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Perspective Series

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Sequence-specific modification of genomic DNA by small DNA fragments

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Small DNA fragments have been used to modify endogenous genomic DNA in both human and mouse cells. This strategy for sequence-specific modification or genomic editing, known as small-fragment homologous replacement (SFHR), has yet to be characterized in terms of its underlying mechanisms. Genotypic and phenotypic analyses following SFHR have shown specific modification of disease-causing genetic loci associated with cystic fibrosis, β-thalassemia, and Duchenne muscular dystrophy, suggesting that SFHR has potential as a therapeutic modality for the treatment of monogenic inherited disease.

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Gene therapy holds a great deal of promise for the future of medical treatment. However, success has been limited, partly because the field is still in its infancy (1, 2). While initial prospects for cDNA-based gene therapy appeared quite promising, the anticipated breakthroughs did not materialize (3). The optimistic projections of success were, somewhat naively, based primarily on in vitro studies and/or model systems that had a limited relationship to the target organs. These projections underestimated the complexity of an intact biological system and the importance of genomic integrity in the cell-specific regulation of gene expression. As a result, a "best guess" approach to gene therapy has developed into an empirical, observation-based approach that is systematically building a foundation of knowledge on which clinical gene therapy will ultimately be based. This investigative foundation building has resulted in the establishment of numerous reagents and strategies intended to make gene therapy both safe and efficacious.

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Nonstandard abbreviations used: small DNA fragment (SDF); small-fragment homologous replacement (SFHR); single-stranded DNA (ssDNA); double-stranded DNA (dsDNA); zeocin resistance (Zeo^r); polyethylenimine (PEI); cystic fibrosis transmembrane conductance regulator (CFTR); sickle globin (β^S-globin); hematopoietic progenitor cell (HPC).

In general, gene therapy has very straightforward goals: to correct the pathogenic phenotype that results from genetic mutation(s), and to ensure that the therapeutic strategy is safe for the patient (see *Goals of gene therapy*). Most current gene therapy approaches are based on the successful introduction and expression of cDNA in cells in vitro (4, 5). These cDNA-based approaches have demonstrated a degree of phenotypic correction; nevertheless, until recently, long-term efficacy in human clinical trials has been elusive.

Two recent studies have provided a glimmer of what the future may hold for cDNA-based gene therapy (6, 7). These studies, as well as previous cDNA-based studies, involved the introduction of WT cDNA that complements the genetic mutation that manifests the disease pathology. One study involved ex vivo treatment of hematopoietic stem/progenitor cells from patients with X chromosome–linked SCID with a recombinant retrovirus containing WT γc cytokine receptor cDNA. Patients were given autologous transplants of genetically modified hematopoietic stem/progenitor cells and now appear to mount a normal immune response to environmental stimuli (6). In another clinical trial, patients with factor VIII deficiency (which results in hemophilia A) were transplanted with autologous skin fibroblasts that had been transfected with a plasmid containing WT factor VIII cDNA (7). Unfortunately, one patient of the SCID clinical trial developed a T cell leukemia that appears to be linked to an insertional mutagenesis event resulting from the integration of the transgene (8).

As alternatives to the cDNA-based approaches to gene therapy, strategies that target the mutant region

of the endogenous gene have been developed. These genome-based approaches circumvent some of the issues (e.g., insertional mutagenesis) that have been associated with viral cDNA-based methods by relying on nonviral DNA delivery vehicles. Gene-targeting strategies also maintain the integrity of the target gene in terms of the relationship between the protein coding sequences and the gene-specific regulatory elements. With the exception of an adeno-associated virus–based approach (9–11), most gene-targeting strategies are oligonucleotide based (12–18). The oligonucleotide-based gene-targeting strategies include those that use triplex-forming oligonucleotides (19–22), RNA/DNA hybrid oligonucleotides (23–26), and small DNA fragments (SDFs) (27–37). This Perspective will focus on one of these oligonucleotide strategies: small-fragment homologous replacement (SFHR) (12, 13, 32, 36). The SDFs used in SFHR are composed of either single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA), contain noncoding sequences, and are essentially homologous to target loci. These SDFs effect homologous exchange between incoming SDF sequences and endogenous (genomic or episomal) sequences, ultimately resulting in phenotypic changes.

SFHR-mediated gene targeting

The SFHR gene-targeting strategy uses ssDNA and dsDNA fragments that are effectively homologous to genomic or episomal cDNA target sequences in order to catalyze intracellular enzymatic mechanisms that mediate homologous exchanges (12) (Figure 1). SFHR has been applied in human epithelial and hematopoietic cells as well as mouse hematopoietic and ES cells (refs. 29, 32, 36, 38; and G. Novelli et al., unpublished observations). As many as 4 bp's have been concomitantly inserted, deleted, and/or altered by SFHR, and to date, studies suggest a broad range of utility in terms of target genes and cell types able to support SFHR. Moreover, SFHR appears to be effective both in vitro and in vivo.

Model systems

A group of independent studies in human airway epithelial cells was used to quantitatively evaluate SFHR and the effectiveness of various DNA delivery systems (39, 40). These studies were focused on the correction of a 4-bp insertion mutation that inactivated the zeocin resistance (Zeo^r) gene carried in both a prokaryotic and a eukaryotic expression vector (31). This insertion mutation both inactivated the Zeo^r gene and eliminated an XmaI restriction enzyme cleavage site. Upon correction, Zeo^r and XmaI cleavage were restored. Using a transient transfection assay in which the vector containing the mutant Zeo^r gene and SDFs comprising WT Zeo^r sequences were cotransfected, up to 4% of the vector recovered from the transfected human cells gave rise to Zeo^r bacteria. Functional assays, restriction digests, and sequence analysis demonstrated that these Zeo^r colonies contained plasmids with an SFHR-corrected WT Zeo^r gene. In addition, these studies indicated that cationic DNA transfer vehicles (liposomes and polyamidoamine dendrimers) can sequester the target DNA and/or the SDF such that it is not readily accessible to the enzymatic machinery that facilitates SFHR-mediated exchange. This was evident from the observation that the frequency of SFHR-mediated exchange was two orders of magnitude higher when bacteria were transformed with plasmids derived from nuclear extracts, as compared with plasmids derived from whole-cell extracts. This is in sharp contrast with studies comparing whole-cell and nuclear plasmid extracts from electroporated epithelial cells, in which no difference in SFHR-mediated modification frequency between the two extraction procedures was observed (31).

Another recent study, using a defective GFP reporter plasmid as the target DNA, allowed real-time measurement of SFHR-mediated functional correction of an episomal target plasmid in live mammalian cells (40). SDFs (442 bp in length) were mixed with polyethylenimine (PEI) and delivered as a complex with a mutant GFP plasmid to variety of different cell lines. Functional cor-

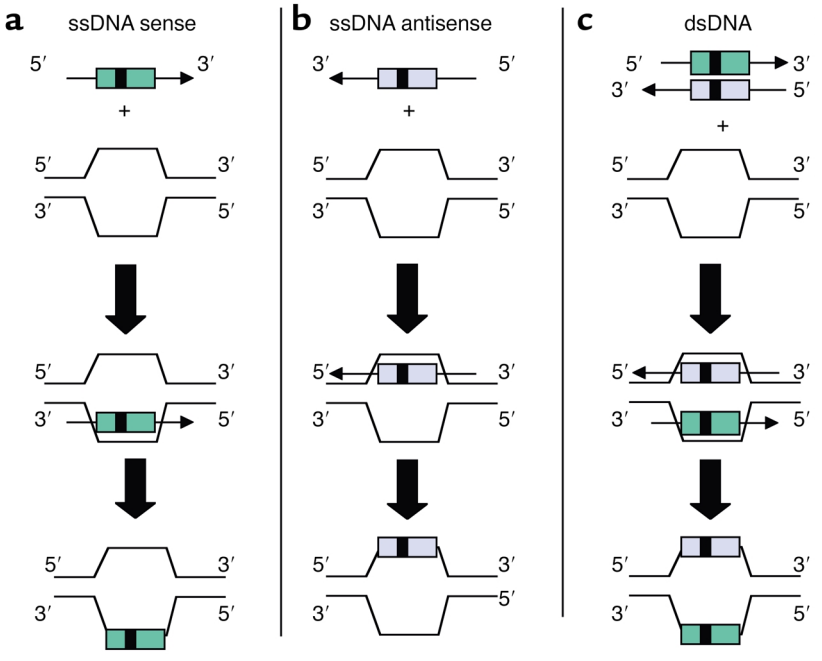

Figure 1
Schematic representation of different SDF configurations possible in SFHR-mediated modification of a target sequence. The SDF can interact with the target sequence as (**a**) sense and/or (**b**) antisense ssDNA, or as (**c**) dsDNA. The ssDNA can be introduced either as individual strands (sense and antisense) or as denatured cDNA strands. The enzymatic mechanisms involved require further elucidation.

Goals of gene therapy

1. Correction of mutant genetic sequences with WT sequences
2. Expression of normal protein and phenotype
3. Long-term correction with minimal application or multiple applications with nontoxic or nonimmunogenic methods of delivery
4. Rapid and efficient delivery

rection of the mutant reporter gene was phenotypically assessed as a restoration of green fluorescence and was measured by FACS analysis of individual cells. Targeted correction efficiencies ranging from 0.1% to 1.2% were observed in five different mammalian cell lines.

In a follow-up study, stable cell lines carrying an integrated GFP gene were created to assess the efficiency of gene conversion (40). Larger SDFs (up to 1,600 bp in length) were more efficient in mediating the homologous exchange of the endogenous mutant GFP gene. However, the efficiency observed in genomic targeting was 100-fold lower than that observed with episomal correction. This may, in part, be due to the delivery of SDFs into the nuclei of target cells, which was observed to be relatively inefficient when PEI-mediated transfection was used.

Model systems such as the Zeo^r and the GFP systems will be extremely useful for defining those parameters critical for optimization of SFHR, as well as for evaluating the effectiveness of cationic systems for the nuclear delivery of DNA. Clearly, extrapolation to other reporter or selectable marker gene systems can have important implications for high-throughput screening for effective DNA delivery systems.

Cystic fibrosis

Over the last decade, numerous in vitro SFHR studies were carried out in human airway epithelial cells, demonstrating both genotypic and phenotypic SFHR-mediated modification of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (12, 14, 27, 29, 32, 37, 41, 42). SDFs were applied in cultured human airway epithelial cells for SFHR-mediated correction of the CFTR gene by direct replacement of the ΔF508 mutation, a 3-bp deletion, with WT sequences (27, 29, 32, 37, 41). Both genotypic and phenotypic measurements (e.g., chloride ion transport) indicated that SFHR-mediated modification occurred in 1–10% of the transfected cells. The studies indicated not only that SFHR could directly modify genomic DNA, but also that multiple bases could be effectively altered at the same time. Moreover, these investigations clearly show that SFHR can facilitate multiple base insertions and deletions, as well as concomitant base transversions in genomic DNA. These in vitro studies resulted in restoration of the WT ion-transport phenotype.

Variations in SFHR-mediated gene-targeting efficiency can also be correlated with cell type and lipid/DNA charge ratio (37, 43). These factors appear to mediate both the internalization of SDF/liposome complexes and their intracellular fate. Previous observations have suggested that an additional element — plasma-membrane lipid composition, a function of the cell type — may define the lipid/DNA charge ratio (43). It also appears that this lipid/DNA charge ratio regulates the mechanism of lipoplex entry into the cells; i.e., lipid-DNA entry into the cells by endocytosis or membrane fusion depends on lipoplex charge ratio. Entry via an endocytic mechanism seems to be essential for the release of the DNA from the lipoplexes and for transport into the nucleus (37, 44). This apparent relationship between endocytic entry and nuclear delivery will likely have important implications for the delivery of any DNA.

Recent studies have also shown that such gene targeting can occur in mouse airway epithelial cells in vivo (28). The data indicate that SFHR-mediated modification occurs in mouse airways and intestine in cells that express mouse CFTR mRNA following transfection with various cationic lipid and dendrimer DNA complexes. These in vivo studies represent the first in a series of preclinical investigations necessary for quantification of SFHR modification and evaluation of its functional efficacy. In addition, these studies support previous in vitro observations showing that 4 bp's can be concomitantly modified even when one bp is distal from the primary mutation.

Sickle cell anemia

In another series of studies, the human β-globin gene was the target for SFHR (36). The aim of this work was modification of the β-globin gene, *HBB*, at codon 6, the site of the mutation responsible for sickle cell anemia. These proof-of-principle studies demonstrated that SFHR-mediated conversion, from A to T at codon 6, could be achieved by transfection of hematopoietic cells with β-globin SDFs comprising the sickle globin (β^S-globin) mutation. The hematopoietic cells were either cell lines — i.e., murine erythroleukemia cells carrying human chromosome 11 (known as A181β) or the human erythroleukemia cell line K562 — or human primitive hematopoietic

Genome-based oligonucleotide gene therapy

Advantages

Maintains gene integrity
Maintains relationship of coding and regulatory sequences
Retains cell-specific expression
Ensures appropriate level and duration of expression
Should be permanent

Disadvantages

May have low frequency
May result in random integration
May stimulate an apoptotic cascade
Degradation of therapeutic DNA may occur

progenitor cell (HPCs). The A181β and K562 cell lines were transfected by electroporation, while the HPCs were transfected by microinjection. These two physical methods of transfection might overcome the inefficient nuclear delivery of DNA fragments that was observed in the model systems described above, in which chemical delivery vehicles were used. Besides demonstrating SFHR-mediated modification of the β-globin gene with SDFs, the studies showed that the β-globin gene in human airway epithelial cells can also be modified with β^S-globin SDFs. This is particularly noteworthy in that these cells do not express the β-globin gene, implying that transcription is not necessary for SFHR-mediated modification.

The studies in HPCs showed that SFHR-mediated conversion of WT β-globin (known as β^A-globin) to the sickle cell β^S-globin was stable in a culture that was expanded from 10^3 to 10^5 cells (36). These studies also indicated that, at a minimum, 1–2% of the cells had been modified by SFHR. This was determined from the fact that 60 of 100 injected cells were viable in the starting population of 10^3 cells. Thus, if only one in 60 cells underwent SFHR-mediated conversion, a minimum of 1–2% of the cells would have been converted.

Duchenne muscular dystrophy

Another target for SFHR-mediated modification has been the Duchenne muscular dystrophy (DMD) analogue in the *mdx* mouse model of DMD (30). In vitro and in vivo application of a WT SDF (603 bp) was used to facilitate a T→C WT conversion of a C→T nonsense mutation in *mdx* exon 23 of the dystrophin gene, *dys*. Multiple applications of the WT SDF and variations in the Lipofectamine transfection complex enhanced the efficiency of SFHR-mediated modification in vitro. Conversion was observed at both the DNA and the RNA levels when the cells and tissue were analyzed by PCR or RT-PCR amplification, respectively. The efficiency of conversion of the *mdx* mutant locus to the WT *dys* was about 15–20% in vitro and up to 0.1% in the tibialis anterior in vivo. Correction in myoblasts from *mdx* mice persisted at least 28 days in culture and up to 3 weeks in vivo. These studies are very encouraging for the potential development of SFHR-based therapies for neuromuscular disorders.

Summary

The ability to correct a mutation in genomic DNA is clearly a desirable goal for gene therapy. There are advantages and limitations to this approach (see *Genome-based oligonucleotide gene therapy*). The advantages include the ability to maintain gene integrity and the relationship between the protein coding sequences and the gene-regulatory elements. This would overcome any potential for inappropriate gene expression either in the amount of protein produced or in the cell type expressing the gene. The limitations to a genomic correction/modification approach include the possibility that the modification would not be efficient enough to produce a viable therapeutic outcome. Previous homologous-recombination studies in mouse ES cells, using large bacterial plasmids to carry large homologous segments of mouse DNA into the ES cells, indicated that the efficiencies of homologous recombination were low ($\leq 10^{-5}$) (45). While these studies were elegant, they were based on the introduction of both homologous (in the form of genomic DNA segments) and nonhomologous (bacterial plasmid and selectable marker gene) dsDNA sequences into ES cells. The homologous regions were often interspersed with nonhomologous sequences and therefore would be thermodynamically less stable in homologous pairing than would pieces of DNA that were effectively homologous to the target genomic DNA. One early study, in which small pieces of dsDNA were injected into ES cells, demonstrated that 1 in 150 cells underwent homologous exchange when the DNA segments were effectively at least 99% homologous to the target genomic DNA (46), although these high frequencies were not always observed in other cell systems using a variety of DNA delivery vehicles (33, 35).

More recently, a number of different oligonucleotide strategies have reported frequencies similar to those indicated following the microinjection of ES cells (12–15, 17, 18). These studies provide evidence to suggest that such strategies might be viable alternatives to cDNA-based gene therapy systems now being evaluated in clinical trials. The SFHR strategy outlined in this overview has demonstrated utility for sequence-specific genome editing of three endogenous genes: *CFTR*, *HBB*, and *dys*. SFHR has demonstrated the capacity to elicit single-base substitutions as well as concomitant insertion or deletion of multiple bases at efficiencies that could approach therapeutic viability.

A critical step in the development of SFHR as a therapeutic intervention will be the elucidation of the molecular mechanism(s) that underlies the homologous replacement observed after the introduction of SDFs. Dissection of the molecular basis of SFHR will be greatly facilitated by assessment of SFHR-mediated modification in cell-free extracts, bacteria, or eukaryotic cells defective in DNA repair, replication, or recombination pathways, coupled with microarray analysis of changes in gene expression after the introduction of SDFs.

SFHR, like other gene-editing strategies, has the potential both to be applied therapeutically and to enhance the understanding of disease through the development of transgenic animal models of disease. Advances in bone marrow–derived stem cell technology (15, 47–53) have suggested opportunities for expanding the range of genetic diseases that can be effectively treated by ex vivo gene targeting in pluripotent stem cells. Preliminary studies in mouse ES cells and in bone marrow–derived stem cells indicate that these cells can be genetically modified by SFHR (G. Novelli et al., unpublished observations). These studies on human cells have significant implications for the potential of using SFHR-modified stem cells for tissue repair in organs damaged by genetic disease–related pathology.

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