GENE DELIVERY

Journal of Controlled Release 155 (2011) 312–316

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Polymer delivery systems for site-specific genome editing

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ARTICLE INFO

Article history:
Received 29 March 2011
Accepted 10 May 2011
Available online 18 May 2011

Keywords:
Gene therapy
Triplex forming oligonucleotides
Peptide nucleic acids
Nanoparticles
Hematopoietic stem cells
Poly(lactic-co-glycolic acid)

ABSTRACT

Triplex-forming peptide nucleic acids (PNAs) can be used to coordinate the recombination of short 50–60 bp "donor DNA" fragments into genomic DNA, resulting in site-specific correction of genetic mutations or the introduction of advantageous genetic modifications. Site-specific gene editing in hematopoietic stem and progenitor cells (HSPCs) could result in the treatment or cure of inherited disorders of the blood such as β-thalassemia or sickle cell anemia. Gene editing in HSPCs and differentiated T cells could also help combat HIV infection by modifying the HIV co-receptor CCR5, which is necessary for R5-tropic HIV entry. However, translation of genome modification technologies to clinical practice is limited by challenges in intracellular delivery, especially in difficult-to-transfect hematolymphoid cells. Here, we review the use of engineered biodegradable polymer nanoparticles for site-specific genome editing in human hematopoietic cells, which represent a promising approach for *ex vivo* and *in vivo* gene therapy.

1. Gene therapy in hematopoietic cells

Hematopoietic stem cells (HSCs) self-renew throughout an individual's lifetime and differentiate into progenitors that populate the diverse components of the human blood and immune system (Fig. 1). Because of these properties, genetic manipulation of HSCs—or, more broadly hematopoietic stem and progenitor cells (HSPCs)—could provide curative treatments for single-gene disorders of the blood or introduce new genomic changes to combat certain infectious diseases. For example, editing of the *β-globin* gene in HSCs and myeloid progenitors could provide treatments for devastating inherited hemoglobinopathies, such as β-thalassemia and sickle-cell anemia. As a further example, editing of the *CCR5* gene in HSCs (or lymphoid progenitors, or mature T cells) could provide a treatment for HIV infection through inhibition of HIV entry into immune cells. The chemokine receptor CCR5 is a major co-receptor used by HIV for entry into T cells, and a naturally occurring deletion mutation in *CCR5* has been shown to confer resistance to HIV [1]. A recent study has indicated that disruption of *CCR5* using zinc-finger nucleases can help reduce HIV infection in a humanized mouse model [2].

Unfortunately, gene modification remains a challenge in hematopoietic cells, especially HSPCs, due to their quiescent nature and relative resistance to both viral and non-viral methods of transfection [3,4]. Only 5–10% of human bone marrow cells are CD34+ (a marker for HSPCs), and of these, only 10–20% express the more primitive HSC phenotypes [5], thus making cell-specific targeting important for *in vivo* gene therapy. Here, we review our recent work showing that nanoparticles—designed for gentle delivery of gene-editing oligonucleotides—can be used to modify disease relevant genes in human hematopoietic cells.

2. Gene modification with triplex-forming oligonucleotides

A major problem with the use of viral vectors for gene modification is the lack of control over the site on the host genome where the viral genome is integrated. To accomplish efficient and safe genome modification, genetic targeting—and, therefore, sequence-specificity—is critically important. Triplex forming oligonucleotides (TFOs) form unique structures by binding with high affinity and specificity in the major groove of duplex DNA. TFOs are capable of catalyzing genomic events including inhibition of transcription and DNA replication, promotion of site-specific DNA damage, and enhancement of recombination [6]. To improve them for use in *ex vivo* or *in vivo* therapies, TFOs can be produced as peptide nucleic acids (PNAs), which contain nucleobases with a polyamide backbone, conferring resistance to intracellular degradation and enhancing their binding affinity to DNA because of reduced electrostatic interactions. Triplex-forming PNAs bind via Hoogsteen and Watson-Crick bonding to a complementary DNA strand, forming a stable PNA/DNA/PNA triple helix. This abnormal structure is then recognized by cells' own DNA repair machinery, sensitizing a site for homologous recombination. Thus, intracellular delivery of a site-specific PNA can induce recombination of a short, single-stranded "donor" DNA molecule with a nearby genomic site [7] (Fig. 2). The advantages of PNA TFOs as a method for gene editing include their gene specificity [8], high binding affinity to DNA, the availability of binding sites throughout the genome, and intracellular stability.

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doi:10.1016/j.jconrel.2011.05.011

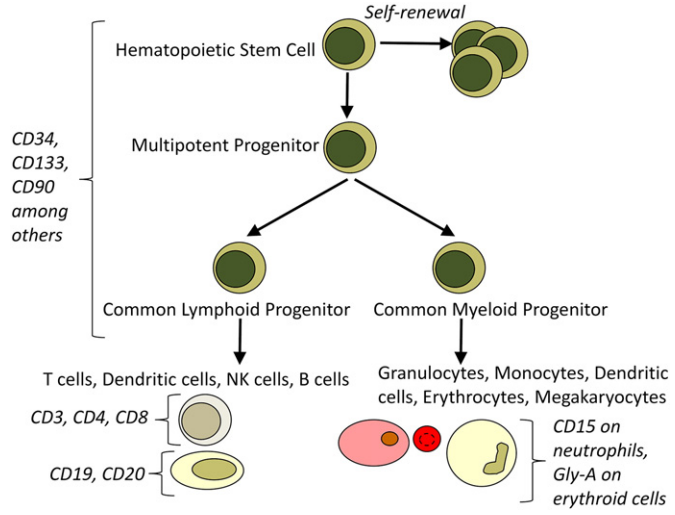

Fig. 1. Hematopoeisis. A schematic of hematopoiesis, the process by which hematopoietic stem cells form the components of the blood and immune system. Some examples of cell surface markers are included.

Triplex-forming PNAs facilitate genomic modification in human hematopoietic cells. For example, triplex-forming PNAs can bind the human *β-globin* gene and stimulate modification at a β-thalassemia associated site in human CD34+ HSPCs without loss of pluripotency [9]. Chin *et al* designed PNAs that mediate recombination at the first position of intron 2 (IVS2-1) of the *β-globin* gene (Fig. 3A), achieving recombination frequencies of 0.1–0.5% in a CHO cell GFP *β-globin* fusion model (Fig. 3B) [9]. Gene correction was verified at the genomic (by sequencing), mRNA (by qRT-PCR), and protein levels. Primary human CD34+ HSPCs transfected with PNA and DNA were found to contain the desired mutation (as determined by allele-specific PCR, or AS-PCR), even after differentiation along erythroid and neutrophil lineages (Fig. 3C) [9]. In addition, the Glazer lab has developed a PNA and DNA combination for modification of the human *CCR5* gene, which confers HIV resistance in THP-1 cells (manuscript in revision)[10]. Transfection in these studies was accomplished via electroporation/nucleofection [11], which although useful for proof-of-principal studies, is relatively toxic to hematopoietic cells and cannot be used *in vivo*. Cationic lipid methods cannot be used for PNA delivery due to their neutral or net positive charge, and other methods of PNA transfection have drawbacks including dependence on complementary or conjugated DNA carriers, direct conjugation to peptides or lipids, and the use of non-biodegradable materials [12–18]. Because of the difficulty of transfection of HSPCs, many prior studies have been performed in cell line reporter systems, which makes it difficult to predict the value of these approaches in a clinical setting.

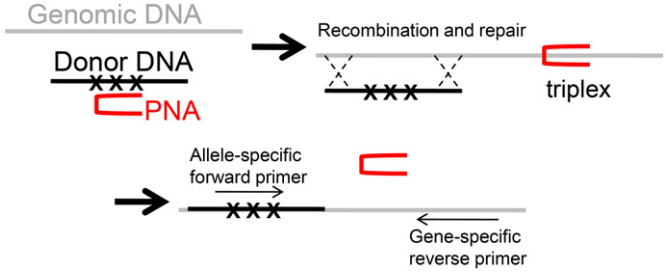

Fig. 2. Genome modification using PNA and DNA. The 50 to 60-mer donor DNA (black) is homologous to the gene target of choice (grey) except for a several base-pair mutation (X X X). The PNA (red) binds near the target and induces homologous recombination of the donor strand into the target. Allele-specific PCR (AS-PCR) can distinguish between modified (mutant) and unmodified (wild-type) genomic DNA.

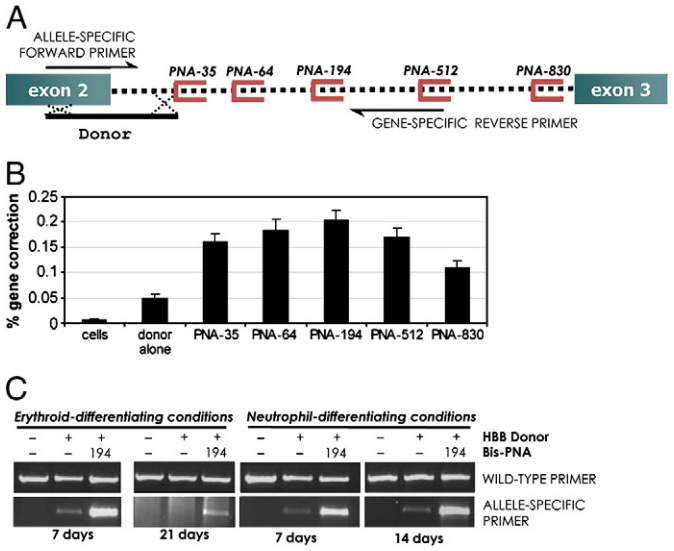

Fig. 3. Triplex-forming PNAs induce recombination at a thalassemia mutation site. (A) PNAs were designed to bind to intron 2 sequences of the human *b-globin* gene at a distance of 35 to 830 bp from the targeted thalassemia mutation at the first position of intron 2. (B) PNA-mediated gene correction frequencies in CHO cells using a GFP-expression assay. (C) Human CD34+ cells treated with PNAs and short donor DNAs (HBB donor) were capable of differentiation into erythroid and neutrophil lineages; both lineages showed targeted gene modification by AS-PCR up to 21 days following oligonucleotide transfection.
Reproduced with permission from [9] (permission pending).

3. PLGA nanoparticles for gene modification

Here, we review our novel solution to the problem of intracellular delivery of PNA and DNA molecules for genome editing. Poly(lactic-co-glycolic acid) (PLGA) is an FDA-approved biocompatible polymer used clinically for delivery of drugs for numerous indications including treatment of prostate cancer (Lupron ® and Trelstar ®). In prior work, we have shown that PLGA nanoparticles can provide reliable intracellular delivery of nucleic acid polymers and oligomers, including plasmid DNA [19,20] and siRNAs for sustained gene silencing [21]. These PLGA nanoparticles are taken up readily by numerous cell types through a likely endocytic mechanism, followed by escape from endosomes and association with exocytic organelles [22]. However, the exact mechanism of endosomal escape and subsequent delivery of nucleic acid cargo to cytoplasm and nucleus is not well understood, and requires continuing study. We have developed methods to formulate nanoparticles containing PNA and DNA (PNA-DNA) for enhanced delivery to HSPCs (Fig. 4). Our studies indicate that PLGA nanoparticles can be used to deliver PNA and DNA for gene modification in hematopoietic progenitors [23].

To design nanoparticles for intracellular delivery of PNA, we first found compositions for PLGA nanoparticles that allowed for their internalization in HSPCs. As in prior work, we used coumarin 6 (C6) as a fluorescent probe, which can be loaded into PLGA nanoparticles. Nanoparticles loaded with C6 can be used to track particle association, internalization, and distribution, because the hydrophobic C6 is not released readily from PLGA particles [24,25]. C6 PLGA nanoparticles were formulated using a single-emulsion solvent evaporation technique we have previously described [21]: the particles are small and spherical (Fig. 4 inset), with C6 loadings of ~4 nmol C6/mg PLGA. When added to cells in culture, C6 PLGA nanoparticles associated with and were taken up by CD34+ HSPCs (Fig. 5A), although a large percentage of particles still remained externally associated without internalization.

These PLGA nanoparticles become associated with HSPCs in the bone marrow after intravenous injection. In a small pilot experiment, unmodified C6 nanoparticles injected intravenously or intraperitoneally