Gene targeting by single-stranded DNA oligonucleotides in mouse embryonic stem cells

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Chapter

7

General Discussion

The generation of knockout mice has provided invaluable knowledge about gene function and human genetic disorders. However, complete abrogation of protein function may not always accurately mimic human disease. With the advance of human genome sequencing many small DNA sequence variations between individuals have been identified. Site-specific modification of the mouse genome provides a powerful tool to functionally characterize the identified mutations and distinguish between neutral polymorphisms and pathogenic mutations. In this thesis, we describe the development of a simple procedure to generate subtle mutations in the genome of mouse embryonic stem (ES) cells using short single-stranded oligodeoxyribonucleotides (ssODNs). Significant progress has been made in unravelling the mechanisms and reaction parameters underlying ssODN-mediated gene targeting. The implications of our findings for routine application of ssODN-mediated targeting and challenges that need to be overcome for future therapeutic applications are discussed here.

Role of MMR proteins in ssODN-mediated gene targeting

In wild-type mouse ES cells, ssODNs could be used to generate subtle gene modifications, but targeting frequencies were too low (~10^{-7}) for routine modification of non-selectable genes. The finding that mismatch repair (MMR) activity strongly hampered ssODN-mediated gene targeting was crucial for the development and application of the technique in mouse ES cells [1,2]. Although inactivation of the MMR system improved the targeting frequency several 100-fold, the mutator phenotype associated with constitutive MMR deficiency appeared to interfere with the pluripotency of ES cells impeding the generation of genetically-modified mice [3]. To allow introduction of a desired sequence alteration by ssODNs, while minimizing the inadvertent effects of constitutive MMR inactivation, we transiently down-regulated the MMR gene *Msh2* 10-fold in wild-type ES cells by RNA interference (**Chapter 2**). Consistent with the anti-mutagenic capacities of a previously generated hypomorphic *Msh2^{low}* ES cell line [4], 10% of the wild-type MSH2 level was sufficient to suppress simple substitutions of 1- and 2-nt. In contrast, complex substitutions of 3- or 4-nt were no longer detected in the MSH2 knockdown cells and could successfully be introduced by ssODNs, reaching frequencies that were up to 40-fold higher than in untreated wild-type cells. This distinction was abolished upon transient suppression of the downstream MMR gene *Mlh1* (**Chapter 3**), allowing effective introduction of all types of nucleotide substitutions ranging from 1- to 4-nt.

Remarkably, ssODN-mediated nucleotide *insertions* were still strongly suppressed in both the MSH2 and MLH1 knockdown cells. As *Msh2$^{-/-}$* ES cells were permissive for 4-nt insertions [2], this suggests that a low level of MMR activity was sufficient to detect and repair nucleotide insertion/deletion loops (IDLs) but not compound base-base mismatches. In *E. coli* it was shown that heteroduplexes with 1-, 2-, and 3-nt IDLs were repaired with equal efficiency as those with G-T mismatches [5]. However, the repair of 4- or 5-nt deletions was marginal or not detectable, respectively, indicating that larger IDLs were poorly detected by the bacterial MMR system. These data are

contradictory to the suppression of 4-nt insertions in our MSH2 and MLH1 knockdown mouse ES cells. This may be ascribed to the fact that bacteria only contain one homodimeric MutS compex, whereas in eukaryotic cells multiple mismatch recognition complexes exist of which MSH2/MSH3 has evolved to specifically target larger IDLs. Indeed, we have previously shown that *Msh2*$^{-/-}$ and *Msh3*$^{-/-}$ ES cells were permissive for 4-nt insertions [2], indicating that MSH2/MSH3 complexes play an important role in suppressing these types of alterations. It is possible that other DNA repair factors could act in concert with the MMR proteins to suppress the ssODN-directed nucleotide insertions. In **Chapter 4**, we have investigated the involvement of various DNA repair proteins in the targeting efficacy of ssODN-mediated substitution of three adjacent nucleotides (ssODN 3N). In these experiments, none of the tested DNA repair proteins appeared to be essential for or detrimental to ssODN-mediated gene targeting. However, the effect of knockdown of these DNA repair proteins was not assessed in the GT-*neo* reporter cell line using the corresponding insertion ssODNs. Therefore, the involvement of other proteins besides MSH2/MSH3 in suppressing nucleotide insertions in the MSH2 and MLH1 knockdown cells cannot be excluded.

Using transient suppression of either MSH2 or MLH1, ssODN-mediated gene modifications could be introduced with frequencies of ~2 x 10^{-5}. However, the dependence on (partial) MMR inactivation may pose a severe limitation to routine application of this method. Although the occurrence of spontaneous mutations is reduced compared to constitutively MMR-defective ES cell lines (**Chapters 2 and 3**), the introduction of undesired mutations along with the ssODN-generated mutation is inevitable. This problem seems less severe in case of transient knockdown of *Msh2*, as ssODN-directed simple nucleotide substitutions were still suppressed. It is therefore likely that the majority of spontaneously occurring base-base mismatches is subject to repair by low MSH2 level. Furthermore, when ssODN-mediated gene targeting is applied in mouse ES cells for the generation of mutant mouse models, most of the additional mutations will be crossed out in subsequent generations of mice, which will avoid confounding effects of unlinked mutations on the phenotype of the ssODN-mediated modification. One could argue that the accumulation of undesired mutations is inherent to the use of ssODN-mediated gene targeting, disqualifying this technology for routine application. However, it should be kept in mind that the majority of the mammalian cell lines that are used for scientific research are derived from tumors and carry many (unknown) genetic and chromosomal aberrations. The generation of independent clones should suffice to exclude possible confounding effects when ssODN-modified cells are used for *in vitro* experiments. Nonetheless, MMR inactivation, albeit transiently, may be problematic for the application of ssODN-mediated gene targeting for therapeutic purposes (discussed below).

Reaction parameters of ssODN-mediated gene targeting

In **Chapters 4 and 5**, we have systematically investigated the role of various parameters in the ssODN-mediated gene targeting procedure in mouse ES cells, including ssODN composition, transcription and replication of the target locus, and involvement of DNA repair proteins. In contrast to previous reports [6-9], our findings demonstrate that transcriptional activity of the target locus is not a prerequisite for successful targeting in mouse ES cells (**Chapter 4**). This implies that genes whose expression is restricted to differentiated cells, may also be accessible

to ssODN-mediated gene targeting in undifferentiated ES cells.

Our data demonstrate that DNA replication has a considerable impact on the efficiency and coordination of the targeting reaction. Reducing the rate of DNA synthesis by hydroxyurea increased the targeting frequency of sense ssODNs 1.5-fold, whereas the targeting frequency of anti-sense ssODNs remained unaffected (**Chapter 5**). The optimal polarity of the ssODN seems to be dictated by the differential accessibility of the DNA strands during replication: ssODNs that correspond to the lagging strand were consistently most effective in *E. coli* [10,11]. This finding could not be implemented into rules for the design of targeting ssODNs in mammalian cells, since the direction of replication through a target region is unknown and may even vary each cell cycle due to the stochastic firing of replication origins [12]. We speculate that in our *neo* reporter system, the sense ssODN, being more effective than the anti-sense ssODN, corresponds in most cases to the lagging strand.

In mouse ES cells, ssODN-mediated targeting frequencies range from 10^{-7} to 10^{-4} and appear to be relatively low compared to those found in other cell types [13-15] (this thesis). Although the experiments described in this thesis have provided more detailed insight into the mechanisms underlying gene targeting by ssODNs, manipulation of the identified reaction parameters did not lead to dramatic improvement of the targeting frequency in mouse ES cells. This could partially be explained by the unique cell cycle distribution of ES cells and the presence of robust genome maintenance pathways. The cell cycle of ES cells is characterized by a short G_1 phase and a high proportion of cells in S phase. As a result, already a relatively large proportion of cells progresses through S phase during ssODN exposure, which may leave little room for improvement by slowing down S phase progression upon hydroxyurea treatment. Additionally, ES cells readily undergo apoptosis upon DNA damage or replication fork stalling, thereby restricting the use of replication inhibitors.

An explanation for the relatively low targeting efficacy in our mouse ES cells could be the use of single copy reporter genes to monitor targeting events, as opposed to the multi copy reporters used in some other studies [16-19]. Olsen *et al.* [17] obtained targeting frequencies of ~4.5% in a CHO-K1 cell line carrying ~60 reporter gene copies and, using the same ssODN, 0.025% in a single copy reporter cell line, confirming an earlier observation from Radecke *et al.* [16]. Accordingly, episomal gene targeting frequencies appeared to be 10- to 100-fold higher than chromosomal gene targeting frequencies in the same cell type [14], again indicating that a high number of target gene copies increased the chance of ssODN-mediated gene correction.
The transfection efficiency of the ssODNs does not appear to be limiting in our experiments, since ~80% of the transfected ES cells have taken up fluorescently labelled ssODNs as determined by FACS analysis. Moreover, in **Chapter 5** we have demonstrated that ES cells with a 10- to 100-fold higher ssODN uptake (bin 4 compared to bin 1) showed only a 4-fold increase in targeting frequency, indicating that the efficacy of ssODN incorporation depends on other factors besides ssODN availability.

Together, these data suggest that several hurdles need to be taken for an ssODN to mediate targeted gene modification: the ssODN needs to find its target site near an advancing replication fork and become extended by the replication machinery; the mismatched heteroduplex that is formed

needs to escape detection by the MMR system and survive until it becomes replicated in the next S phase to stably fix the planned modification in the genome. Therefore, the outcome of an ssODN-mediated gene targeting reaction may actually be the sum of many different smaller events that each have a small chance of success.

Viability of targeted cells

Over the last few years, several reports have demonstrated that not all cells were able to expand after being modified by ssODNs. Particularly cells that were targeted by PTO-modified ssODNs appeared to arrest in the G_2 phase of the cell cycle due to double-stranded DNA breaks (DSBs) and subsequent activation of a DNA damage checkpoint [18-21]. In **Chapter 5**, we have investigated the effect of gene modification by unmodified ssODNs on cell viability. Using anti-sense ssODNs, we noticed that the number of targeted cells appeared to decrease over time. This decrease could not be explained by a reduction in cell viability, since the colony-forming ability of targeted cells recovered shortly after ssODN exposure was only marginally lower than that of EGFP-expressing control cells. We postulated that semi-conservative replication was responsible for the gradual decrease of cells modified by anti-sense ssODNs. This hypothesis was supported by time-lapse imaging of ES cells that had become EGFP-positive upon correction by anti-sense ssODN 1m (Figure 1). As shown in Figure 1B, the EGFP fluorescence of a single ssODN-modified G_2 cell was transmitted to both daughter cells (56 hrs; Figure 1). Due to semi-conservative replication only one of these cells carried the corrected DNA template and therefore the fluorescent signal in the other daughter cell gradually faded. Consequently, after the second cell division (at 69 hrs; Figure 1), only one out of four daughter cells was able to produce progeny that stably expressed EGFP fluorescence (third cell division at 88 hrs; Figure 1). Importantly, our data demonstrated that ssODN-directed gene modification did not impair the proliferative capacity of ssODN-modified cells *per se*. Although a mildly reduced survival was observed in ssODN-modified cells, this was not due to the induction of genomic DSBs caused by exposure to or incorporation of unmodified ssODNs, but may rather reflect toxic effects of EGFP (over)expression (**Chapter 5**).

Although many others in the ssODN-mediated gene targeting field encourage the use of PTO-modified ssODNs because of the higher targeting frequencies that were obtained in short term assays [22], we would rather advocate the use of unmodified ssODNs. At this moment, it is still unclear how PTO-modified ssODNs induce genomic DSBs. Olsen *et al.* [19] showed that the DSB level was specifically elevated in cells that were modified by ssODNs, indicating that the incorporation of PTO-modified ssODNs itself was toxic. However, the modified locus could be transcribed to produce green-fluorescence, arguing against local DNA damage. We have demonstrated that DSBs were also induced in cells that were exposed to PTO-modified ssODNs without being modified (**Chapter 5**), which is in agreement to the results of Bonner *et al*. [21] and indicates that high intracellular levels of ssODN may induce DNA damage.

Stable outgrowth of the targeted cells is essential for successful ssODN-mediated modification of endogenous genes, since the modified cells need to be isolated without the use of selection markers. In **Chapter 6**, we describe a highly sensitive PCR protocol to identify ssODN-modified cells in a background of unmodified cells. This procedure enabled us to clonally purify many independent ssODN-directed mutants

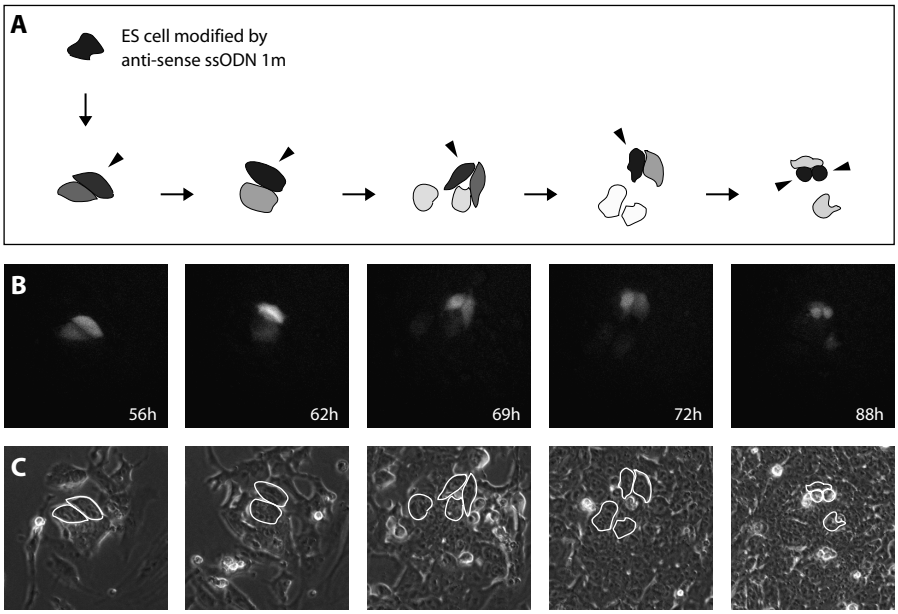

Figure 1. Time-lapse imaging of EGFP-positive ES cells corrected by anti-sense ssODN 1m.
*(**B**) Images of EGFP-positive ES cells taken at 56 h, 62 h, 69 h, 72 h and 88 h after exposure to anti-sense ssODN 1m. Corresponding bright field images are shown in (**C**). For clarity, a graphic of the cells, including the originally targeted G_2 cell, is given in (**A**). The level of EGFP expression is indicated by shades of grey. The ES cell carrying the corrected DNA strand(s) is indicated by an arrowhead.*

through several rounds of limiting dilution, again indicating that cells that were targeted by unmodified ssODNs showed similar growth characteristics as the untargeted cells in the population. If this were not the case, the limiting dilution procedure would have been nearly impossible, since the number of ssODN-modified cells would progressively decline throughout the cloning procedure.

Prospects for gene therapy

We have established a reliable protocol for ssODN-directed gene modification in mouse ES cells and were the first to successfully apply this technique to generate mutant mice (**Chapter 2**). The possibility to site-specifically alter endogenous genes may make ssODN-mediated gene targeting an attractive tool for therapeutic applications. The procedure does not require the laborious construction of a homologous targeting vector and is based on standard cell culture and molecular biology techniques. Virtually any codon of interest in the genome can be substituted and clonal mutant cell lines can be obtained within 2-3 months, depending on the efficacy of the subcloning procedure. Nevertheless, several challenges need to be overcome before ssODN-mediated gene targeting may be exploited for gene therapeutic purposes. First, *in vivo* delivery of the ssODNs to the cell type of interest may be problematical and require much optimization for each cell type specifically. Complexing the ssODNs with receptor ligands may facilitate organ- or tissue-specific delivery of ssODNs. For example, the carrier YEEE-K_{18}, which is a ligand for the asialoglycoprotein receptor tagged with a polylysine

chain, has been used to target ssODNs to the liver and kidney of mice by tail vein injection [23]. Second, only replicating cells appear to be amenable to modification by ssODNs. This excludes most somatic cells as target cell for gene therapy since they are quiescent and reside in the G_0 phase of the cell cycle. Third, even if the above criteria could be met, the low targeting frequency may be the greatest obstacle for effective therapeutic application. With the current frequencies, at least 10^6 cells need to receive ssODNs in order to obtain a few modified cells. These conditions could easily be achieved in cell culture experiments, but might be nearly impossible in a living organism. Andrieu-Soler *et al.* [24] demonstrated that ssODNs could be used to correct the *rd1* point mutation and promote the survival of photoreceptor cells in retinas of *rd1* mutant mice. Although the ssODNs could be delivered with repeatable efficiency to the mouse photoreceptor cells *in vivo*, the number of corrected photoreceptor cells remained insufficient to obtain clinical improvement. In addition, Bertoni *et al*. [25] showed that intramuscular ssODN injection resulted in correction of the *mdx*^{5cv} splice site mutation in the dystrophin gene, leading to stable dystrophin expression in muscle fibers for up to three months after ssODN injection. However, muscle cells are multinucleated syncytia and dystrophin expression from a single nucleus may only protect a small region of the fiber. Therefore, the rescue of a single muscle fiber requires the correction of multiple nuclei along the length of the fiber, which may be difficult to achieve due to the low targeting efficiencies. Thus, a sustained beneficial effect may only be accomplished when ssODN-modified cells have acquired a proliferative advantage over non-corrected mutant cells. Lastly, the suppression of MMR activity, which is required for effective ssODN-mediated gene targeting in mouse ES cells, may be highly objectionable for gene therapeutic applications. At the moment, we cannot accurately determine how many undesired mutations will be accumulated in ssODN-modified cells during the brief period of MMR suppression, or whether these mutations actually predispose cells to tumor formation. These issues need to be addressed in detail before this technology can enter into a clinical setting. As for all therapies, toxicity and risk of the treatment will have to be balanced with the severity of the disease and the prognosis of the patient.

Ex vivo application of ssODN-mediated gene targeting in, *e.g.*, induced pluripotent stem (iPS) cells may overcome some of the limitations mentioned above and become the preferred strategy towards therapeutic application. Such cells can be derived from somatic cells upon ectopic expression of four reprogramming factors, Oct4, Klf4, Sox2 and c-Myc [26]. The iPS cells resemble ES cells in many aspects, including morphology, growth characteristics and pluripotency. Therefore, the protocol that we have developed for ssODN-mediated gene modification in mouse ES cells may also be applicable in iPS cells, although further optimization will obviously be required. Recently, zinc-finger nucleases (ZFNs) have been used to stimulate homology-directed gene targeting by generating a DSB in the target site [27]. This approach has proven successful for transgene insertion in human ES and iPS cells [28,29] and the introduction of single base changes in the endogenous *IL2R*γ locus in human T cells [30], denoting the potential of this technology for therapeutic modification of human cells. Nevertheless, several issues regarding ZFN safety need to be addressed before gene therapy comes within reach. Reliable assays need to be developed for the screening of new ZFNs to assess their affinity and sequence-specificity in order to prevent off-target cleavage. In ad-

dition, mutagenesis due to DSB repair by the erroneous non-homologous end-joining pathway may interfere with precise modification of the genome. Future research may contribute to a better understanding of the capabilities and limitations of site-specific sequence gene modification directed by ZFNs and ssODNs and reveal whether these techniques could potentially be applied for the treatment of human genetic disease.

Concluding remarks

Over the last two decades, significant advance has been made in unravelling the basal mechanisms of ssODN-mediated gene targeting. The finding that DNA replication plays a crucial role in the coordination of the targeting reaction in bacteria [10,11], yeast [31,32], and various mammalian cell lines [18,33] (this thesis), has been a major breakthrough. Although the field has been troubled by the publication of conflicting data and concerns about reproducibility [34,35], many of the reported discrepancies could actually be ascribed to differences in experimental setup and in some cases to misinterpretation of results. Several of these confounding factors have now been identified, including the use of episomally located genes as target for ssODN-mediated modification and, related to that, the use of cell lines carrying different copy numbers of the target gene; the time after which gene targeting events have been recovered; chemical composition of the ssODN; and the MMR status of the reporter cell line. Recognizing these differences and more careful interpretation of the results may improve the reputation of the field. Hopefully, the experiments described in this thesis will contribute to the further development of ssODN-directed gene modification for both experimental and therapeutic applications.

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Appendices

Nederlandse Samenvatting
English Summary
Curriculum Vitae
Publications
Dankwoord