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Oligonucleotide-Directed Gene Correction in Epidermis

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Oligonucleotide-directed gene alteration produces a targeted DNA sequence change in the genome of mammalian cells. The advantage of this approach is that expression of the corrected gene is regulated in the same way as a normal gene. Reliable, sensitive, and standardized assays played a critical role in the measurement of gene correction frequency among different cell types and in evaluating the structure–activity relationship of oligonucleotides. Mechanistic studies using these assays have become critical for understanding the gene repair process and setting realistic expectations on the capability of this technology. The epidermis is an ideal tissue where oligonucleotides can be administered locally and the treated sites can be monitored easily. But given the low frequency of gene correction, general selection procedures and amplification of corrected cells via epidermal stem cells are ultimately needed to make the gene repair technology practical. Recent data suggest that the *in vivo* application of oligonucleotides may be capable of gene correction in epidermal stem cells and the subsequent expansion of the corrected cells may result in an apparent high-level and long-lasting gene repair. Advances in oligonucleotide delivery and targeting of epidermal stem cells will be required for potential application of oligonucleotides toward treatment of genodermatoses.

Key words: chimeric RNA–DNA oligonucleotide/epidermal stem cells/gene targeting/single-stranded oligonucleotide

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Genetic defects in human cells can be corrected by gene therapy in two ways, gene replacement and gene targeting. In gene replacement therapy, a therapeutic gene is transferred to the cell or affected organ usually by virus-mediated gene transfer. But expression of the transferred gene is constitutive and unregulated, a situation that may be harmful in certain cases. Moreover, gene replacement cannot rescue a dominant mutation and the use of viral vectors presents safety concerns including immune reactions and the generation of replication competent virus. In contrast, gene targeting is designed to produce pre-defined alterations in the genome. By repairing the mutant gene *in situ*, normal gene regulation is preserved and the potential is there to treat dominant disorders.

Several gene-targeting strategies have been developed to correct mutations through homologous recombination process (Table I). Through the use of traditional gene-targeting strategies via double-stranded DNA, it has become possible to replace or delete genetic information in chromosomes (Thomas and Capecchi, 1987; Mansour *et al*, 1988; Capecchi, 1989). The application of this strategy to embryonic stem cells, however, has been possible by selecting rare successful targeting products. In another approach, triple-helix-forming oligonucleotides (TFO) have been utilized to make a specific sequence change in the

genome by exploiting its specific recognition of the target DNA sequence (Knauert and Glazer, 2001; Vasquez and Glazer, 2002). A small-fragment homologous replacement strategy utilized a 500 base single-stranded DNA to generate a homologous replacement in mammalian cells (Goncz *et al*, 1998, 2002). A recent innovation is the use of adeno-associated viral vectors, which have a single-stranded DNA genome, to modify homologous chromosomal sequences (Russell and Hirata, 1998). Although each of these methods was shown to work in limited cases, the low absolute frequency of homologous recombination remains a serious limitation.

My laboratory has focused on the development of an experimental strategy that centers on the site-specific correction of single-point mutations by using chimeric RNA–DNA oligonucleotides (RDO) and relatively short single-stranded oligodeoxynucleotides (ODN). One objective is to establish reliable, sensitive, and standardized assays to measure frequencies of gene repair and to use these assays in mechanistic studies (Igoucheva *et al*, 1999, 2000, 2001, 2002, 2003). Such studies have become critical for understanding the gene repair process and setting realistic expectations on the capability of this technology. The other objective is the application of gene repair toward potential gene therapy in the epidermis (Alexeev and Yoon, 1998; Alexeev *et al*, 2000, 2002). The epidermis is an ideal tissue where oligonucleotides can be administered locally and the treated sites can be monitored easily. In renewing tissues such as the epidermis, a gene therapy approach must be

Abbreviations: ODN, oligodeoxynucleotides; RDO, RNA–DNA oligonucleotides

Table I. Comparison of gene-targeting vectors

	Mechanism	Frequency	Application
Double-stranded DNA	HR + selection	10^{-6} – 10^{-5}	ES cells, knockout mouse
Triplex DNA	Triplex + DNA repair	10^{-4} – 10^{-2}	Targeted mutagenesis
Small fragment replacement	HR + DNA repair	10^{-3} – 10^{-2}	Correction of point mutations and large insertions and deletions
AAV-mediated gene targeting	HR + DNA repair	10^{-5} – 10^{-2}	Correction of point mutation and large insertion and deletions
RNA–DNA oligonucleotide	HR + DNA repair	10^{-5} – 10^{-1}	Repair of point and frameshift mutations
Single-stranded oligonucleotide	HR + DNA repair	10^{-4} – 10^{-3}	Repair of point and frameshift mutations

targeted toward the stem cell population and should require identification, enrichment, and targeting of stem cells to ensure the continued presence of the corrected gene. Epidermal gene therapy is in an early stage and yet holds great promise for its ultimate clinical application.

Structure of Targeting Oligonucleotides

RNA–DNA chimeric oligonucleotide An oligonucleotide composed of a contiguous stretch of RNA and DNA residues was developed to facilitate correction of single-base mutations in mammalian cells (Yoon *et al*, 1996). The original design of the chimeric RDO consisted of a double-hairpin capped duplex comprising a 25-nucleotide-long DNA stretch (DNA-strand) containing the mismatch, paired to a fully complementary 2'-O-methyl RNA stretch (RNA-strand) with a pentameric DNA interruption in the middle (Fig 1A). In order to protect the molecule from RNase H and exonuclease, ribose sugars were 2'-O-methylated and RDO was folded into a double-hairpin structure containing four T residues in each loop and a five-base-pair GC clamp.

Mechanistic studies from our group and others showed that the DNA-strand of RDO is responsible for the gene repair activity (Gamper *et al*, 2000b; Igoucheva *et al*, 2001). Extension of DNA by recombinase is considered to be critical for homology search and this extension is not possible for RNA because of the 2' hydroxyl group in the RNA (Shibata *et al*, 2001). Indeed, strand-pairing reactions indicated that RNA was a poor substrate for recombination (Kirkpatrick and Radding, 1992). Thus, RDO is expected to have a lower strand pairing activity than single-stranded DNA (ODN). On the other hand, the RNA-strand of RDO was shown to stabilize a putative intermediate made by RecA substrates (Gamper *et al*, 2000a). RDO made a stable intermediate (double D-loop) whereas ODN made a very unstable intermediate (D-loop) that dissociated rapidly (Fig 1A). But the question remains whether one can extrapolate the conclusions drawn from the RecA studies toward understanding the mechanism of ODN-directed gene correction in living cells. In contrast to the RecA studies, our mechanistic studies indicate that ODN made stable intermediates via resection of D-loops by nucleases present in mammalian cells (Fig 1B) (Igoucheva *et al*, 2002, 2003).

Single-stranded oligodeoxynucleotides In the 1980s, several groups reported that homologous ODN of 20–70

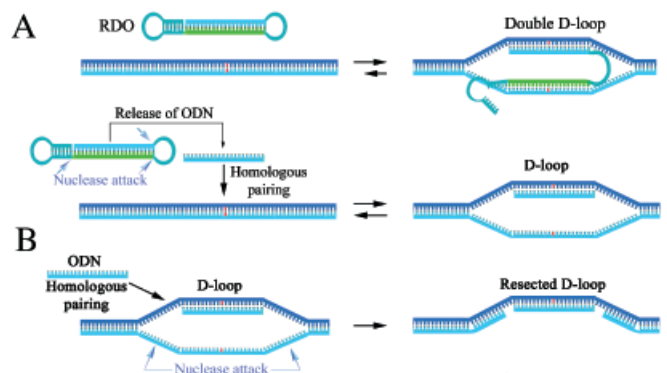


Figure 1

Oligonucleotide repair strategies. (A) The chimeric RNA–DNA oligonucleotide (RDO) consists of a double-hairpin-capped duplex comprising a 25-nucleotide long DNA stretch (DNA-strand) containing a mismatch to the base present in the target DNA (red) and a fully complementary 2'-O-methyl RNA stretch (RNA-strand). Two different models are presented. One model predicts the formation of a double D-loop between a RDO and the homologous target DNA, based on the RecA studies (*top panel*). The stability of double D-loop is attributed to the RNA-strand. Another model predicts the processing of a RDO by nucleases and/or helicases and the strand pairing of the exposed active domain of RDO (DNA-strand) to the targeted sequence, based on the mechanism studies in mammalian cells (*bottom panel*). (B) The initial step for gene correction by single-stranded oligodeoxynucleotide (ODN) is postulated to be the formation of a joint molecule (D-loop). Subsequently, a D-loop is further processed to a resected D-loop by nuclease cleavages. According to this model, ODN has a better strand pairing activity and makes a more stable intermediate than RDO.

nucleotides containing a single mismatch to the target DNA caused a sequence-specific alteration in mammalian cells and yeast (Simon and Moore, 1987; Moerschell *et al*, 1988; Campbell *et al*, 1989; Yamamoto *et al*, 1992). The large variation in frequencies of gene alteration made it difficult to use RDO in mammalian cells in a reproducible manner and prompted the use of ODN. In light of the structure–activity result that the DNA-strand of RDO was responsible for DNA repair, the use of ODN in gene modification has been re-examined (Gamper *et al*, 2000b; Igoucheva *et al*, 2001). Modifications were made to protect both ends of ODN from exonuclease degradation by incorporation of three to four residues of 2'-O-methyl RNA or phosphorothioate residues. ODN with the 2'-O-methyl RNA modification were shown to alter target DNA sequences in reaction with mammalian nuclear extracts, in the episomes and chromosomes of mammalian cells (Igoucheva *et al*, 2001). It was also shown that the gene correction rate in living cells was highly

dependent on the length and polarity of ODN. The highest activity was shown by an ODN in antisense orientation with a homology length of 45 nucleotides containing a mismatch in the middle to the targeted sequence. These results led us to propose a link between transcription and gene repair in mammalian cells (Igoucheva *et al*, 2001). These findings were later confirmed in yeast (Liu *et al*, 2002).

Oligonucleotide-Directed Gene Repair in Mammalian Cells

The feasibility of gene correction by RDO was first demonstrated in 1996 by correction of a point mutation in alkaline phosphatase cDNA in CHO cells (Yoon *et al*, 1996). Gene correction restored enzymatic activity; thus enabling visualization of cells that gained the alkaline phosphatase positive phenotype as red cells. Moreover, gene correction was confirmed by both functional enzyme activity and DNA sequence. This study provided the basis for subsequent applications of RDO for modification of chromosomal genes. Since then, RDO has been shown to either correct or cause a specific point mutation in episomal and genomic DNA in mammalian cells, yeast, and plants (Liu *et al*, 2003; Igouchev *et al*, in press; Kren and Steer, 2002). Gene repair has also been reported *in vivo* in a variety of tissues including the liver, lung, muscle, and skin. Frequencies approaching 40% reported in several studies (Kren *et al*, 1998) have created expectations that oligonucleotide-directed gene repair would be a quick and easy method to use without selection procedures. It has become clear that the reported high frequencies were caused by artifacts as a result of assays solely based on PCR analysis. The very reagents used for gene-targeting experiments are nucleic acids containing the base for the desired alteration. These molecules are degraded inside the cell and contaminate the preparation of genomic DNA. During amplification, these fragments are incorporated into the PCR products, thus erroneously inflating the frequency of correction (Yoon *et al*, 2002). In light of these difficulties, functional assays scoring phenotypic changes upon gene correction has become critical to measure the frequencies in different target cells reproducibly.

Importance of functional assays Reliable, sensitive, and standardized assays played a critical role in the measurement of gene correction frequency among different cell types and in evaluating the structure–activity relationship of oligonucleotides (Igoucheva *et al*, 1999; Igoucheva *et al*, 2002). Toward these goals, two assay systems utilizing mutant tyrosinase and mutant *LacZ*, have been established in which phenotypic changes could be detected upon gene correction. Both systems have provided clear evidence of gene correction by RDO and ODN in the episome and chromosome of several mammalian cells: melanocytes (Alexeev and Yoon, 1998; Alexeev *et al*, 2002), CHO-K1 (Igoucheva *et al*, 1999, 2000, 2001, 2003), primary human keratinocytes (Yoon *et al*, 2002), and mouse embryonic stem cells (Pierce *et al*, 2003).

In 1998, we demonstrated that an RDO corrected a point mutation in murine tyrosinase gene, resulting in inheritable

restoration of enzymatic activity, melanin synthesis, and pigmentation changes in albino melanocytes by using clonal analysis (Alexeev and Yoon, 1998). Use of natural pigmentation in gene conversion studies offers a distinct advantage over other strategies: live cells can be monitored for pigmentation change, cloned, and characterized by their genotype. Two important aspects of this study were: (i) permanent genotypic and phenotypic changes were verified by clonal analysis of the corrected cells and (ii) this was the first report documenting a large variation in the level of gene alteration by RDO using the same system. Among 100 experiments performed, only three yielded a high frequency approaching 15% of gene correction. Appearance of pigmented cells was detected in only 10% of experiments. How can one explain the large variation observed in RDO-mediated gene repair in a given cell line and the same lot of synthesized RDO? The delivery and quality of RDO, two favorite candidates for the reported variation among different systems, are not sufficient to contribute the large variation seen in our experiments. We propose that the active domain (DNA-strand) needs to be exposed at the right time and at the right locale to the target DNA for successful gene correction (Fig 1A). Since the processing of RDO by nucleases or helicases to expose the active domain is random, the subsequent strand-pairing reaction to the target DNA is also random and difficult to control.

Relying on the pigmentation change, RDO and ODN were compared in correcting the tyrosinase gene in albino melanocytes (Alexeev *et al*, 2002). The frequency of ODN-directed gene alteration (2×10^{-4} – 1×10^{-3}) was comparable with RDO. ODN, however, showed much more reproducible gene correction rates than RDO. Furthermore, ODN exhibited a consistent frequency of gene correction in the genome of CHO-K1 cells with an integrated mutant *LacZ* gene (data from over 100 experiments). Other groups also reported more consistent and higher gene repair rates of ODN than RDO (Gamper *et al*, 2000b; Liu *et al*, 2001). Also, ODN is easier to synthesize and thus would become more accessible for researchers interested in using this technology.

In 1999, we established a mutant *LacZ* vector that contains a single point mutation in the *LacZ* gene (G1651A), which results in a loss of enzymatic activity caused by an amino acid substitution (E523K) (Igoucheva *et al*, 1999). When cells containing this mutant reporter gene are corrected by gene targeting, the enzymatic activity of the mutant β -galactosidase is restored and corrected cells can be visualized by histochemical staining. This mutant *LacZ* system has been instrumental in developing gene repair strategies by providing an easily detectable and measurable marker for gene correction in biochemical studies using nuclear extracts, episomes, and chromosomes of mammalian cells (Igoucheva *et al*, 1999, 2000, 2001, 2002, 2003). The mutant *LacZ* system has the sensitivity to measure a low frequency because simple counting of the number of the blue bacterial colonies or blue-stained mammalian cells can be used to score gene correction event.

Delivery of ODN to tissue culture cells Chemical or physical means of gene delivery are attractive alternatives to viral vectors for gene therapy since they appear to be

relatively safe and do not place size limitations on the therapeutic agent (Niidome and Huang, 2002). But efficient delivery of macromolecules, such as nucleic acids and proteins, to the right locale has been difficult to achieve so far. The use of liposomes as delivery agents for DNA and other polynucleotides is a natural extension of their application as drug delivery agents. The use of positively charged liposomes reduces the negative charges of the DNA molecule facilitating its delivery through the cell membrane. DNA also induces cationic liposome fusion, perhaps by bridging two liposomes, drawing them closer and destabilizing the bilayer. This fusogenic property provides the opportunity for the DNA to enter the new liposome, or to associate with the net negative charge on a cell membrane to deliver their DNA. The efficiency of liposome delivery appears to depend on many variables: cell type; lipid membrane composition; relative ratio of the lipid to DNA; endocytosis of ODN; release of ODN from endosome; nuclear transfer; and the stability of the DNA-lipid complex during these processes. Delivery of ODN requires optimization for each cell type by testing many available liposomes. It has been difficult to deliver ODN by liposome to the majority of human primary cells. In contrast, highly efficient delivery of ODN was achieved in human primary keratinocytes, even in the absence of liposome (Nestle *et al*, 1994). Keratinocytes have the ability to take up ODN probably by receptor-mediated endocytosis or macropinocytosis. The exposure of normal human keratinocytes to ODN induced expression of several genes, including IL-1 α and follistatin in a non-sequence-specific manner (Mirmohammadsadegh *et al*, 2002). The mechanism of this efficient ODN delivery in keratinocytes has not yet been fully elucidated although internalized ODN has been speculated to bind to an intracellular receptor (e.g., Toll-like receptor 9), which mediates signaling.

Frequency of gene targeting in mammalian cells Initially, efficient ODN-directed gene alteration in keratinocytes was expected since large amounts of ODN can be delivered to the nucleus. Keratinocytes in tissue culture, however, exhibited a very low level of gene correction by ODN. In 1998, we reported that human primary keratinocytes did not show any detectable level of gene conversion by PCR-based RFLP analysis (Santana *et al*, 1998). In 2001, another group communicated the failure of gene targeting in human keratinocytes (van der Steege *et al*, 2001). The primary reason for these failures is that assays used to detect gene repair, the PCR-based RFLP analysis by us and the immunofluorescence by van der Steege *et al*, are simply not sensitive enough to detect the low frequency of gene alteration. To measure a low frequency, human primary keratinocytes were transduced by a retrovirus containing the mutant *LacZ*. Using these keratinocytes, a low level of gene correction in human keratinocytes, 5–10 blue cells per 10⁶ cells, was observed, in spite of their efficient nuclear uptake of oligonucleotide (Yoon *et al*, 2002).

If oligonucleotides are to be used for gene targeting, how can we make it more practical? Low rates of homologous recombination, on the order of 10⁻⁵, were overcome by the ingenious use of selectable markers in gene-targeting vectors (Capecchi, 1989). But it has been difficult to devise a general selection strategy, because positive and negative

selections used in gene-targeting vectors cannot be incorporated into ODN. We hypothesized that cells competent in ODN-mediated alteration of one gene might also be competent in alteration of another gene. Based on this concept, a selection strategy was developed to identify cells that have undergone a gene modification by the use of two ODN, one targeting a gene of interest and the other targeting a defective selectable marker gene that manifests a phenotypic change upon gene alteration. Our results indicate that if two oligonucleotides are present within the nucleus of a "repair-competent" cell, then dual targeting events could possibly occur with a relatively high frequency (Alexeev *et al*, 2002). Thus, the absolute frequency remains at the same level, but the probability of finding cells with the desired gene alteration is increased by first selecting cells according to the phenotypic change. This exemplifies one approach to overcome the low frequency of gene correction, which is essential to make this technology more applicable.

Oligonucleotide-Directed Gene Repair in Animal Model

***In vivo* delivery** The epidermis is well suited to many localized *in vivo* delivery methods including topical application, intradermal injection, gene gun and *in vivo* electroporation. Topical application of the cationic liposome-DNA mixture to mouse skin in the anagen stage of hair cycle resulted in efficient *in vivo* DNA delivery to hair follicles (Alexeev *et al*, 2000; Domashenko *et al*, 2000). Topical application of antisense ODN in ointments resulted in a rapid and prolonged uptake in keratinocytes in the epidermis (Mehta *et al*, 2000). Short oligomers of arginine attached to a variety of small molecules showed efficient delivery of these conjugates into the epidermis, overcoming the stratum corneum barrier (Wender *et al*, 2000).

Gene gun delivery utilizes an adjustable electric discharge to generate a shock wave which accelerates DNA-coated gold particles into target cells or tissues, resulting in gene transfer (Lin *et al*, 2000). These gold particles can penetrate through the cell membrane, carrying the bound DNA into the cell by a receptor-independent pathway. Gene gun delivery of the reporter plasmid encoding *LacZ* gene resulted in dermal expression (Williams *et al*, 1991) and a promoter-specific expression in skin (Lin *et al*, 2001). *In vivo* gene gun delivery, however, is limited by the degree of penetration into the tissue and transient expression.

Combining electroporation *in vivo* together with intramuscular injection of plasmid DNA enhanced gene expression much higher than the intramuscular injection alone (Aihara and Miyazaki, 1998). By using various types of electrodes, efficiency of gene transfer was further increased, resulting in 100–10,000-fold increases in expression (Mir *et al*, 1999). During electroporation, multiple pulses are delivered to the target area where DNA is pre-injected. The introduced electric field causes transient pores in the cell of the tissue and allows uptake of molecules into the cells. Delivery of the reporter plasmid encoding *LacZ* gene by electroporation to hairless mice showed extensive expression of β -galactosidase in the dermis and hair follicles (Maruyama *et al*, 2001; Zhang *et al*, 2002).

To determine the most efficient delivery method, fluorescein-conjugated oligonucleotides were complexed with liposomes and delivered to murine skin (Alexeev *et al*, 2000). The intensity and localization of fluorescence was compared among different methods. Fluorescence was detected in the epidermis and hair follicles upon topical application (Fig 2A). Intradermal injection resulted in much more intense fluorescence than topical application in the dermis and hair follicles, but not in the epidermis (Fig 2B). The *in vivo* electroporation method increased efficiency of ODN delivery more than a 1000-fold, in comparison to the intradermal injection alone (Fig 2C). The fluorescence was distributed evenly through the epidermis as well as the dermis in the injected area and associated with all components of hair follicles. Thus, each method delivers a different amount of ODN to different compartments of skin.

***In vivo* gene correction** In contrast to the low level of gene correction by RDO and ODN in cultured keratinocytes, a much higher level of genotypic correction was observed from the skin biopsies *in vivo* (Alexeev *et al*, 2000). Five intradermal injections of RDO designed to correct a single point mutation in the tyrosinase gene of albino BALB/c mouse skin resulted in 20–30 pigmented hairs per 25 mm² injected area that contains approximately 1000 hair follicles, indicating phenotypic correction of 2%. In contrast, DNA sequence analysis of the same skin biopsies from the 5-mo-old mice exhibited 5%–30% of gene correction. It is reasoned that the large discrepancy between phenotypic and genotypic correction frequency could arise from the unique regenerating property of epidermis: (i) keratinocytes represent the majority of cells in skin and the regenerating property of skin comes from the stem cells of keratinocytes; (ii) melanocytes are very specialized cells involved in pigmentation, consisting of less than 1% of the cells in skin; (iii) because the tyrosinase gene is transcribed exclusively in melanocytes and not in other cells, the corrected keratinocytes will not express tyrosinase nor make melanin; (iv) phenotypic change represents the number of corrected melanocytes, whereas genotypic change represents the genetic alteration in all cells in skin; and (v) since keratinocytes constitute the majority of skin cells, the frequency of genotypic correction in skin biopsies reflects that of keratinocytes.

How can keratinocytes, which exhibit a low-level gene correction in tissue culture condition, manifest a high level *in vivo*? One possibility is that high frequencies are generated by the multiple injections performed during a 5-mo-period. Repetitive injections, however, can cause at most a linear increment and are not sufficient to explain the observed high frequencies. Another possibility is that oligonucleotides

are capable of gene correction of epidermal stem cells and expansion of the corrected stem cells may result in an apparent high-level and long-lasting gene repair in epidermis that has been observed.

To test whether *in situ* application of oligonucleotides can alter genes in epidermis, RDO and ODN were made to introduce a dominant mutation in the keratin 17 gene and administered to neonatal murine skin by multiple intradermal injections. In the injected areas, several morphological changes were observed, consisting of twisted hair shafts, broken hair follicles at the sebaceous gland level, occasional rupture of hair bulb, and epidermal cysts (Fan and Yoon, 2003). These phenotypic changes are remarkably similar to those found in the K17 null mice (McGowan *et al*, 2002), but transient either due to the compensation of K17 by other keratins or the replacement of the mutated cells by the normal surrounding cells during hair growth. These results indicate that *in situ* application of oligonucleotides to skin can produce genotypic and phenotypic changes in keratinocytes (Fan and Yoon, 2003), as well as in melanocytes (Alexeev *et al*, 2000). If the rate of gene alteration can be improved, oligonucleotide-directed gene repair may have an impact in cutaneous gene therapy.

Mechanism of ODN-Directed Gene Targeting

The initial step for gene correction by oligonucleotides was postulated to be the formation of an intermediate joint molecule, in which an oligonucleotide is paired to the homologous DNA by proteins involved in recombination (Fig 1B). The next step is the processing of intermediates by DNA repair processes, leading to the correction of the target sequence.

Formation of intermediates in gene repair process Strand pairing has long been considered to be the rate-limiting step in the process of gene correction by oligonucleotides. But recent data from my laboratory indicated that this might not be true in some cases (Igoucheva *et al*, 2002, 2003). Mechanistic studies utilized mammalian extracts to measure strand pairing and gene correction activity simultaneously. Mammalian nuclear extracts promoted strand pairing of supercoiled DNA and its homologous oligonucleotides as an initial step (Igoucheva *et al*, 1999). Contrary to the presumed notion that strand pairing is rate limiting, two nuclear extracts (mouse embryonic fibroblasts isolated from isogenic p53^{+/+} and p53^{-/-} mice) made the same amount of joint molecules but showed vastly different functional gene repair activity (Igoucheva *et al*, 2002). Another result also supports the notion that strand pairing is not rate limiting in

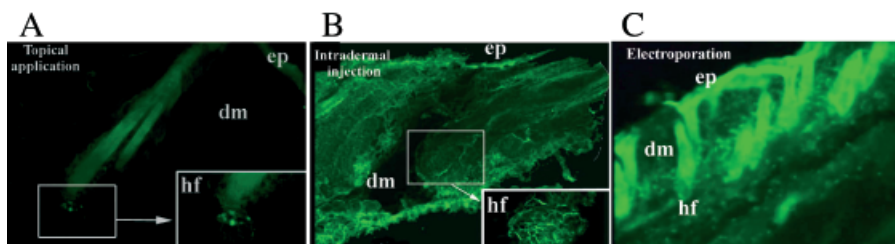


Figure 2
Delivery of fluorescein-conjugated oligonucleotides to murine skin. (A) Confocal fluorescence micrograph of the frozen murine skin section in which oligonucleotide was applied topically, (B) injected intradermally, and (C) *in vivo* electroporated after intradermal injection. Abbreviations: ep, epidermis; hf, hair follicle; dm, dermis.

episomal gene correction. When a known amount of pre-formed intermediates were introduced into living cells, only a very small fraction of these molecules achieved gene correction (Igoucheva *et al*, 2003). Since these intermediates were relatively stable in living cells, the rate-limiting step appears to be a subsequent reaction, rather than the initial strand pairing. More studies are needed to investigate the strand-pairing event of chromosome since the structure of target is complex and affected by a milieu of biological events.

Processing of intermediates in gene repair process A bigger piece of the puzzle is the processing of joint molecules (D-loops), which leads to modification of the target sequence. It has been postulated that the intermediate would be further processed either by resolution of cross-junctions and/or by DNA repair pathways. Because D-loops contain mismatches to the target DNA, proteins involved in DNA mismatch repair have been suggested to participate in the processing of joint molecules. Contrary to this conventional belief, recent data suggest that mismatch repair in fact has an antagonistic effect in ODN-directed repair (Dekker *et al*, 2003).

Perhaps more attention should be given to the structural perturbation caused by D-loop formation rather than a mismatch within the D-loop. Transient formation of a D-loop in living cells could be considered as another class of DNA structural changes that can elicit a plethora of DNA repair activities. A clear example of such a phenomenon was shown by TFO, which bind in the major groove of double-stranded target with a minor structural change. In spite of this minor structural change, TFO binding induced significant increases in both DNA repair (nucleotide excision repair) and recombination activities around the binding site (Knauert and Glazer, 2001). In comparison, D-loop formation is expected to make a much greater change in base stacking, tilting, and thus creating considerable torsional stress at both junctions. What is clearly emerging is that ODN can cause a transient but major structural change in DNA, which in turn elicits multiple DNA repair pathways in conjunction with transcription and replication.

Transcription affects both formation and processing of intermediates Recently, importance of transcription in gene repair by ODN was shown in mammalian cells in both episome and chromosome (Igoucheva *et al*, 2001, 2003). During this study several important observations have been made: (i) gene repair by ODN occurs much more efficiently when the target gene is actively transcribed; (ii) antisense is more active than sense ODN when the gene is actively transcribed; and (iii) a point mutation in transcriptionally repressed genes cannot be efficiently repaired by ODN. These results imply that one needs to select actively transcribing genes for ODN-directed gene targeting and are in agreement with conclusions made in yeast (Liu *et al*, 2002).

The mechanism linking gene repair and transcription was investigated by combining a biochemical approach and a cell-based assay to measure the functional activity of intermediates (Igoucheva *et al*, 2003). Transcription plays a role in both formation and processing of intermediates but in the opposite direction (see Fig 3). Transcription favors the for-

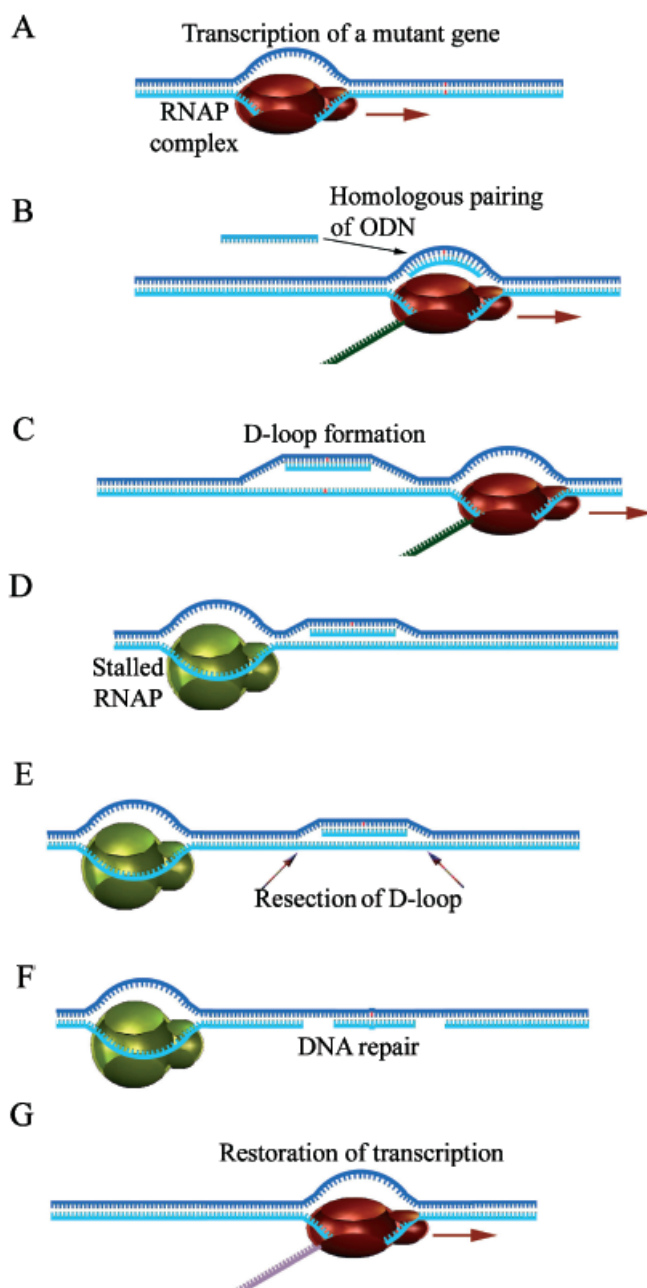


Figure 3
Hypothetical model for ODN-directed gene repair. (A–C) During transcriptional elongation, a portion of the non-transcribed strand forms a transcription bubble that is accessible for the strand pairing by antisense ODN to form a D-loop. In contrast, the transcribed strand is occupied by the RNA polymerase complex (RNAP), limiting the accessibility of sense ODN to the transcribed strand. (D) During the next round of transcription, trafficking of RNAP is interrupted by the presence of the D-loop. (E) The D-loop is converted to the resected D-loop by nucleases. (F) The presence of stalled RNAP may serve as a signal to direct the repair to the transcribed strand. (G) Restoration of transcription.

mation of intermediate by antisense ODN. The non-transcribed strand DNA present in the transcriptional bubble is accessible for strand pairing by antisense ODN, whereas the transcribed strand is occupied with the RNA polymerase complex, limiting strand pairing by sense ODN. On the other hand, transcription favors the processing of

intermediate formed by sense ODN, suggesting that ODN-directed gene repair may be linked to transcription-coupled repair. When DNA damage occurs in the transcribed strand, transcriptional elongation is arrested and the presence of stalled RNA polymerase at a lesion on the transcribed strand serves as a signal to direct repair to the transcribed strand. The central role in the recruitment of DNA repair systems to the transcribed strand has been attributed to transcription repair coupling factors, such as Cockayne Syndrome A (CsA) and B (CsB), a family of ATPases with chromatin remodeling activities (van Gool *et al*, 1997; Citterio *et al*, 2000). These coupling factors displace the stalled RNA polymerase transiently and recruit proteins involved in DNA repair to the site of DNA damage (Citterio *et al*, 2000). Multiple DNA repair systems are coupled to transcription, including nucleotide excision repair, base excision repair and mismatch repair. Thus, transcription plays an important role in many different types of DNA repair pathway, perhaps including ODN-directed repair pathway. Our recent studies indicate that the primary reason for efficient gene repair exhibited by the antisense ODN is an increased formation of an intermediate during active transcription (Igoucheva *et al*, 2003). The enhanced activity of antisense ODN holds for different mismatches, insertions and deletions (Igoucheva *et al*, 2001; Liu *et al*, 2001), indicating a general transcription-driven mechanism. This effect is remarkable since antisense ODN can actually suppress the expression of the corrected gene, thus lowering its apparent correction rate when compared with the sense ODN.

Future Directions

A great potential for gene alteration will undoubtedly be in stem cells. The epidermis continuously regenerates via division of keratinocytes that are renewed throughout the adult life span by proliferation of epidermal stem cells. In continually renewing tissues, the stem cell population is present to provide a source of rapidly proliferating transit amplifying and differentiating cells. Stem cells have both the capacity for self-renewal, that is, the ability to generate additional stem cells and thus be long-lived, and the capacity to generate progeny that are fated to proliferate and differentiate. During *in vivo* experiments, we found a surprisingly high frequency of correction in skin biopsies and hypothesized that oligonucleotides may be capable of gene correction in epidermal stem cells. Transgenic mice that express a high level of the mutant β -galactosidase in epidermis, recently generated in my laboratory, will be ideal to test this hypothesis. In addition, gene correction in epidermal stem cells in the mutant *LacZ* mouse might mark the cells in a similar manner as the retroviral marking of these cells (Ghazizadeh and Taichman). Histological analysis of tissue sections from these mice may yield information on the lineages of epidermal stem cells.

A group of inheritable disorders characterized by fragility of skin, epidermolysis bullosa, are caused by point mutations in several genes maintaining the integrity of cutaneous structure. Separation of tissue occurs between dermal and epidermal junction, resulting in recurring painful blisters. Mutations in some keratin genes cause cytolysis of kera-

tinocytes. Thus, if one can correct genes in the epidermal stem cells, corrected cells will have a proliferative advantage and may compensate for the mutated cells. In this case, a small percentage of the corrected target cells may ameliorate the phenotype of disease. But with the ultimate goal of treating human patients with genetic disease, we must consider the issue of delivery. Topical formulation, cationic lipid technology, electroporation, and ultrasonic deliveries are promising experimental techniques but need further improvements. The accessibility of the epidermis presents an opportunity to test many localized administration methods and monitor phenotypic changes.

Mechanistic studies to identify the rate-limiting step and critical proteins involved in the gene correction process continue to play an important role in the development of ODN-directed gene alteration. Introduction of a specific double-strand break at the targeted loci and modulation of cellular activities to increase homologous recombination will be necessary to make ODN-directed gene alteration practical. We are constantly reminded that the expectation of oligonucleotide-directed gene repair as "a quick and easy method to use without selection procedures" was generated by initial studies lacking solid mechanistic understanding. Systematic mechanistic studies together with the development of reliable, sensitive, and standardized assays to measure gene correction are essential to develop this technology for the practical use.

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