nucleoprotein filament. Supported by Rad54, the Rad51 filament can engage in a homology search and invade the homologous duplex to form a DNA joint called a D-loop. The 3' end of the strand invasion intermediate is extended by DNA synthesis, which can be followed by capture of the second DSB end. Dissolution of the joint molecules and religation of the strands finally results in error-free repair of the lesion. (B) Proteins involved in HR may also promote invasion or annealing of the ssODN to its homologous target region in a structure that resembles a D-loop. Resection of the chromosomal template by endonucleases (indicated by arrowheads) may result in incorporation of the ssODN into the target locus. Adapted from Symington et al. [51].

The impact of overexpression of the HR proteins Rad51p, Rad52p and Rad54p was investigated in *S. cerevisiae* [42,52]. Overexpression of wild-type Rad51p enhanced the ssODN-mediated targeting frequencies

approximately 5- to 15-fold in both studies, while overexpression of Rad52p and Rad54p gave inconsistent results. Overexpression of a mutant Rad51p protein with either increased single-strand binding activ-

ity or increased interaction with the Rad54p protein, enhanced the ssODN-mediated targeting frequency approximately 10- and 100-fold, respectively [53]. In addition, co-injection of ssODNs with hRAD51 and hRAD54 increased the efficiency of targeted mutagenesis into mouse zygotes [54]. Since injection of ssODNs alone did not lead to detectable targeting frequencies, the effect of Rad51 depletion by co-injection with anti-Rad51 antibodies could not be determined. In *S. cerevisiae*, ssODN-mediated gene targeting was still efficient in *rad51*- or *rad52*-deficient strains although the frequencies were 2- to 4-fold lower than in a wild-type strain [55,56]. Together, these findings suggest that HR proteins may contribute to ssODN-mediated gene targeting by promoting strand invasion of the homologous double-stranded target region.

However, in *E. coli* ssODN-mediated recombination was independent of RecA, the bacterial ortholog of Rad51 [18]. Moreover, Huen *et al.* [22] showed that ssODN-mediated gene repair was still highly efficient in bacterial strains that were deficient for various recombination proteins. Instead, ssODN-mediated gene targeting strongly depended on the bacteriophage λ Red-recombination system, consisting of the Gam, Exo and Beta proteins [18,20-23]. While all three proteins were necessary for homologous recombination of linear duplex DNA, only the Beta protein was required for recombination of short ssODNs [18,21]. Red Beta is a Rad52-like protein that binds ssDNA and promotes the annealing of complementary single strands, but cannot promote strand invasion of duplex DNA [57]. Therefore, Beta may promote annealing of the ssODN to the ssDNA regions that are transiently present near the replication fork. Episomal expression of the phage Red Beta protein in *S. cerevisiae* led to a 9-fold increase in targeting frequency [42]. In addi-

tion, stable expression of the phage Red Beta protein in mouse ES cells led to detectable levels of ssODN-mediated gene correction, while no correction was observed in the parental mouse ES cell line [21]. We have shown that transient knockdown of various HR proteins (Mre11, Rad50, Rad51, XRCC2, Rad52, Rad54) had no significant effect on the targeting frequency in mouse ES cells, indicating that these proteins are neither essential for nor detrimental to ssODN-mediated gene targeting in these cells [25].

Taken together, these data suggest that strand invasion of the ssODN is not a critical step in the targeting process. Rather, the opportunity for ssODNs to anneal to their chromosomal complement at ssDNA regions that are present near the replication fork represents a major bottleneck. Recombination proteins such as Rad51, Rad52 or the annealing protein Red Beta may bind the ssODN and stabilize the heteroduplex that is formed upon annealing of the ssODN to its target locus. The auxiliary effect of the HR proteins on the targeting frequency varied greatly between yeast on the one hand and bacteria and ES cells on the other hand. Perhaps these differences could be explained by the different activity of the HR pathway in these cell types: HR is the predominant DSB repair pathway in *S. cerevisiae*, whereas DSBs in mammalian cells are mainly repaired via non-homologous end-joining (NHEJ). Additionally, the longer ssODNs (~70 nt) that are used for gene targeting in yeast [42,52,53], may be more efficiently coated by the HR proteins than the shorter ssODNs (~40 nt) that were used in mouse ES cells [25].

MISMATCH REPAIR SYSTEM

Upon annealing of the ssODN to its single-stranded complement, DNA mismatches are formed that are recognized by the DNA mismatch repair (MMR) system. In eukaryotic cells, mismatches can be recognized by either one of two heterodimeric protein complexes: MSH2/MSH6 (MutS α) and MSH2/MSH3 (MutS β) that have specific and partially redundant mismatch recognition capacities (reviewed in [58,59]). The MSH2/MSH6 heterodimer mainly recognizes single base-base mismatches and small loops of one or two nucleotides, while the MSH2/MSH3 complex has more affinity for larger loops of two to five unpaired nucleotides (Figure 4). Upon mismatch binding, the MSH heterodimeric complexes activate a eukaryotic MutL homolog, consisting of the MLH1 protein complexed with PMS2 (MutL α), PMS1 (MutL β) or MLH3 (MutL γ). Of these complexes, MutL α has the most important role in MMR and functions to coordinate the events from mismatch binding to degradation and resynthesis of the nascent DNA strand. Nucleolytic degradation is initiated at a gap or nick that is generated either 5' or 3' of the mismatched nucleotide. In prokaryotes, strand discrimination is mediated by the MutS/MutL-activated MutH endonuclease, which generates a nick into the transiently unmethylated nascent DNA strand.

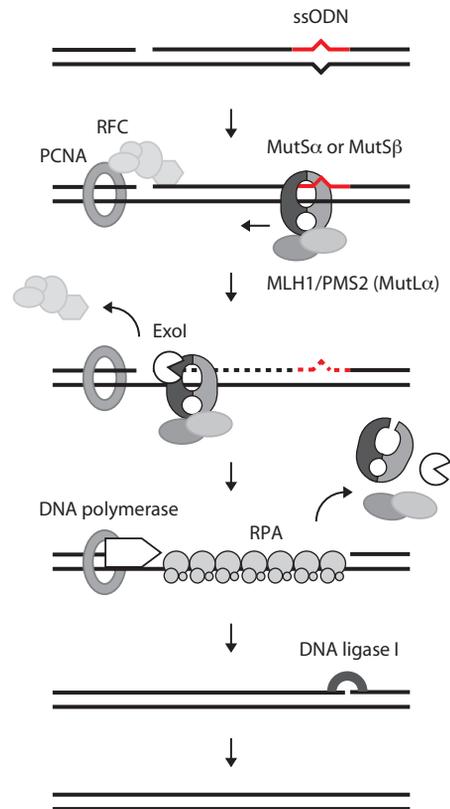


Figure 4 *Suppression of ssODN-mediated gene modification by the DNA mismatch repair system.* Annealing of the ssODN (indicated in red) to its complementary target region leads to the formation of DNA mismatches that can be recognized by either MutS α (MSH2/MSH6) or MutS β (MSH2/MSH3). MutS α mainly recognizes single base-base mismatches and small loops of one or two nucleotides, whereas MutS β has more affinity for larger loops of two to five unpaired nucleotides. Upon mismatch binding and recruitment of the MLH1/PMS2 heterodimer (MutL α), the complex undergoes an ATP-driven conformational switch, which allows sliding away from the mismatch. The MutS α - or MutS β -activated exonuclease ExoI subsequently degrades the newly synthesized DNA strand containing the ssODN-induced mismatch in a 5'-3' direction. Once the mismatch is removed, ExoI activity is inhibited and DNA polymerases are activated to fill the gap. PCNA and RFC play an important role in coordinating the mismatch recognition and DNA synthesis steps. Repair synthesis is completed when DNA ligase I seals the remaining nicks.

In eukaryotic MMR, the endonuclease activity of MutL α may be responsible for inducing nicks into the discontinuous DNA strand [60]. Strand discontinuities in the leading strand and the Okazaki fragments in the lagging strand may serve as the signal that targets the newly synthesized strand for MMR. The MutH- or MutL α -induced nicks provide entry points for the MutS/MSH-activated exonuclease ExoI that removes the DNA strand carrying the mismatch in a 5'-3' direction. The resulting single-stranded gap is stabilized by RPA. Once the mismatch is removed, ExoI activity is inhibited and DNA polymerases are activated to fill the gap. Repair synthesis is completed when DNA ligase I seals the remaining nick. In addition to the MutS and MutL complexes, other factors of the replication machinery may play a role in coordinating MMR. Proliferating cell nuclear antigen (PCNA) is a homotrimeric sliding clamp that is loaded onto the 3' ends of newly synthesized DNA by replication factor C (RFC), where it functions as a processivity factor for the replicative polymerases. In eukaryotes, PCNA plays a central role in targeting the MMR proteins to the replication fork, but is also involved in the mismatch recognition and DNA synthesis steps in MMR (Figure 4).

Dekker *et al.* [45] have demonstrated that the MMR system strongly impaired the efficacy of ssODN-mediated gene targeting in mouse ES cells. ssODN-mediated substitution, insertion or deletion of a single nucleotide was 150- to 700-fold more effective in cells lacking the central MMR gene *Msh2* than in wild-type cells, yielding targeting frequencies of $\sim 2 \times 10^{-5}$ [45,61]. Consistently, inactivation of the MMR pathway in *E. coli* through deletion of *mutS*, *mutL*, or *mutH* enhanced the targeting frequency two- to several hundred-fold [19,20,22,23]. Costantino *et al.* [19] demonstrated that *mutS*-deficiency differentially

affected the targeting frequency of ssODNs generating different types of mismatches upon annealing: the efficacy of ssODNs generating a G/G mismatch improved most strongly (~ 300 -fold), that of ssODNs generating T/C or A/G mismatches improved moderately (~ 50 -fold), while the efficacy of ssODNs generating C/C mismatches was not significantly altered. Conversely, in MMR-proficient *E. coli* strains ssODNs generating a C/C mismatch performed better than ssODNs generating a G/G mismatch [20,22], implicating that C/C mismatches escape detection by the MMR machinery. These findings are corroborated by a similar hierarchy in the recognition and repair efficiencies of different base-base mispairs by the MutS/L/H MMR system: G/T, G/G, A/A and A/C mismatches and loops of less than 4 nt are repaired most efficiently; G/A, T/T and C/T mismatches are repaired with intermediate efficiency; C/C mismatches are repaired with poor efficiency [62-64]. Together, this suggests that the level of suppression by the MMR system depended on the type of sequence alteration that was introduced by the ssODN.

The effect of MMR deficiency on the strand bias was assessed in *E. coli* strains that carried a reporter gene in opposite orientations relative to the direction of replication [19,20,22,23]. Thus, in one strain, the ssODN could function as a leading strand ssODN, while in the other strain as a lagging strand ssODN. These experiments demonstrated that the replication-directed strand bias was independent of ssODN sequence and MMR status. In all cases, highest targeting frequencies were obtained when the ssODN corresponded in sequence to the lagging strand, in both MMR-proficient and MMR-deficient strains. Although the effect of the MMR system has been most extensively studied in *E. coli* and mouse ES cells, the use of several MMR-defective human cell lines

Table 1 MMR characteristics of cell lines used to study ssODN-mediated gene targeting.

Cell line	Origin	MMR status*	MMR protein defect ^a	References
DLD-1	Human colon carcinoma	–	hMSH6	27-30, 49, 82, 85, 86
HCT116	Human colon carcinoma	–	hMLH1 , hPMS2 ^b , hMSH3 ^b	28, 83, 85, 87
HEK293T	Human embryonic kidney epithelium	–	hMLH1 , hPMS2 ^b , hMLH3	40, 46, 76
HEK293	Human embryonic kidney epithelium	+		37, 43
HeLa	Human cervical carcinoma	+		31, 32, 77
CHO-K1	Chinese hamster ovary	+		14, 24, 26, 38, 40, 48, 74
Mouse ES	Inner cell mass of mouse blastocyst	+	(mMSH2, mMSH3, mMSH6)^c	21, 25, 44, 45, 61, 67, 109

* MMR status “+” indicates MMR proficient, “–” indicates MMR deficient (these cell lines are unable to repair both base-base mismatches and insertion/deletion loops).

^a The primary alteration of MMR protein expression is reported in bold.

^b Lack of hMLH1 leads to proteolytic degradation of hPMS2. The hMSH3 gene in HCT116 cells is mutated as a consequence of the MMR defect.

^c Mouse ES cells are MMR proficient; the reported MMR defects were experimentally introduced to study their effect on ssODN-mediated gene targeting. Adapted from Cannavo et al. [110].

strongly suggests that the MMR system also imposes a significant barrier to effective application of ssODN-mediated gene targeting in human cell lines (Table 1).

Besides the correction of DNA polymerase errors during replication, the MMR system also prevents recombination between homologous but non-identical sequences (homeologous recombination) [65]. Since ssODN incorporation takes place during replication, it is most likely that the MMR system prohibits ssODN-mediated gene modification by its mismatch repair function.

The specific repair and recognition capacities of the MSH2/MSH6 and MSH2/MSH3 protein complexes could be exploited to allow the introduction of specific sequence

alterations in mouse ES cells (Table 2). Ablation of both heterodimeric MutS complexes by deletion of the *Msh2* gene allowed effective introduction of 1-, 2-, 3- and 4-nt substitutions, as well as +1 and +4 nt insertions [61]. Deletion of the *Msh3* gene only eliminates the MutS β complex, leaving the MutS α complex consisting of the MSH2 and MSH6 proteins unaffected [66]. Since 4-nt insertion loops are mainly suppressed by the MSH2/MSH3 branch of MMR, these could effectively be introduced into *Msh3*-deficient ES cells [61], whereas nucleotide substitutions were still suppressed by the activity of the MSH2/MSH6 complex [67]. On the other hand, *Msh6*-deficiency only allowed the introduction of 1-, 2-, 3- and 4-nt substitutions, while nucleotide insertions were still suppressed by the activity of

the remaining MSH2/MSH3 complex [67]. Similarly, Kow *et al.* [56] reported that +7 nt insertions could efficiently be introduced into an *msh3*-deficient yeast strain, while +1 nt insertions were suppressed by residual MSH2/MSH6 activity. Conversely, *msh6*-deficient cells were permissive for +1 nt insertions, while +7 nt insertions were suppressed. The targeting frequencies of the insertion ssODNs in the *Msh3*-deficient ES cell line [61] and the substitution ssODNs in the *Msh6*-deficient ES cell line [67] were on average 5-fold lower compared to those in the *Msh2*-deficient ES cells. This could be explained by the partial redundancy in MMR functions: the MSH2/MSH3 complex may suppress nucleotide substitutions to some extent in an *Msh6*-deficient background, whereas the MSH2/MSH6 complex may modestly suppress nucleotide insertions in an *Msh3*-deficient background.

Although MMR deficiency strongly improved the efficacy of ssODN-mediated gene targeting, general application may be hampered by the mutator phenotype associated with MMR deficiency [65,68-72] (Chapter 3) (Table 2). It has been postulated that mismatches that are poorly detected by the MMR system could be introduced into MMR-proficient cell lines with a reasonable efficacy [61]. Indeed, ssODNs encoding complex mismatches, such as 3- or 4-nt substitutions and 4-nt insertions, led to detectable levels of gene alteration in wild-type ES cells, but frequencies were still rather low ($\sim 10^{-6}$) and general rules for ssODN design could not be established.

Alternatively, temporary inhibition or down-regulation of MMR proteins may limit the mutagenic effects of MMR deficiency. In *E. coli*, a short-time treatment with the ade-

Table 2 ssODN-directed nucleotide alterations allowed in MMR-defective mouse ES cells.

MMR gene defect	Nucleotide substitutions	Nucleotide insertions	Mutator phenotype (MSI high/low?) ^b		Refs
			Mononucleotide	Dinucleotide	
<i>Msh2</i>	1, 2, 3, 4 nt	+1 nt, +4 nt	high	high	45, 61
<i>Msh3</i>	Not efficient ^a	+4 nt	low	none	61, 67
<i>Msh6</i>	1, 2, 3, 4 nt	Not efficient ^a	moderate	low	67
<i>Mlh1</i>	ND	ND	high	high	NA
MSH2 RNAi	3, 4 nt	Not efficient ^a	moderate (transient)	moderate (transient)	67
MLH1 RNAi	1, 2, 3, 4 nt	Not efficient ^a	moderate (transient)	moderate (transient)	Chapter 3

^a The targeting frequency was similar to that in wild-type ES cells ($\sim 10^{-7}$).

^b MSI, microsatellite instability. Adapted from Wei *et al.* [111].

ND, not determined; NA not applicable.

nine analog 2-aminopurine (2-AP) increased the targeting frequency in an MMR-proficient strain by ~10-fold, which was ~10-fold lower than that in an MMR-deficient strain [19]. Transient down-regulation of MMR expression by RNA interference may also generate a time window that allows effective gene alteration by ssODNs, while preventing the accumulation of inadvertent mutations. Transient knockdown of MSH2 by vector-based RNAi for 4-5 days in mouse ES cells allowed the introduction of 4-nt substitutions with frequencies that were comparable to those in *Msh2*-deficient cells [67]. MMR function was not completely abrogated in these cells: approximately 10% of residual MSH2 activity remained and was sufficient to suppress simple nucleotide substitutions and nucleotide insertions (Table 2). The mutator phenotype, assessed by the occurrence of slippage errors in simple sequence repeats, was slightly elevated but remained far below that in constitutively MMR-defective cells, as has been demonstrated previously for an *Msh2*^{low} ES cell line carrying a hypomorphic *Msh2* allele [73]. In the same system, transient down-regulation of MLH1 made cells permissive for all nucleotide substitutions ranging from 1- to 4-nt, whereas nucleotide insertions were still suppressed (Chapter 3). After MLH1 knockdown for 4-5 days, slippage rates were approximately 3-fold higher compared to those after MSH2 knockdown. Importantly, the genetically modified ES cells that were obtained after transient down-regulation of MSH2 or MLH1 retained their pluripotency and could contribute to the mouse germ line, resulting in the generation of mutant mice [67] (Chapters 2 and 3).

This approach was recently extended to other mammalian cell lines, showing that transient down-regulation of MSH2 by siRNA led to a 2-fold increased targeting frequency in CHO-K1 cells [74], whereas stable knockdown of MSH2 increased the

targeting frequency 20- to 35-fold in human hepatocytes [75]. In addition, inducible repression of hMLH1 resulted in a 3-fold increase in targeting frequency in a doxycycline-regulatable HEK293T α cell line [76]. Together, these data suggest that transient down-regulation of the MMR genes is a feasible approach to improve ssODN-mediated gene targeting frequencies in a variety of cell lines.

OLIGONUCLEOTIDE PROPERTIES AND CELLULAR RESPONSES

Several properties of the ssODN itself, such as the length of the ssODN and the position of the desired sequence alteration(s) within the ssODN, may have an effect on the targeting efficacy. In mammalian cell lines, the optimal length appears to be 25- to 45-nt [14,25,43,45,77], while in bacteria and yeast ssODNs of 70- to 120-nt are used [19-21,52]. In general, ssODNs with centrally-located base alterations were most effective, while ssODNs with base alterations located more towards the 3' end performed better than those closer to the 5' end of the ssODN [21,55,77,78]. Gene correction frequencies dropped dramatically when the position of the mismatch was shifted to the very end of the ssODN. Hegele *et al.* [79] showed that ssODNs with sequence alterations at two different positions could lead to simultaneous exchange when the alterations clustered together within a region of 14-nt, confirming previous data using an episomal target in yeast [80]. Again, when the second mismatch was placed more towards the terminus of the ssODN, it was less frequently co-introduced with the centrally-located first mismatch. These data suggest that the ends of the ssODN are susceptible to degradation by cellular exonucleases resulting in removal of the desired sequence alterations. Accordingly, Radecke *et al.* [81]

demonstrated that ssODNs with standard phosphodiester backbones and non-canonical 5'-OH and 3'-H ends were still effective in ssODN-mediated gene correction in contrast to nuclease-resistant ssODNs with non-canonical ends. This implies that nucleolytic activity had been responsible for the formation of canonical 5'-phosphate and 3'-OH ssODN ends.

Chemical modifications, such as 2'-*O*-methyl uracil residues [14], phosphorothioate (PTO) linkages [12], locked nucleic acids (LNA) [46] and thymine-adenine (TA) clamps [47], have been added to protect the ssODNs against nucleolytic degradation (Figure 5). Yet, besides improving the bioavailability of the ssODNs, these modifications may also have deleterious side effects. While PTO-modified ssODNs initially generated more corrected cells than unmodified ssODNs, the potential of these cells to form viable colonies was decreased compared to cells corrected by unmodified ssODNs [24,43] (Chapter 5). Olsen *et al.* [26] demonstrated that the majority of the cells that were initially corrected by PTO-modified ssODNs arrested in the G₂ phase of the cell cycle and only 1-2% of these cells were able to expand. This G₂ arrest was due to the presence of unrepaired genomic DSBs, leading to the activation of the ATM/ATR pathway and phosphorylation of histone H2AX [76,82,83]. Although the mechanism underlying this ssODN-mediated toxicity is still largely unknown, the observed DNA damage response seemed to be induced particularly by ssODNs containing protective PTO linkages (Chapter 5). Papaioannou *et al.* [74] demonstrated that the position of the protective PTO linkages differentially affected the cellular response. The G₂ arrest was less pronounced when cells were exposed to ssODNs carrying internal PTO linkages as opposed to ssODNs that were end-protected by PTO linkages. In this study it was shown that gene correc-

tion by LNA-protected ssODNs induced a similar accumulation of cells in G₂ as PTO-protected ssODNs [74]. On the other hand, Andrieu-Soler *et al.* [46] reported that gene correction by LNA-modified ssODNs did not preclude clonal expansion of the corrected cells. Consistently, we have shown that 55% of the mouse ES cells that were corrected by unmodified or LNA-modified ssODNs were able to form colonies, while only 20% of the cells corrected by PTO-modified ssODN survived (Chapter 5). The effect of 2'-*O*-methyl uracil residues on the viability of corrected cells has not been investigated as yet. However, Igoucheva *et al.* [84] demonstrated that exposure to ssODNs protected by 2'-*O*-methyl uracil residues had no effect on the cellular response, while the introduction of plasmid DNA induced many changes in the expression levels of genes involved in ATM/ATR signalling, cell cycle arrest, apoptosis and genome stability and repair pathways.

Several attempts have been made to counteract the apparent loss of targeted cells due to DNA damage caused by chemically-modified ssODNs [85-87]. These strategies were based on inhibition of the proliferating non-corrected cells by thymidine [86] or monastrol [85] to prevent dilution of the arrested corrected cells in the total population. Although these treatments seemed to enrich for corrected cells, actual recovery of these cells may be hampered by the senescent state that was induced by prolonged thymidine treatment [86].

Rather than counteracting the toxic side effects of PTO-modified ssODNs, the use of unmodified ssODNs may be a more promising strategy for the isolation of viable and stably targeted cells. Recently, we have demonstrated that exposure to or integration of unmodified ssODN did not induce a substantial amount of DSBs in mouse ES cells (Chapter 5). The viability of corrected

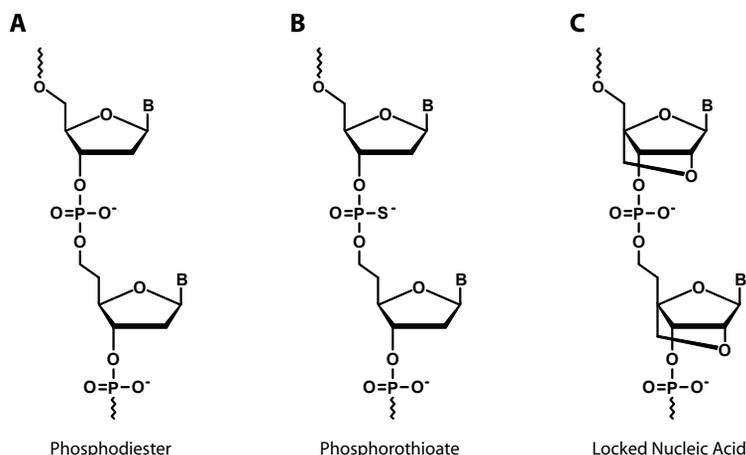


Figure 5 Backbone and base modifications of DNA oligonucleotides.

(A) The backbone of unmodified ssODNs consists of normal phosphodiester bonds in which the 3'-carbon atom of one deoxyribose molecule is linked to the 5'-carbon of another. (B) In phosphorothioate (PTO) internucleotide bonds, one of the non-bridging oxygen atoms is replaced by a sulfur atom. This backbone modification renders the internucleotide linkage more resistant to nucleolytic degradation. (C) Locked nucleic acid (LNA) nucleosides are conformationally restricted nucleic acid analogues in which the ribose ring is "locked" by a methylene bridge connecting the 2'-oxygen atom and the 4'-carbon atom. The bridge "locks" the ribose in the 3'-endo structural conformation, which enhances base stacking and backbone pre-organization.

cells was only mildly affected, which facilitated the long-term recovery of these cells. As a result, unmodified ssODNs yielded higher targeting frequencies than PTO-modified ssODNs in long-term assays [25,45], although PTO-modified ssODNs initially seemed to be superior in short-term assays [24,74].

The optimal polarity of the ssODN has been the subject of debate for several years. Some reports demonstrated a preference for targeting by sense ssODNs [17,19,25,42], whereas others showed a preference for targeting by anti-sense ssODNs [24,31,38, 40,83]. Recently, we have demonstrated that the measured efficacies of sense and anti-sense ssODNs depended on the time of readout (Chapter 5). Anti-sense ssODNs performed best when the targeting frequencies were determined at 24 hrs after

ssODN exposure, while sense ssODNs were most effective after 8 days. We proposed that the often-observed preference for targeting by anti-sense ssODN is the consequence of the early time points that were used to determine the targeting frequency. The correction frequency of mutant *EGFP* or *LacZ* reporter genes is usually determined at 24 hrs after ssODN exposure by flow cytometry or (fluorescence) microscopy. Engstrom *et al.* [28] reported that corrected EGFP-positive cells could be detected as early as 8 hrs after ssODN exposure. Replication-dependent integration of anti-sense ssODNs into the transcribed strand allows immediate transcription of the corrected *EGFP/LacZ* strand and production of green fluorescence or β -galactosidase activity. In contrast, incorporation of sense ssODNs into the non-transcribed strand requires the genetic alteration to be transferred to

the transcribed strand before *EGFP/LacZ* can be expressed. We found this is only accomplished 48 hrs after ssODN exposure. Furthermore, due to semi-conservative DNA replication in subsequent cell cycles, the DNA strand that is initially corrected by anti-sense ssODN becomes diluted, leading to a decrease in the number of green fluorescent cells upon prolonged culturing. Therefore, to accurately determine the optimal polarity of the ssODN, we recommend that the targeting frequencies should also be determined at later time points (*e.g.* after 8 days) to minimize the confounding effects of ssODN toxicity and dilution due to semi-conservative replication (Chapter 5). As discussed earlier, the actual strand bias may be the result of the differential accessibility of the leading and lagging strand during replication and not be related to the polarity of the ssODN with respect to the direction of transcription.

DSB REPAIR BY ssODNs AND ZFN TARGETING

As discussed before, the longer ssODN that are generally used in yeast to introduce site-specific sequence alterations may become integrated into the genome with the help of HR proteins. This is corroborated by the finding that ssODN-mediated gene targeting frequencies could be stimulated several 1,000-fold by the presence of a DSB, suggesting that the ssODN can be used as template for homology-directed recombinational repair [88]. Storici *et al.* [89] demonstrated that exogenous ssODNs of 81-95 nt could be used to delete a previously integrated counterselectable reporter cassette in *S. cerevisiae*. The ssODNs were designed to have homology to regions of 40 nt on both sides of the integration site of the reporter cassette. Annealing of the ssODN will bring the distant regions on ei-

ther side of the cassette into close proximity, which may promote excision of the cassette through resolution of the recombination intermediate. Site-specific mutagenesis could be accomplished by introducing a centrally-located point mutation into the ssODN. With these ssODNs colonies could be obtained that had lost the reporter cassette and acquired the desired mutation. The efficiency of this “delitto perfetto” strategy was relatively low ($\sim 3 \times 10^{-6}$), but could be stimulated more than a 1,000-fold by the introduction of a site-specific DSB, reaching levels of ~ 0.3 -1% [88]. In this approach, ssODNs were designed to have 40 nt of homology to chromosomal sequences on both sides of an I-SceI cleavage site. When the regions of homology were placed adjacent (<15 bp) to the break, the ssODN could be used to introduce site-specific mutations. When the regions of homology were placed distantly from the break (4.7 to 11.9 kb away), ssODN-mediated repair of the I-SceI-induced DSB resulted in chromosomal deletions. Similar results were obtained in mammalian cells, where ssODNs of 80-96 nt spanning a defined DSB yielded targeting frequencies of $\sim 0.25\%$, which was approximately 20-fold higher than ssODN-mediated gene targeting without DSB induction [81,90]. Importantly, after ssODN-mediated DSB repair, cells were able to expand and repair frequencies remained stable for up to 14 days [81,90].

Radecke *et al.* [81] showed that DSB-directed gene modification did not occur through physical incorporation of the ssODN into the target locus, suggesting a homology-directed repair mechanism. Although in yeast the repair process strongly depended on Rad52, strand invasion proteins such as Rad51 or Rad54 were not required and even inhibited ssODN-mediated DSB repair [91]. Therefore, it was postulated that ssODN-mediated DSB repair occurred through several rounds of strand annealing and

DNA synthesis rather than recombinational repair [81,91]. According to the “bridge” model, one ssODN anneals to each side of the break, whereas in the “template” model, the ssODN first anneals to one end of the break and serves as a template for DNA synthesis. Subsequently, the DNA strand that has been copied from the ssODN pairs with a homologous sequence on the other side of the break in a second annealing event. The DSB-directed gene modification approach has been used to generate nucleotide deletions ranging from 2-nt up to 16-kb, 10-nt insertions and single nucleotide substitutions near a defined DSB site, depending on the position of the ssODN homology arms, in both yeast and human cell lines [81,88,90,91]. In these reporter systems DSBs were induced by the site-specific I-SceI endonuclease, thereby limiting the application to reporter genes that had been engineered to contain the 18-bp I-SceI recognition site.

The targeting of endogenous genes may be greatly facilitated by the development of chimeric zinc-finger nucleases (ZFNs). ZFNs consist of a non-specific *FokI* nuclease domain fused to a specific DNA binding domain composed of tandem Cys₂His₂ zinc-finger binding motifs [92] (Figure 1A). Donor-free delivery of ZFNs could be used for the targeted disruption of endogenous genes due to inaccurate rejoining of the DSB ends by the non-homologous end-joining (NHEJ) pathway [93]. On the other hand, introduction of specific sequence alterations can be achieved by delivery of a homologous donor DNA carrying the desired sequence alteration along with the ZFNs. The donor DNA is then used as a template for HR-directed repair of the ZFN-induced DSB. Successful ZFN-mediated gene targeting has been demonstrated in different mammalian cell lines [94-97], *Drosophila melanogaster* [98], *Caenorhabditis elegans*

[99] and zebrafish [100]. In human cell lines, ZFN targeting in combination with linear double-stranded donor DNA resulted in the introduction of single base changes in the endogenous *IL2R γ* locus in ~20% of the cells, while the targeting frequency without DSB induction was $\sim 2 \times 10^{-6}$ [94]. Notably, approximately 7% of the cells became homozygous for the donor-specified genotype. Even in human ES or induced pluripotent stem (iPS) cell lines, in which HR-driven gene targeting occurs at very low frequencies, ZFN targeting could be efficiently applied to generate transgene insertions at frequencies ranging from 5-94% [95,101] or chromosomal translocations at frequencies of 10^{-5} - 10^{-6} [102].

Although these results are very promising, off-target cleavage by the ZFNs may cause unpredictable genotoxic effects and is therefore a potential limitation of ZFN targeting. Yet, these adverse effects may be reduced by limiting the timing and duration of ZFN expression or by increasing the ZFN specificity by increasing the number of zinc-finger modules, *e.g.* ZFNs consisting of 4-6 zinc-fingers have a recognition site of 24-36 bp [93]. Another drawback is the construction of a homologous donor template that is required for the introduction of site-specific sequence alterations by ZFN targeting. Construction of such a donor template can be laborious, even though the homology arms of the donor DNA are relatively short (~500-750 bp for each arm). Possibly, synthetic ssODNs could function as an alternative donor template for the repair of ZFN-induced DSBs, as has been shown for I-SceI-induced DSBs [81,88,90,91].

Although the presence of a DSB strongly stimulated the targeting efficacy, DSBs are highly toxic lesions that may lead to chromosomal instability if left unrepaired. Instead of using the ssODN or double-stranded donor DNA as a template for HR-directed

repair, the broken chromosome ends may be sealed via the error-prone NHEJ pathway, which constitutes the major DSB repair pathway in mammalian cells. Hockemeyer *et al.* [101] showed that 6-8% of the clones that were heterozygous for an integrated transgene after ZFN targeting carried NHEJ alterations at the non-targeted allele. In addition, sequence analysis after ssODN-mediated DSB repair revealed that although 78-96% of the loci were faithfully corrected, some loci contained additional mutations due to insertion of DNA sequences derived from either the ssODNs or co-transfected plasmids [81]. This insertional mutagenesis may be an effective tool for targeted gene disruption, but could also seriously hamper the application of DSB-induced gene targeting for therapeutic purposes.

Recently, Olsen *et al.* [76] compared the cellular responses following gene targeting by ssODNs and gene targeting by ZFNs

in combination with a donor DNA plasmid. ZFN-directed correction of a single copy chromosomal target gene was 100-fold more effective than gene correction by ssODNs (0.26% and 2.6×10^{-5} , respectively). Importantly, cells corrected by ZFNs displayed a normal cell cycle distribution and low levels of DNA damage, in contrast to cells corrected by PTO-modified ssODNs. The high targeting efficacy and the potential to generate biallelic alterations by ZFN targeting may be particularly advantageous for the introduction of site-specific sequence alterations for therapeutic purposes. Nevertheless, several issues regarding ZFN safety need to be addressed before gene therapy comes within reach, including the affinity and sequence-specificity of the ZFN and possible mutagenesis due to DSB repair by the NHEJ pathway. The design, production and testing of customized ZFN pairs may be an additional bottleneck for wide application of the ZFN technology.

CONCLUDING REMARKS

Over the last decade significant advance has been made in unravelling the mechanisms of ssODN-mediated gene targeting and the various parameters that impinge on the targeting process. It has been established that the genetic information from the ssODN is incorporated into the target locus during replication rather than being transferred via template-based repair. This has several implications for the applicability of ssODN-mediated gene targeting. First, only actively replicating cells can be used to introduce the site-specific sequence alterations. Second, incorporation of exogenous DNA could be potentially dangerous for gene therapeutic purposes. Since targeting frequencies are relatively low (ranging from 10^{-6} to 10^{-3}), random incorporation of the ssODN in a replication-dependent manner may also occur infrequently. However, the occurrence of undesired integration of (parts of) the ssODN is difficult to detect due to the short sequences that may have been introduced and can therefore not be excluded.

We have shown previously that DNA MMR raises a strong barrier to ssODN-mediated gene targeting in mouse ES cells and that this problem can be overcome by transient down-regulation of the central MMR genes *Msh2* or *Mlh1* using vector-based RNAi (Chapters 2 and 3). The development of specific MMR inhibitors could greatly facilitate and broaden the application of ssODN-mediated gene targeting to other cell types in which RNAi-mediated MMR down-regulation is not possible.

During the last few years it has become increasingly clear that the addition of protective groups to improve the intracellular stability of the ssODN strongly affects the cellular response to ssODN-mediated gene targeting. Particularly PTO-modified ssODNs have been shown to induce DNA damage

upon incorporation into the genome and were deleterious for cell survival. Unmodified ssODNs may yield lower targeting frequencies, but we have shown that the targeted cells have a higher potential to form viable colonies (Chapter 5). Stable outgrowth of the targeted cells is an important determinant of the success rate of ssODN-mediated modification of endogenous genes, since the modified cells need to be isolated without the use of selection markers. We have developed a highly sensitive PCR protocol to detect ssODN-modified cells in a background of unmodified cells, thereby allowing clonal isolation of the modified cells through several rounds of limiting dilution [103].

Yet, several hurdles need to be overcome before ssODN-mediated gene targeting can be translated to *in vivo* applications. The delivery of the ssODNs to the cell type of interest and the low targeting frequency are great challenges that need to be further addressed. Nevertheless, the stable correction of dystrophin gene mutations in muscle fibers [104] or in myoblasts isolated from *mdx^{scv}* mice [105] or correction of a *rd1* stop mutation in the mouse retina [106] hold great promise for therapeutic applications. The possibility to reprogram differentiated human and mouse somatic cells into induced pluripotent stem (iPS) cells [107,108] may shift the focus of future research towards *ex vivo* application of ssODN-mediated gene modification. The therapeutic potential of these iPS cells would greatly benefit from the development of efficient techniques for site-specific correction of the underlying genetic defects. It is tempting to speculate that in-depth insight into the parameters governing ssODN-directed gene modification in ES cells may pave the way for customized gene therapy using iPS cells.

OUTLINE OF THIS THESIS

Gene targeting by ssODNs is a promising technique for the generation of subtle mutations in the genome of various cell types. The aim of this thesis was to study the role of various parameters in the targeting process and improve the efficacy of ssODN-mediated gene modification in mouse ES cells. We have shown previously that ssODN-mediated gene targeting was only effective in MMR-deficient cells. Unfortunately, the mutator phenotype associated with MMR deficiency greatly hampered general application of this technique [65].

In **Chapter 2**, we demonstrate that transient down-regulation of the MMR gene *Msh2* by RNAi allows effective substitution of four adjacent nucleotides in wild-type ES cells. This strategy was successfully applied to generate the first mutant mouse line that was created via ssODN-mediated gene targeting. In **Chapter 3**, we show that transient down-regulation of the MMR gene *Mlh1* made cells permissive for all nucleotide substitutions, ranging from one to four nucleotides. The occurrence of frameshift mutations at simple sequence repeats was assessed to gain insight into the level of spontaneous mutagenesis resulting from transient and constitutive MMR suppression.

In **Chapter 4**, we systematically investigated the role of ssODN composition, transcription and replication of the target locus, and DNA repair pathways to gain more insight into the parameters governing ssODN-mediated gene targeting in mouse ES cells.

Chapter 5 describes the generation of a novel *EGFP* reporter cell line that was used to provide more information about the stability of the introduced sequence alteration, possible toxicity of the ssODN and the viability of targeted cells. We show that unmodified ssODNs did not induce a substantial amount of DNA damage and only mildly affected the viability of targeted cells. Short and long term analyses revealed that ssODNs are incorporated into the genome during DNA synthesis.

A detailed protocol for ssODN-mediated gene targeting in mouse ES cells is given in **Chapter 6**. A summary of the findings, concluding remarks, and implications for future research are presented in **Chapter 7**.

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