RETRACTION

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Retraction for “Triplex-forming oligonucleotide-orthophenanthroline conjugates for efficient targeted genome modification,” by Fabio Cannata, Erika Brunet, Loïc Perrouault, Victoria Roig, Slimane Ait-Si-Ali, Ulysse Asseline, Jean-Paul Concordet, and Carine Giovannangeli, which appeared in issue 28, July 15, 2008, of Proc Natl Acad Sci USA (105:9576–9581; first published July 3, 2008;10.1073/pnas.0710433105); the undersigned authors wish to note the following. “During efforts to extend this work, we have been unable to reproduce the mutation data shown in this paper (Fig. 3C and Figs. S4 and S5 B and C). The first author of the paper admitted to an investigation committee having falsified the corresponding sequence data. Consequently, the conclusion concerning the induction of mutations by the orthophenanthroline-triplex forming oligonucleotide conjugate (OP-19merTFO/LNA) in 10% of cells is no longer supported by available evidence and the other data concerning the cellular activity of OP-19merTFO/LNA conjugate should be reexamined. The undersigned authors therefore retract the paper and the first author approves this retraction. We apologize for any inconvenience this may have caused.”

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Triplex-forming oligonucleotide–orthophenanthroline conjugates for efficient targeted genome modification

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The inefficiency of gene modification by homologous recombination can be overcome by the introduction of a double-strand break (DSB) in the target. Engineering the endonucleases needed, however, remains a challenging task that limits widespread application of nuclease-driven gene modification. We report here that conjugates of orthophenanthroline (OP), a DNA cleaving molecule, and triplex-forming oligonucleotides (TFOs), known to bind specific DNA sequences, are synthetic nucleases efficient at stimulating targeted genome modification. We show that in cultured cells, OP-TFO conjugates induce targeted DSBs. An OP-TFO with a unique target was highly efficient, and mutations at the target site were found in ~10% of treated cells, including small deletions most likely introduced during DSB repair by nonhomologous end joining. Importantly, we found that when homologous donor DNA was co-transfected, targeted gene modification took place in >1.5% of treated cells. Because triplex-forming sequences are frequent in human and mouse genes, OP-TFO conjugates therefore constitute an important class of site-specific nucleases for targeted gene modification. Harnessing DNA-damaging molecules to predetermined genomic sites, as achieved here, should also provide inroads into mechanisms of DNA repair and cancer.

The controlled introduction of genome sequence modifications is still a formidable challenge to the study of gene function and to many biotechnological and therapeutic applications (1, 2). Indeed, efficient gene targeting procedures have made yeast and mouse privileged models for genetic analysis of a wide range of biological problems, and targeted genome modification in other systems would be highly valuable. More than 10 years ago, it was reported, that by using the rare-cutting Iceu endonuclease, a double-strand break (DSB) stimulates gene targeting by more than two orders of magnitude (3–5). More recently, designed zinc finger nucleases were shown to stimulate efficient introduction of targeted point mutations in cultured human cells, marking an important milestone toward gene correction for the therapy of genetic diseases (6). The possibilities opened by nuclease-driven gene modification have been exemplified in several experimental systems (2, 6–10), and major applications in basic and applied research are expected (2).

The site-specific endonucleases needed have been obtained so far by extensive protein engineering, either by modifying the specificity of natural endonucleases with up to 30-bp-long recognition sequences (11–13) or by designing zinc finger nucleases (6). In the latter, the nuclease domain of the FoxI restriction enzyme is redirected to novel sequences by fusion to two zinc finger domains engineered to bind 9- or 12-bp sequences separated by a 6-bp spacer (6, 14). Obtaining high sequence specificity is necessary when developing endonucleases for targeted gene modification to minimize off-target cleavage and cellular toxicity (1). Recent studies of zinc finger nucleases have highlighted the importance of assaying off-target cleavage activity and led to redesign the FoxI domain to minimize homodimerization of zinc finger nuclease subunits and prevent the corresponding off-target cleavage (15, 16). Although the methods used to date allow, in principle, the targeting of any chosen sequence, they remain challenging to carry out, and different types of site-specific endonucleases, such as synthetic endonucleases, are therefore of high interest for nuclease-driven gene modification (1).

We have recently shown that chemically-modified triplex-forming oligonucleotides (TFOs) provide an attractive alternative to proteins for designing sequence-specific DNA ligands. TFOs bind to specific oligopyrimidine–oligopurine sequences through Hoogsteen hydrogen bonding and are promising tools for genome manipulation (17–19). By using oligonucleotides with locked nucleic acids (LNAs), DNA-binding affinities in the nanomolar range, which are comparable with those of DNA-interacting proteins, can now be obtained in physiological conditions (20, 21). Consequently, an LNA-containing TFO (TFO/LNA) strongly bound to its chromosomal DNA target in a sequence-specific manner (22) and inhibited transcription at its target site in cultured cells, whereas a nonmodified TFO could not. Previous studies by our lab and others have shown that conjugating a molecule with DNA cleavage activity to a TFO results in sequence-specific cleavage in vitro (23), but to our knowledge, no targeted cleavage activity has been reported in cells. By conjugating orthophenanthroline (OP) to TFO/LNAs, we show here that the resulting OP-TFO/LNA conjugates are highly specific endonucleases in the chromosomal context and that they can be used for efficient targeted gene modification in human cells.

Results

Intracellular Activity of OP-TFO/LNA Conjugates. First, we checked that, as expected from previous studies using OP-TFO conjugates with a closely related chemical structure, conjugates of OP and TFO/LNAs have sequence-specific double-strand cleavage activity in vitro (Fig. 1). Cleavage took place at positions adjacent to the OP moiety of the OP-TFO/LNA conjugate at the duplex-triplex junction. We next investigated the intracellular cleavage activity of three OP-TFO/LNA conjugates with, respectively, 57,
and H11032 et al. We next sought to GACC 5’ PNAS Fig. 20841 in vitro supporting (0) NH See Retraction February 16, 2010 information (SI) Fig. S1 to 1–4 phosphoH2AX foci per cell [Fig. 2] have a unique target site on chromosome 7, predominantly led matin accessibility (25). OP-19-mer TFO/LNA, predicted to predicted target sequences, most likely because of limited chro- finding that 16-mer TFO/LNA binds only to a subset of its phoshoH2AX focus approxi- mately corresponded to that of nontransfected cells (~10% of cells, based on detection of fluorescently labeled molecules, Fig. S2). Taken together, these findings suggest that OP-19-mer TFO/LNA is a highly efficient and specific synthetic endonu- clease. Finally, after treatment by OP-22-mer TFO/LNA, with no predicted target site in the human genome, a large majority of cells (~80%) had no foci: It corresponded to nontransfected cells (~10%) and to cells where the OP-22-mer TFO/LNA was inactive (~70%), consistent with the absence of genomic target sites (Fig. 2A); only a few cells exhibited phosphoH2AX foci, possibly because of low-efficiency binding at partially homolo- gous sites or to minor dissociation of OP from conjugates inside cells. Conjugating OP to TFO/LNAAs therefore drastically re- stricted OP cleavage activity to a limited number of sites and, importantly, OP-TFO/LNA conjugates induced DSBs in numbers consistent with specific cleavage at their predicted target sites in the human genome.

Cleavage Specificity of OP-19-Mer TFO/LNA. We next sought to directly examine whether OP-19-mer TFO/LNA induced DSBs at its predicted target site in the human genome. First, we examined whether the target site was encompassed within a phosphoH2AX focus. Using ChIP, we found that in cells treated with OP-19-mer TFO/LNA, phosphoH2AX was selectively en- riched at the target locus compared with a GAPDH control sequence. In contrast, in agreement with the high number of phosphoH2AX foci observed by immunofluorescence, phosphoH2AX levels were approximately equivalent at both genomic sites in cells treated with free OP (Fig. 3A). Next, we indirectly quantified DSBs by real-time PCR using primers flanking the OP-19-mer TFO/LNA target site. A reduction in the relative amount of the corresponding DNA fragment was detected 10 h after treatment with OP-19-mer TFO/LNA and increased up to 24 h, whereas the OP-22-mer TFO/LNA had no significant effect (Fig. 3B). Importantly, no reduction in the efficiency of PCR could be detected when the OP-19-mer TFO/LNA was added to DNA prepared from nontreated cells immediately before PCR (data not shown). The 80% reduction detected at 24 h is therefore most likely explained by efficient double-strand lesions at the target site. At later time points, however, the relative amount of target sequence was no longer diminished (Fig. 3B), a finding that could be explained by cell death or efficient DNA repair. No major induction of cell death, however, was detected between 24 and 48 h in OP-19-mer TFO/LNA-treated cells (data not shown). Because DSBs are predominantly repaired by error-prone nonhomologous end joining in mammalian cells (25, 26), we then examined the sequence of the DNA fragment at 72 h after treatment in OP-19-mer TFO/LNA- and control-treated cells (Fig. 3C, Fig. S3). In OP-19-mer TFO/LNA-treated HeLa cells, 215 of 247 sequences were wild type. Absence of DNA damage is expected in nonpermeabilized cells (~10% of cells, Fig. S2). Given the high cleavage efficiency attested to by our analysis of phosphoH2AX levels and PCR quantification, a large propor- tion of wild-type sequences likely results from accurate repair after DNA damage, as described for IScel cleavage (25). Importantly, 32 of 247 sequences exhibited mutations at or near the triplex target site that corresponded to the predicted site of cleavage of OP-TFO/LNAs (which was also confirmed in vitro, Fig. 1 and Fig. S4) and therefore likely reflect error-prone repair of the double strand breaks inflicted by the OP moiety of the conjugate. Among these, 18 were small deletions, confirming that a DSB had occurred at the target site and been repaired by

1, and no predicted target site in the human genome (Fig. 1B). Molecules were transfected into human cultured cells by strep- tolysin O (SLO)-mediated permeabilization, and 24 h after treatment, sites of DSB processing were detected by phosphoH2AX immunohistochemistry (24). Free OP was used at 5 μM as a positive control, and, as expected, it was toxic, and surviving cells contained abundant phosphoH2AX foci (Fig. 2A). OP-16-mer TFO/LNA clearly demonstrated intracellular cleavage activity. The number of phosphoH2AX foci detected per cell was signif- icantly lower than expected from its 57 triplex-forming target sites in the human genome but was consistent with our previous finding that 16-mer TFO/LNA binds only to a subset of its predicted target sequences, most likely because of limited chromatin accessibility (25). OP-19-mer TFO/LNA, predicted to have a unique target site on chromosome 7, predominantly led to 1–4 phosphoH2AX foci per cell [Fig. 2A and supporting information (SI) Fig. S1]. We further checked in this case that phosphoH2AX foci colocalized with 53BP1, another protein known to be recruited at DSBs, thus confirming that phosphoH2AX foci corresponded to DSBs (Fig. 2B). After treatment by OP-19-mer TFO/LNA or OP-16-mer TFO/LNA, the proportion of cells with no phosphoH2AX focus approxi- mately corresponded to that of nontransfected cells (~10% of cells, based on detection of fluorescently labeled molecules, Fig. S2). Figures 1 and S4 show the cleavage pattern obtained on a synthetic 29-bp-long target is depicted; the bands consistent with specific cleavage at their predicted target sequence of the DNA fragment at 72 h after treatment in OP-TFO/LNA target site (5’ AAAAAAAGGGGGG- CAGAG 3’) is located on chromosome 7.

OP-20mer CTOLNA OP-19mer TFO/LNA OP-16mer TFO/LNA

- 5900 bp - 3900 bp - 2000 bp

Fig. 1. In vitro cleavage activity of OP-TFO/LNAs. (A) Schematic representation of the cleavage pattern obtained on a synthetic 29-bp-long target is depicted; vertical bars indicate the sites of cleavage observed on each strand; the length of the bars represents the relative abundance of the fragments (see details in Fig. S4). The oligopyrimidine–oligopurine target sequence of the OP-16-mer TFO/ LNA conjugate is indicated in bold. OP-TFO/LNA conjugate structure is schemat- ically shown above the target sequence. (B) Double-strand cleavage assay on a plasmid substrate. pcDNA-T/S was incubated with the indicated OP-TFO/LNAs and specific cleavage evaluated by agarose-gel electrophoresis after linearization by SacII. The presence of cleavage products of the expected length (indicated to the right of the gel) indicates that cleavage has taken place at the specific target site. (C) Sequences of OP-TFO/LNAs used in this work are represented: in upper-case letters, DNA; in lower-case letters, LNA nucleotides. For each TFO/LNA, the total number of predicted target sequences in the human genome is given in paren- theses. The OP-19-mer TFO/LNA target sequence (5’ AAAAGAAAAAGGGGGG- GAGAG 3’) is located on chromosome 7.

OP-19m TFO/LNA

OP-16m TFO/LNA

OP-20m CTOLNA

19mer TFO/LNA 10mer TFO/LNA 16mer TFO/LNA 22mer TFO/LNA 20mer CTOLNA

19mer TFO/LNA 10mer TFO/LNA 16mer TFO/LNA 22mer TFO/LNA 20mer CTOLNA

- 5900 bp - 3900 bp - 2000 bp
rejoining of the DNA ends. Interestingly, the remaining 14 were substitutions, which are not usually found after repair of DSBs produced by enzymatic cleavage and that may result here from the necessary processing of more complex DNA lesions before ligation (26, 27). In contrast, no mutation was detected in sequences from control-treated cells (either not treated or treated with 19-mer TFO/LNA oligonucleotide not conjugated to OP; one mutation in 233 clones and no mutation in 47 clones, respectively). Experiments were repeated three times, and similar frequencies were always found (Fig. 3C). The induction of mutations depended on the concentration of OP-19-mer TFO/LNA (Fig. S5). In addition, mutation frequency was stable over time, indicating that no overt toxicity was associated with treatment by OP-19-mer TFO/LNA (Fig. S5). Finally, similar results were obtained in immortalized fibroblasts (28) (Fig. 3C and Fig. S3). Taken together, these results demonstrate that OP-19-mer TFO/LNA induced highly efficient cleavage at its target site at 24 h after treatment, and subsequent repair of the targeted double-strand breaks likely took place by nonhomologous end joining, generating site-specific mutations.

**Efficient Gene Correction Stimulated by an OP-TFO/LNA Conjugate.**

Finally, we wanted to test the ability of OP-TFO/LNA conjugates to direct targeted gene modification. We mutated the chromophore-coding domain of the eGFP cDNA by inserting either the OP-19-mer TFO/LNA target sequence or the recognition site for I\(_{\text{Sce}}\)I, which is already known to stimulate targeted gene modification. The corresponding mutant eGFP cDNAs were then integrated at the unique FRT site of a HeLa-FRT cell line (29), producing cell lines HeLa/mutGFP-I\(_{\text{Sce}}\)I and HeLa/mutGFP-I\(_{\text{Sce}}\)I respectively. After nuclease treatment, the homology-directed repair of the mutant eGFP gene by cotransfected wild-type eGFP donor DNA can be detected by eGFP expression (Fig. 4A). The resulting cells therefore allow for quantification of mutant eGFP correction by fluorescence activated cell sorting (FACS) (6, 30). Because a single copy of the transgene is integrated, counting green fluorescent cells provides an accurate measure of the number of transgene copies corrected. We checked that OP-19-mer TFO/LNA and I\(_{\text{Sce}}\)I induced targeted DSBs as expected (see Fig. S6). After cotransfection of wild-type eGFP cDNA (lacking cis-regulatory elements for eGFP expression) together with OP-19-mer TFO/LNA or an I\(_{\text{Sce}}\)I expression plasmid, green-fluorescent cells could be detected by fluorescence microscopy, and quantification by FACS indicated that they represented \(\approx 1.7\%\) and \(1\%\) of cells, respectively (Fig. 4B and C). After sorting of green fluorescent cells, it was further confirmed by DNA sequencing that the expected correction of mutant eGFP sequence had taken place (Fig. S7). Importantly, we found that 3 weeks after OP-19-mer TFO/LNA transfection, the percentage of green
fluorescent cells was not significantly changed (Fig. S7A), which indicates that the targeted gene modification was stably propagated during cell division and confirmed that OP-19-mer TFO/LNA treatment was not associated with any overt cell toxicity.

Discussion

In conclusion, OP-TFO/LNAs appear to be highly efficient tools for targeted genome modification. In cells treated with OP-19-mer TFO/LNA alone, ~10% of target sequences carried mutations, which were small deletions and point mutations (Fig. 3C), whereas in cells cotransfected with donor DNA, correction of the inactive eGFP sequence took place in >1.5% of cells, at an efficiency similar to that obtained after cleavage with I-SceI (Fig. 4C). Although TFOs alone or TFOs conjugated to psoralen, a DNA cross-linking agent, have been shown to be mutagenic and to stimulate gene modification by single-strand oligonucleotides, efficiencies have been at least one order of magnitude lower than described here, with a TFO conjugated to a cleaving agent. The mechanism of gene modification involved in the former studies is different. We therefore tested the 19-mer TFO/LNA oligonucleotide alone in our assay and found that it did not stimulate correction of the mutant eGFP reporter by donor DNA (data not shown). Genome sequence modification driven by OP-TFO/LNA nucleases, as reported here, therefore constitutes an important alternative to previous triplex-based strategies.

Importantly, OP-TFO/LNA synthetic nucleases were found to be highly specific in the genomic context. Given that OP-19-mer TFO/LNA induced DSBs in 80% target sequences (Fig. 3A), the majority of DSBs detected by phosphoH2AX labeling (Fig. 2A) likely corresponded to targeted cleavage. Similarly, OP-16-mer TFO/LNA induced DSBs at high efficiency in the three target sites that we tested (data not shown), and DSBs detected by phosphoH2AX labeling (Fig. 2A) likely resulted mainly from targeted cleavage. Such low-level off-target cleavage is critical for stable propagation of modified cells. In this respect, the OP-19-mer TFO/LNA therefore appears to exhibit limited unwanted genotoxicity, as described with the second-generation zinc finger nucleases, and cells carrying the wanted genome modification could be readily isolated and propagated (Fig. 4C and Fig. S7). Potential advantages of synthetic nucleases such as OP-19-mer TFO/LNA compared with protein-based nucleases include relative ease of design, production, and utilization and should be confirmed by targeting additional sequences.

Triplex-forming sequences are frequent in the human genome, and many are present in a given gene (with approximately one every 1,000 bp) (31). Triplex-forming sequences should therefore provide convenient targets for many studies. However, as reported here, the targeting of breaks by OP-TFO/LNA conjugates should be systematically evaluated by counting...
different synthetic sequence-specific DNA ligands in the near future (32, 33). Conjuncting sequence-specific DNA ligands to OP may therefore prove a powerful approach to the design of site-specific endonucleases for targeted gene modification. More generally, harnessing DNA-damaging molecules of chosen chemistry to predetermine genomic sites with high efficiency, as achieved here, could also provide inroads into mechanisms of DNA repair and cancer.

**Experimental Procedures**

**Synthesis of the Orthophenanthroline–Oligonucleotide Conjugates.** The linkage of the phenanthroline to the LNA-modified oligonucleotides was performed via the formation of a thioether linkage by reaction of iodoacetamidophenanthroline, obtained after a procedure that was adapted from a published work on phosphodiester oligonucleotides (34) with a thiol function present at the end of an hexamethylene linker attached to the 5′ end of the oligonucleotides (obtained from Eurogentec).

OP-TFO/LNA transfection into HeLa/luc cells and immortalized fibroblasts was performed by SLO-mediated permeabilization (Fig. S2). These two cell lines were described in refs. 22 and 28, respectively. The protocol used is a modification of a previously described SLO-based method (35). Briefly, cells were washed twice in HBSS without Ca2+ ( Gibco) and 2 × 10⁵ cells were resuspended in 100 μl of HBSS. An optimized amount of SLO and 10 μl of OP-TFO/LNA (100 μg) were added for a 15-min incubation at 37°C to allow SLO-induced permeabilization. DMEM (0.9 ml) containing 1.8 mM CaCl2 was then added to reseal cells.

**Fluorescence Immunolabeling.** Treated cells were cultured on Lab-Tek Chamber Slides and then fixed with 4% formaldehyde for 10 min. Immunolabeling was performed with primary Ab against phosphoH2AX (monoclonal clone JW301; Upstate) or 53BP1 (Upstate), followed by secondary Ab Cy5-labeled anti IgG-Fc-specific mouse (The Jackson Laboratories) or FITC-labeled anti IgG-Fc-specific rabbit antibodies. Slides were mounted by using a mounting medium for fluorescence (Vector), and observation of immunolabeled cells was performed by using a fluorescence microscope (DMIR; Leica).

**Quantification of Double-Strand Lesions by Quantitative Real-Time PCR.** Primers flanking the OP-19-mer TFO/LNA target site were used for real-time PCR amplification. For quantification, the amount of DNA was calculated by comparison with a standard curve that was obtained by amplification (in the same conditions as the test reaction) of serial dilutions of a DNA sample from untreated cells, as described (21). Data were always normalized on a control region unrelated to the target sequence (see Table S1). Analyses were performed with Mx3005P Real-Time PCR System (Stratagene).

**Target Sequence Analysis After OP-19-mer TFO/LNA Transfection.** A region containing the OP-19-mer TFO/LNA target site was amplified by PCR with Phusion polymerase (Fynzyme) starting from HeLa cells or immortalized fibroblasts at 72 h after treatment. After amplification, DNA was purified (QiaQuick; Qiagen), and a polyA tail was added according to the manufacturer’s procedures; then, DNA was ligated into the pGEM-T vector (Promega). Plasmid DNA from individual colonies was sequenced at the Genome Express Sequencing Center.

**Mutated eGFP Gene Correction Assay.** pFRT-mutGFP-19TF and pFRT-mutGFP-Iscel were constructed in pcDNA-FRT by insertion of a mutated eGFP cDNA containing, respectively, a 19-mer TFO target sequence and Iscel recognition site and each introduced as a single-copy transgene at the FRT site of HeLa-FRT97 cells by cotransfection with a Flp recombinase expression vector (Invitrogen). Cells (400,000) were transfected with 1,500 ng of the donor plasmid and 500 ng of Iscel expression plasmid or 1 μM OP-19-mer TFO/LNA by using Superfect (Qiagen). Green cells were counted and sorted by FACS 1 week after treatment and subsequently expanded for 2 weeks before DNA sequence analysis and 2nd cell sorting (Fig. S7).

**In Vitro Cleavage Assay and ChIP.** These methods are described as SI Text.

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![Fig. 4](image-url)
sorting. Immortalized fibroblasts were a kind gift of J-P. de Villartay (Hôpital Necker, Paris). This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique,


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