Mouse Retina Has Oligonucleotide-Induced Gene Repair Activity

Vincent T. Ciavatta, Staci A. Padove, Jeffrey H. Boatright, and John M. Nickerson

Purpose. To test the ability of murine retinal nuclear extracts to support in vitro oligonucleotide-mediated gene repair (OMGR)—a prerequisite to repairing endogenous gene lesions underlying inherited diseases of the neurosensory retina.

Methods. An in vitro reaction assayed whether retinal extract and oligonucleotides could correct a mutation in an antibiotic (tetracycline) resistance gene in a plasmid. The in vitro gene repair reaction combined plasmid, repair oligonucleotide, and nuclear extract. Site-specific repair of the tetracycline gene point mutation was revealed in a bacterial readout system in which plasmid from the in vitro reaction was recovered and electroporated into *Escherichia coli*. Colony growth on tetracycline indicated repair of the point mutation. To confirm site-specific nucleotide repair, plasmids were sequenced or subjected to restriction fragment length polymorphism (RFLP) analysis.

Results. To quantify repair incidence, tetracycline-resistant colonies were normalized to ampicillin-resistant colonies. A repair oligonucleotide composed of RNA and DNA that contains one mismatched base relative to the target DNA induced an estimated 1 in 10^4 plasmids to be converted to wild type. If the extract was eliminated or boiled before reaction, no tetracycline-resistant colonies grew. Repair incidence increased with the concentration of retinal nuclear extract and oligonucleotide. Creating single-strand breaks in the plasmid caused a twofold increase in repair incidence.

Conclusions. These in vitro assay data suggest that murine retina nuclei contain all the DNA repair factors necessary for OMGR, a finding that is prerequisite to attempting endogenous gene repair in mouse retina. (*Invest Ophthalmol Vis Sci.* 2005; 46:2291–2299) DOI:10.1167/iovs.04-1220

In vivo correction of a recessive point mutation that underlies an inherited disease is a possible strategy for gene therapy that has gathered support from experienced molecular biologists and gene therapy researchers.[1–3] Such a strategy avoids potential gene silencing or random integration associated with gene augmentation approaches. In addition, in vivo correction of a point mutation preserves the proper genomic context of the repaired gene, leaving regulation of expression undisturbed. Notable strategies for repair of point mutations include single-stranded, short-fragment homologous replacement,[4] viral vector gene targeting (Dejneka NS, et al. *IOVS* 2003;44: ARVO E-Abstract 1080), guided homologous recombination with triplex helix formation,[5] and oligonucleotide-mediated gene repair (OMGR). Successful in vivo OMGR, with the use of an RNA/DNA oligonucleotide (RDO) in liver,[6] skin,[7] and muscle[3,8] tissue, prompted us to explore the applicability of this technique to the neural retina.

The OMGR strategy involves an oligonucleotide designed to be complementary to regions flanking the point mutation, but noncomplementary to the mutant base (Fig. 1). The oligonucleotide is envisioned to provide a template for insertion of the proper wild-type base in the target DNA and must therefore be delivered to the nuclei of target cells. The strategy was originally described in 1996 with the repair of an episomal mutant alkaline phosphatase gene in CHO cells[9] and has since been demonstrated in several cell-free (in vitro) systems[10]; cell culture systems, including mouse embryonic stem (ES) cells[11]; and animal models of genetic disease.[6–8]

Although not wholly defined, the process through which OMGR replaces the mutant base in the target DNA has been described as multistep, comprising heteroduplex formation between oligonucleotide and target DNA, mismatch recognition, and DNA repair.[12] These steps are likely to involve proteins from different DNA repair pathways. For instance, in mammalian cells, heteroduplex formation seems to require the homologous recombination protein RAD51,[13] and a key protein of mammalian mismatch repair (MMR), MSH2, also appears to play a role.[10] In addition, in yeast, proteins of the nucleotide excision repair (NER) pathway, such as the RAD1/RAD10 5′ endonuclease complex, were shown to be necessary for OMGR.[14] Last, transcriptional activity of the target DNA has been demonstrated to be beneficial for single-stranded oligodeoxyribonucleotide (ssODN)–mediated gene repair.[15] This finding suggests that elements of the transcription-coupled DNA repair pathway may be involved in repair of mismatches formed between an incoming oligonucleotide (ssODN or RDO) and the endogenous DNA. Therefore, some OMGR mechanistic requirements have been identified, including involvement of different DNA repair mechanisms. However, the generalized model is not well defined, and it is not clear whether a universal mechanism exists for all cell types.

Despite the undefined mechanism, OMGR has been demonstrated on several levels. For proof-of-principle, the technology has been shown to be effective for correction of point mutations in genomic and/or episomal DNA in cells from bacteria,[2] yeast,[14] plants,[16,17] and mammals.[18] In vivo OMGR has been documented in animal models of human disease. Examples include repair in muscle tissue (mouse[3] and canine[8] dystrophin genes), skin (tyrosinase gene),[7] and liver, including the UDP glucuronosyltransferase gene,[6] the factor IX gene,[19] and the apolipoprotein (ApoE2) gene.[20] All these examples

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Corresponding author: John M. Nickerson, Department of Ophthalmology, Emory University, Room B5602, 1365B Clifton Road, NE, Atlanta, GA 30322; litjn@emory.edu.

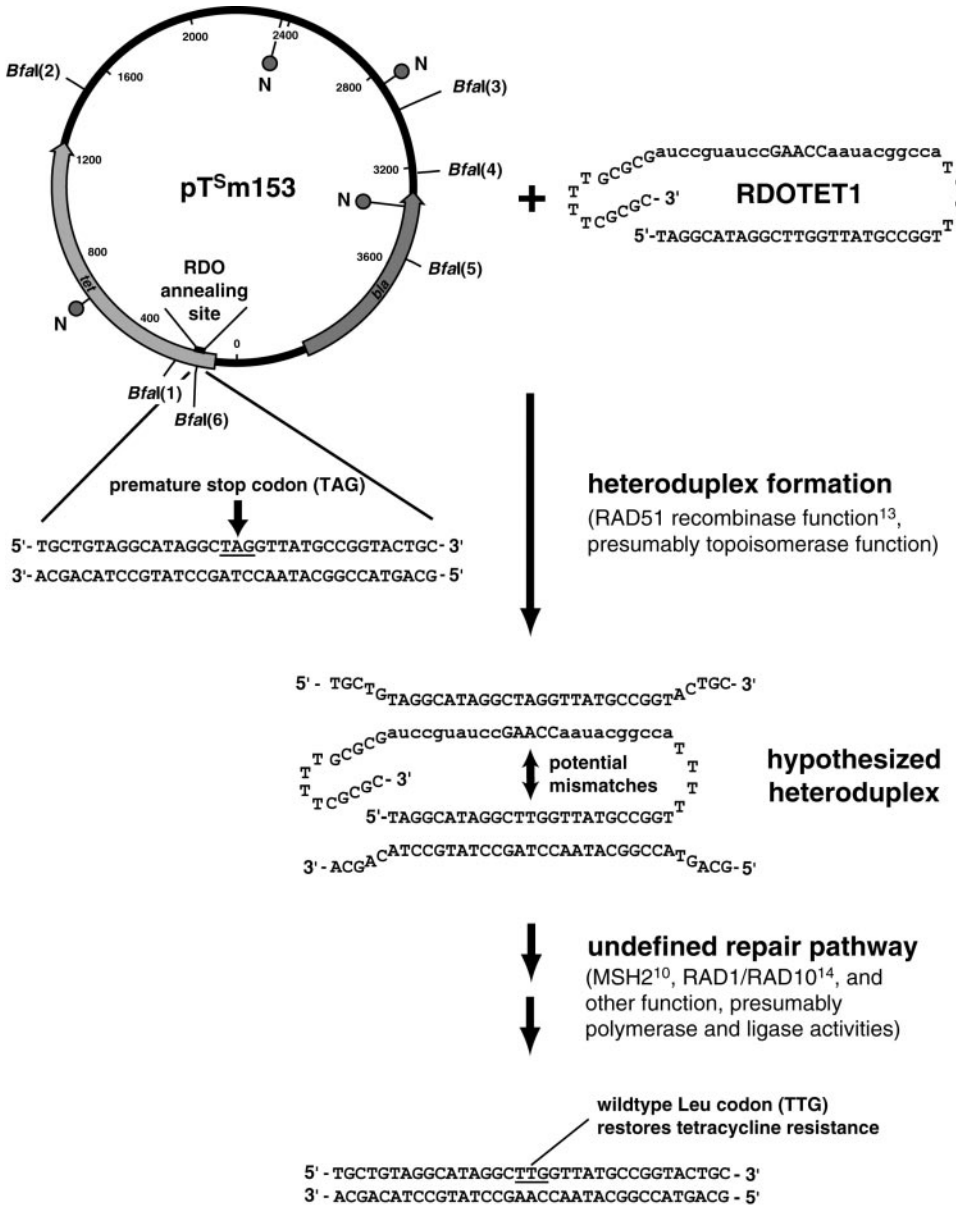

FIGURE 1. A model of RNA/DNA oligonucleotide (RDO)-mediated gene repair. In vivo OMGR is meant to repair point mutations in target DNA (e.g., genomic or mitochondrial DNA). For consistency with the in vitro assay, the plasmid (pT^Sm153) is shown as the target. The *tet* and *bla* genes for tetracycline and ampicillin resistance, respectively, the potential RDO annealing site, *Bfa*I restriction sites, and potential N.*Bst*NBI nicking sites (denoted by the letter N; recognition sequence 5′-GACTCNNNN†N-3′ are labeled. †Site of the break. *Lines* indicating nicking sites touch the strand that will be nicked (e.g., assuming the outer strand runs 5′ to 3′ in the clockwise direction, hydrolysis at the N.*Bst*NBI sites at ~nt 600 would break the phosphodiester bond of the *tet* gene's sense strand). RDO, shown as RDOTET1, is designed to be complementary to the target DNA, but forms a mismatch in the middle of the complementary region. The heteroduplex is a hypothetical structure. However, mechanistic studies have revealed that repair involves recombination proteins such as RAD51, which probably facilitate heteroduplex formation between RDO and target DNA, and MMR proteins (e.g., MSH2) and exonucleases (e.g., RAD1/RAD10).

include evidence of gene correction at the DNA and protein levels.

Our goal was to assess the applicability of OMGR to cells of the neural retina. As suggested by the involvement of DNA repair proteins such as RAD51, MSH2, RAD1, and RAD10, OMGR appears to rely on a cell's endogenous ability to repair damage to DNA. Evidence of UV-induced DNA synthesis in the retina[21,22] and presence of repair proteins in *rd1* mice retinas[23] suggests that retinal cells possess some DNA repair capabilities. Therefore, prerequisite to the application of OMGR to eye disease, it must be determined whether retinal DNA repair capabilities support this gene repair strategy. Because retinal and, more generally, neuronal tissues had not been tested for OMGR capability, the strategy of Cole-Strauss et al.,[10] in which the repair capability of HuH-7 cell-free extracts was assessed, was adapted to assay neural retinal nuclear extracts. Results indicate that murine retina nuclei possess the requisite DNA repair activities for supporting OMGR.

MATERIALS AND METHODS

Preparation of Nuclear Protein Extract

Mice were maintained and euthanized in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Nuclear proteins were extracted from liver and retina by the method of Andrews and Faller[24] with the modification that tissues were homogenized with a pellet pestle while in buffer A, and only leupeptin and aprotinin (2 μg/mL, each) were used as protease inhibitors. Sixteen to 20 retinas or 100 mg of liver tissue were processed per preparation. Protein concentrations in recovered extracts were estimated (BCA Protein Assay; Pierce, Rockford, IL), and single-use aliquots were stored at −80°C.

Plasmid DNA and Oligonucleotides

Plasmid pT^Sm153, a derivative of pBR322 (Fig. 1) was used as a target for gene repair. This plasmid contains the *bla* and *tet* genes for ampicillin and tetracycline resistance, respectively. The *tet* gene has a

T-to-A substitution at nucleotide (nt) 153 and a silent mutation at nt 325 (A-to-G substitution). The substitution at nt 153 converts the wild-type leucine codon (TTG) to a termination codon (TAG). Bacterial cells harboring $pT^{S}m153$ are then resistant to ampicillin (Amp^{R}), but sensitive to tetracycline (Tet^{S}). The silent mutation at nt 325, which converts the wild-type leucine codon (CTA) to another leucine codon (CTG), provides a convenient way to differentiate between corrected $pT^{S}m153$ (i.e., A at nt 153 instead of T) and potential pBR322 contamination. For use in repair reactions, greater than 500 μg of $pT^{S}m153$ was grown and purified (MaxiPrep column; Qiagen Inc., Valencia, CA), and the sequence was verified by antibiotic sensitivities, restriction digestion, and DNA sequencing.[25]

For plasmid nicking, 50 μg $pT^{S}m153$, 5.0 μL N.*Bst*NBI (10 U/μL) (New England Biolabs, Beverly, MA), 10 μL 10× buffer 3 (New England Biolabs), and 7.5 μL deionized H_2O were combined and incubated for 16 hours at 55°C. Nicking reactions were terminated by extracting with an equal volume of Tris-saturated phenol-chloroform-IAA (25:24:1). Traces of phenol were removed with a column (Qiaquick; Qiagen Inc.), according to the manufacturer's instructions.

All RDOs were synthesized at the Emory University Microchemical Facility. They were purified by ion-pair HPLC and ion-exchange chromatography to remove contaminating oligonucleotides bearing depurinated bases. Purified material was examined by capillary electrophoresis and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Only batches >95% pure were used. For PAGE analysis of RDOTET2, oligonucleotides were denatured as described in Manzano et al.[26] and separated on a 15% polyacrylamide TBE (Tris-borate-EDTA)-urea gel (Criterion; Bio-Rad, Hercules, CA). Gels were stained with a nucleic acid stain (1× SYBR Green II; Molecular Probes, Eugene, OR) in 1× TBE for 20 minutes.

For the in vitro DNA repair assay (Fig. 2), single-use aliquots of all reagents were prepared and stored at −20°C (HEPES, dithiothreitol, β-mercaptoethanol, BSA, spermidine, calf thymus DNA, and plasmid DNA) or −80°C (oligonucleotide, extract, $MgCl_2$, dNTPs, rNTPs, phosphocreatine, and creatine phosphokinase). The standard repair reaction and plasmid recovery were performed as described in Chen et al.[27] with the following exceptions: All reactions consisted of 1 μg plasmid DNA, 1 μg RDO, 10 μg nuclear extract, 12.5 mM phosphocreatine, and 15 μg calf thymus DNA (except extract and RDO concentration response experiments), reactions were stopped with 50 μL of 20 μM EDTA, and purified plasmid was resuspended in 10 μL deionized H_2O instead of 0.5× TE (Tris-EDTA) buffer. One-millimeter gap cuvettes and an electroporator (Electro Square Porator, model ECM 830; BTX, San Diego, CA) programmed for five pulses, 2250 V/pulse, 100 μs/pulse, and 200-ms intervals were used for transforming 2 μL of recovered DNA into 20 μL electrocompetent *recA*$^{-}$ *Escherichia coli* (DH10B; Invitrogen, Carlsbad, CA). The *E. coli* was incubated at 37°C for 1 hour in SOC medium without antibiotics (0.5% yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose), diluted, and spread on 100 × 15 mm Luria-Bertani (LB) plates containing 100 μg/mL ampicillin (10^4- and 10^5-fold dilutions for plasmid from reactions with nuclear extract, 10^5- and 10^6-fold

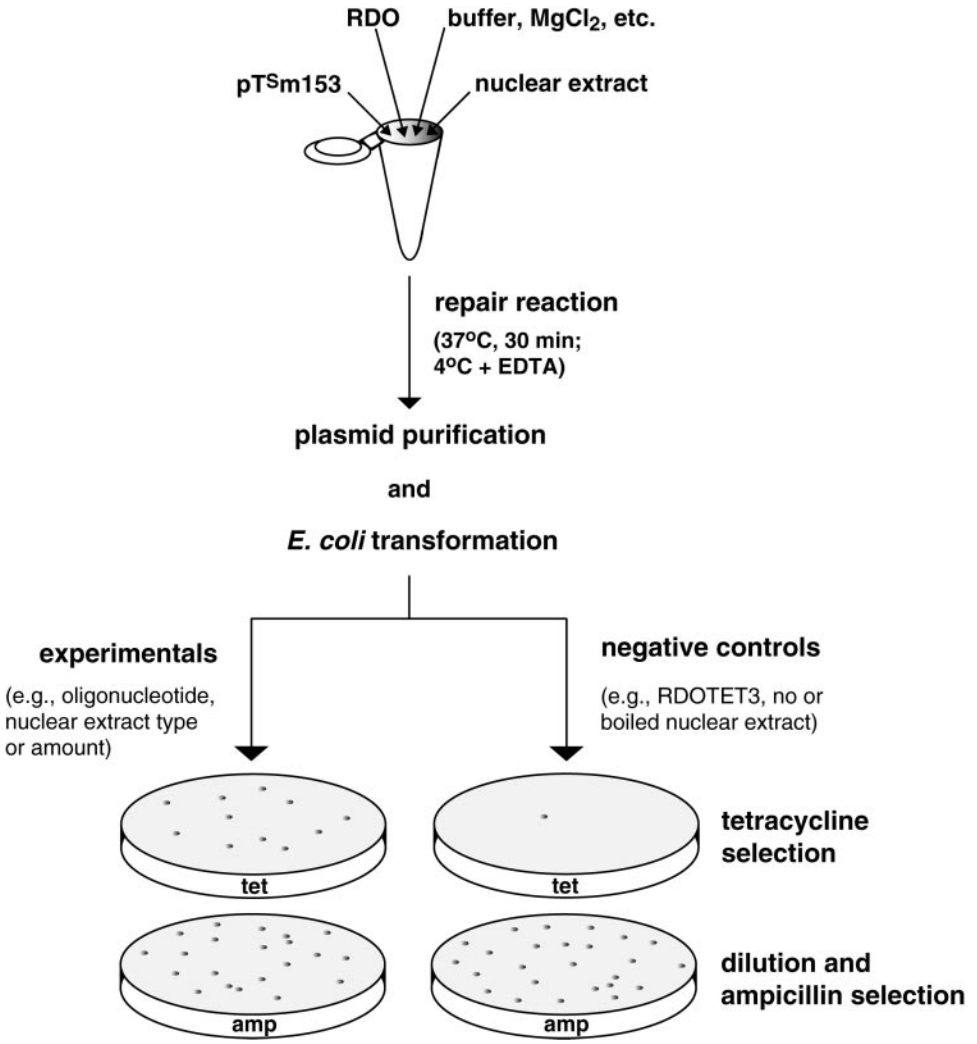

FIGURE 2. Diagram of in vitro repair assay. Plasmid, repair oligonucleotide, and nuclear extract were combined with an assay buffer and incubated at 37°C for 30 minutes. Placing the reaction tube on ice and adding EDTA terminated the reaction. Plasmid was recovered by phenol-chloroform extraction and ethanol precipitation and then electroporated in *recA*$^{-}$ *E. coli*. Electroporated cells were selected on tetracycline- or ampicillin-containing LB medium, and the incidence of gene repair was subsequently taken from the ratio of Tet^{R} colonies to Amp^{R} colonies. Base conversion was corroborated as described in Figure 4.

dilutions for plasmid from reactions without nuclear extract). Two 200-μL aliquots of the remaining undiluted cells were spread on two separate 100 × 15 mm LB plates containing 12.5 μg/mL tetracycline. The LB+ampicillin and LB+tetracycline plates were incubated at 37°C for 12 to 16 and 36 to 40 hours, respectively, and colonies were counted manually. The ratio of Tet^R colonies to Amp^R colonies is an estimate of the fraction of repaired plasmids and is therefore reported as a quantitative assessment of extract repair capability (henceforth reported as Tet^R/million Amp^R). Where given, *P* values represent the probability of accepting the null hypothesis that there was no difference between two means as determined by Student's *t*-test (one-sided *t*-tests, assuming unequal variances).

To confirm site-specific gene repair, plasmids recovered from selected tetracycline-resistant colonies were digested with *Bfa*I, because *Bfa*I site 6 (Fig. 1) is no longer present in repaired plasmids. Restriction fragments were separated by electrophoresis through 1.0% agarose in 1× TAE (Tris-acetate-EDTA) and stained with 1× nucleic acid stain (SybrGreen I; Invitrogen). Recovered plasmid was sequenced across nucleotides 153 and 325 using 5′-TGTTTGACAGCTTATCATCG-3′ as a primer.

Results

Support of Oligonucleotide-Mediated pT^Sm153 *tet* Gene Repair

To mimic the primary structure of RDOs shown capable of repairing point mutations in vivo,[6,8,19] an RDO that bears two mismatches to pT^Sm153 was used to evaluate mouse retinal nuclear extracts (RDOTET1; Fig. 3A). In standard assay conditions, repair incidence with extracts prepared from BALB/c retinas averaged 12 Tet^R/million Amp^R (Table 1). Positive control reactions performed with mouse liver nuclear extract resulted in an average repair incidence of 32 Tet^R/million Amp^R. In contrast, substituting an unrelated oligonucleotide ($RDO_{unrelated}$) for RDOTET1, eliminating the nuclear extract, or boiling the nuclear extract before addition to in vitro reaction all resulted in <0.08 Tet^R/million Amp^R. This frequency ($\sim 10^7$) is consistent with a basal bacterial spontaneous mutation incidence[25] (i.e., independent of gene repair activity in the in vitro cell-free reaction).

Six single Tet^R and six $Amp^R(Tet^S)$ colonies were picked and restreaked on selective media to assure that they were stably Tet^R and $Amp^R(Tet^S)$. All colonies among the dozen restreaks had the anticipated antibiotic resistances (data not shown). Plasmids recovered from one $Amp^R(Tet^S)$ colony and six Tet^R colonies were subsequently digested with *Bfa*I enzyme, and all digests revealed the expected bands (Fig. 4A). Finally, conventional dideoxy sequencing revealed the expected nucleotide at positions 153 (A for Tet^S, T for Tet^R) and 325 (G for all; Fig. 4B).

Because the ultimate goal is to apply this gene repair technology in vivo, an increased incidence of repair, above that which was observed from RDOTET1 in the standard reaction, was sought. According to Gamper et al.,[28] mismatch between the all-DNA portion of the RDO and the target DNA is critical to gene repair, whereas a mismatch between the RNA/DNA chimeric portion and target DNA is not essential. Furthermore, Igoucheva et al.[13] demonstrated that an RDO containing a single mismatch on the all-DNA portion had an approximately fivefold higher incidence of gene repair than the double-mismatch RDO (e.g., RDOTET1). Accordingly, an RDO (RDOTET2) in which the all-DNA side bears a single mismatch to pT^Sm153 was synthesized (Fig. 3B).

Use of RDOTET2 in the in vitro reaction with BALB/c or C57BL6 retinal nuclear extract resulted in 54 and 135 Tet^R/million Amp^R, respectively (Table 2). Repair incidence increased as the amount of retinal nuclear extract (Fig. 5A) or RDOTET2 (Fig. 5B) increased. RDOTET2 was prepared on three occasions, and these preparations varied in their ability to induce gene repair (the 54 and 135 Tet^R/million Amp^R reported are data from the best-performing RDOTET2 preparation). When these oligonucleotides were analyzed on a denaturing 15% polyacrylamide gel, it was noted that the degree of oligonucleotide degradation was inversely related to the amount of repair (Fig. 6). Augmenting the RDOTET1 data, these RDOTET2 data emphasize that targeted repair of a point mutation in pT^Sm153 occurred when a proper oligonucleotide and soluble nuclear extract from mouse retina were used.

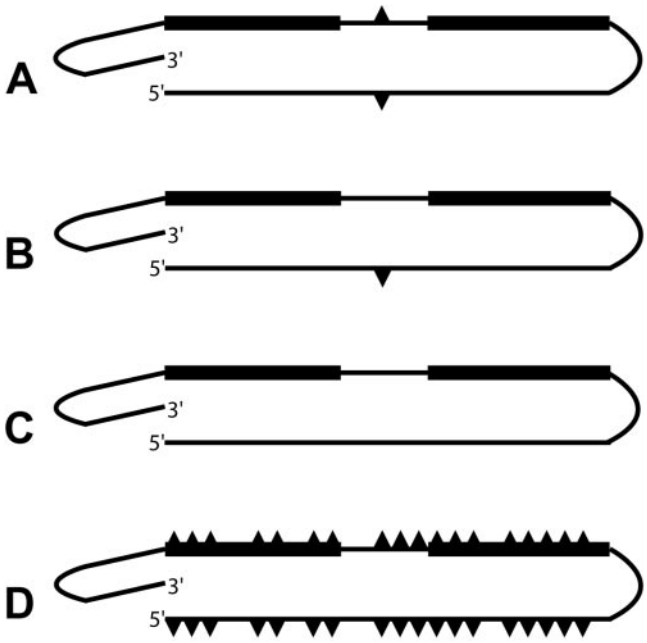

Figure 3. Theoretical RDO two-dimensional secondary structures. *Thick lines*: regions of 2-*O*-methyl-RNA. *Parallel lines*: self-complementarity and complementarity to the target. *Triangles*: site of mismatch between oligonucleotide and plasmid. With respect to the target site in pT^Sm153, note that RDOTET1 has two mismatches, RDOTET2 has a single mismatch located on the all-DNA portion of the oligonucleotide, RDOTET3 has no mismatches, and RDO unrelated has many mismatches. The mismatches created by RDOTET1 and RDOTET2 presumably are candidates for repair. Sequences: (*lowercase letters*: regions of 2-*O*-methyl-RNA, potential targeting mismatches are *italic*). (**A**) RDOTET1 (mismatches with template and nontemplate strands of pT^Sm153) 5′-TAGGCATAGGCT*T*GGTTATGCCGGTTTTTaccggcauaaCC*A*AGccuaugccuaGCGCGTTTTCGCGC-3′; (**B**) RDOTET2 (mismatch with template strand of pT^Sm153) 5′-TAGGCATAGGCT*T*GGTTATGCCGGTTTTTaccggcauaaCCTAGccuaugccuaGCGCGTTTTCGCGC-3′; (**C**) RDOTET3 (no mismatches with pT^Sm153) 5′-TAGGCATAGGCTAGGTTATGCCGGTTTTTaccggcauaaCCTAGccuaugccuaGCGCGTTTTCGCGC-3′; (**D**) $RDO_{unrelated}$ (random complementarity to pT^Sm153) 5′-ACTGCGGAAACTGTAAGTTTGGATTTTTTaauccaaacuTACAGuuuccgcaguGCGCGTTTTCGCGC-3′.

Effect of Single-Stranded Breaks in Target DNA on Gene Repair Incidence

It was observed that the transformation rate, in general, (as judged by the number of ampicillin-resistant colonies) was lower for reactions that contained nuclear extract (Table 1) and that this decrease was extract dose dependent (Table 3).

TABLE 1. Repair Results from In Vitro Assay with RDOTET1

Treatment	Oligo	Nuclear Extract	Amp^R (Millions)	Tet^R/Million Amp^R	*n*
1	RDOTET1	Retina (BC)	68.0 ± 12.1	11.66 ± 11.59	5
2	RDOTET1	None	209.0 ± 104.5	0.071 ± 0.017	4
3	None	Retina (BC)	64.0 ± 24.0	0.0*	3
4	RDOTET1	Liver (BC)	19.5 ± 25.0	31.7 ± 17.0	5
5	RDOunrelated	Retina (BC)	86.0 ± 15.1	0.017 ± 0.029	3

Oligonucleotides used were RDOTET1, previously shown to induce repair of pT^Sm153 with liver nuclear and mitochondrial extracts,[27] and RDOunrelated, a nonspecific oligonucleotide used as a negative control (Fig. 3). For each treatment, transformation and repair are reported (mean ± SD of *n* replicates) as the number of Amp^R colonies and the quotient of Tet^R colonies per million Amp^R colonies (Tet^R/million Amp^R), respectively. The Amp^R column shows that transformation tended to be lower for reactions that contained nuclear extract. Average repair incidence with retina nuclear extract (11.66, treatment 1) was greater than the no-extract control case (0.071, treatment 2; $P = 0.045$; *t*-test) and the nonspecific oligonucleotide case (0.017, treatment 5; $P = 0.0441$; *t*-test). Similarly, average repair incidence with liver nuclear extract (31.7, treatment 4) was greater than both the no-extract ($P = 0.0071$; *t*-test) and nonspecific oligonucleotide ($P = 0.0071$; *t*-test) cases. BC, extract was prepared from BALB/c mice.

* - no Tet^R colonies were detected among 7×10^7 Amp^R colonies.

This suggests that nuclear extract affects (damages) the supercoiled plasmid DNA so as to decrease its ability to transform *E. coli*. Because repair incidence also increased as the amount of nuclear extract increased (Fig. 5A), it was hypothesized that damage to the plasmid DNA enhances OMGR, perhaps by helping to recruit DNA repair proteins to the heteroduplex by a mechanism analogous to the stimulation of MMR offered by single-stranded breaks (nicks).[29] To test this hypothesis, pT^Sm153, which has four potential N.*Bst*NBI sites (Fig. 1), was treated with N.*Bst*NBI enzyme before the in vitro repair reaction. In support of the hypothesis, reactions with nicked plasmid resulted in lower transformation and higher repair incidences than reactions with non-nicked plasmid (Table 4).

DISCUSSION

An in vitro assay was used to assess the applicability of OMGR to postmitotic retinal cells. The assay had been used to demonstrate repair capabilities of extracts prepared from HuH-7 cells[10] and rat liver nuclei and mitochondria.[27] With this assay, we showed that mouse retinal nuclear extract is sufficient to support gene repair when incubated with the proper RDO for a given target DNA. Omitting the extract or boiling the extract (thereby denaturing heat-sensitive proteins present in the extract) for 10 minutes before addition to the reaction reduced gene repair to undetectable levels. Likewise, RDOs either lacking a mismatch to the target nucleotide or bearing only random sequence complementa-

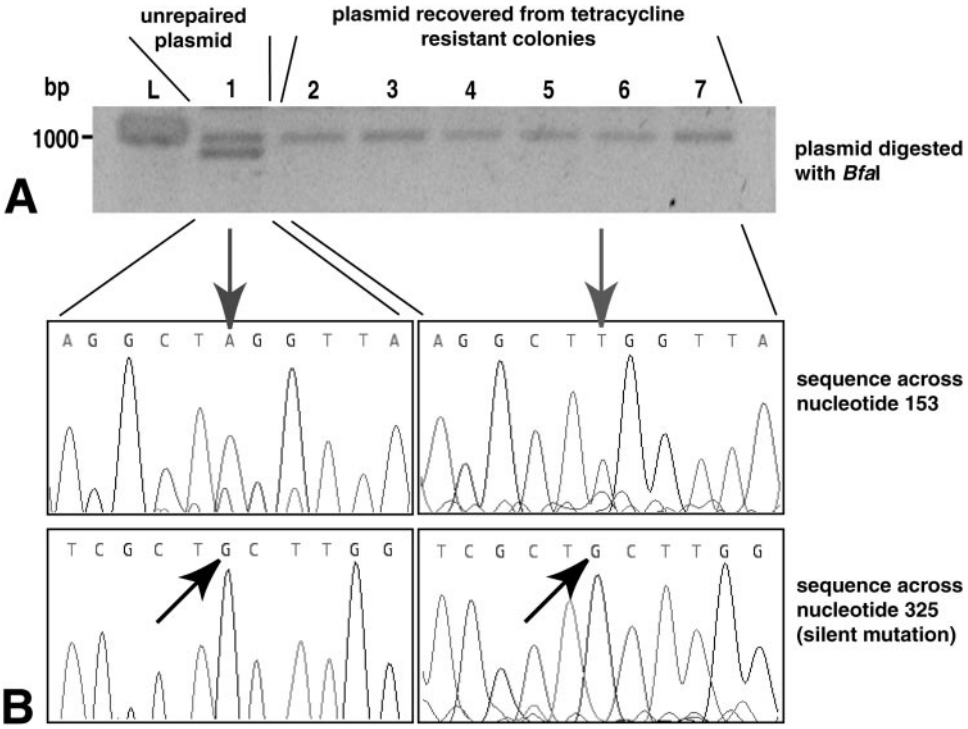

FIGURE 4. Corroborating gene repair evidence. (**A**) *Bfa*I restriction of plasmids recovered from Tet^R and Tet^S colonies. RFLP analysis of pT^Sm153 should produce a 958-bp band between the restriction at *Bfa*I (*lane 5*) and *Bfa*I (*lane 6*) and a 1035-bp band between *Bfa*I (*lane 5*) and *Bfa*I (*lane 1*), whereas RFLP analysis of repaired plasmid can only result in a 1035-bp band between the restriction at *Bfa*I (*lane 5*) and *Bfa*I (*lane 1*)—that is, *Bfa*I (*lane 6*) is eliminated on proper repair. Seven plasmids were partially digested with *Bfa*I. Consistent with anticipated RFLP patterns, digest of a plasmid recovered from a Tet^S colony produced two bands of ~1000 bp, one slightly greater (~1035) and one slightly lesser (~958) (*lane 1*), whereas digests of plasmids recovered from six Tet^R colonies all showed bands slightly greater than 1000 bp (~1035) (*lanes 2–7*). (**B**) Sequence of gene repair (nt 153) and silent mutation (nt 325) sites in plasmids recovered from Tet^S and Tet^R colonies. The two chromatograms on the top confirm that plasmids recovered from Tet^R colonies have a thymine residue at nt 153, which was previously occupied by an adenine residue in the unrepaired pT^Sm153. The commonly used pBR322 contains an adenine at nt 325, whereas pT^Sm153 has a guanine at nt 325. Therefore, that guanine was detected at nt 325 for all plasmids tested (*arrows* in the chromatograms at the *bottom* of the figure) indicates tetracycline resistance was not due to contamination by wild-type pBR322, but was instead achieved through site-specific repair of pT^Sm153 nt 153 from A to T.

TABLE 2. Repair Results from In Vitro Assay with RDOTET2

Treatment	Oligo	Nuclear Extract		Tet^R/Million Amp^R	*n*
		Type	Amount (μg)		
1	RDOTET2	Retina (BC)	10	53.9 ± 26.2	8
2	RDOTET2	Retina (B6)	10	135.2 ± 40.8	9
3	RDOTET2	—	—	0	9
4	RDOTET2	Boiled (BC)	10	0	3
5	—	Retina (B6)	10	0	3
6	RDOTET2	Liver (BC)	5	95.75	2
7	RDOTET3	Retina (BC)	10	0	3
8	RDOTET3	—	—	0	3

Oligonucleotides used were RDOTET2 and RDOTET3 (Figure 2). For each treatment, repair is reported (mean ± SD of *n* replicates) as the quotient of Tet^R colonies per million Amp^R colonies (Tet^R/million Amp^R). Full-complement reactions using RDOTET2 with either BALB/c or C57BL6 retina nuclear extract resulted in 53.9 and 135.2 Tet^R/million Amp^R, which were greater than repair from RDOTET1 with BALB/c extract (Table 1; $P = 0.0013$ and 3.0×10^{-6}, respectively; t-tests). Extract positive control (i.e., substituting liver extract for retina extract) also resulted in measurable gene repair (treatment 6). No-extract control (treatment 3), boiling the extract prior to reaction (treatment 4), omitting RDOTET2 (treatment 5), or substituting RDOTET3 (treatments 7 and 8) did not produce any Tet^R colonies among greater than 1×10^7 Amp^R colonies. BC, extract was prepared from BALB/c mice; B6, extract was prepared from C57BL6 mice.

rity to the target DNA were ineffective. Consistent with in vitro repair using extracts from HuH-7 cells[30] and DT40 and HeLa nuclei,[13] our retinal nuclear extract data indicate that an RDO in which the central base on the all-DNA side forms a single mismatch with the target DNA (RDOTET2; Fig. 3B) is more effective than the original RDO design (RDOTET1; Fig. 3A). Gamper et al.[30] suggested that RDOs in which the chimeric strand bears a mismatch to the target DNA lead to a mutagenic, template-independent pathway. Such an effect may have detracted from repair of the *tet* gene by RDOTET1. In addition, because fully self-complementary RDOs (e.g., RDOTET1) are expected to have significantly higher melting temperatures than RDOs with an internal mismatch[31] (e.g., RDOTET2), it should be more energetically favorable for RDOTET2 than RDOTET1 to participate in heteroduplex formation with the target DNA. Consequently, a possible increase in heteroduplex formation may have contributed to the increased repair observed with RDOTET2. Last, as noted by others,[32] a mechanistic role of the RDO and extract is supported by the observation that repair incidence was dependent on the concentration of extract and repair oligonucleotide.

Outcomes from this work suggest that damage to the target DNA enhances its repair. First, increasing extract in the in vitro reaction resulted in lower transformation (Table 3), but higher repair (Fig. 5A). Second, liver extracts were more damaging to the plasmid DNA than were retinal extracts, as indicated by the lower transformation (Table 1), yet reactions containing liver extracts nearly always resulted in higher repair than did those with retinal extracts. Last, in

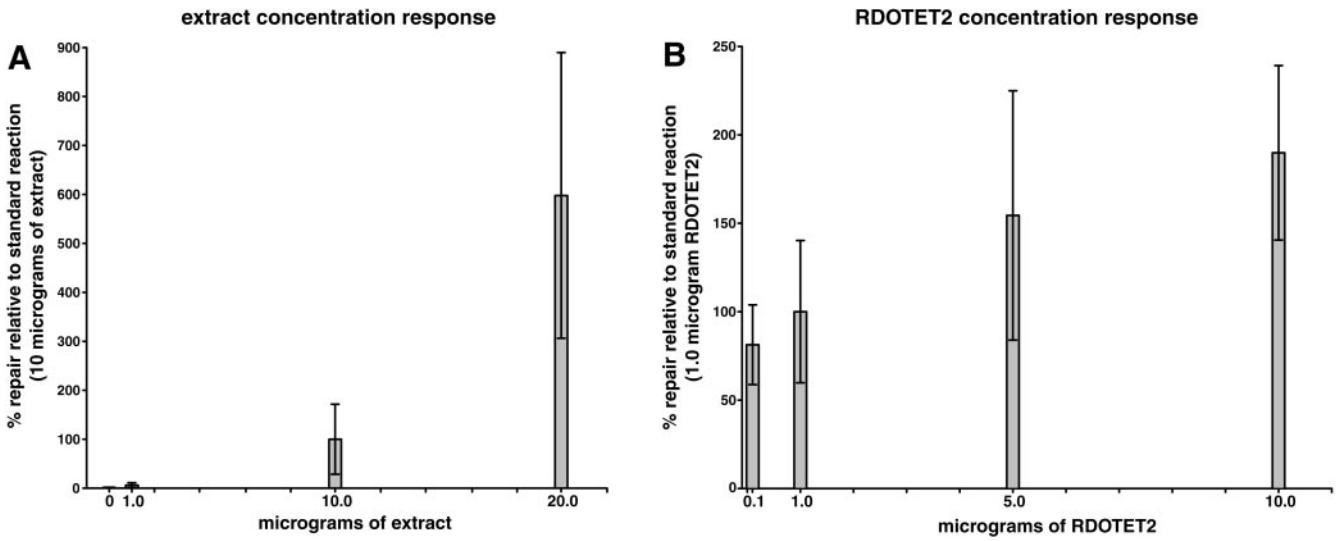

FIGURE 5. In vitro gene repair response to concentration of extract and RDOTET2. (**A**) Extract concentration response. Standard repair reaction conditions were used except the amount of BALB/c mouse retinal nuclear extract was varied at 0.0, 1.0, 10.0, and 20.0 μg. Bars represents (mean Tet^R/million Amp^R$_{(Nth\ extract\ dose)}$/mean Tet^R/million Amp^R$_{(10\ \mu g\ extract\ dose)}$) × 100%, where $n = 0$, 1, 10, or 20 μg extract. Error bar magnitudes represent the scale-corrected standard deviations of Tet^R/million Amp^R$_{(Nth\ extract\ dose)}$ which were determined from (SD of Tet^R/million Amp^R$_{(Nth\ extract\ dose)}$) × (height of the *n*th bar/100). (**B**) Oligonucleotide (RDOTET2) concentration response. Standard repair reaction conditions were used, except the amount of RDOTET2 was varied at 0.1, 1.0, 5.0, and 10.0 μg. Data are from one representative experiment in which three replicates were performed for each RDOTET2 concentration. Bars represent the averages of the three replicates normalized against the 1.0-μg oligonucleotide case, which was set to 100%. Error bar, SD normalized accordingly.

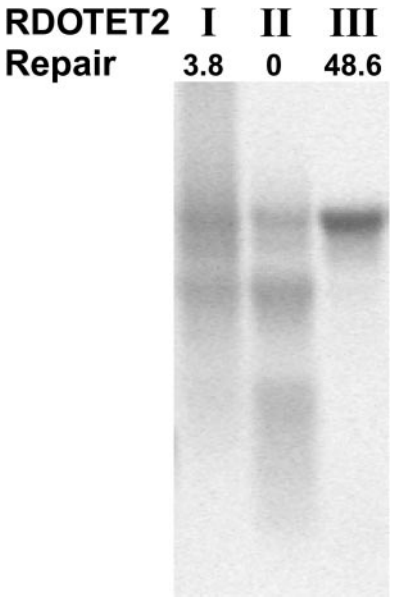

FIGURE 6. Oligonucleotide degradation and effect on gene repair. Image of a 15% denaturing polyacrylamide gel of 3 preparations of RDOTET2: I, II, and III. Preparations I and III were tested side by side in the standard in vitro gene repair assay with BALB/c retinal nuclear extract. III resulted in greater repair ($P = 0.0024$; *t*-test) at 48.6 ± 22.9 ($n = 6$) than I, which produced 3.8 ± 0.58 ($n = 3$). Two experiments with 0.1, 1.0, 5.0, and 10.0 μg of II per reaction produced no repair.

vitro repair experiments in which $pT^{S}m153$ was treated with nicking enzyme (N.*Bst*NBI) showed a decrement in transformation, but a twofold increase ($P = 0.0065$; *t*-test) in repair of nicked over non-nicked plasmid (Table 4). In addition to these observations, Suzuki et al.[33] showed that 5 mM bleomycin, an antitumor drug known to cause single- and double-stranded breaks in DNA,[34] enhanced gene repair in cultured melan-c cells. Strand breaks may enhance repair due to easier heteroduplex formation for relaxed (nicked) target DNA relative to supercoiled target DNA. Alternatively, the effect of single-stranded breaks on OMGR may be analogous to the enhancement of MMR,[29] in which free 5′ ends and abundant proliferating cell nuclear antigen (PCNA) are suggested to function as strand discrimination signals that augment repair of the lagging strand during DNA replication.[35] Selectively damaging the target DNA or inducing expression of the proper MMR proteins may therefore be plausible strategies for enhancing OMGR in vivo.

Retinal nuclear extract from C57BL6 mice produced a greater incidence of repair than that from BALB/c mice (135.2 ± 40.8 and 53.9 ± 26.2, $P = 0.0001$; *t*-test) when tested with RDOTET2 (preparation III). According to the DNA damage hypothesis (i.e., DNA nicking enhances repair by OMGR), it might be expected that C57BL6 retinal nuclear extract was more damaging than BALB/c extract. However, there was no difference in the average number of Amp^{R} colonies resulting from reactions treated with BALB/c or C57BL6 extracts (3.92×10^{6} and 3.52×10^{6}, respectively). These data suggest that C57BL6 extract was no more damaging than BALB/c extract and that some other factors are responsible for the higher repair offered by C57BL6 extracts. For example, Igoucheva et al.[32] demonstrated that nuclear extract from cells that exhibit high homologous recombination rates or are deficient in p53 resulted in higher gene repair rates. Therefore, it may be that variations in the type and/or amount of DNA repair proteins in C57BL6 and BALB/c retinas account for the difference in in vitro gene repair incidence. Future experiments will pursue the reasons for strain differences of OMGR success.

OMGR has attracted negative attention, as many researchers have found it difficult or impossible to achieve gene conversion.[36] Experiments with RDOTET2 were likewise not always successful in our hands. Specifically, three separate preparations (I, II, and III) of RDOTET2 were synthesized and used. Preparations I and III were effective in converting Tet^{S} to Tet^{R}, but III was more effective than I, and II was completely ineffective (Fig. 6). Despite identical synthesis and purification procedures, denaturing PAGE analysis revealed that III migrated largely as a tight band, whereas I and II were largely degraded (Fig. 6). Because PAGE analysis was performed only after several failed experiments with II, it cannot be determined if the smeared appearance of I and II is the result of suboptimal synthesis and purification or if degradation occurred after purification. Irrespective of the cause of degradation, proper RDO synthesis, purification, and storage are essential as demonstrated in the current study and as published by one other group.[26] We therefore suggest that any researcher attempting OMGR should show the quality of their oligonucleotides.

There are reasons to be encouraged that the in vitro gene correction demonstrated in this study indicates potential for in vivo gene correction and that such in vivo correction could have a therapeutic effect. For example, using the in vitro assay, Chen et al.[27] reported a measurable in vitro repair incidence when using rat liver nuclear extract (approximately 500 Tet^{R}/million Amp^{R}). Correspondingly, a *therapeutic* effect, as indicated by conversion of lethal Crigler-Najjar type I syndrome to the manageable Crigler-Najjar type II syndrome, was achieved after an estimated 20% gene conversion in the Gunn rat.[6] This 20% in vivo gene conversion and similar gene conversion frequencies reported in other applications of OMGR to animal models (e.g., 2% and 10% for the mouse[3] and canine[8] dystrophin genes, respectively, and 25% for the mouse ApoE2 gene[20]) may seem low. However, an individual who maintains use of only approximately 10% of their foveal cones is expected to maintain acuity greater than legal blindness (20/200).[37] Consequently, the amount of gene repair necessary to achieve a therapeutic effect for some retinal degenerative diseases may lie within range of what is achievable through OMGR. This possibility argues for further exploration of OMGR as a mode of ocular gene therapy.

TABLE 3. Number of Amp^{R} Colonies as a Function of the Amount of Retinal Nuclear Extract Used in the In Vitro Reaction

Extract Amount (μg)	Amp^{R} (millions)	*n*
0.0	11.1 ± 1.63	3
1.0	1.92 ± 0.59	3
10.0	0.8 ± 0.95	3

Data from a typical retina extract concentration response experiment (i.e., the same master mix, extract source, plasmid recovery, and transformation procedure were used for all samples). For each treatment, transformation is reported (mean ± SD of *n* replicates) as the number of Amp^{R} colonies. Note that Amp^{R} decreased as extract amount increased.

TABLE 4. A Comparison of In Vitro Repair of Nicked and Non-nicked pT^Sm153

Extract	pT^Sm153	Amp^R (in Millions)	*n*	Tet^R/Million Amp^R	*n*
Yes	Nicked	0.77 ± 0.54	7	267.2 ± 101.5	7
Yes	Non-nicked	3.21 ± 1.21	5	132.6 ± 31.0	5
No	Nicked	10.20 ± 5.08	4	0.14 ± 0.14	5
No	Non-nicked	18.42 ± 4.31	4	0.030 ± 0.059	4

RDOTET2 and C57BL6 retina nuclear extracts were used in the standard repair reaction with pT^Sm153 that had been either treated or not treated with N.*Bst*NBI enzyme. Repair and transformation are reported (mean ± SD of *n* replicates) as the quotient of Tet^R colonies per million Amp^R colonies (Tet^R/million Amp^R) and the number of Amp^R colonies, respectively. Nicked plasmid was repaired at a greater frequency than was non-nicked plasmid (267.2 ± 101.5 versus 132.6 ± 31.0, $P = 0.0065$; *t*-test) and nicked plasmid resulted in a lower transformation rate than non-nicked plasmid, irrespective of whether reactions had nuclear extract (0.767 ± 0.54 versus 3.21 ± 1.21, $P = 0.0042$; *t*-test) or did not have nuclear extract (10.2 ± 5.08 versus 18.42 ± 4.31, $P = 0.0243$; *t*-test).

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References

1. Porteus MH, Cathomen T, Weitzman MD, Baltimore D. Efficient gene targeting mediated by adeno-associated virus and DNA double-strand breaks. *Mol Cell Biol*. 2003;23:3558–3565.
2. Metz RP, DiCola M, Kurihara T, et al. Mode of action of RNA/DNA oligonucleotides: prOMGRess in the development of gene repair as a therapy for alpha(1)-antitrypsin deficiency. *Chest.* 2002; 121(suppl 3):91S–97S.
3. Rando TA, Disatnik MH, Zhou LZ. Rescue of dystrophin expression in mdx mouse muscle by RNA/DNA oligonucleotides. *Proc Natl Acad Sci USA*. 2000;97:5363–5368.
4. Kapsa R, Quigley A, Lynch GC, et al. In vivo and in vitro correction of the mdx dystrophin gene nonsense mutation by short-fragment homologous replacement. *Hum Gene Ther*. 2001;12:629–642.
5. Maurisse R, Feugeas JP, Biet E, et al. A new method (GOREC) for directed mutagenesis and gene repair by homologous recombination. *Gene Ther*. 2002;9:703–707.
6. Kren BT, Parashar B, Bandyopadhyay P, Chowdhury NR, Chowdhury JR, Steer CJ. Correction of the UDP-glucuronosyltransferase gene defect in the Gunn rat model of Crigler-Najjar syndrome type I with a chimeric oligonucleotide. *Proc Natl Acad Sci USA*. 1999;96:10349–10354.
7. Alexeev V, Igoucheva O, Domashenko A, Cotsarelis G, Yoon K. Localized in vivo genotypic and phenotypic correction of the albino mutation in skin by RNA-DNA oligonucleotide. *Nat Biotechnol*. 2000;18:43–47.
8. Bartlett RJ, Stockinger S, Denis MM, et al. In vivo targeted repair of a point mutation in the canine dystrophin gene by a chimeric RNA/DNA oligonucleotide. *Nat Biotechnol*. 2000;18:615–622.
9. Yoon K, Cole-Strauss A, Kmiec EB. Targeted gene correction of episomal DNA in mammalian cells mediated by a chimeric RNA.DNA oligonucleotide. *Proc Natl Acad Sci USA*. 1996;93: 2071–2076.
10. Cole-Strauss A, Gamper H, Holloman WK, Munoz M, Cheng N, Kmiec EB. Targeted gene repair directed by the chimeric RNA/DNA oligonucleotide in a mammalian cell-free extract. *Nucleic Acids Res*. 1999;27:1323–1330.
11. Pierce EA, Liu Q, Igoucheva O, et al. Oligonucleotide-directed single-base DNA alterations in mouse embryonic stem cells. *Gene Ther*. 2003;10:24–33.
12. Andersen MS, Sørensen CB, Bolund L, Jensen TG. Mechanisms underlying targeted gene correction using chimeric RNA/DNA and single-stranded DNA oligonucleotides. *J Mol Med*. 2002;80:770–781.
13. Igoucheva O, Alexeev V, Yoon K. Nuclear extracts promote gene correction and strand pairing of oligonucleotides to the homologous plasmid. *Antisense Nucleic Acid Drug Dev*. 2002;12:235–246.
14. Igoucheva O, Alexeev V, Pryce M, Yoon K. Transcription affects formation and processing of intermediates in oligonucleotide-mediated gene alteration. *Nucleic Acids Res*. 2003;31:2659–2670.
15. Rice MC, Bruner M, Czymmek K, Kmiec EB. In vitro and in vivo nucleotide exchange directed by chimeric RNA/DNA oligonucleotides in *Saccharomyces cerevisiae. Mol Microbiol*. 2001;40:857–868.
16. Beetham PR, Kipp PB, Sawycky XL, Arntzen CJ, May GD. A tool for functional plant genomics: chimeric RNA/DNA oligonucleotides cause in vivo gene-specific mutations. *Proc Natl Acad Sci USA*. 1999;96:8774–8778.
17. Zhu T, Peterson DJ, Tagliani L, St Clair G, Baszczynski CL, Bowen B. Targeted manipulation of maize genes in vivo using chimeric RNA/DNA oligonucleotides. *Proc Natl Acad Sci USA*. 1999;96: 8768–8773.
18. Cole-Strauss A, Yook K, Xiang Y, et al. Correction of the mutation responsible for sickle cell anemia by an RNA-DNA oligonucleotide. *Science*. 1996;273:1386–1389.
19. Kren BT, Bandyopadhyay P, Steer CJ. In vivo site-directed mutagenesis of the *factor IX* gene by chimeric RNA/DNA oligonucleotides. *Nat Med*. 1998;4:285–290.
20. Tagalakis AD, Graham IR, Riddell DR, Dickson JG, Owen JS. Gene correction of the apolipoprotein (Apo) E2 phenotype to wild-type ApoE3 by in situ OMGR. *J Biol Chem*. 2001;276:13226–13230.
21. Pichler L, Hofer H, Gumpelmayer TF. AutoradiOMGRaphic studies of DNA-repair in the retina of rabbits. *Invest Ophthalmol*. 1974; 13:290–293.
22. Rapp LM, Jose JG, Pitts DG. DNA repair synthesis in the rat retina following in vivo exposure to 300-nm radiation. *Invest Ophthalmol Vis Sci*. 1985;26:384–388.
23. Menu dit Huart L, Lorentz O, Goureau O, Leveillard T, Sahel JA. DNA repair in the degenerating mouse retina. *Mol Cell Neurosci*. 2004;26:441–449.
24. Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res*. 1991;19:2499.
25. Sambrook J, Russell DW. Plasmids and their usefulness in molecular cloning. In: *Molecular Cloning: A Laboratory Manual.* 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001:1.1–1.30.
26. Manzano A, Mohri Z, Sperber G, et al. Failure to generate atheroprotective apolipoprotein AI phenotypes using synthetic RNA/DNA oligonucleotides (chimeraplasts). *J Gene Med*. 2003;5:795–802.
27. Chen Z, Felsheim R, Wong P, et al. Mitochondria isolated from liver contain the essential factors required for RNA/DNA oligonucleotide-targeted gene repair. *Biochem Biophys Res Commun*. 2001;285:188–194.
28. Gamper HB, Parekh H, Rice MC, Bruner M, Youkey H, Kmiec EB. The DNA strand of chimeric RNA/DNA oligonucleotides can direct gene repair/conversion activity in mammalian and plant cell-free extracts. *Nucleic Acids Res*. 2000;28:4332–4339.
29. Holmes J Jr, Clark S, Modrich P. Strand-specific mismatch correction in nuclear extracts of human and Drosophila melanogaster cell lines. *Proc Natl Acad Sci USA*. 1990;87:5837–5841.
30. Gamper HB, Cole-Strauss A, Metz R, Parekh H, Kumar R, Kmiec EB. A plausible mechanism for gene correction by chimeric oligonucleotides. *Biochemistry*. 2000;39:5808–5816.

31. Li J, Bourdelat-Parks B, Boatright JH, Wartell RM. Targeting degradation of RNA by RNase H using DNA hairpins. *Biochemistry*. 2003;42:10945–10954.
32. Igoucheva O, Peritz AE, Levy D, Yoon K. A sequence-specific gene correction by an RNA-DNA oligonucleotide in mammalian cells characterized by transfection and nuclear extract using a lacZ shuttle system. *Gene Ther*. 1999;6:1960–1971.
33. Suzuki T, Murai A, Muramatsu T. Low-dose bleomycin induces targeted gene repair frequency in cultured melan-c cells using chimeric RNA/DNA oligonucleotide transfection. *Int J Mol Med*. 2003;12:109–114.
34. D'Andrea AD, Haseltine WA. Sequence specific cleavage of DNA by the antitumor antibiotics neocarzinostatin and bleomycin. *Proc Natl Acad Sci USA.* 1978;75:3608–3612.
35. Pavlov YI, Mian IM, Kunkel TA. Evidence for preferential mismatch repair of lagging strand DNA replication errors in yeast. *Curr Biol*. 2003;13:744–748.
36. Taubes G. Gene therapy: the strange case of OMGR. *Science*. 2002;298:2116–2120.
37. Geller AM, Sieving PA. Assessment of foveal cone photoreceptors in Stargardt's macular dystrophy using a small dot detection task. *Vision Res*. 1993;33:1509–1524.