Research Signpost
Trivandrum
Kerala, India

Recent Advances in Pharmaceutical Sciences VII, 2017: 51-68 ISBN: 978-81-308-0573-3
Editors: Diego Muñoz-Torrero, Montserrat Riu and Carles Feliu

4. Polypurine Reverse Hoogsteen Hairpins as a tool for gene repair and editing

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Abstract. We describe the design and use of repair-PPRHs and editing-PPRHs as a new methodology either to correct a point mutation or to edit a genomic fragment of the *dihydrofolate reductase* gene in Chinese Hamster Ovary (CHO) cells. Repair-PPRHs are formed by a PPRH core, following the Reverse Hoogsteen bonds rules, covalently connected to a repair tail, which is homologous to the mutated region of the dsDNA except for the repaired nucleotide. Several point mutations in the endogenous *dhfr* gene have been successfully repaired in mammalian cells using repair-PPRHs, including a deletion, an insertion, and single and double substitutions in different regions of the gene. All repaired colonies showed high levels of DHFR protein and activity, and the corrected nucleotide was confirmed in all DNA sequences. Editing-PPRHs are formed by a PPRH core, covalently connected to a sequence tail homologous to the upstream and downstream regions of the DNA fragment to be edited. All edited colonies showed high levels of DHFR protein and activity, and the edition was confirmed in all DNA sequences.

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Introduction

Mutations are a natural process that alters the DNA and are constantly taking place in the genome. It is estimated that tens of thousands of changes happen daily in the DNA of a human cell [1]. Not all the mutations result in functional impairment, but in some cases, small changes in the DNA sequence can provoke an enormous impact on an entire living being. Therefore, these mutations need to be reversed by the DNA repair machinery that fixes DNA damage such as mismatched nucleotides, DNA cross-links, bulky adducts and splicing broken DNA strands back together.

Depending on the cells affected, mutations can be classified in two groups: inherited mutations, when they affect germ cells, and the alteration can often be passed on to offspring; and acquired mutations, that can spontaneously arise during the life of an organism in somatic cells. Mutations of the latter can result from normal metabolic activities including DNA replication errors, spontaneous lesions such as depurination and deamination of the DNA, and the generation of reactive oxygen species (ROS), but can also result from environmental factors such as physical or chemical mutagens [2–4] (Fig. 1).

Point mutations are a type of mutations that typically refer to an alteration of a single or a few adjacent base pairs in a DNA sequence. They usually take place during DNA replication, although other endogenous and

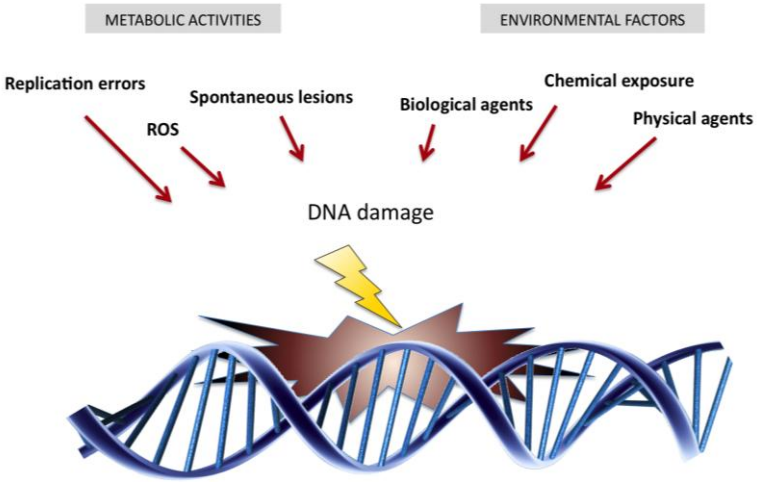

Figure 1. Causes of DNA damage.

exogenous agents can be implicated. Some point mutations are beneficial or have no effect. Polymorphisms for instance, are mutations that generally do not cause functional damage under basal conditions. Nonetheless, these alterations can also be detrimental for gene function at various levels. If the mutation occurs in the promoter region of a gene, the expression of this gene may be altered. If the alteration is caused in a coding region, the activity may change and in the case of insertions or deletions a frame shift can be produced, thus changing the whole peptide or provoking the appearance of a nonsense mutation originating a truncation of the protein. In addition, if the mutated base pair is found near or in the intron-exon junction, it can result in a splicing alteration of the mRNA.

DNA damage may lead towards a large variety of lesions, including mismatches, chemical adducts or single- and double-strand breaks (DSBs). Therefore, different repair pathways have evolved, each focused on a particular type of lesion.

If DNA damage affects terminally differentiated cells, DNA damage repair will ensure the integrity of the transcribed genome. However, if DNA damage occurs in dividing cells, "cell cycle checkpoints" will detect the damage by sensor proteins, and by means of different protein complexes, signal transducers and effector proteins. These effector proteins will lead to the repair of DNA or will temporarily stop the proliferating cells in their cell cycle progression to provide enough time to the DNA repair machinery to act. Some of these important cell cycle checkpoint proteins are ataxia telangiectasa mutated (ATM) and ATM and Rad3 related (ATR) that act as signal transducers. In response to DNA damage in G1, for example, these proteins will phosphorylate p53, which acts as a transcription factor for p21, leading to an inhibition of both cyclinE/Cdk2 and cyclinA/Cdk2 complexes, and therefore an inhibition of G1/S transition, thus preventing the synthesis of damaged DNA [5–8]. However, the specific pathway that will be activated is determined by the type of DNA damage. When repair processes fail and DNA damage cannot be repaired, cells may become senescent or can be conducted to programmed cell death or apoptosis. Apoptosis is conducted by different protein factors such as the anti-apoptotic protein Bcl-2, inhibited directly or indirectly by p53 [9]. If any of these processes do not work properly, there may be an unregulated cell division that can lead to the formation of a tumor, which could become cancerous.

DNA damage checkpoints can halt cell proliferation, but the repair machinery is required to prevent the transduction of mutations to daughter

cells. DNA-damage-signaling and DNA repair are believed to be linked and operate collectively [10,11]. As mentioned before, since there is a wide diversity of possible lesions, a large variety of DNA repair mechanisms have evolved, such as direct reversal repair, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and DSB repair (Fig. 2).

Gene augmentation therapy (GAT) is one of the most studied strategies to treat diseases caused by point mutations; it consists of introducing copies of the wild type gene in the affected cells to obtain the functional protein in sufficient amounts to restore the normal phenotype. This strategy is especially available for recessive diseases, since the mutated gene does not interfere with the normal product, and the amount of this product does not need a rigorous regulation to recover a normal phenotype. However, it presents some drawbacks, as random gene integration in the genome, and the loss of endogenous regulator elements of the gene. As an alternative, a different philosophy for gene repair was developed to correct point mutations in their endogenous loci using different types of oligonucleotides. These strategies consisted of targeting

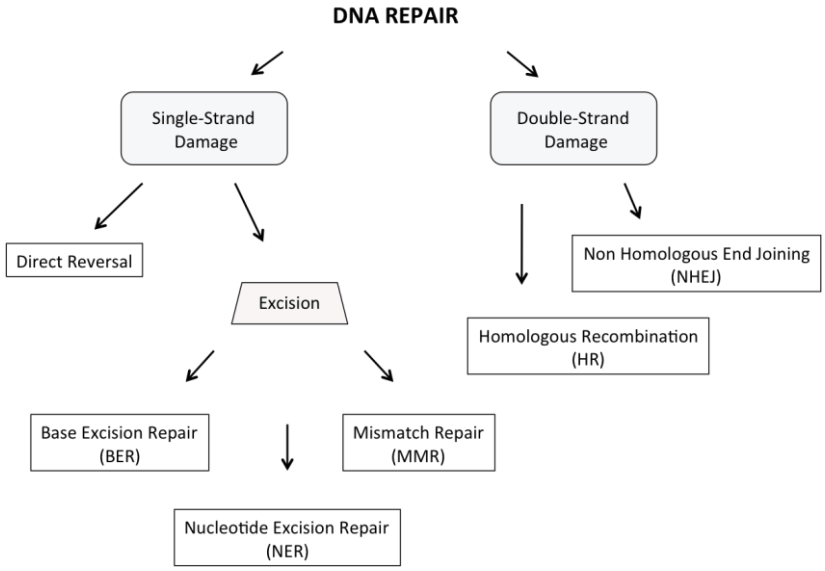

Figure 2. Scheme of DNA repair responses.

the genomic DNA with an oligonucleotide complementary to the DNA sequence, except for the corrected nucleotide. In the last years, different approaches have emerged in this direction, such as chimeric RNA-DNA oligonucleotides, single-stranded oligonucleotides (ssOs), bifunctional triple-helix-forming oligonucleotides (TFBO), or peptide nucleic acids (PNAs).

Programmable endonucleases such as zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9), are artificial proteins composed of a sequence specific DNA-binding domain fused to a nuclease, that are able to provoke double strand breaks (DSBs) in the genome, thus stimulating the cellular DNA repair-mechanisms, including error-prone non-homologous end joining (NHEJ), in the absence of a homologous DNA template, and homologous recombination (HR), in the presence of a synthetic repair template [12]. These site-specific nucleases have shown to edit DNA to disrupt, introduce, invert, or delete genes [13]. Although to a lesser extend, these tools are also being studied to correct point mutations [13,14].

1. Polypurine reverse Hoogsteen Hairpins

PPRHs are non-modified DNA molecules formed by two antiparallel polypurine strands linked by a pentathymidine loop that allows the formation of intramolecular reverse-Hoogsteen bonds between both strands. These hairpins bind to polypyrimidine stretches in the DNA via Watson-Crick bonds, while maintaining the hairpin structure (Fig. 3). It was demonstrated that PPRHs, upon binding their polypyrimidine target in a dsDNA, were able to displace the polypurine strand of the target duplex configuration [15,16].

Because the polypyrimidine domains can be found in both strands of the DNA, PPRHs can be designed to target either one of the strands of genomic DNA. PPRHs directed against the template strand of the DNA are called template-PPRHs, while the ones targeting the coding strand of the DNA are called coding-PPRHs, which are also able to bind transcribed mRNA, since it has the same sequence and orientation than the coding strand of the DNA. Therefore, PPRHs can act as antigene and antisense oligonucleotides depending on the strand they target (Fig. 4). PPRHs were first described for gene silencing [15–20].

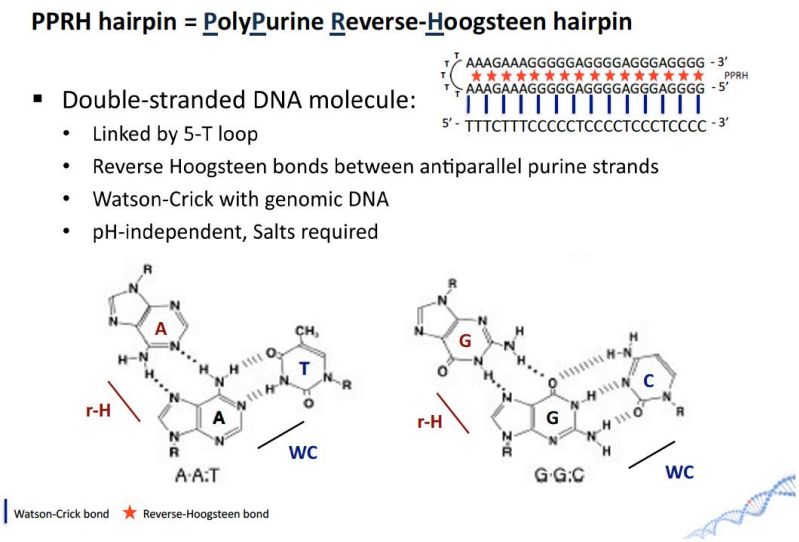

Figure 3. PPRH characteristics and structure.

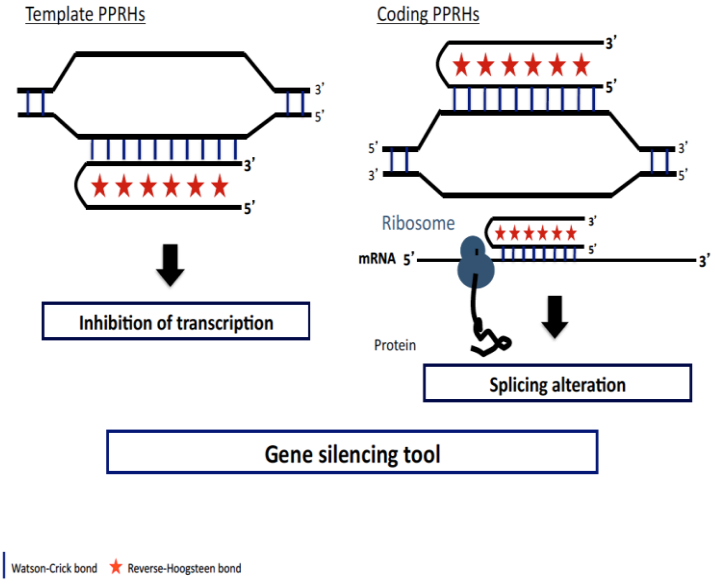

Figure 4. PPRHs for gene silencing.

2. Repair-PPRHs

Repair-PPRHs are Polypurine reverse Hoogsteen hairpins bearing an extension sequence at one end homologous to the DNA strand to be repaired but containing the wild type nucleotide instead of the mutation. We made a successful first attempt to correct a point mutation at the endogenous *locus* of the *dhfr* gene. Homologous recombination was found to play an important role in the mechanism for gene correction by repair-PPRHs [21].

Next we wanted to expand the use of repair-PPRHs and improve the methodology to correct a representative collection of different types of mutations (substitutions, double substitutions, deletions, and insertions) at an endogenous *locus* in a mammalian genome. To achieve this goal, we again used the *dhfr* gene as a model because it is a selectable marker that readily allows for the identification of repaired clones, and because of the availability of an extensive collection of endogenous mutants obtained by UV irradiation, and different chemicals such as *N*-hydroxy-aminofluorene [22–26].

To test the potential of repair-PPRHs in different types of point mutations, we used a collection of various *dhfr* mutant cells; all derived from the parental cell line UA21 [27], which carries only one copy of the *dhfr*

Table 1. Compendium of different mutant cell lines subjected to correction using Repair-PPRHs.

Cell line	Position	Alteration	Base change	Coding change (normal termination is at 562)
DA5	541 (exon 6)	Deletion (-G)	GAA > -AA	Opal at 584 in exon 6
DA7	235 (exon 3)	Substitution	GAG > TAG	Amber in place
DI33A	493 (exon6)	Insertion (+G)	GGG / GGGG	Opal at 505
DP12B	370 – 2 (intron 4)	Substitution	ag > tg	Exon 5 skipped Opal at 504
DU8	136 +1 (exon2/ intron2)	Double substitution	G/gt > A/at	Exon 2 skipped Opal at 139
DF42	541 (exon 6)	Substitution	GAA > TAA	Ochre in place

gene. The natural stop codon is found at nucleotide 562 of the protein-coding region. All mutants produce termination codons either by a direct base substitution or indirectly due to frame shift by single base insertions or deletions or by exon skipping (Table 1). These mutant cells produce no functional DHFR enzyme and so are unable to grow in a DHFR selective culture medium without glycine, hypoxanthine and thymidine (-GHT).

Table 1 shows the characteristics of the different cell lines subjected to correction with Repair-PPRHs. The mutated bases are represented in the coding strand with a 5' to 3' orientation. Position numbers are referred to the translational initiation site (ATG). For mutations that occurred in introns, the position relative to the nearest exon is given, where + indicates downstream of the exon and - means upstream of the exon.

Specific repair-PPRHs for each mutant cell line were devised by attaching to the end of one strand of the PPRH core, a sequence tail homologous to the point mutation region of the target, except for the mutated nucleotide, which was corrected (Table 2). This extended tail was added to provide the PPRHs with the ability to repair the mutation. We searched for

Table 2. Sequences of the repair-PPRHs against the *dhfr* gene.

Sequence	Total length	Name	Cell line
5' CATCAAGTATAAATTT**G**AAGTCTATGAGAAGAAAGGCTAACAGAAAGA 3' GAGAAGAAAGGCTAACAGAAAGA 5T	76 nt	**HpDE6rep**	DA5 / DF42
5' GATATAATTAAGACAGGAAA 3' GAATGAACTCGAGA**G**ATGACTCTTGATATAATTAAGACAGGAAA 5T	69 nt	**HpDE3rep**	DA7
5' GCCTGGCTGATTCATGGCTTCC**T**TTAAAATAAAATAGAAGG 3' AAAATAAAATAGAAGG 5T	62 nt	**HpDI4-1rep**	DP12B
5' CTGATTCATGGCTTCC**T**TTAAAATAAAATAGAAGGATAGTGAGAGGAA 3' ATAGTGAGAGGAA 5T	66 nt	**HpDI4-2rep**	
5' TGATTCATGGCTTCC**T**TTAAAATAAAATAGAttttttGAAAGACAAAGAGACTAGAAGA 3' GAAAGACAAAGAGACTAGAAGA 5T	85 nt	**SDR-HpDI4-3rep**	
5' GGACTTCAGAAAGGACCCCTGGGTACCTGGAAAAGAAAAGGA 3' GGAAAAGAAAAGGA 5T	61 nt	**HpDE6-4rep**	DI33A
5' CCTTTTCTTTTCCAGGTACCCAGGGGTCCTTTCTGAAGTCCAGGAGGAAAAAGGCA 3' AGGAGGAAAAAGGCA 5T	76 nt	**HpDE6-5rep**	
5' CTTAACCCCAAATTA**CC**TTCCACTGAGGAGGTGGTGG 3' GAGGAGGTGGTGG 5T	55 nt	**HpDE2-1rep**	DU8
5' CCTCAGTGGAA**GG**TAATTTGGGGTTAAGATGAGGA 3' AAGATGAGGA 5T	50 nt	**HpDE2-2rep**	
5' ATCAAGTATAAATTT**G**AAGTCTATGAGAAGAttttttAGAGATGGGAGCAGGTGGAGGA 3' AGAGATGGGAGCAGGTGGAGGA 5T	85 nt	**LDR-HpDE6-1rep**	DF42

polypyrimidine regions near to the point mutations and proceeded according to the rules of PPRH construction [16,17]. When encountering purine interruptions in the polypyrimidine stretches, we chose the WT-PPRH strategy, which includes the base complementary to the target interruption in the PPRH core [28].

Table 2 shows the names and sequences of the repair-PPRHs as well as the cell line used. The corresponding corrected nucleotide in the repair-PPRHs is shown in bold and bigger size. Bulleted symbols represent reverse-Hoogsteen bonds.

In all of the experiments, DHFR mRNA levels, protein levels, enzyme activity levels and DNA sequences were determined as follows. Different numbers of cells, ranging from 1,000 to 150,000 were plated and the corresponding repair-PPRHs were transfected using 2 to 5 μg of DNA. Six random cell colonies surviving in –GHT medium from different experiments were expanded individually, and the targeted DNA region was PCR-amplified and sequenced. Cells were subsequently analyzed for DHFR mRNA, protein, and enzyme activity levels. DHFR protein levels in the repaired cells were measured by Western blot performed with 100 μg of total protein extracts and were normalized to tubulin levels. Protein levels in the repaired colonies were referred to those of the positive control UA21. DHFR activity was determined by the incorporation of 2 μCi of 6-[^{3}H] deoxyuridine to the DNA. Cells were collected and lysed with SDS after 24 h. Radioactivity was counted in a scintillation counter. DHFR mRNA levels were measured using qRT-PCR and were normalized to APRT. DHFR mRNA levels of the repaired colonies were also referred to the positive control UA21.

3. Correction of single point mutations using Repair-PPRHs

The first cell line subjected to correction by repair-PPRHs was DA5, where the deletion of a guanine in exon 6 of the *dhfr* gene results in a frame shift that generates a premature opal stop codon (TGA). The repair-PPRH used (HpDE6rep) contained three pyrimidine interruptions, and its hairpin core was extended with 25nt at the 5' end including the missing guanine. We confirmed the presence of the corrected nucleotide in all repaired colonies analyzed. DHFR mRNA levels in the repaired cells were higher than in the mutant DA5 cell line. The protein was restored in all cases, and it showed high levels of DHFR activity (Fig. 5). The next step was to test whether repair-PPRHs were also able to correct substitutions. Thus, we chose DA7 cells that contain a substitution of a guanine by a thymine in exon 3, producing an amber stop codon (TAG) in situ. A 20nt

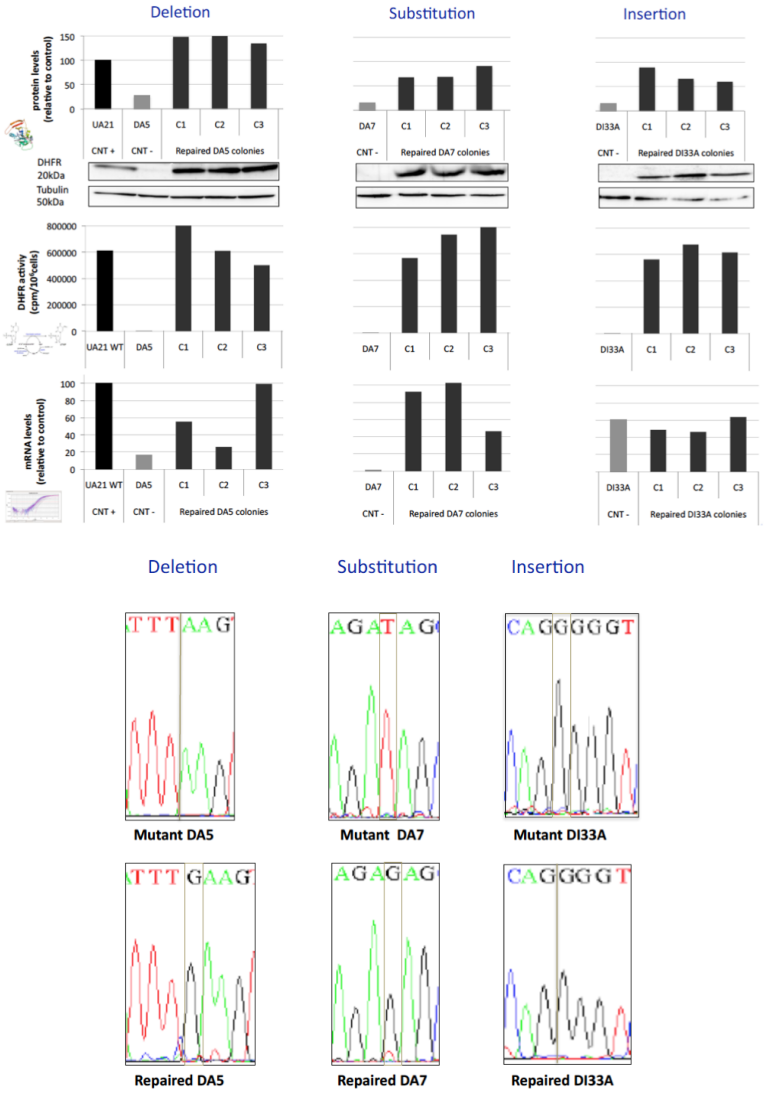

Figure 5. Correction of a deletion, a substitution and an insertion in *dhfr* mutant cell lines. DHFR protein levels, DHFR activity, DHFR mRNA levels and DNA sequences in repaired cells are shown. UA21 cells bearing a copy of the *dhfr* wild type gene are used as a positive control whereas the mutant cell line corresponds to the negative control.

polypyrimidine sequence in the template strand upstream of the point mutation was used to design HpDE3rep. The sequence tail was extended from the 3' end of the hairpin core, with the wild type guanine instead of the thymine present in the mutant. After the isolation of the surviving colonies, we confirmed the corrected guanine in the DNA sequence, high levels of mRNA, protein, and activity compared to DA7 cells (Fig. 5).

Insertions are another type of point mutation very detrimental due to the disruption of the reading frame of a sequence. We chose the DI33A cell line to test whether repair-PPRHs were able to correct an insertion of a guanine in dhfr exon 6. Two repair-PPRHs were designed.

HpDE6-4rep using a 14nt pyrimidine sequence found in the coding strand upstream of the mutation, and HpDE6-5rep using a 15nt sequence in the template strand downstream of the mutation. Both repair-PPRHs succeeded in correcting the mutation by introducing the missing guanine in the DNA sequence, and thus restoring DHFR protein and its activity [29]. The amount of DHFR mRNA of repaired cells was similar to those of the mutant cells (Fig. 5).

Substitutions can also be found in non-coding regions, without changes in the amino acid sequence. However, an alteration in an intron may affect splicing and lead to a frame shift in the subsequent downstream amino acid sequence. Hence, the DP12B mutant cell line was chosen to test the capacity of repair-PPRHs to correct a substitution of an adenine by a thymine at the penultimate position of intron 4 that causes the skipping of exon 5 and a subsequent opal premature stop codon in exon 6. We searched for polypyrimidine sequences in both DNA strands near the point mutation, finding different domains. HpDI4-2rep originated from a 13nt polypyrimidine sequence in the template strand, 18nt upstream of the mutation. The second repair-PPRH target was 22nt, contained one purine interruption and was found 41nt upstream of the mutation. In this case, it was not feasible to design a repair tail directly attached to the hairpin core, because the total repair-PPRH sequence would exceed a length of 100nt in the synthesis of oligonucleotides. Therefore, we attempted a different design in which the hairpin core and the repair tail were connected by 5 thymidines instead of the whole intervening sequence between them. This PPRH was called SDR-HpDI4-3rep, for Short-Distance-Repair hairpin, since it skipped 26 nucleotides of the intron. Upon transfection, surviving colonies were obtained in selective medium with HpDI4-2rep and SDR-HpDI4-3rep. These colonies were isolated and analyzed, and DHFR mRNA levels were comparable to the mutant cells. The DNA sequence was corrected and the protein was restored showing DHFR activity (Fig. 6) [29].

Our results showed that repair-PPRHs could correct different types of single nucleotide mutations. The next challenge would be to repair double point mutants. Thus, DU8 cells were selected to test the ability of repair-PPRHs to correct the tandem mutation of two nucleotides. DU8 cells contain a substitution of 2 nucleotides, Gg > Aa, involving the last nucleotide of exon 2, and the first nucleotide of intron 2. This change does not involve a nonsense mutation in situ, but provokes the skipping of exon 2, which disrupts the reading frame. As a consequence, an opal stop codon appears prematurely. Two different repair-PPRHs were designed for this approach: HpDE2-1rep, located 7nt upstream of the mutation, and HpDE2-2rep, 12nt downstream of the mutation. The structure of HpDE2-1rep is that of a hairpin core of 13nt, containing two pyrimidine interruptions, followed by a 24nt tail bearing the corrected nucleotides. In HpDE2-2rep the hairpin core contains 10nt, with one interruption, ending in a 25nt repair tail. Both repair-PPRHs succeeded in correcting the double point mutation at all levels (Fig. 6) [29].

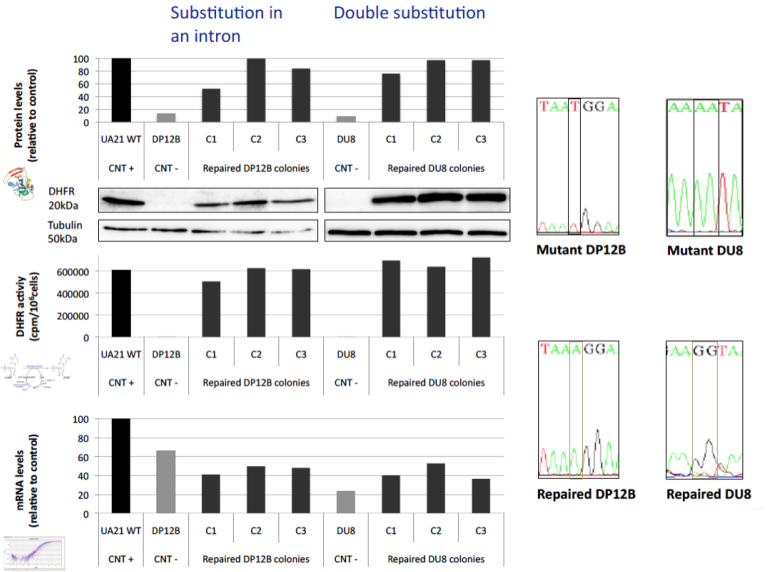

Figure 6. Correction of a substitution in an intron and a double substitution in *dhfr* mutant cell lines. DHFR protein levels, DHFR activity, DHFR mRNA levels and DNA sequences in repaired cells are shown. Experimental conditions are the same as described before. UA21 cells bearing a copy of the dhfr wild type gene are used as a positive control whereas the mutant cell line corresponds to the negative control.

One of the limitations of this methodology could reside in finding homopurine domains relatively close to the point mutation. To solve this, we designed a Long Distance Repair-PPRH (LDR-PPRH) which contains a hairpin core hundreds of nucleotides away from the location of the mutation, linked by 5 Ts to the repair tail. This approach was tested in DF42 cells, containing a substitution of a guanine by a thymine in exon 6 resulting in an ochre stop codon (TAA). We designed a repair tail at the location of the point mutation, and a hairpin core targeting a sequence located 662nt downstream. This repair-PPRH was called LDR-HpDE6-1rep, formed by a hairpin core of 22nt in each homopurine strand containing three pyrimidine interruptions, and a tail of 31nt. In parallel, we also tested HpDE6rep, a regular repair-PPRH, and we obtained similar results

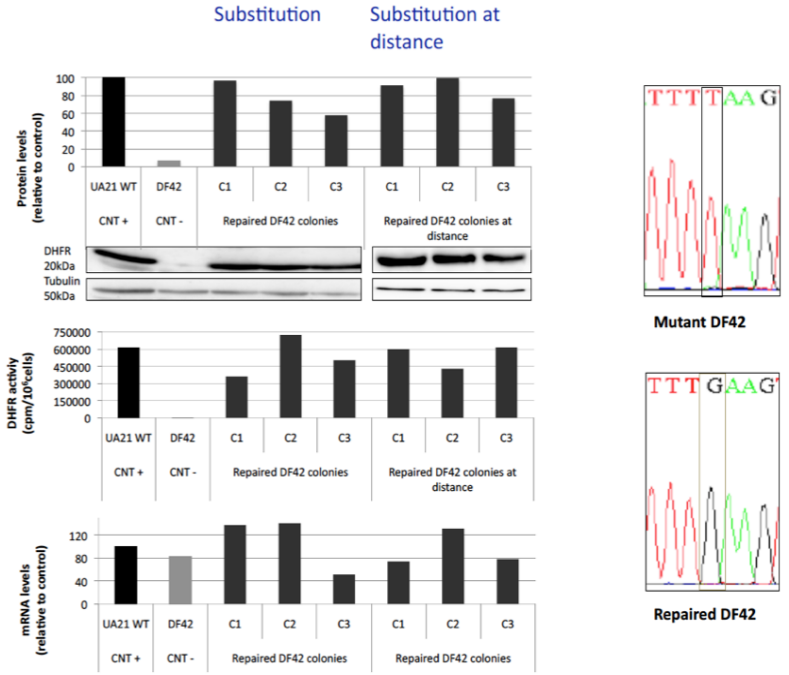

Figure 7. Correction of a G substitution in DF42 cell line. DHFR protein levels, DHFR activity, DHFR mRNA levels, and DNA sequences in repaired cells are shown. Experimental conditions are the same as described before. UA21 cells bearing a copy of the *dhfr* wild type gene are used as a positive control whereas the mutant cell line corresponds to the negative control.

for both approaches. The levels of mRNA in the repaired cells were similar to those of the mutant. However, repaired colonies recovered DHFR protein with high activity, and the nucleotide was corrected in the DNA sequence (Fig. 7) [29].

4. Editing-PPRHs

Duchenne muscular dystrophy (DMD) is a progressive and fatal degenerative muscle disease caused by mutations due to large deletions (approx. 65%) in the DMD gene encoding for the dystrophin protein. Accordingly, the resulting reading frame involves an aberrant dystrophin translation, causing the absence of the protein essential for the muscle. This leads to an irreversible damage of muscle fibers that are replaced by adipose tissue. A variant of the disease, the Becker muscular dystrophy (BMD), results in a much milder phenotype. This disease is also caused by mutations in the dystrophin gene, but they do not completely disrupt the reading frame of the protein, and thus allow the production of a reduced version of a partially functional protein. In DMD one or several exons are deleted, and this mutation interferes with the assembly of the full-length mRNA. This fact led to the development of a therapeutic strategy for DMD called "exon skipping strategy", in which antisense deoxyoligonucleotides (aODNs) are used to mediate the elimination of the mutated exon, alone or with additional adjacent exons, to restore the reading frame of the protein. In these conditions, the expression of a shorter but functional dystrophin protein is induced, simulating the BMD phenotype [30]. Theoretically, exon skipping could be used to treat approximately 90%, 80%, and 98% of DMD patients with deletion, duplication, and nonsense mutations, respectively [31]. Several aODNs developed using different chemical modifications such as 2'OMethyl (Prosensa Inc., Switzerland) and morpholinos (PMOs) (AVI Biopharma, UK) are currently in Phase II or Phase III trials to validate the effectiveness of this therapeutic approach.

In order to explore the capability of PPRHs to cause Exon-skipping at the DNA level to be applied to the DMD gene as a possible therapeutic tool, we probed the potential of PPRHs for this purpose using a gene with a clean metabolic selection. In this regard, the editing abilities of PPRHs were explored using a stably transfected DHFR mutant with duplication of Exon-2 of the *dhfr* gene that causes a frameshift abolishing DHFR activity. Chen and Chasin [32] developed this model generating NB6 cells, carrying that minigene with 2 Exons 2 of the *dhfr* gene (D22) (Fig. 8).

Those cells are auxotrophic for glycine, hypoxanthine and thymidine. However, strategies that induce Exon-skipping of that minigene within NB6 cells and recover prototrophy for one carbon metabolism. Therefore, Exon-skipping can be positively detected and selected by growing NB6 cells in –GHT medium. This approach was used to test the capability of PPRHs to cause Exon-skipping at the DNA level.

The sequence corresponding to the pD22 minigene was analyzed for polypurine target regions and the corresponding editing-PPRHs were designed, by attaching to the end of one strand of the PPRH core, a sequence tail homologous to the upstream and downstream regions of the PstI restriction site in the original *dhfr* minigene pDCH1P (Fig. 9).

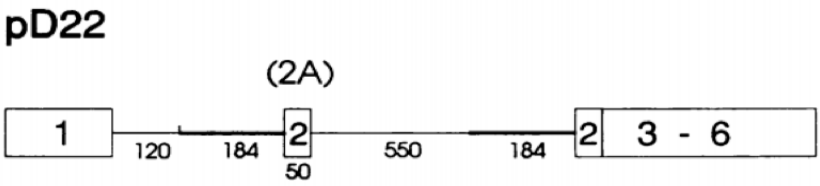

Figure 8. Structure of the pD22 *dhfr* minigene. The Chinese hamster *dhfr* minigene pDCHlP containing the six exons of the gene, intron 1, about 900 bp of the 5' flank, and the first of the two major polyadenylation sites in exon 6 was used to construct pD22 in which a 0.8-kb PstI-BstEII genomic DNA fragment containing exon 2 and flanks was cloned into the unique PstI site in intron 1 of pDCH1P.

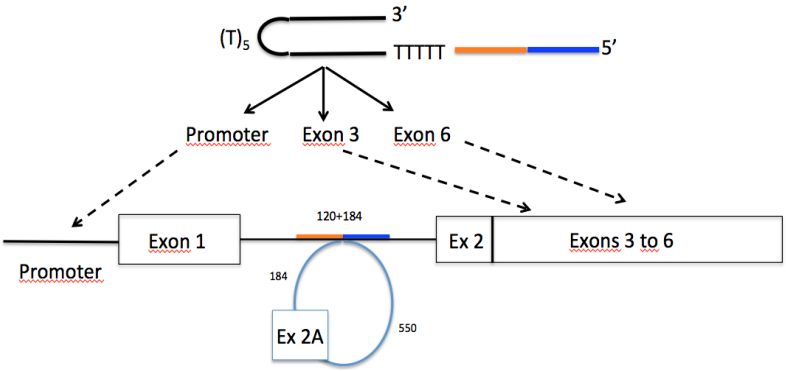

Figure 9. Structure of the different Editing-PPRH used to edit the extra exon 2 present in the *dhfr* minigene pD22 stably transfected in NB6 cells.

150,000 NB6 cells were plated and the corresponding editing-PPRHs were transfected using 2 to 5 µg of DNA. Random cell colonies surviving in –GHT medium from different experiments were expanded individually, and the targeted DNA region was PCR-amplified and sequenced. Cells were subsequently analyzed for DHFR protein, and enzyme activity levels. As it can be seen in Fig. 10, in all the clones analyzed, DHFR protein was restored, and it showed high levels of DHFR activity. Furthermore, the DNA sequencing results proved that the *dhfr* sequence in all the surviving clones corresponded to the wild type *dhfr* minigene with just one copy of Exon 2 (data not shown).

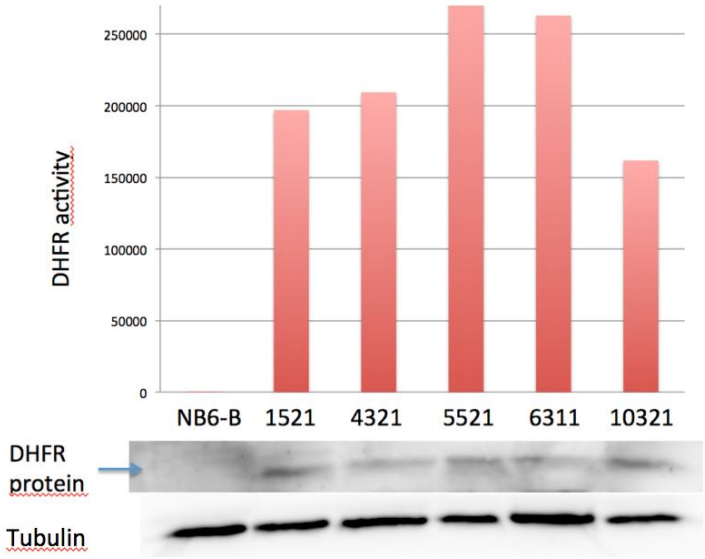

Figure 10. DHFR activity and protein levels. Experimental conditions as described. DHFR activity and protein levels in the edited colonies were compared to those of the control NB6-B cells.

5. Conclusion

We provide evidences that repair-PPRHs have the ability to correct different types of mutations in mammalian cells. Therefore, our method may offer an alternative, simple, and powerful tool for gene therapy to correct many disorders caused by point mutations. In addition, we show that

editing-PPRHs represent an alternative method to ZFN, TALEN and CRISPR/Cas9 site specific nucleases for efficient editing, without the difficulty in constructing and delivering exogenous enzymes, the off-target effect caused by the nucleases, and the non-homologous end joining effects stimulated after a DNA double strand break.

Acknowledgements

The work was supported by Grants SAF2011-23582 and SAF2014-51825-R from "Plan Nacional de Investigación Científica" (Spain). Our group holds the Quality Mention from the "Generalitat de Catalunya" 2014-SGR96. A.S. was recipient of a FI fellowship from the "Generalitat de Catalunya". A.J. is recipient of a FPU fellowship from the "Ministerio de Educación".

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