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ENGLISH SUMMARY

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. Such cells can be used to generate mice carrying (disease-causing) sequence variants in their genome. This strategy makes use of short single-stranded DNA oligonucleotides (ssODNs) that are identical to the target gene except for the codon to be modified.

A comprehensive overview of the current insights into the mechanisms and reaction parameters underlying ssODN-mediated gene targeting is given in **Chapter 1**. Previously, we have shown that the mismatch repair (MMR) system strongly impairs the introduction of genetic alterations in mouse ES cells. Transfer of the genetic information requires base pairing between the ssODN and the chromosomal target locus resulting in the formation of DNA mismatches. The MMR system will recognize and remove these mismatches, thereby preventing stable inheritance of the desired genetic alteration. Inactivation of the MMR system improved the targeting frequency several 100-fold, but could not be used due to the mutator phenotype associated with constitutive MMR deficiency.

In **Chapter 2**, we have investigated whether transient down-regulation of MMR gene expression by RNA interference could generate a time window that allowed effective gene alteration by ssODNs, while preventing the accumulation of inadvertent mutations. We show that transient down-regulation of the MMR gene *Msh2* allowed effective substitution of four adjacent nucleotides in wild-type ES cells. In these cells, simple nucleotide substitutions and nucleotide insertions were still suppressed by 10% of residual MSH2 activity. The occurrence of spontaneous mutations was only mildly elevated in the MSH2 knockdown cells and remained far below the mutation frequencies found in constitutive *Msh2*-deficient cells. We have successfully applied this strategy to generate the first mutant mouse line that was created via ssODN-mediated gene targeting.

This work was further extended in **Chapter 3**, where the effect of suppression of *Mlh1*, a downstream MMR gene, on the ssODN-mediated gene targeting frequency was investigated. We show that transient suppression of MLH1 made cells permissive for all nucleotide substitutions ranging from one to four nucleotides, thereby alleviating the suppression of simple substitutions in the MSH2 knockdown strategy. 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. Although MLH1 knockdown resulted in somewhat higher mutation frequencies than MSH2 knockdown, the ssODN-modified ES cells retained pluripotency and could contribute to the mouse germ line. Our data demonstrate that both the MSH2 and the MLH1 knockdown strategies could be used for the generation of mutant mice.

The basal mechanism of ssODN-mediated gene targeting is not yet fully understood and many cellular processes besides the MMR system may be involved. In **Chapter 4**, we have studied the involvement of transcription, DNA replication and DNA repair pathways to gain more insight into the parameters governing ssODN-mediated gene targeting in mouse ES cells. Our findings were most compatible with a replication-dependent model in which the ssODN anneals to its chromosomal complement within the context of a replication fork. The single-stranded DNA regions that are present during DNA synthesis of the lagging strand may enhance ssODN annealing. This differential accessibility may explain why in our reporter system ssODNs in the sense orientation performed better than ssODNs in the anti-sense orientation. Following annealing, the ssODN may serve as a primer for DNA synthesis and become extended by the endogenous replication machinery, resulting in incorporation of the ssODN into the nascent DNA strand.

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 demonstrate that ssODN composition can strongly affect the cellular response to ssODN-mediated gene targeting. Unmodified ssODNs did not induce a substantial amount of DNA damage and only mildly affected the viability of targeted cells. In contrast, ssODNs that were end-protected by phosphorothioate linkages strongly reduced the viability of targeted cells by inducing DNA double-strand breaks. The use of unmodified ssODNs rather than end-protected ssODNs allows stable outgrowth of targeted cells and may facilitate routine application of this technique. Time

course analyses reveal that ssODNs are most likely incorporated into the genome during DNA synthesis. Semi-conservative replication and cell division lead to a dilution of targeted cells, which explains why the number of targeted cells that could be recovered after ssODN-mediated gene targeting declines over time.

A detailed protocol for ssODN-mediated gene targeting in mouse ES cells is given in **Chapter 6**. We have exploited the specific repair and recognition capacities of the MMR protein complexes to allow the introduction of specific sequence alterations. We found that ssODN-mediated nucleotide substitutions could effectively be obtained upon transient suppression of MSH2 or MLH1, allowing substitution of any codon of interest in the genome. Insertions of four nucleotides were effective in ES cells deficient for MSH3. These cells do not show an overt mutator phenotype and could therefore be used for the generation of knockout alleles.

In conclusion, the studies described in this thesis have contributed to a better understanding of the mechanisms underlying gene modification by ssODNs in mouse ES cells. Various parameters that impinge on the targeting process have been identified, which resulted in the development of a generally applicable protocol for ssODN-mediated gene targeting. The possibility to site-specifically alter endogenous genes may make ssODN-mediated gene targeting an attractive tool for therapeutic applications. Future research may focus on the capabilities and limitations of this technique and reveal whether it could potentially be applied for the treatment of human genetic disease.