Precise Genome Modification Using Triplex Forming Oligonucleotides and Peptide Nucleic Acids

Raman Bahal, Anisha Gupta, and Peter M. Glazer

Abstract Many genetic disorders are caused by single base pair mutations which lead to defective protein synthesis. In addition to gene replacement therapy, modification of genomic DNA sequences at specific sites has been employed to manipulate the function and expression of various genes, which are implicated in various genetic disorders. On this front, triplex technology has been used to alter the expression of different genes by correcting mutations site specifically via homologous recombination (HR) or targeted mutagenesis based mechanisms. In this chapter we will discuss the advances made in triplex technology involving triplex forming oligonucleotides (TFOs) and peptide nucleic acids (PNAs) for site specific genome editing.

Keywords TFOs • PNA • Recombination • Repair • Mutagenesis • Genome modification

Introduction

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Triplex Forming Oligonucleotides

In addition to Watson–Crick base pairing, knowledge of alternative binding interactions between the nucleobases known as Hoogsteen base pairing led to the design of another scaffold for the molecular recognition of DNA known as TFOs. The process of triple helix formation was first suggested by Pauling and Corey in 1953 whereas the formation of triplex structures was first reported in 1957 by Felsenfield et al. [1]. Through RNA diffraction studies it was observed that stretches of poly(U) polyuridylate and poly(A) (polyadenylate) sequences hybridize in a 2:1 binding ratio in presence of divalent magnesium ions [1]. The high density of target homopurine sequences in the genome coupled with the sequence specificity of TFOs makes them attractive molecules to target individual genes and modulate gene function [2].

Binding Code

In the case of polypyrimidine TFOs binding to polypurine DNA targets, 'pyrimidine motif' T binds AT, and C binds GC (forming T–A–T and C^+–G–C base triplets) via Hoogsteen hydrogen bonding (Fig. 1) resulting in parallel orientation with respect to the polypurine site of the target duplex [3,4]. The orientation of the 'purine motif' was also demonstrated by Peter Dervan and his co-workers, wherein G binds GC and A binds AT (forming A–A–T and G–G–C base triplets) through reverse hydrogen bonding resulting in antiparallel orientation with respect to the polypurine site in target B-DNA duplex [5–7]. Through X-ray diffraction [8], nuclear magnetic resonance [9], and chemical probing studies [10], it was revealed that TFO binding to the genomic DNA leads to helical distortions [11], which evoke the repair system of cells leading to manifold applications by inducing mutagenesis and enhancing homologous recombination.

Chemical Modifications to Improve TFO Binding Affinity and Stability

The binding of TFOs to duplex DNA is influenced by a number of different factors including ionic conditions, sequence composition and accessibility to the target site [12]. In the case of polypyrimidine TFOs that bind in a parallel orientation, the N3 of the cytosine must be protonated in order to form optimum Hoogsteen hydrogen

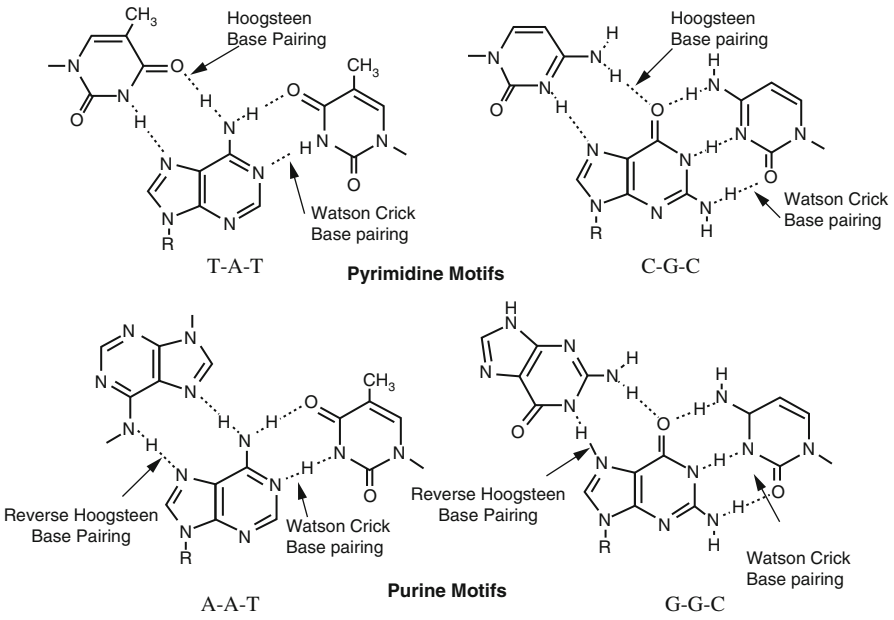

Fig. 1 Motifs for triple helix formation. (*Top*) In the pyrimidine motif the third strand binds parallel to the purine strand of DNA via Hoogsteen bonds. (*Bottom*) In the purine motif, the third strand binds antiparallel to the purine strand via reverse Hoogsteen hydrogen bonds. The canonical base triplets are shown for each motif

bonds with N7 of the guanine [13]. Hence, the pH dependence of pyrimidine TFOs limits their utility in intracellular targeting. On the other hand, polypurine TFOs bind to the target DNA in an antiparallel orientation without any dependence on pH. However G-rich purine TFOs are limited in application because the guanine rich sequences tend to form G-quadruplexes at physiological conditions in presence of high K^+ concentrations [14–17]. The intracellular K^+ promotes the aggregation of TFOs due to the formation of stable secondary structures known as G-tetrads. In order to form stable triplex structures, continuous polypurine runs are required [18]. The interruptions by pyrimidines or single base pair mismatches can significantly destabilize the binding of TFO. Because TFOs are limited to homopyrimidine or homopurine sequences, triplex binding could not be extended to the human genome sequences containing mixed sequence ds-B-DNA. Furthermore the charge repulsions due the three polyanionic strands and the accessibility to the binding site in the cellular environment pose significant challenges in success of the TFO design [19].

Hence, chemical modification of TFOs is essential to increase triplex stability and to protect TFOs from enzymatic degradation. Many efforts have been made to optimize the TFO design. In case of pyrimidine rich TFOs, the replacement of cytosine with 5-methyl cytosine or pseudoisocytosine have been done to overcome the dependence on pH [20,21]. Different cytosine substitutions including 8-oxoadenine [22], 7,8-dihyro-8-oxoadenine [23], 6-oxocytidine [24], 8-oxo-2′deoxyadenosine

[25,26] and 8-aminoguanine [27] have improved the binding affinity. Likewise the modification of thymidine to 2′deoxyuridine (dU), 5-propargylamino- and 2′-aminoethoxy,5-propargylamino-dU bis-substituted derivatives have shown to improve triplex binding stability [28–32]. Modified sequences comprising of 2′-deoxy-6-thioguanosine or 7-deaza-2′-deoxyxanthosine in the G-rich TFOs have been shown to inhibit the G-Quartets formation [15–17]. Many different modifications in the sugar involving 2′-O-ribonucleotides [33,34], and 2'-O-aminoethyl ribose [35–37] have been shown to confer nuclease resistance and promote triplex stability. Different chemical modifications at the backbone or bases have been also explored to improve the binding affinity and biological applications of the TFOs. It has been demonstrated that by replacing the sugar phosphate backbone with morpholino oligonucleotides or by cationionc phosphoramidate linkages such as N,N-diethethyleneamine (DEED) or N,N-dimethyl-aminopropyl amine, binding affinity of the TFO can be increased in vitro [38–41]. Further, by replacing the backbone to uncharged, achiral peptide units in case of PNAs, significant progress has been made in the area of triplex research [42–44]. The different studies involving PNA as a triplex forming agent are discussed in detail in another section of this chapter.

Triplex Mediated Genome Modification

Targeted Mutagenesis via Triplex Formation

By inducing mutations at specific sites in the genome, triplex technology can be employed to induce heritable changes in gene function and expression. The TFOs have the potential to invoke the DNA repair by directly binding with a segment of a gene or by delivering a mutagen at the site of repair. The conjugation of psoralen (pso), a photoreactive mutagenic agent to a TFO has been shown to induce damage to the DNA [45–48]. Upon irradiation with UVA light, psoralen intercalates into the DNA and covalently crosslink thymines on both strands [49]. The characterized mutations in mammalian cells involved T:A to A:T transversions [46,50–52].

Plasmid Based Assays for Detecting Mutagenesis in Mammalian Cells

To analyze TFO induced mutagenesis, a reporter system based on supF gene was constructed. The supF reporter gene cloned into an SV40 vector encodes an amber suppressor tyrosine tRNA and contains a TFO binding site. Upon treatment with pso-TFO and UV A irradiation, the vector was transected into monkey COS-7 cells. The mutation frequencies were quantified by isolating the plasmids and co-transform in bacterial cells. The results suggested that 6 % of the plasmids underwent targeted mutagenesis out of which 55 % were T:A to A:T transversions [45]. Furthermore, it was shown that a TFO alone without conjugating to a mutagen can also induces site directed mutagenesis [53].

TFO Induced Mutagenesis at Chromosomal Sites in Mammalian Cells

TFOs have been shown to induce mutagenesis at chromosomal target sites in cell culture. Pso-TFOs induced mutations at the endogenous hypoxanthine phosphoribosyl transferase (*hprt*) gene in CHO cells have been reported by Majumdar et al. [54]. They showed that 80 % of the mutations occurred at the triplex target region. Vasquez et al. also constructed a transgenic mouse containing multiple copies of lambda vector containing a 30-bp triplex target site within the supF mutation reporter gene (Fig. 2) [55]. Upon treatment of the cells with pso-TFOs, a tenfold induction of site specific mutations above the background were observed. The mutations were observed both in presence and absence of UV A irradiations suggesting that the TFO alone has the potential to induce mutations.

TFOs in Homologous Recombination

The modification of human genome by HR is widely employed in several different applications. However the use of HR is limited due to low frequency in mammalian cells and random integration at non-targeted sites (reviewed in ref [56]). In order to improve the frequency of HR, several different approaches have been employed which include DNA damage near the target site by UV irradiation, alkylation and psoralen induced cross linking or double-strand DNA breaks by endonucleases [57–59]. TFO directed DNA damage either by formation of a triplex alone or by a mutagenic agent has been shown to improve recombination and gene modification frequencies. The studies involving different TFOs in genome modification via HR are summarized in Table 1.

Intramolecular Recombination

To detect the enhancement in homologous recombination, different reporter constructs have been used. Our lab has used Pso-TFOs to induce recombination between tandem mutant copies of a reporter gene in plasmids and in chromosomal sites [60]. In order to study targeted recombination via TFO directed intrastrand crosslinks, Faruqi et al. used SV40 shuttle vectors with two tandem supF genes containing different point mutations and a TFO binding site between the mutated genes [61]. Later, Luo et al. studied a similar construct in chromosomal sites in mice [62]. The TFO-induced recombination was detected through beta galactosidase screening assay using bacterial colonies. The studies involved the use of unconjugated TFOs as well as TFOs conjugated to psoralen. It was observed that the recombination events stimulated by intrastrand crosslinks via TFO conjugated psoralen occurred at a higher frequency in comparison to unconjugated TFOs. Further, the mechanisms involved in these intrachromosomal recombination events were studied. It was observed the increase in triplex induced recombination was dependent on

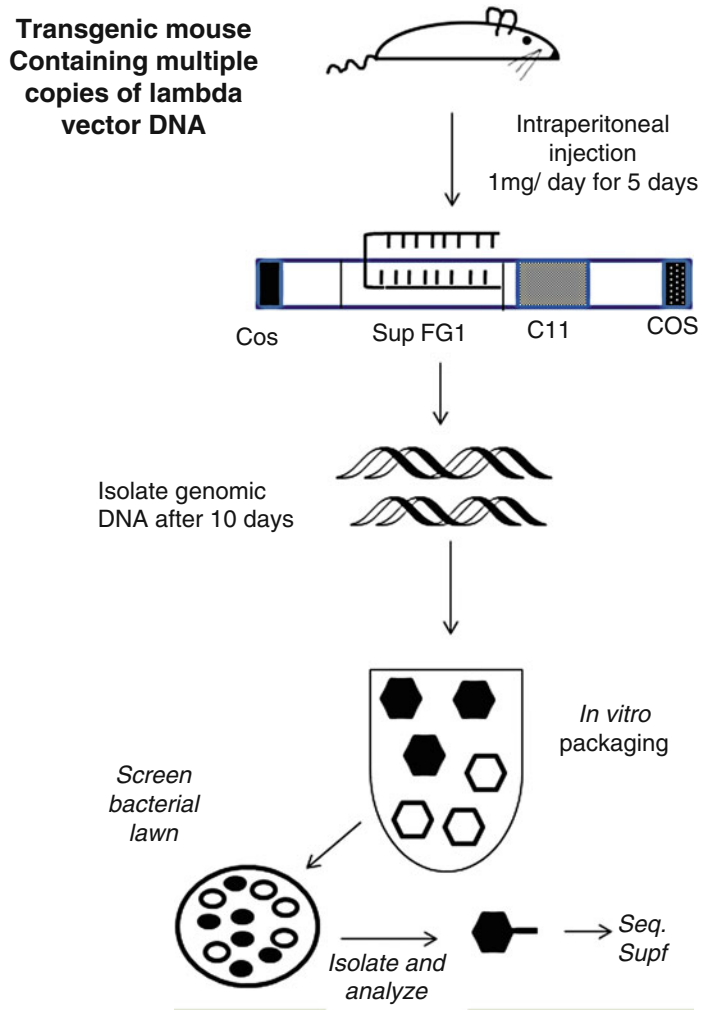

Fig. 2 Experimental protocol for chromosomal mutations detection after TFO administration in mice. Transgenic mice containing many copies of chromosomally integrated A supFG 1 vector, which contains the 30 bp triplex binding site was used. TFOs were injected intraperitoneally. After treatment, tissues were harvested, and genomic DNA was isolated and analyzed. In vitro packaging of the phage vector led to mutagenesis detection via plating on a bacterial lawn. If no mutations occur, the plaques will appear *blue* in the presence of IPTG and X-Gal. If a mutation does occur, the resulting plaques will be *white*

nucleotide excision repair (NER) pathway [61]. No significant recombination was observed in cell lines deficient in Xeroderma Pigmentosum Group A (XPA) repair factor [63]. The psoralen conjugated TFO directed recombination was partially dependent on XPA demonstrating the involvement of multiple repair pathways.

Table 1 HR induced by TFOs in mammalian cells

TFO	Type of recombination	Reporter gene	Recombination frequency (%)	Cell line
AG-30	Intramolecular	supF (bacterial suppressor tRNA)	O.37	COS-7
Pso-AG30	Intramolecular	supF	0.58	COS-7
AG30	Intrachromosomal	TK (thymidine kinase)	1.2	LTK-
AG30 + 51 mer donor DNA	Intermolecular	Fluc (Firefly luciferase gene)	0.05	CHO derived
22 mer purine rich TFO	Intermolecular	supF	~0.05	Jurkat T lymphoblastoid
Psoralen conjugated 19mer purine TFOs	Intra molecular	supF	0.002	HeLa

Intermolecular Recombination

Chan et al. used an approach based on TFOs etethered to donor DNA fragments homologous to the target site (except at the base pair to be corrected) and were able to demonstrate intermolecular recombination events [64]. In this strategy, the formation of the triplex induces repair and sensitizes the target site for recombination [65]. The TFO domain also positions the donor DNA for recombination. Subsequent studies have shown that the conjugation of the donor DNA to the TFOs not necessary [66–69].

Peptide Nucleic Acids (PNAs)

One of the major issues related with DNA based TFOs is enzymatic degradation. In the past, in order to improve the enzymatic stability, numerous synthetic nucleic acid analogues have been developed. One such promising class of nucleic acid mimic is known as PNA. Structurally, PNA is a homomorphous unit of DNA where phosphodiester backbone is replaced with an uncharged aminoethylglycine backbone [42]. Due to its achiral, neutral polyamide backbone, PNAs are resistant to enzymatic degradation (Fig. 3). PNA hybridizes with complementary DNA/RNA targets in a sequence specific manner to form highly stable duplexes (PNA/DNA or PNA/RNA). PNAs also form triplex complexes where one strand, invades the DNA duplex through Watson-Crick base pairing and another strand is capable of forming Hoogsteen bonds with the PNA–DNA duplex [70]. Two PNA strands linked by a flexible linker forms a clamp and form highly stable complexes with the target dsDNA.

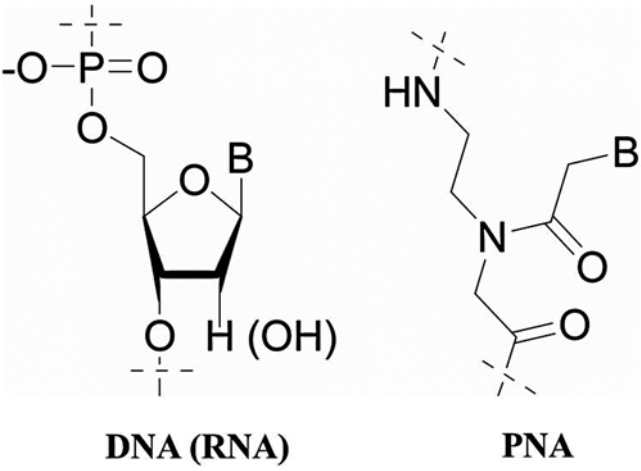

Fig. 3 Chemical structure of DNA (RNA) and PNA

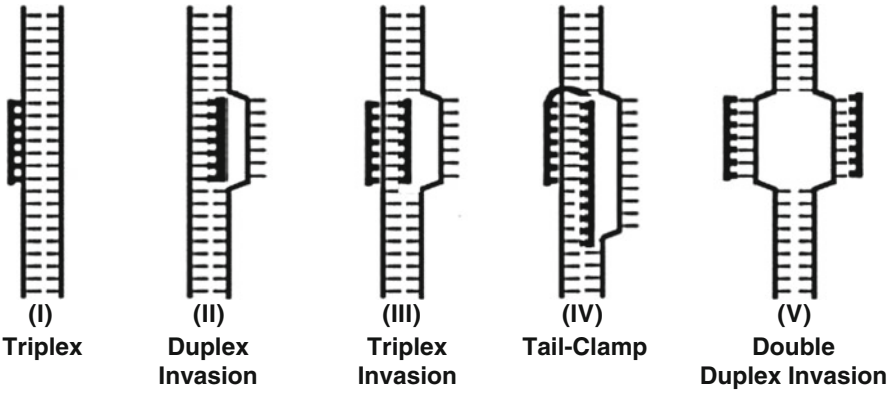

Fig. 4 Different binding modes of PNA with dsDNA

Recently it was found that PNA has potential to invade certain regions of genomic dsDNA through strand invasion based mechanisms. The mechanism by which PNA binds to dsDNA relies on the sequence composition of the target region. At least five different binding modes have been established (Fig. 4). For cytosine-rich PNA, binding takes place in the major groove through Hoogsteen base-pairing in a 1:1 ratio. Homopurine PNA also binds dsDNA in a 1:1 ratio, but through Watson-Crick base-pairing resulting in local displacement of the homologous DNA strand, which creates D-loop formation [71]. Homopyrimidine PNA, on the other hand, binds dsDNA in a 2:1 (PNA:DNA) stoichiometry in which one strand forms Watson-Crick base pairs whereas the second strand forms Hoogsteen base-pairing, resulting in a PNA_2–DNA triplex and a locally displaced D-loop. A combined homopyrimi-

dine/homopurine binding mode has also been exploited in the development of "tail-clamp" hairpins for recognition of partially mixed-sequence containing dsDNA [44]. Peter Nielsen and coworkers have demonstrated a fifth binding mode that relies on the use of pseudo-complementary (pcPNAs) PNAs to bind simultaneously to both strands of the DNA double helix. This strategy is also known as double duplex invasion strategy [70]. This approach possess greater flexibility in sequence selection, but it also complicates the targeting strategy by use of two separate strands of pcPNAs for binding because of their propensity to interact with each other, which greatly limits its utility.

PNAs have demonstrated enormous potential by their activity as transcriptional regulators, inhibitors of protein binding and DNA polymerization blockers. However cellular delivery of PNAs has been the major issue for its therapeutic application.

Use of PNA in Repair and Recombination

Due to high binding affinity with their complementary base pairs, PNAs have potential to induce mutagenesis and HR based repair mechanisms. In the past, Faruqi et al. employed a single dimeric PNA to target a site in the supFG1 mutation reporter gene within a chromosomally integrated recoverable λ phage shuttle vector in mouse fibroblasts [72]. The designed PNA forms a clamp via both double- and triple-helix formation within 8- or a 10-bp site in the supFG1 gene and induce mutations at frequencies in the range of 0.1 %, tenfold above the background [72].

Rogers et al. have done comprehensive studies to demonstrate that dimeric bis-PNAs have potential to promote site directed recombination [73]. Generally, bis-PNAs undergo both strand invasion as well as triplex formation, which lead to formation of clamp structures on target dsDNA. In this study, they have shown that a bis-PNA conjugated to a 40-nucleotide donor DNA is able to induce site directed recombination. PNA-donor DNA conjugates were prepared by employing maleimide based chemistry. The PNA–DNA conjugate mediated sequence specific changes within the supFG1 reporter gene in vitro in human cell free extracts, resulting in correction of a mutation at a frequency at least 60-fold above background [73]. Similarly induced site-specific recombination also achieved by using bis-PNA and the donor DNA together without any covalent linkage. The bis-PNA and the bis-PNA-donor DNA conjugate were also found to induce DNA repair with high specificity in the target plasmid. It was shown that both PNA-induced recombination as well as repair was found to be dependent on the nucleotide excision repair factor, XPA (Xeroderma pigmentosum complementation group A protein) [73]. Due to the formation of clamp structures with duplex DNA, PNA creates a helical distortion that strongly provokes DNA repair and thereby sensitizes the target DNA sites to recombination.

Wang et al. designed a series of dimeric PNAs to form clamps at a 10 bp homopurine/homopyrimidine site of the E. coli supFG1 gene [74]. To study the recombination frequency based mechanisms, mouse fibroblasts with 15 chromosomally

integrated copies of the lambda phage shuttle vector containing the supFG1 gene were employed. SupFG1 gene mutagenesis determined by counting colonies after infection of E. coli ClacZ125 (am) bacteria. It was demonstrated that, dimeric PNAs induced mutations in the chromosomal supFG1 gene in the mouse cells at a frequency of 0.1 % (tenfold above background) [74]. Sequence analysis also substantiates that the majority of mutations were located within the PNA binding site. Further exploring the utility of PNAs for site-specific gene modification various groups have shown that PNAs conjugated with DNA modifying agents, such as benzophenone, anthraquinone, or psoralen, can be used for DNA modification in vitro.

In another studies, dimeric bis-PNAs-psoralen conjugates were employed. The designed PNA-psoralen conjugates were able to bind the target site on the supFLSG3 reporter gene by triplex invasion- complex formation and directed site-specific photoadduct formation by the conjugated psoralen [75]. The formation of photoadduct was confirmed by in vitro assays and mutagenesis in the targeted gene was assayed using SV 40 based episomal shuttle vector assay. The photo adducts directed by PNAs conjugated to psoralen induced mutations at frequencies in the range of 0.46 % (6.5-fold above the background). For intracellular gene targeting in the episomal shuttle vector, 0.13 % of psoralen-PNA induced mutation frequency was achieved (3.5-fold higher than the background). In this study it has been demonstrated that most of the induced mutations were deletions and single-base-pair substitutions at or adjacent to the targeted PNA-binding and photoadduct-formation sites [75]. This set of study further support the development of PNAs as tools for gene-targeting applications.

Above, different types of binding modes of PNA to dsDNA have been discussed. In addition to bis PNAs, pcPNAs were used for intracellular gene targeting at mixed sequence sites. It has been well reported in literature that due to steric hindrance, pcPNAs are unable to form pcPNA–pcPNA duplexes but can bind to complementary DNA sequences by Watson–Crick pairing via double duplex-invasion complex formation. It has been demonstrated that psoralen-conjugated pcPNAs can deliver site-specific photoadducts and mediate targeted gene modification within both episomal and chromosomal DNA in mammalian cells without possessing any off-target effects [76]. Psoralen-pcPNA mutations were single-base substitutions and deletions found at the predicted pcPNA-binding sites. No mutations were induced by the individual pcPNAs alone nor did complementary PNA pairs of the same sequence cause mutations [76].

PNAs combined with donor DNAs have the potential to induce gene correction based on triplex-induced homologous recombination mechanism and have been tested in several disease models including beta-thalassemia [69]. Generally, splice-site mutations in the β-globin gene lead to aberrant transcripts and decreased functional β-globin, causing beta-thalassemia. It was shown that bis-PNAs when co-transfected with recombinant donor DNA fragments containing the corrected mutation site, can promote single base-pair modification at the start of the second intron of the β-globin gene, the site of a common thalassemia-associated mutation. Green fluorescent protein-β-globin fusion gene was used to detect the restoration of proper splicing of transcripts. It was also shown that recombination frequencies can

be enhanced when the PNAs/DNA were used with the lysomotropic agent, chloroquine [69].

Later on, Lonkar and coworkers also demonstrated that pcPNAs, when co-transfected with donor DNA fragments, can promote single base pair modification at the start of the second intron of the β-globin gene [68]. Gene editing was detected by examining the restoration of proper splicing of transcripts produced from a green fluorescent protein-beta globin fusion gene. In addition it was also found that pcPNAs stimulate recombination in human fibroblast cells dependent on the nucleotide excision repair factor, XPA. These results signify that pcPNAs can be used as tools for site-specific gene modification in mammalian cells without sequence restriction.

In addition to β-globin gene, genome modification has been also demonstrated in the CCR5 gene. CCR5 encodes a chemokine receptor required for HIV-1 entry into human cells, and individuals carrying mutations in this gene are resistant to HIV-1 infection. Schleifman et al. has shown that transfection of human cells with bisPNAs/donor DNA targeted to the CCR5 gene, can introduce stop codons mimicking the naturally occurring CCR5-delta32 mutation, produced 2.46 % targeted gene modification [67]. In a series of experiments, CCR5 modification was confirmed at the DNA, RNA, and protein levels [67]. It was also shown that introduction of stop codon confer resistance to infection with HIV-1. This work underscores that PNA induced genome modification can be used as a therapeutic strategy for CCR5 knockout in HIV-1-infected individuals.

Further Rogers et al. have shown that through conjugation of a triplex-forming peptide nucleic acid (PNA) to the transport peptide, antennapedia (Antp), successful in vivo chromosomal genomic modification of hematopoietic progenitor cells can be achieved [77]. In addition, hematopoietic progenitor cells still possessed the differentiation capabilities even after gene modification. This strategy obviates the use of transfection-based protocols for PNA/donor DNA.

McNeer et al. proposed new methods for intracellular delivery of PNA/donor DNA molecules for genome editing by using poly(lactic-co-glycolic acid) (PLGA)-based nanoparticles [78]. PLGA is an FDA-approved biocompatible polymer and is used clinically for delivery of drugs for numerous indications including the treatment of prostate cancer (Lupron and Trelstar). The previous work has shown that PLGA nanoparticles can be used for intracellular delivery of nucleic acid polymers and oligomers, including plasmid DNA and siRNAs for gene-silencing studies [79]. PLGA nanoparticles are formulated by using double-emulsion solvent-evaporation technique. PLGA nanoparticles encapsulate tcPNA (Fig. 5), DNA (DNA was neutralized using spermidine as a counter ion), or both tcPNA and DNA (in which the lysines conjugated to the PNA both on C and N termini served as the counter ion for the DNA). Further, treatment of human CD34+ HSCs with nanoparticles containing nucleic acids in dosages of 0.25–2 mg PLGA/mL was performed. It has been found that cells treated with nanoparticles show greater cell recovery and viability as compared to nucleofection protocols. Nanoparticle treatment also led to much higher rates of recombination, corresponding to at least a 60-fold increase in modified and viable cells.

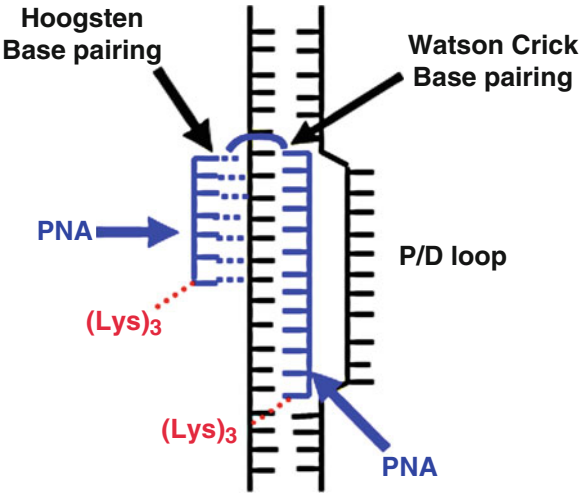

Fig. 5 Lysine conjugated tail clamp PNA binding to dsDNA

It has been successfully shown that PNA/donor DNA delivered by nanoparticle-based approach caused site-specific gene editing of human cells in vivo in hematopoietic stem cell-engrafted NOD-scid mice. Intravenous injection of particles containing PNAs/DNAs produced modification of the human CCR5 gene in hematolymphoid cells in the mice. Deep-sequencing results confirmed in vivo modification of the CCR5 gene at frequencies in the range of 0.1–0.5 % in hematopoietic cells in the spleen and bone marrow. At the same time, off-target modification in the homologous CCR2 gene was two orders of magnitude lower. Application of nanotechnology in the site-specific gene editing by using PNA offers numerous advantages. Firstly, this approach provides a framework for development of a flexible system for direct in vivo genomic modification of HSCs. Second; this work suggests a versatile method for targeted drug delivery to human hematopoietic populations.

tcPNAs targeting always requires homopurine or homopyrimidine rich regions for effective targeting.

In another strategy we have shown that new generation chemically modified gamma PNAs (γPNAs) can be used to target mixed sequence genomic DNA and induce genome modification [80]. In γPNAs, stereogenic chiral center has been induced at the gamma position of PNA backbone (Fig. 6) [81–83]. The presence of chiral center at gamma positions results into preorganized conformation of PNAs which further boost its binding affinity with complementary sequences containing DNA or RNA [84,85].

In order to demonstrate proof of concept, a transgenic mouse model containing a β-globin/EGFP fusion gene, consisting of intron 2 of human β-globin inserted within the GFP coding regions have been employed [86,87]. The intron region contains the IVS2-654 (C → T) mutation which is a common cause of thalassemia in individuals of Southeast Asian heritage. This mutation leads to a cryptic splice site that causes incorrect splicing of the intron and prevents expression. Using nanopar-

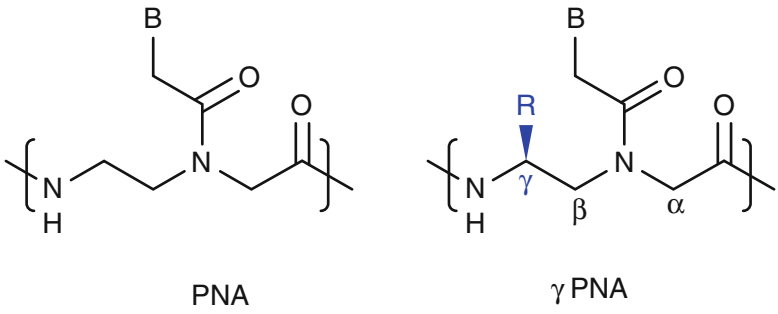

Fig. 6 Chemical structure of PNAs and γPNAs

ticulate based delivery systems we have shown that single-stranded γPNAs designed to target genomic DNA site near IVS2-654 mutation along with donor DNA, induced site-specific gene editing at frequencies of 0.8 % in mouse bone marrow cells treated ex vivo and 0.1 % in vivo via IV injection, without detectable toxicity. Moving one step ahead we have shown that γPNAs provide a new tool for induced gene editing based on Watson-Crick recognition without sequence restriction.

Summary

Overall TFOs and PNAs have played an important role in genome modification by implying different strategies. Promising results have been attained both in ex vivo as well as in vivo studies. By utilizing information at chemistry/biology interface, researchers have come up with many strategies to increase the gene editing frequency. Though PNAs possess promising chemical properties in comparison to TFOs, nonetheless, lot of work still need to be done in order to attain gene-editing frequencies, which are clinically relevant.

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