

DIVISION OF MOLECULAR BIOLOGY

Genetic instability and deregulated cell cycle control are hallmarks of human cancer. Our research involves both aspects focusing on (1) the role of DNA mismatch repair and the Fanconi anemia genome maintenance pathway in mutation avoidance and (2) the role of cell cycle checkpoints in tumor suppression. The principle tools include gene modification in murine embryonic stem cells (ESC) and analyses of the phenotypic consequences in ESC, mutant mice and cell lines derived thereof.

DNA MISMATCH REPAIR

Lynch syndrome/HNPCC (hereditary non-polyposis colorectal cancer) is caused by inherited defects in DNA mismatch repair (MMR). The primary function of MMR is correction of DNA replication errors, which is initiated by MSH2/MSH6 or MSH2/MSH3 protein complexes that recognize mismatches in DNA. Subsequent steps involve recruitment of another protein complex, MLH1/PMS2, activation of exonucleolytic activity to remove the error-containing DNA strand and resynthesis of a new strand. Mismatches can also arise by replication of damaged bases such as O⁶-methylguanine. This lesion elicits futile cycles of mismatch repair, ultimately leading to cell death. Thus, DNA MMR acts anti-mutagenic and mediates the toxicity of methylating agents.

Oligonucleotide-directed gene modification We have shown that single-stranded oligo-deoxyribonucleotides (ssODNs) can be used for subtle gene modification in ESC. The ssODN are identical to the chromosomal target sequence except for one or a few centrally located nucleotides that comprise a desired modification. However, the incomplete base pairing between the ssODN and its chromosomal complement activates the MMR system, leading to abortion of the gene modification reaction (Dekker et al., *NAR* 2003;31:e27). We found that ESC can be rendered permissive for 'oligo targeting' by transient suppression of MSH2 protein level by RNA interference (Aarts et al., *NAR* 2006;34:e147).

To study the mechanism of oligo targeting we generated ESCs carrying single-copy chromosomally-located neomycin (neo) or EGFP reporter genes that were disabled by a mutation in the start codon. The appearance of G418-resistant or green-fluorescent cells serves as readout for ssODN-mediated restoration of the start codon. The highest targeting frequencies were obtained with ssODNs of 35-40 nucleotides. Remarkably, the efficacy of oligo targeting was not affected by any DNA repair mechanism other than MMR, nor by transcription of the target locus (Aarts and Te Riele, *JCMM* 2010;14-6b:1657). Using the EGFP reporter we found that anti-sense ssODN yielded green-fluorescent cells already 24h after exposure, however, the frequency of targeted cells declined upon prolonged culturing to stabilize at day 4. In contrast, with sense ssODN, green fluorescent cells appeared at 48h and the frequency of targeted cells remained stable upon culturing (Aarts and Te Riele, *NAR* 2010;38:6956). These observations support a model in which the ssODN physically integrates into the host genome during DNA replication (figure 1).

Remarkably, ssODN containing phosphorothioate (PTO) linkages (to protect them from degradation) induced DNA double-strand breaks impeding proliferation of targeted cells. Unmodified ssODN were harmless to cells and yielded higher targeting efficiencies than PTO-ssODN.

Unclassified variants of MMR genes We have developed a protocol for ssODN-directed gene modification to target in principle any site in the ESC genome (Aarts et al., *Methods Mol Biol* 2009;530:79-99). We use this technology to recreate suspected variants of MMR genes in ESC in order to assess their capacity to sustain MMR functions. MMR gene mutations affecting a single codon are widespread in the human population and in (suspected) Lynch syndrome patients but the consequences of single amino acid substitutions are often difficult to predict. We have thus far analyzed five of such 'Variants of uncertain significance' (VUS) of MSH2 and found one fully and another partially abrogating MMR activity. As we use



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Publications

Aarts M, Te Riele H. *Parameters of oligonucleotide-mediated gene modification in mouse ES cells.* *J Cell Mol Med* 2010;14-6b:1657-67

Aarts M, Te Riele H. *Subtle gene modification in mouse ES cells: evidence for incorporation of unmodified oligonucleotides without induction of DNA damage.* *Nucleic Acids Res* 2010;38:6956-67

Aarts M. *Gene targeting by single-stranded DNA oligonucleotides in mouse embryonic stem cells.* PhD thesis, VU University Amsterdam, April 2010

Aarts M, Te Riele H. *Progress and prospects: oligonucleotide-directed gene modification in mouse embryonic stem cells: a route to therapeutic application.* *Gene Ther* 2010;doi:10.1038/gt.2010.161

Van Harn T, Foijer F, van Vugt M, Banerjee R, Yang F, Oostra A, Joenje H, Te Riele H. *Loss of Rb proteins causes genomic instability in the absence of mitogenic signaling.* *Genes Dev* 2010;24:1377-88

Vormer TL. *Tumor suppression by retinoblastoma proteins: interacting proteins and cooperating pathways.* PhD thesis, VU University Amsterdam, November 2010

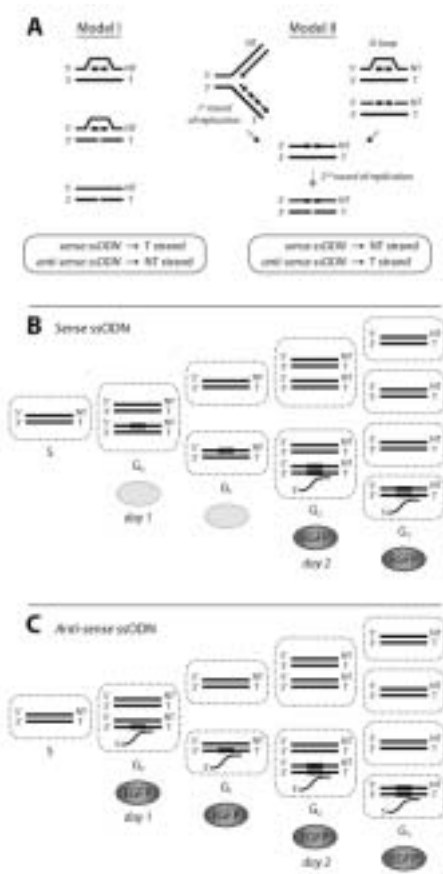


Figure 1. ssODN-mediated correction of mutant EGFP.

(A) Model I: the sense ssODN (fat line with white cross) anneals to its chromosomal complement and serves as a template for repair of the transcribed strand (white x), allowing immediate EGFP expression.

Model II: the sense ssODN integrates into the non-transcribed strand during replication or recombination (D-loop). A second round of replication is needed to alter the transcribed strand and allowing EGFP expression.

Our findings are compatible with Model II.

NT, non-transcribed strand; T, transcribed strand. (B) Incorporation of sense ssODNs (fat line) into the non-transcribed strand during S phase requires an extra round of replication to transmit the corrected EGFP sequence to the transcribed strand (day 2).

(C) Incorporation of anti-sense ssODNs (fat line) into the transcribed strand immediately provides a template for EGFP production (day 1). However, subsequent semi-conservative replication will dilute the number of EGFP-positive cells, theoretically four-fold.

mouse ESC, the latter could be introduced into the germ line of mice. We found this variant to induce tumour formation as strongly as a full Msh2 knockout, identifying it as a deleterious mutation. Three variants showed normal MMR capacity, which is remarkable in view of the evolutionary conservation of the affected amino acids.

THE FANCONI ANEMIA PATHWAY

Another example of cancer predisposition by inherited defects in DNA repair is Fanconi anemia (FA), a recessive disorder characterized by malformations, progressive anemia and high incidence of AML and epithelial tumors. FA is caused by bi-allelic defects in either one of 13 genes, FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N. At the cellular level, malfunctioning of these genes causes hypersensitivity to cross-linking agents as manifested by G₂ arrest, chromosomal aberrations and cell death.

To assess the significance of the FA pathway in suppression of cancer, we have generated Fancf- and Fancm-deficient mice. FANCF and FANCM are part of the FA core complex that mono-ubiquitinates FANCD2 and FANCI. Both, Fancf and Fancm deficiency caused hypogonadism in mice and hypersensitivity to cross-linking agents in mouse embryonic fibroblasts (MEFs), thus phenocopying other FA mouse models. However, Fancm deficiency also caused features atypical for FA, including under-representation of females and decreased overall and tumor-free survival (Bakker et al., Hum Mol Genet 2009;18:3484). The latter may be correlated to the role of FANCM in the suppression of spontaneous sister chromatid exchanges as we observed in MEFs. Thus, FANCM may function both inside and outside the FA core complex to maintain genome stability. The Fancf and Fancm knockout alleles have been crossed into cancer prone Apc^{+/+} mice to study whether defects in the FA pathway accelerate tumorigenesis.

CELL CYCLE CHECKPOINTS

Loss of G₁/S control is a frequent if not mandatory event in tumor development. G₁/S control relies on the pocket proteins pRB, p107 and p130 that collectively regulate the activity of E2F transcription factors. We use MEFs devoid of pocket proteins (TKO MEFs) to study residual cell cycle control mechanisms and to identify events that promote oncogenic transformation.

Growth-factor independence TKO MEFs still rely on mitogens for survival and proliferation. In the absence of mitogens, many TKO MEFs undergo apoptosis whereas the surviving fraction arrests in the G₂ phase of the cell cycle. G₂ arrest was effectuated through inhibitory interactions of the cyclin-dependent-kinase inhibitors p21^{CIP1} and p27^{KIP1} with Cyclins A and B1 (Fojier et al., Cancer Cell 2005;8:455). G₂ arrested cells showed high levels of DNA double strand breaks, which were only partially repaired when TKO cells were stimulated to enter mitosis by mitogen re-addition. Moreover, mitotic chromosomes showed a 'railroad' appearance indicative of defects in centromeric sister-chromatid cohesion (Van Harn et al., Genes Dev 2010;24:1377). Furthermore, we found aneuploidy in cell cultures derived from TKO cells that had been transiently mitogen deprived. These results suggest that combined lack of pocket proteins and mitogenic signaling can lead to genomic instability, which may contribute to tumor progression.

Anchorage independence We have also shown that pocket protein ablation was not sufficient to sustain anchorage-independent growth, even not upon expression of RAS^{V12}, although TKO MEFs were immortal. Apparently, a cell cycle mechanism still operates to restrict proliferation of these cells in soft agar. By performing a gain-of-function screen for genes that permit anchorage-independent growth of RAS^{V12}-expressing, pocket protein deficient MEFs, we identified the immortalizing oncogene TBX2 that was shown to suppress the p53 pathway through downregulation of p19^{ARF} (Vormer et al., MCB 2009;28:7263). Furthermore, we have developed a new technique to enzymatically produce shRNA libraries from cDNAs. Screening of these libraries yielded several novel suppressors of anchorage-independent growth.