Gene Therapy for Autosomal Dominant Disorders of Keratin

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Dominant mutations that interfere with the assembly of keratin filaments cause painful and disfiguring epidermal diseases like pachyonychia congenita and epidermolysis bullosa simplex. Genetic therapies for such diseases must either suppress the production of the toxic proteins or correct the genetic defect in the chromosome. Because epidermal skin cells may be genetically modified in tissue culture or *in situ*, gene correction is a legitimate goal for keratin diseases. In addition, recent innovations, such as RNA interference in animals, make an RNA knockdown approach plausible in the near future. Although agents of RNA reduction (small interfering RNA, ribozymes, triplex oligonucleotides, or antisense DNA) can be delivered as nucleotides, the impermeability of the skin to large charged molecules presents a serious impediment. Using viral vectors to deliver genes for selective inhibitors of gene expression presents an attractive alternative for long-term treatment of genetic disease in the skin.

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Gene-based therapies depend on several critical elements. First, one must have a disease gene. "Having a disease gene" implies having an understanding of the disease-causing mutations and how they contribute to pathology. Second, one must have a therapeutic gene, which may be a wild-type (normal) version of the disease gene or may be another gene that suppresses the disease phenotype, for example, one whose product prevents apoptosis. Third, gene therapy requires an efficient delivery system. This delivery system may be a virus or a formulated nucleic acid, but should permit specific expression of the therapeutic gene according to cell type. Finally, one should have an animal model that recapitulates both the genetic and the phenotypic characteristics of the disease and permits testing of potential treatments. Animal models include knockout or transgenic mice and spontaneous mutations leading to a disease of domestic animals that is analogous to the human disorder. In this review, we will use these criteria to evaluate the prospects of gene therapy for dominant keratin diseases, emphasizing innovations such as transcriptional silencing and RNA interference as the most promising genetic therapeutics in the near term.

Keratin Mutations—Disease Genes

Mutations in keratin genes cause oral, ocular, epidermal, and hair-related diseases (Corden and McLean, 1996; Fuchs and Cleveland, 1998; Coulombe and Omary, 2002; Kirfel *et al*, 2003; Porter and Lane, 2003). The study of these keratin diseases has contributed to our understanding of the function of intermediate filaments in epithelial cells, but the large number of these genes, more than 50, indicates that the study of keratin biology will continue to be fruitful for many years to come (Hesse *et al*, 2004). Pairs of type I and type II keratins are co-expressed in a developmentally regulated, tissue-specific manner and form heterodimers that are assembled into filaments. These intermediate filaments are the main stress-bearing cytoskeletal components within the affected epithelial cells. Consequently, the pathology associated with keratin mutations is determined by the expression pattern of the defective proteins. For example, type I keratin K16a and type II keratin K6a are co-expressed in the palmoplantar epidermis and the orogenital squamous epithelium. They are also induced by wounding. Missense mutations in either of these proteins may result in pachyonychia congenita (PC), which is characterized by hypertrophic nail dystrophy and palmoplantar keratoderma, although not by defects in wound healing. Most of the disease-causing mutations in keratin are missense mutations, and most affect conserved sequences at the termini of the central rod domains of the molecules (Coulombe and Omary, 2002). These regions are probably important for correct assembly of the keratin filaments.

Nearly all of the human keratin disorders are associated with dominant mutations. Such an inheritance pattern is typically caused either by *haploinsufficiency* or by toxic *gain-of-function* mutations. Haploinsufficiency implies that a single normal allele does not provide a sufficient amount of protein to support cell viability or function. A common example is polycystic kidney disease associated with premature termination mutations in the PKD1 gene that affect about one in 1000 people of European decent. Most

Abbreviations: AAV, adeno-associated virus; dsRNA, double-stranded RNA; LTR, long terminal repeat; ODN, oligodeoxynucleotides; PC, pachyonychia congenita; RISC, RNA inducible silencing complex; shRNA, short hairpin RNA; siRNA, small interfering RNA; TFO, triplex-forming oligonucleotides; ZFN, zinc-finger nuclease

patients have loss-of-function mutations in one allele of PKD1. Toxic gain-of-function mutations are caused by either missense or chain termination mutations that result in the accumulation of abnormal proteins. In some cases, these proteins lead to death of the affected cells. Such is the case with rhodopsin mutations leading to autosomal dominant retinitis pigmentosa that lead to the apoptotic death of rod photoreceptor cells. Dominant mutations may also disrupt the assembly of multimeric protein complexes. Examples include cardiac myosin heavy chain mutations leading to familial hypertrophic cardiomyopathy and collagen I defects leading to osteogenesis imperfecta. Keratin diseases such as epidermolysis bullosa simplex and PC are dominant for this reason: missense mutations interfere with the assembly of multimeric keratin filaments.

Gene Therapy for Dominant Diseases—Why Supplementation Will Not Suffice

The genetic approach to treating such a dominant disorder is more complex than that for a simple recessive disease caused by the lack of a particular protein. In the case of toxic mutations, it does not suffice to replace a missing function by delivering a "healthy" gene. Rather, the therapy must block the production of the defective gene product. The first approach for blocking protein production employed antisense RNA and DNA, and this was followed shortly thereafter by the use of ribozymes. Such approaches to therapy fall into two categories—*nucleotide* therapies and *gene* therapies. The first class includes antisense oligonucleotides, triplex-forming oligonucleotides (TFO), and short double-stranded interfering RNA. The second class comprises genes that encode nucleotide inhibitors such as small interfering RNA (siRNA) and ribozymes and also genes for proteins that may repress the transcription of specific genes or enhance directed gene repair. Although this approach to gene therapy has lagged behind that for recessive diseases, several new technologies and delivery systems are making gene silencing for keratin disorders seem plausible.

Epidermal Stem Cells

The accessibility of the skin and the characterization of its stem cells will facilitate the development of gene-based therapies for epidermal keratin diseases (Watt, 2000; Potten and Booth, 2002). Nevertheless, the compartmentalization of the epidermis and its continuous self-renewal pose obstacles for long-term therapy that can be overcome only by genetic transduction of epidermal stem cells. The bulge region of adult hair follicles contain multipotent progenitor cells that can reconstitute a wounded epidermis and respond to morphogenic stimuli by forming hair follicles, sebaceous glands, and epidermis (Oshima *et al*, 2001; Panteleyev *et al*, 2001). But using retroviral tagging, Ghazizadeh and Taichman demonstrated that the contribution of hair follicles to the epidermis was restricted to the rim of epidermis surrounding the follicle and that the interfollicular epidermis was independent of follicular stem cells. Their work suggests that there may be multiple stem cell lineages within the epidermis and that some of these have restricted cell-fate in the absence of injury (Ghazizadeh and Taichman, 2001). The epidermis of the mouse is comprised of functionally distinct proliferative units, consisting of a stem cell in the basal layers of the dermis and its dividing cell progeny, which give rise to a column of differentiated keratinocytes (Gambardella and Barrandon, 2003) (Fig 1). In the mouse, this "column" can be two to four cells. Long-term gene therapy for keratin diseases must target these stem cells and yet deliver genes that are appropriately regulated until the progeny of these cells differentiate (Ghazizadeh *et al*, 2002). In contrast, oligonucleotide-based gene silencing should affect the differentiated keratinocytes themselves. Such treatments will require periodic re-application. These issues have been discussed in earlier excellent reviews (Khavari and Krueger, 1997; Somani *et al*, 1999; Spirito *et al*, 2001).

Gene therapies for dominant disease can alter gene expression at two levels: at the level of the gene (chromatin) or at the level of RNA (transcript) (Fig 2). Approaches at the gene level encompass *gene correction*, *gene ablation* and *gene silencing*. At the RNA level, ribozymes, siRNA, or antisense DNA can be used to cleave mutant transcripts or to block their translation into protein. Interposing at the level of gene should be superior to intervening at the level of the transcript, as the expression of the disease-causing allele can be blocked completely and perhaps permanently.

Gene Correction—The Gold Standard of Gene Therapy

Correcting the mutation that leads to disease is the ultimate objective of gene therapy (Richardson *et al*, 2002). This approach is superior to gene supplementation (i.e., adding an

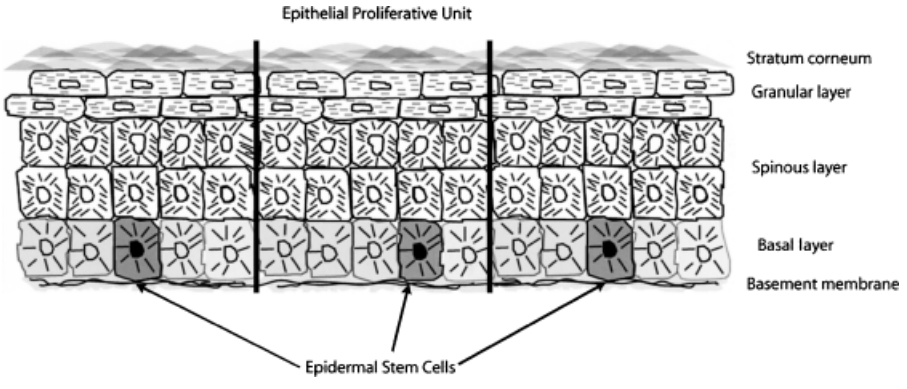

Figure 1
Columnar organization of the epidermis into discrete units with individual stem cells. Based on Alonso and Fuchs (2003).

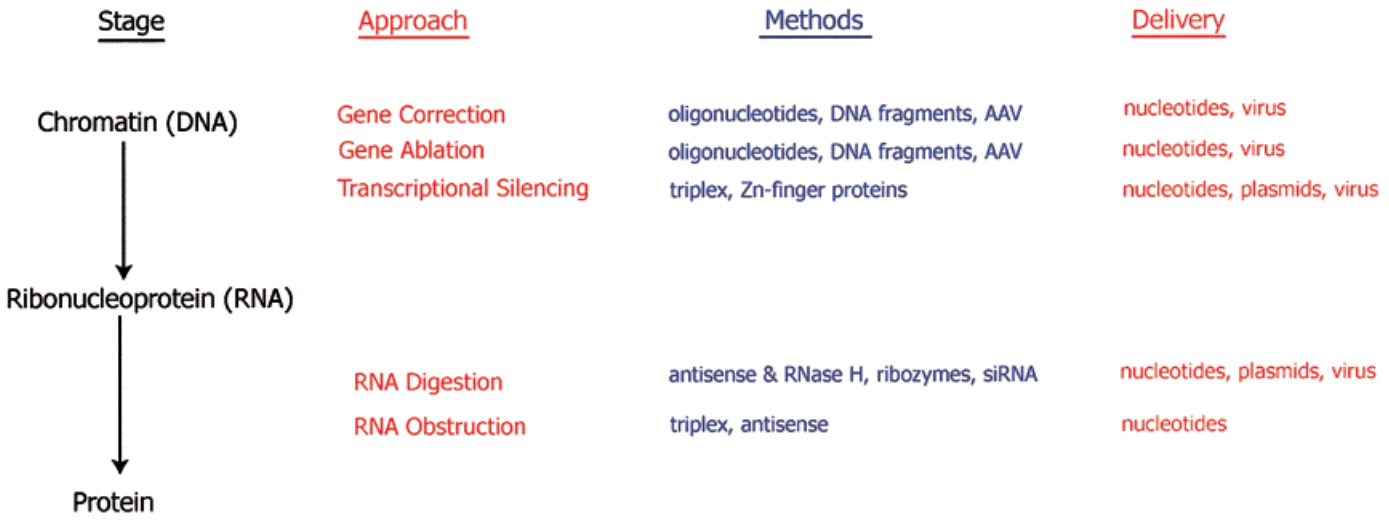

Figure 2
Gene therapy for dominant diseases should either correct a mutant gene or suppress the synthesis of its product. Such therapies target either chromatin in the nucleus (DNA) or ribonucleoprotein in the cytoplasm (RNA). At the DNA stage, the ideal approach is gene correction, which may be mediated by oligonucleotides, DNA fragments or adeno-associated virus (AAV). Alternatively, genes may be knocked out using the same approaches or silenced using triplex-forming oligonucleotides or zinc-finger transcription factors. In the cytoplasm, antisense RNA (with RNase H) or ribozymes or siRNA can be used to digest transcripts and lead to their degradation. Alternatively, antisense DNA or triplex-forming oligonucleotides can interfere with the translation of the mRNA.

extra wild-type cDNA via virus-mediated transduction) because the repaired gene will reside at the normal chromosomal locus and be regulated by its own promoter and regulatory elements. Despite this consensus among gene therapists, this "gold standard" has been difficult to achieve. Several approaches have been attempted in tissue culture and even in animals. These include using RNA–DNA oligonucleotides, single-stranded DNA oligonucleotides, homologous replacement with small DNA fragments, TFO, and repair with the single-stranded adeno-associated virus (AAV). As the mechanism by which mutations are corrected (recombination, primed replication, mismatch repair, etc.) has not been elucidated, this approach has been referred to as "gene targeting" or "gene correction." Without genetic selection, the frequencies of gene repair of endogenous chromosomal genes by these methods is generally low (10^{-6}–10^{-5} per cell), especially *in vivo*. But the ability to induce double-stranded breaks in the target gene using site-specific endonucleases or TFO has increased the probability of successful gene repair.

Oligonucleotide-Based Gene Targeting

RNA–DNA oligonucleotides (also called RDO or chimeroplasts) are designed to induce single nucleotide changes in homologous DNA sequences in mammalian cells. The original design of RDO contained two strands of about 25 nucleotides identical in sequence to the target except at the site of the host mutation; one strand containing both ribo- and deoxyribonucleotides and a complementary strand containing only DNA (Cole-Strauss *et al*, 1999). This method has been particularly successful in correcting mutations in episomes for which selection was possible, but has also been used for the modification of chromosomal genes. For example, RDO were used to convert the factor IX gene in hepatocytes and for the repair of the sickle-cell hemoglobin in lymphoblasts. Subsequently, it was determined that short DNA oligonucleotides of 40–50 nucleotides have the same capacity for inducing DNA repair as do RDO. Oligodeoxynucleotides (ODN) have been used to correct an integrated copy of the *lacZ* gene in mouse embryonic stem cells (Pierce *et al*, 2003) and to modify the c-kit and the tyrosinase gene in single melanocytes from albino mice (Alexeev *et al*, 2002). In this case, the co-conversion of the tyrosinase gene gave a visual marker by which cells undergoing repair events could be screened. Transcription apparently facilitates the formation of intermediates in ODN-directed repair, and the non-transcribed strand is more frequently the target of correction, as antisense ODN appear more potent than those with sense strand polarity (Igoucheva *et al*, 2003).

Although high levels of gene repair were reported in the liver, the reproducibility of gene repair by RDO has been questioned because some researchers have failed to achieve a significant level of gene alteration. As pointed out by Yoon *et al* (2002), the supposition that gene correction using oligonucleotides would be quick and easy was naive. Large amounts of oligonucleotides are employed, and quality control is essential. Highly sensitive assays are required, and these must be properly controlled. Different cell types have different repair competence, probably based on the activities of recombination and repair enzymes. Given the low frequency of oligonucleotide-directed gene alteration, a selection procedure is essential to make the gene-repair technology practical. This implies an *ex vivo* approach will be required in which stem cells are manipulated, and those with repaired genes are expanded and re-introduced into the tissue.

Homologous Replacement with DNA Fragments

As an alternative to oligonucleotide-stimulated gene repair, small fragment homologous replacement uses double-

stranded DNA fragments to restore a normal sequence to mutated genes. In this method, 400–800 bp segments of DNA are introduced into cells, typically using electorporation (Goncz *et al*, 2002; Gruenert *et al*, 2003). This DNA contains the wild-type sequence and is intended to replace or repair the mutant gene, although the exact mechanism is not known. In a model system, a 4 bp insertion in the Zeocin resistance gene was corrected, concomitantly restoring antibiotic resistance and an *Xma*I restriction site (Colosimo *et al*, 2001). Homologous replacement has been used to correct the dystrophin gene in the *mdx* mouse model of muscular dystrophy (Kapsa *et al*, 2001), the β-globin gene in human hemopoietic progenitor cells (Goncz *et al*, 2002) and the cystic fibrosis transmembrane conductance regulator in human airway epithelial cells from cystic fibrosis patients (Sangiuolo *et al*, 2002). As with ODN-stimulated gene repair, homologous replacement requires introduction of large amounts of DNA, and efficiency is low (1 correction event per 10^5–10^7 cells).

Targeted Correction with AAV

Because of the low rate of gene correction using DNA fragments and oligonucleotides, Russell and Hirata (1998) and Russell *et al* (2002) have used AAV to stimulate repair. AAV has demonstrated considerable advantages for conventional gene therapy (Flotte, 2004), but because it efficiently delivers single-stranded DNA to the nucleus, it can also serve as a donor for normal genetic information. For the type I collagen (COL1A1) gene, up to 1% of normal human fibroblasts were corrected using gene targeting by AAV (Hirata *et al*, 2002). AAV-mediated gene targeting can introduce 1 or 2 bp substitutions and even insertions up to 1.5 kb (Hirata and Russell, 2000; Inoue *et al*, 2001). Thus, in cells the frequency of correction using AAV is much higher (~10,000-fold) than that typically obtained by other gene targeting methods. It should be pointed out that these results were obtained in tissue culture and rely on phenotypic selection for correction events. In the absence of selection, much lower correction frequencies (0.006%) were observed in primary mouse fibroblasts and in the tibialis muscles of mice (Liu *et al*, 2004). Nevertheless, Chamberlain *et al* (2004) used AAV vectors and selection for G418 resistance to specifically disrupt a mutant allele of the COL1A1 gene in mesenchamal stem cells from patients with osteogenesis imperfecta. As heterozygous mice that carry disrupted keratin genes showed no obvious structural and functional defects of the skin, it is likely that such a targeted disruption approach could be used to block the expression of mutant keratin 6a or keratin 16 in stem cells from patients with PC-1.

Two technologies have been used to increase repair by oligonucleotides, TFO and site-specific endonucleases. Both triplex-forming ODN and site-specific endonucleases generate double-strand breaks near the mutation site and therefore increase recombination repair. TFO can also be used to block the transcription of DNA.

TFO

Triplex DNA was first recognized in 1957, but only during the past 15 y has this structure been exploited to interfere with gene function. TFO are single-stranded oligonucleotides that form triple-stranded DNA by binding in the major groove of polypurine: polypyrimidine runs in the DNA duplex. The TFO binds to the purine strand of duplex DNA with high affinity and according to a recognized binding "code." Mismatches in this code generally destabilize the triple helix, but because triplex formation does tolerate some sequence variation, much effort has been directed toward developing nucleotide analogues that would extend the third-strand-binding code so that triple helix formation would not be restricted to homopurine or homopyrimidine runs (reviewed in Gowers and Fox, 1999).

TFO have been used in a variety of ways as sequence-specific, DNA targeting reagents (reviewed in Knauert and Glazer, 2001). TFO have been linked to reactive moieties to produce site-specific cleavage of DNA and to block enzymes that cleave or modify DNA. For example, by interfering with RNA polymerase binding and mRNA elongation, they can inhibit transcription.

Gene Targeting Using TFO

Heritable alterations in DNA by TFO were first demonstrated using a TFO linked to a psoralen molecule (Takasugi *et al*, 1991). Site-specific mutations were identified initially in triplexes formed *ex vivo* and subsequently in triplexes formed in episomal and then chromosomal DNA targets. These psoralen-TFO experiments established the concept that DNA-binding molecules can be used to direct site-specific, heritable genome modification. Equally important, they showed that cell and nuclear membranes and the packaging of DNA into chromatin presented no absolute barriers to gene targeting with TFO. In the course of those studies, it became apparent that TFO alone, even in the absence of linkage to a psoralen, could induce site-specific mutations. Mechanistic studies demonstrated that TFO binding to DNA stimulates DNA repair synthesis, and that intact DNA repair pathways are required for the production of TFO-induced mutations (Knauert and Glazer, 2001).

The potential utility of TFO as gene therapy reagents was greatly expanded by the recent discovery that TFO stimulate recombination (reviewed in Seidman and Glazer, 2003). TFO stimulate intrastrand, chromosomal DNA recombination at high frequency (1%) following intranuclear injection but at considerable lower frequency when delivered to the culture medium (Luo *et al*, 2000). TFO increase the frequency of donor oligonucleotide-directed, sequence-specific base changes in DNA in close proximity to the triplex (Chan *et al*, 1999). The concomitant delivery of a homologous "donor" oligonucleotide and an adjacent TFO raises the frequency of donor-mediated, sequence-specific changes at least 5-fold. In studies that have been done to date, higher frequencies of targeting were observed if the donor was covalently tethered to the TFO than when the oligonucleotides were unlinked, and double-stranded donors gave higher frequencies of correction than single-stranded donors.

It is important to acknowledge several potential obstacles to general use of TFO as gene targeting reagents, and to recognize that progress is being made in addressing

each of those issues (Seidman and Glazer, 2003). Even with synthetic oligonucleotide mimetics, the binding code limits the sequences that can be targeted, and intracellular pH and ion concentration conspire to reduce binding coefficients below those achieved in ideal solution. Physical, chemical, and metabolic barriers limit delivery and biological effectiveness of oligonucleotides, as indicated by the intranuclear injection experiments cited above. Finally, all gene targeting methods are prone to a low frequency of error, which may be introduced during recombination-mediated repair or by non-homologous end joining. Errors introduced by TFO appear to be limited to the vicinity of the TFO-binding site.

The existence of a triplex site at the beginning of the K6a gene makes it a reasonable model in which to explore and expand the usefulness of TFO-mediated gene targeting. The ideal treatment for PC would be one that selectively repairs or inactivates the mutant K6a allele, leaves the normal K6a allele intact, incurs no risk of genomic alteration in sequences other than the targeted sequence, and can be accomplished with reasonably high efficiency, so that a lasting effect can be achieved after a limited number of treatments. But it is possible that gene targeting could inactivate the wild-type allele only, which for some genes would make the disease phenotype worse.

Several considerations recommend a TFO-mediated gene inactivation strategy rather than a correction strategy. First, complete inactivation of K6a would probably be curative, as there appear to be highly related genes with redundant function; animals lacking a single K6 gene have no clinical phenotype. Second, the inactivation strategy should work in individuals harboring mutations anywhere in this gene. Third, as discussed elsewhere in these *Symposium Proceedings*, there are reasons to believe that an improvement in phenotype might occur if even fewer than 100% of keratinocytes had their mutant allele inactivated; moreover, the K6a gene might be one of those genes for which cells having one wild-type and one inactivated allele might have a selective growth advantage over cells that retain a mutant allele. Fourth, biologically active oligonucleotides can be given to humans with few serious risks or side effects. Finally, a correction strategy was dismissed for the following reasons. There is no polypurine run in the vicinity of the known mutations in the human K6a gene. Furthermore, as all gene targeting strategies have some inherent error rate, if an error, rather than a correction, were introduced at one of those critical, helix-coding regions of a keratin gene, an exacerbation in (dys)function might occur; errors in the noncoding 5′ end of the coding sequence are much less likely to have deleterious effects.

The fortuitous polypyrimidine/polypurine run at the beginning of exon 1 in the K6a gene suggests a gene inactivation strategy that utilizes a TFO to stimulate oligonucleotide-mediated introduction of a stop codon early in the coding sequence of K6a (see Fig 3).

Gene Targeting Stimulated by Double-Strand Breaks—Zinc-Finger Nucleases (ZFN)

On the supposition that gene repair is mediated by homologous recombination, several groups have used site-specific endonucleases to cleave double-stranded DNA near the site of mutation and to induce correction by double-strand break repair (Resnick and Martin, 1976). The rare cutting endonuclease from yeast, I-SceI, stimulates homologous recombination at the HPRT locus (Brenneman *et al*, 1996), and delivering this enzyme increases the level of plasmid-based gene correction events to 1% (Donoho *et al*, 1998). This approach also promotes gene correction by AAV. Miller *et al* (2003) engineered a site for the I-SceI endonucleases within the *lacZ* gene and integrated this modified marker into the genome of human cell lines using a retrovirus. Delivering the gene for Sce-I with a second retrovirus increased the frequency of AAV-mediated gene repair in this system by 60–100-fold. Similar results were obtained by Porteus *et al* (2003) who repaired a chromosomally integrated but defective allele of GFP.

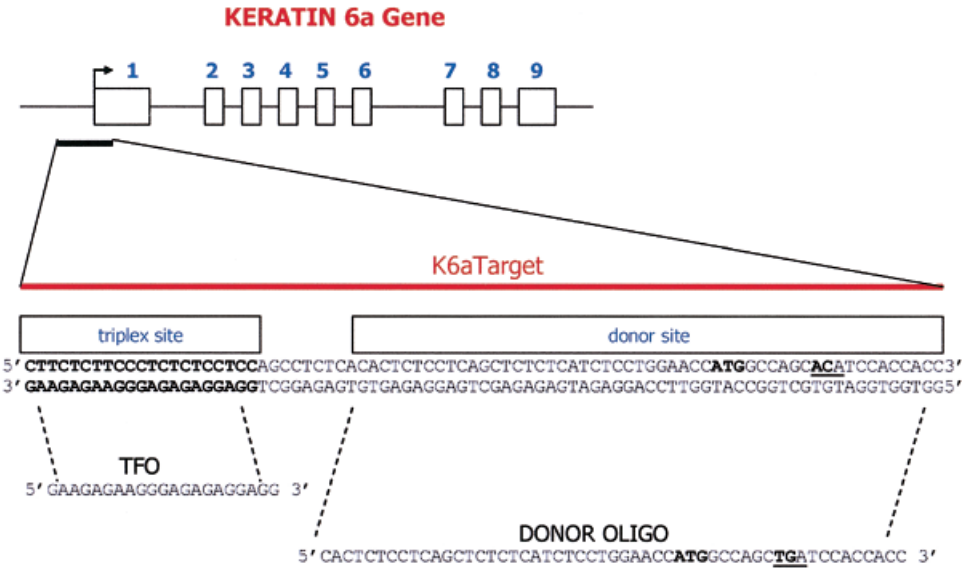

Figure 3
The introduction of a premature stop codon in K6a using a triplex-forming oligonucleotide (TFO) and donor oligonucleotide. The TFO and donor oligos can be uncoupled or tethered together by a flexible linker.

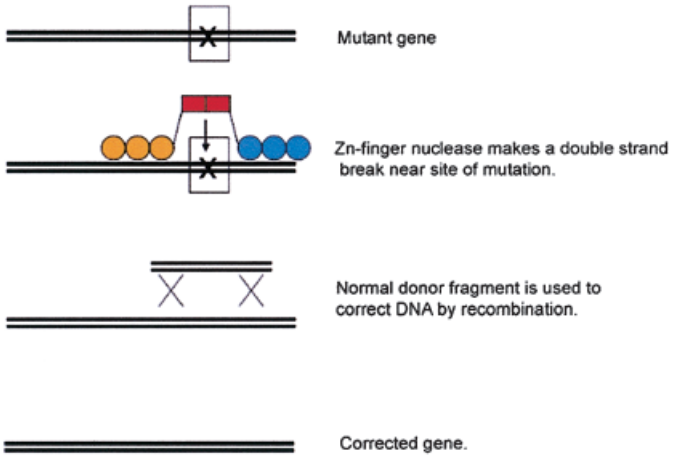

Figure 4
Targeted gene repair using a zinc-finger endonuclease. Although this scheme shows the chromosomal sequence as the nuclease target, the donor sequence may also be cut.

To make this double-strand break repair relevant to gene therapy, two groups have recently shown that site-specific endonucleases can be engineered using zinc-finger DNA-binding domain (Bibikova *et al*, 2003; Porteus and Baltimore, 2003). The Cys2–His2 zinc-finger is comprised of 30 amino acids that fold into a $\beta\beta\alpha$ structure stabilized by the coordination of a zinc cation. It is one of the most common protein motifs encoded in the human genome. This structure recognizes a 3-nucleotide sequence in the major groove of double-stranded DNA and makes contacts mainly in one strand. Several research groups have used the principles of rational design or selection on phage displays to generate zinc-finger domains with novel specificities (Dreier *et al*, 2000, 2001; Liu *et al*, 2002). In this way, zinc-finger domains have been identified with high affinity for all of the 5′GNN3′ and many of the 5′ANN3′ trinucleotides.

This adaptability of zinc-finger domains has been employed to generate sequence-specific nucleases for gene targeting. For example, Bibikova *et al* (2003) designed a pair of zinc-finger domains linked to a non-specific nuclease (*Fok* I) that must dimerize in order to cleave the DNA (Smith *et al*, 2000) (Fig 4). Each zinc-finger domain recognizes a 9 bp DNA sequence, so that the total recognition sequence is an unambiguous 18 bp. Coupling the zinc-finger domains to the nuclease using a spacer sequence provides a high specificity ZFN. They used an 8 kb mutant donor fragment homologous to the Drosophila *yellow* (*y*) gene and containing the cut site for the ZFN. When expression of the donor fragment and both components of the dimeric nuclease were co-expressed in embryos, a high frequency (2%) of yellow progeny was recovered, and most of these were the result of homologous recombination. Porteus and Baltimore (2003) have also used a ZFN to stimulate targeted repair of the GFP gene in a human cell line. In a promising recent development, Urnov *et al* (2005) used ZFN to achieve correction of a SCID mutation in the *IL2R*γ gene in 18% of treated human cells in the absence of phenotypic selection.

The stimulation of recombination by double-strand breaks is a property of all nucleated cells, so that the use of designed ZFN to improve the frequency of gene repair should be widely applicable. As noted above, gene repair must occur in stem cells in order to be sustained in tissue. It is unlikely that single base-pair substitutions can be discriminated by ZFN, so that cells in which the mutant chromosome has been repaired must be selected and transplanted into the patient. To identify and expand these cells, some selectable marker, for example an antibiotic resistance gene, may be included in the flanking wild-type donor DNA. This gene would be co-transferred with the wild-type keratin sequence at some frequency, a phenomenon called co-conversion (Orr-Weaver *et al*, 1988). As including a selectable marker decreases the sequence identity between the mutant chromosome and wild-type donor DNA, the frequency of gene repair can be expected to drop significantly.

Zinc-Finger Proteins for Transcriptional Silencing

Zinc-finger proteins can be used to regulate gene expression in a variety of ways (reviewed in Jamieson *et al*, 2003). In a common application, activation or repression domains are combined with site-specific DNA-binding domains to produce novel transcriptional regulators. Typically, the p65 domain from the transcription factor NF-κB or the VP16 domain from herpes simplex virus is used for activating transcription. Zinc-finger DNA-binding domains can also be used to inhibit transcription by adding the KRAB domain, which creates a zinc-finger transcriptional repressor that mimics the naturally occurring KRAB proteins (Thiesen *et al*, 1991). Designing a zinc-finger transcription factor involves identifying an accessible region of the promoter. In practical terms, this means identifying nuclease sensitive and hypersensitive sites in the upstream (5′) portion of the gene. These DNA sequences can be used as bait to identify zinc-finger-binding domains in a phage display library of such motifs (Rebar and Pabo, 1994). Three or more zinc-finger domains can then be assembled to recognize a 9 bp site. Dissociation constants should be in the low nanomolar range. As with the ZFN, dimerization domains can be used to join two zinc-finger proteins, and to increase the recognition specificity. Creating these proteins requires several areas of expertise and is not off-the-shelf technology.

Zinc-finger proteins have been used in this way to stimulate transcription of the erythropoietin (Epo) and vascular endothelial growth factor genes (Snowden *et al*, 2003). Zinc-finger repressors have reduced transcription of *CHK*2 gene (Tan *et al*, 2003) (which encodes a protein phosphatase required for cell cycle progression) and the peroxisome proliferator activated receptor-γ. As inhibitors of gene expression, zinc-finger repressors have potential in blocking synthesis of toxic gain-of-function proteins, such as mutant keratins. They are at least as potent as another fashionable technology, siRNA. The catch is that transcriptional repressors will limit production of *both* mutant and normal keratin alleles, as the mutations leading to disease do not affect the promoter region. Blocking total production of a specific keratin may be acceptable, if another filament protein can replace it in the affected cell lineage. As

indicated, mice deficient for K6a and K6b are phenotypically normal because of the presence of a third K6 gene. Alternatively, a synthetic transcription factor can be employed to block production of both alleles of an affected gene, for example, and a wild-type cDNA can be supplemented using a different regulatory sequence that permits cell-specific expression. Such a strategy might be pursued most effectively *ex vivo* followed by re-introduction of genetically modified cells.

Blocking Expression at the RNA Level—Antisense Technologies

Although gene correction and transcriptional silencing have theoretical appeal, blocking gene expression by preventing translation may be as effective. More importantly, technology for knocking down mRNA transcripts—antisense oligonucleotides, ribozymes, and RNA interference—are closer to application in the near future. Each of these methodologies has been exhaustively reviewed, so that in this summary we will only indicate the strengths and weaknesses of each approach as it applies to PC and other inherited keratin diseases.

Antisense ODN

The use of antisense ODN, now 25 y old, is the oldest of these technologies (reviewed in Crooke, 2004). Antisense technology exploits oligonucleotide analogs (typically around 20 nucleotides) that bind complementary RNA sequences through conventional base pairing, resulting in the destruction or inactivation of the target RNA. Although some antisense oligonucleotides block gene expression by preventing protein synthesis, it is clear that the most active ODN lead to the RNase H-mediated degradation of target mRNA. RNase H degrades the RNA strand of an RNA–DNA duplex. It has been identified in all eukaryotic cells and is encoded by some viruses. DNA sequences as short as four nucleotides activate RNase H.

The design of antisense ODN is important. Antisense ODN are typically added as modified oligonucleotides to increase their stability in cells and tissues, but oligonucleotides with sugar modifications resembling RNA, such as 2′-fluoro or 2′-methoxy, do not serve as substrates for RNase H. In addition, some backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates are not substrates of the enzyme whereas phosphorothioates are excellent substrates. But some chimeric oligonucleotides that bind to RNA do activate RNase H. For example, oligonucleotides comprising 2′-*O*-methoxyethyl phosphonate sequences flanking a five-nucleotide stretch of deoxyoligonucleotides bind to their target RNA and activate RNase H. Such molecules were shown to have essentially identical potency and specificity as siRNA in a head to head comparison using four different human mRNA and 80 different hybridization positions (Vickers *et al*, 2003).

Phosphorothioate-modified ODN have been used successfully to block the expression of a variety of viral and cellular genes in tissue culture and in animals (Dean *et al*, 1996; Dean and Bennett, 2003). Antisense oligonucleotides have also been employed to modify the splicing patterns of human β-globin pre-mRNA as a potential therapy for thalassemia (Suwanmanee *et al*, 2002). ODN are attractive as therapeutics because several antisense compounds are already approved and on the market and especially because their formulation and pharmacodynamics have been studied extensively (Crooke, 2004). Of particular relevance to PC is that antisense ODN can be applied in a skin cream and permeate the epidermis and dermis in animals and in humans. Epidermal hyperproliferation in psoriasis has been reversed by the use of antisense ODN specific for the insulin-like growth factor I mRNA (Wraight *et al*, 2000). Alicaforsen (Isis Pharmaceuticals, Carlsbad, CA), an inhibitor of intercellular adhesion molecule 1, accumulated throughout the dermis and epidermis after topical administration and has shown promise in a Phase II clinical study undertaken by Isis Pharmaceuticals. It should be noted that the efficiency of topical delivery is very low, however.

Ribozymes

Ribozymes are catalysts comprised of RNA (Doudna and Cech, 2002). Eight naturally occurring ribozymes have been discovered, and each is involved in cleaving and/or forming a phosphodiester bond in RNA or DNA, sometimes as part of the replication cycle of an RNA virus or satellite RNA, sometimes as a step in autocatalytic splicing and sometimes as part of a retrotransposition event. *In vitro* selection has resulted in additional RNA and DNA enzymes capable of catalyzing novel reactions, including the formation of peptide bonds and the aminoacylation of tRNA. The small hammerhead and hairpin ribozymes derived from tobacco ringspot virus satellite have found the greatest number of applications for gene therapy. These ribozymes can catalyze hydrolysis of second RNA molecules at a variety of cleavage sites: Hammerhead will cleave after the dinucleotide UX, as long as X is not a guanosine. Hairpin ribozymes cleave in the sequence BNGUC, where B is not adenosine, and N is any nucleotide. Such sequences are found in any mRNA. Once a target RNA is cleaved by a ribozyme, it is rapidly degraded by cellular nucleases. Like antisense ODN, ribozymes have been applied for inhibiting the expression of dominant disease genes, the replication of RNA and retroviruses and the expression of oncogenes (Lewin and Hauswirth, 2001).

Ribozymes have a theoretical advantage and two practical disadvantages with respect to antisense oligodeoxyribonucleotides. The advantage is that they exhibit enzymatic turnover and therefore can block the accumulation of an mRNA in sub-stoichiometric amounts. In the one study in which ribozymes and antisense inhibitors were directly compared, ribozymes reduced the target RNA between 2-fold and 10-fold more efficiently than antisense RNA depending on the target site (Hormes *et al*, 1997). The disadvantages are that ribozyme RNA is unstable and relatively expensive to synthesize, whereas DNA is stable and cheap. The use of modified nucleotides does stabilize ribozymes significantly, but at a cost of catalytic activity.

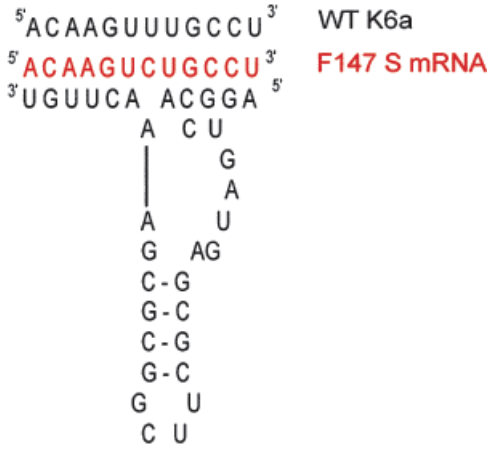

Figure 5
A hammerhead ribozyme specific for the F174S allele of K6a, which leads to pachyonychia congenita. The target sequence is shown in red, and the wild-type sequence is above it in black.

Synthesis of large amounts of RNA with necessary protective groups on their 2′ carbons and on the 3′ termini, is, at least by current standards costly (dollars per residue), compared with synthesis of DNA, either modified or straight (cents per residue). Consequently, ribozymes may have limited application as *nucleotide* therapeutics relative to antisense ODN and siRNA. Instead, ribozymes will be more effective as *gene* therapeutics. Following delivery with a recombinant virus or other delivery vector, ribozymes can be produced continually from within the target cell.

Because they are easy to design, hammerhead ribozymes are the most commonly used for gene therapy and hypothesis testing (Fritz *et al*, 2002). Basically one chooses an RNA sequence of 12 or 13 nucleotides with the target sequence (UX) in the middle and designs an antisense RNA complementary to that sequence, leaving the "X" unpaired and resulting in two hybridizing arms of the ribozyme (Fig 5). The 22 nucleotide catalytic domain is attached to antisense sequence so that both hybridizing arms have six nucleotides or the 5′ arm has five nucleotides and the 3′ arm of the ribozyme has six. Using a longer hybridizing sequence increases the affinity of the ribozyme for its target but reduces the enzymatic turnover, because it retards release of the cleaved product. Such ribozymes function as stoichiometric inhibitors. Similar considerations apply to the hairpin ribozyme, but the target site is more highly conserved.

Although design of hammerheads and hairpins is straightforward, finding highly active ones is not. Hammerhead target sites preceded by the trinucleotides GUC, AUC, CUC, and UUC and followed by the dinucleotides UU or UA tend to be the best substrates (Shimayama *et al*, 1995; Clouet-d'Orval and Uhlenbeck, 1997). Hybridizing arms that form base pairs with nucleotides in the ribozymes catalytic domain (and not with the target) lead to inactive ribozymes. In such short RNA, stable alternative structures can be predicted reliably using folding programs such as Mfold 3.0 (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/). Although most non-viral mRNA are not highly structured, some ribozyme target sites may be buried within a stable secondary structure element such as a hairpin loop. Computer prediction of the structure of long RNA (>100 nucleotides) are unreliable, but stable local folding can be identified. In the end, experimental verification of the accessibility of the target site must be obtained using RNA structure assays (e.g., sensitivity to nuclease or single-strand-specific modifying reagents) or by testing the ribozymes on full-length mRNA *in vitro* or in cells. As catalytic turnover defines a ribozyme's utility, multiple turnover (substrate excess) kinetic assays using oligonucleotide substrates are employed to identify the ribozymes with the highest activity, particularly at low magnesium concentrations. This is not a technology that lends itself to a kit.

Ribozymes do have an important advantage compared with antisense ODN and siRNA—they are more highly sequence specific. A single nucleotide substitution within four residues of a hammerhead cleavage site will block activity completely. In antisense oligonucleotides that are 20–25 nucleotides in length, single mismatches will lower affinity marginally but not prevent inhibition of a wild-type target. Similarly, 19–23 nucleotide siRNA are frequently insensitive to as many as three mismatches with a non-cognate target RNA. Consequently, should a ribozyme target site be located near the site of a disease mutation, allele-specific ribozymes could be generated to selectively eliminate the product of the disease gene. A ribozyme specific for the F174S mutation of K6a is shown in Fig 5.

The fact that ribozymes can be allele specific does not necessarily make them ideal for treating a dominant genetic disease unless there is a predominant allele causing most of that disease and unless there is a cleavage site associated with that mutation. This is not the case for the keratin diseases. Consequently, it may be desirable to develop a ribozyme that cleaves both mutant and wild-type keratin mRNA. Such a ribozyme should cleave a site not affected by the majority of disease mutations (i.e., away from boundaries of rod domain). Because the genetic code is degenerate, it is possible to produce a ribozyme-resistant cDNA for the keratin target and use this as part of an "ablate and switch" approach to reduce the level of toxic protein and replace it with normal protein (Fig 6). This method was suggested for rhodopsin mutations by Millington-Ward *et al* (1997) and has been tested in hepatocytes to replace a dominant-negative form of α-1-antitrypsin (Zern *et al*, 1999). It should also be applicable to RNA replacement using siRNA. As noted above, as mice deficient in keratin 6 exhibit no clinical phenotype, the "replacement" part of this strategy with the ribozyme- or siRNA-resistant gene may be unnecessary.

RNA Interference

The inhibition of gene expression (RNAi) using double-stranded RNA (dsRNA) has stimulated a revolution in molecular genetics, first in plants and in the nematode *Caenorhabditis elegans* and more recently in mammalian cells. This amazing scientific story has been amply recounted. Several recent papers (Dorsett and Tuschl, 2004; Mittal, 2004; Dillon *et al*, 2005; Huppi *et al*, 2005; Shankar *et al*, 2005) review the use of siRNA in mammalian cells. A further paper reviews the relationship between micro-RNA (miRNA)

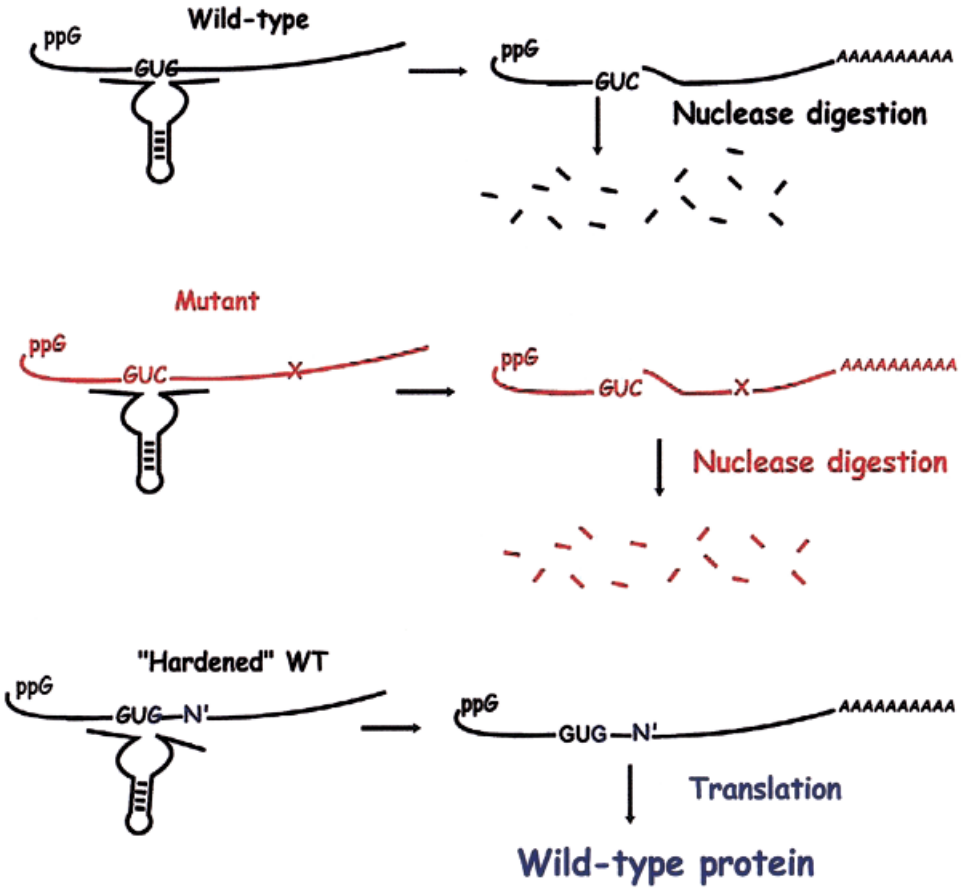

Figure 6
An RNA replacement strategy using ribozymes. A ribozyme is designed to cleave both mutant and normal RNA, leading to the turnover of these transcripts by cellular nucleases. Silent mutations are introduced into a cDNA making it ribozyme-resistant ("hardened WT"). The genes for the ribozyme and the hardened wild-type are introduced together. This same strategy can apply to siRNA induced RNA interference.

and the RNA interference apparatus (Murchison and Hannon, 2004).

Long dsRNA is toxic to mammalian cells through the protein kinase R and the 2′-5′ oligoadenylate pathways (together referred to as the interferon response). These pathways lead to an arrest of protein synthesis and to apoptotic cell death and are a component of our defense mechanism to prevent the spread of viruses. For this reason, long dsRNA fragments are not used to induce specific RNA interference in mammalian cells. When a short dsRNA ($<$30 bp) is artificially introduced into mammalian cells, it may have one of three effects. If it forms a perfect (or nearly perfect) match with the sequence of an endogenous mRNA on one of its two strands, it will lead to degradation of that mRNA using a ribonucleoprotein complex called RISC (RNA inducible silencing complex). Degradation of the cellular RNA is what is meant by RNA interference. If the dsRNA contains short segments of sequence disparity from the target RNA, it may block translation of that transcript, particularly if one strand of the dsRNA can bind in the 3′ untranslated region of the mRNA. An additional impact of the dsRNA may be the induction of transcriptional silencing through chromatin modification. This phenomenon has been well studied in plants and in fission yeast but is thought to occur in human cells as well.

dsRNA also arises endogenously in mammalian cells through the processing of micro-RNA. Micro-RNA are produced as RNA stem loops and are processed by an endonuclease called Dicer into 21–23 nucleotide duplexes with 5′ phosphate groups and 3′ single-stranded overhangs of two or three nucleotides. These dsRNA associate with RISC that unwinds the duplex RNA in an ATP-dependent reaction. There are over 200 miRNA encoded in the human genome, and, based on analogy with nematodes, these are thought to be important regulators of development and differentiation. Micro-RNA block the translation of their target mRNA. Most bind in the 3′ UTR of these targets (in the few cases in which those genes have been identified) and form discontinuous helices.

RNA interference, using exogenous siRNA, exploits the endogenous miRNA machinery. There is no fundamental difference between the two pathways: miRNA mutated to form a perfect match with the target RNA lead to cleavage by RISC, and introduced dsRNA that contain mismatches can block translation. Furthermore, *Drosophila* and other animals that maintain a specific RNAi pathway for defense have multiple Dicer enzymes. Mammals have only one—that used by miRNA and usurped by geneticists introducing siRNA.

siRNA and Short Hairpin RNA (shRNA)

Short interfering RNA can be introduced to cells and animals either as RNA or as DNA clones that code for the dsRNA. Usually, when clones are used, they produce shRNA that is processed by Dicer to form siRNA in the cell. For tissue culture work and temporary dosing to tissue,

direct delivery of siRNA has distinct advantages: Short RNA molecules are easily synthesized and are commercially available in pure form. dsRNA is more stable than single-stranded RNA and can be rendered even more stable by chemical modifications in the sense strand of the duplex. Such modifications have the added advantage of increasing the incorporation of the antisense strand of the duplex into RISC, therefore increasing cleavage of the intended mRNA. Using RNA also permits accurate dosing of the cells, as an exact amount of nucleic acid is added. The disadvantage of siRNA treatment is that large amounts of RNA are required (typically 10–100 nM final concentration) and the effect is temporary (three to four cell generations). Using a high dose of siRNA may saturate Dicer and other components of the miRNA apparatus and lead to non-specific effects, by preventing the activity endogenous miRNA. Identifying highly active siRNA should reduce this side effect.

Plasmids and viral vectors can be used to deliver shRNA, which are usually produced under the control of RNA polymerase III (pol III) promoters. These promoters generate large amounts of short RNA, but most is sequestered in the nucleus away from the cytoplasmic RISC. Consequently, some investigators have switched to pol II promoter systems for the expression of shRNA (Xia *et al*, 2002) or have disguised their siRNA hairpins as micro-RNA to facilitate transport to the cytoplasm (Boden *et al*, 2004). Delivery with viral vectors has the advantages that hard-to-transfect primary cells can often be infected and that expression of the RNA hairpin may remain stable over many cell generations, particularly if an integrating virus is employed. The massive effort focused on tissue-specific targeting of viral vectors should facilitate both systemic and site-specific delivery of shRNA. The use of plasmid or viral vectors also facilitates the generation of shRNA libraries, which are of use in functional genomics. In addition, delivery as DNA provides a relatively inexpensive way to screen a series of siRNA for their potency as inhibitors, compared with RNA duplexes, which currently cost over $250 each. As even modified duplex RNA is degraded in tissue, vector-based therapies will provide a continuous supply of siRNA, although the dosage may be difficult to estimate. Some hairpin RNA have been shown to stimulate the 2′5′ oligoadenylate pathway, even if they are kept short, and this represents a serious disadvantage to the delivery of siRNA as shRNA. In general, such "off target" effects are a pitfall of all RNAi methods and must be controlled for in experiments.

Designing siRNA

Most artificial siRNA contain 19 nucleotides of duplex RNA plus two unpaired nucleotides at the 3′ end (Fig 7). Adding 5′ phosphate is optional, as the duplex will be phosphorylated once inside the cell. RNA hairpins may be a bit longer, and are generally connected by a loop of six or more nucleotides based on the structure of a naturally occurring miRNA. (Most investigators feel that the loop sequence is of limited importance.) There are no absolute rules for selecting target sites for siRNA, but there are some guidelines. These are based on the lessons derived from hundreds of siRNA molecules tested in cells (Reynolds *et al*, 2004; Ui-Tei *et al*, 2004) and from the discovery that siRNA are loaded onto RISC asymmetrically (Khvorova *et al*, 2003). In general, any part of the mRNA can serve as a target, but regions of extremely high (>50%) or low (<30%) guanosine plus cytosine (G + C) content should be avoided. Sequences containing "G quartets" or stretches of four or more As are also poor substrates. Although global RNA folding programs are poor predictors of mRNA structure, and thus are useless in siRNA design, tight hairpins that form within the target RNA will be mimicked in the strands of the siRNA and may block RNA interference.

Other guidelines for siRNA design reflect the bias of RISC: There should be high thermal stability (high G + C) at the 5′ end of the sense strand (the strand with the same polarity as the mRNA) and low stability (high A + U) at the 3′ end of the sense strand in order to promote incorporation of the antisense strand into RISC (which proceeds in the 5′ to 3′ direction). Low thermal stability in the middle of the duplex seems to promote RISC-mediated cleavage of the target RNA, so a U is preferred at position 10. In practice, these guidelines are a useful starting place, but an empirical approach is required. Several companies (e.g., Imgenex, Ambion, Invitrogen) have developed plasmid vectors that permit the cost-efficient generation of siRNA by *in vitro* transcription. This allows the investigator to use standard transfection methods to walk down the mRNA and identify which potential siRNA work best. At that point, chemically synthesized duplex RNA can be bought or an RNA hairpin cloned in a viral vector. The substantial commercial support and user support for this technology makes RNAi accessible to any investigator with tissue culture skills.

Pitfalls of RNAi

The problems with RNAi have been widely discussed by its practitioners. These generally fall under the heading "off target" effects. Such effects have several possible sources, including induction of the interferon response, incorporation of the sense strand into RISC, and the cleavage of an RNA with partial sequence identity to the intended target. As few as eight or nine consecutive base pairs at the 5′ terminus of RISC-associated RNA is sufficient to mediate cleavage. Consequently, some have proposed using microarray analysis in association with each siRNA experiment to monitor potential off-target effects. A more practical approach, and one that applies to the other antisense technologies, is to employ two or more siRNA (ODN, ribozymes) for each target mRNA and to determine if the same concentration-dependent physiological consequences are observed. If two inhibitors of different sequence composition have identical results, then off-target events are a less likely explanation. Another way to avoid inducing non-specific effects with siRNA is to screen for the most potent inhibitors. Those that lead to substantial mRNA reduction at low nanomolar concentrations are less likely to lead to secondary effects by blocking normal miRNA-mediated processes. Because Dicer and RISC may be saturable, it is better to use one highly active inhibitor than to combine a series of mediocre ones.

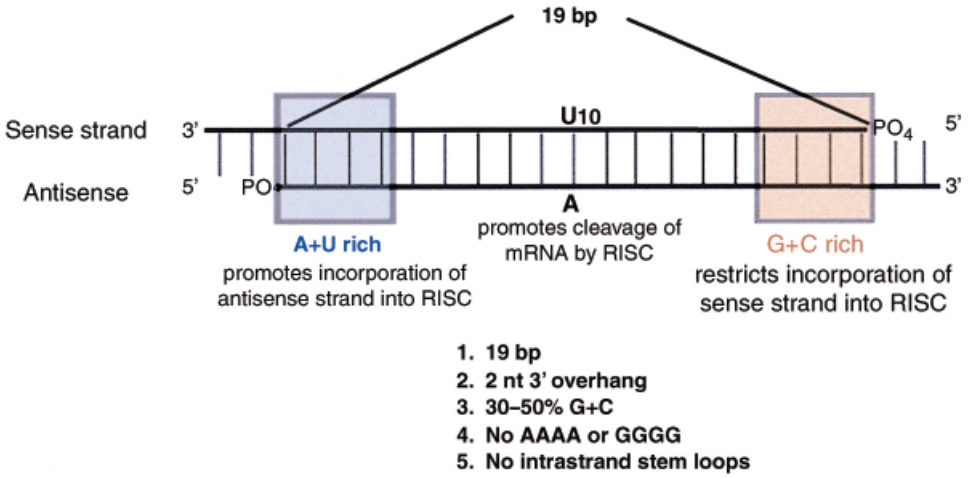

Figure 7
Guidelines for design of siRNA. See Mittal (2004).

Delivery of Genes as Nucleotides

The stratum corneum acts as the major barrier to penetration of molecules across the skin, and large negatively charged molecules such as antisense ODN, TFO, or siRNA will not easily penetrate it (Wraight and White, 2001). Topical application of simple aqueous solutions of oligonucleotides results in little penetration into the viable epidermis of human skin. Transfection of the skin using plasmids can be achieved by compromising the stratum corneum using intense brushing, adhesive glue stripping, or chemical depiliation. Specific formulations of nucleic acids including nanoparticles (Cui and Mumper, 2002) and lipid vesicles (Baca-Estrada *et al*, 2000) do increase their transfer to deeper layers of the skin (Maruyama *et al*, 2001; Babiuk *et al*, 2002; Prokop *et al*, 2002; Raghavachari and Fahl, 2002), but transfer is most efficient in hair follicles or in sites of wounding. Ironically, cationic phospholipids used to improve DNA delivery to cultured cells make delivery less efficient to skin *in vivo*.

As a consequence, techniques such as electorporation, microinjection, and particle bombardment have been tested in animals and in people (Maruyama *et al*, 2001; Meuli *et al*, 2001; Peachman *et al*, 2003). Oligonucleotide administration thus remains a critical area for research. Although antisense ODN, TFO and stabilized siRNA all have tremendous potential as topical agents for blocking the expression of dominant-negative keratin proteins, their exploitation demands a safe and tolerable delivery system. As it can be discontinued, topical delivery of therapeutic nucleotides is inherently safer than viral-mediated gene therapy. For this reason, and because it will require life-long administration, this approach will be attractive to the pharmaceutical industry.

Viral Gene Delivery

Viruses have been selected by nature for gene delivery to cells. For this reason viral vectors provide the most effective delivery vehicles for transmitting therapeutic genes into the body. Vectors based on adenovirus type 5 (Ad5) are efficient at gene delivery to the skin, and despite their inflammatory and immunogenic properties, can lead to expression of therapeutic genes over a period of weeks in wounded epidermis (Doukas *et al*, 2001; Gu *et al*, 2004). AAV, which like adenovirus can infect both dividing and quiescent cells, will also infect keratinocytes, and wild-type AAV will replicate in a helper independent fashion in differentiating cells (Meyers *et al*, 2000; Galeano *et al*, 2003). But in the absence of the viral rep protein, AAV vectors do not usually integrate into chromosomal DNA and are diluted out of replicating cells *in vivo*. They may therefore be unsuitable for long-term transduction of the epidermis (but see Hengge and Mirmohammadsadegh, 2000). Repetitive administration of Ad5 or AAV is precluded by the immune response to the vector.

For these reasons, retroviral vectors are the best current delivery system for long-term expression of therapeutic genes in the epidermis. Retroviral vectors integrate, express exogenous genes and provide an efficient transfer tool for human gene therapy applications (Mulligan, 1993). Vectors based on murine leukemia virus (MLV) have been used for transduction of epidermal skin cells *in vivo* (Ghazizadeh *et al*, 1999). Transcription in these vectors is dependent on the viral promoter/enhancer in the 5′ viral long terminal repeat (LTR), permitting constitutive expression of the therapeutic gene in keratinocytes. But as mentioned above, for diseases like PC, which affect the upper layers of the skin, if genes are transferred to stem cells, it might be important to keep therapeutic genes silent until these cells differentiate. Otherwise, keratin isoforms may be inappropriately expressed in the basal layers of the epidermis and stem cells may have reduced capacity for self-renewal. Although attempts have been made to obtain tissue specificity by inserting a tissue-specific promoter internally within retroviral vectors or by deleting viral enhancer elements, these approaches frequently lower viral titers and fail to achieve cell type specificity. Ghazizadeh *et al* (2002) achieved targeted gene expression in a stratum-specific manner in the epidermis using the upstream regulatory elements of the human involucrin gene to replace the viral enhancer element in the U3 region of the LTR. Involucrin is a precursor protein used to assemble the cornified cell envelope and is expressed in the suprabasal layers of the stratifying epithelia. Ghazizadeh *et al* achieved good viral titers (2×10^6 per mL)

and demonstrated expression of a marker gene (GFP) in epidermal cell lines and in the suprabasal layers of raft cultures of human keratinocytes. Infection of mouse skin with this vector led to long-term (20 wk) stratum-specific expression of GFP in the mouse epidermis.

HIV-1-derived lentiviral vectors can efficiently infect slowly dividing and non-dividing cells, including stem cells (Rubinson *et al*, 2003), and demonstrate more stable expression than MLV-derived vectors (Zaiss *et al*, 2002). In order to reduce the risk of contaminating preparations of recombinant virus with infectious HIV-1, helper plasmids contain deletions for many genes required for productive infection. In addition, current lentiviral vectors are "self-inactivating"; they contain deletions of promoter elements in the 3′ LTR that become incorporated into the 5′ LTR upon replication. At least one lentivirus vector has been approved for use in human therapy (VRX496 from VIRxSYS of Gaithersburg, Maryland) Lentivirus vectors have been used successfully for long-term gene delivery to the skin (Baek *et al*, 2001). Although gene therapy vectors based on feline immunodeficiency virus and other non-primate lentiviruses are being developed (Poeschla, 2003), these are currently of lower infectious titer than HIV-1-based viruses and they have not been demonstrated to be safer for use in humans.

In a recent and exciting application of lentiviral gene delivery to the skin, Siprashvili and Khavari (2004) generated a series of vectors in which gene expression and persistence in the skin are regulated by the administration of steroid hormones. To control the expression of the transferred gene they used promoters containing a series of glucocorticoid response elements. To control the persistence of the provirus elements in the skin, they included loxP sites in the LTR and genes for a Cre-estrogen receptor fusion protein in some of the vectors. Upon activation of the fusion protein with a 4-hydroxytamoxifen cream, most of the provirus was excised from host genomic DNA, leaving an inactive LTR behind. These investigators used a single intradermal injection to deliver a recombinant virus encoding the Epo gene with a view to using the skin as a depot for the systemic delivery of proteins. Upon topical glucocorticoid induction, a rapid increase in hematocrit was observed, indicating a therapeutic effect. More importantly, when the animals were allowed to recover for 30 d and the hematocrit returned to basal levels, a second round of stimulation led to a similar increase in plasma Epo and hematocrit was obtained, indicating stable introduction of a regulated genetic element.

Non-Viral Delivery of Genes

The major compartments comprising the skin are easily isolated, expanded *in vitro* and grafted back to the donor following therapeutic manipulation. This is the basis for the epithelial transplants currently used in the treatment of burns. Such modified epithelia can be monitored and removed if cessation of treatment is indicated. Therefore, direct delivery of plasmid DNA encoding therapeutic genes may be possible *ex vivo* followed by autologous transplant to the patient. To achieve stability, such genes must be delivered to stem cells and stably expressed, implying integration into active chromatin of the patient's DNA. Several of these non-viral technologies, including retrotransposition (e.g., the Sleeping Beauty transposon) and rare cutting bacterial integrases (e.g., ϕC31) have been piloted for use in inherited skin disease (Ortiz-Urda *et al*, 2002, 2003). This approach would be useful for the long-term delivery of several of the therapeutic genes described above, including zinc-finger repressors, and genes encoding ribozyme or shRNA. In addition, cultured keratinocytes and their progenitor cells are ideal for gene correction or gene ablation strategies using oligonucleotides, zinc-finger recombinases, or TFO. Cells containing a correction or disruption of the disease-causing allele could be identified and then expanded. But *ex vivo* gene transfer followed by tissue expansion and grafting will be expensive and time consuming. In addition, grafting of genetically modified cells is based on the assumption that the processes of adhesion, growth and differentiation of keratinocytes will not be modified by the expression of the transferred gene. As these processes are strictly regulated by the sequential expression of a series of genes from the basal to the upper layers of the epidermis, the therapeutic gene must not interfere with this highly regulated developmental program of proliferation and differentiation (Ghazizadeh *et al*, 2002).

Animal Models of Keratin Diseases

Developing animal models for PC and other keratin disorders is the subject of another paper in these *Symposium Proceedings*. Such models are critical for understanding the pathology of rare diseases and for testing the efficacy and safety of therapies (Coulombe and Omary, 2002; Porter and Lane, 2003). Models comprised of human skin grafted onto immune deficient mice have been useful in reproducing the pathological effects of mutation and determining whether these can be corrected by gene transfer. These models do not permit study of the immune responses to therapy, which are critical factors for treatment of homozygous recessive diseases, and are also important when using viruses or even naked DNA to restrain expression of a dominant disease gene.

Mouse models in which disease genes have been disrupted may be useful for mimicking recessive loss-of-function mutations (Wojcik *et al*, 2001; McGowan *et al*, 2002), but such models cannot recapitulate disease associated with dominant-negative missense mutations (Cao *et al*, 2001). For these, transgenic or knockin models are required.

For testing therapies that depend on nucleotide sequence the *genotype* as well as *phenotype* must be duplicated. From the genetic standpoint, it might be more interesting to produce the human mutation within the context of the homologous mouse keratin gene (if that can be identified). Constructing such a knockin model permits detailed investigation of the pathophysiology of the disease, using a mutated gene that is appropriately regulated at its normal chromosomal locus. For the gene therapist trying to develop an antisense therapy or to target gene correction, such a mouse model provides a useful proof-of-concept test. Nevertheless, to test the efficacy of a therapy to be used in humans, the *human* disease gene must be

corrected or suppressed. For a valid test of these nucleotide sequence-specific therapies, a human disease gene (as a knockin or a transgene) must be the cause of pathology. Only then can the tool to be used in people be tested.

Conclusions

There are several clear and plausible pathways to successful gene therapy for diseases caused by dominant keratin mutations. The obstacles along these pathways may be economic as well as scientific: given the rarity of these diseases, they will not become targets for pharmaceutical companies or for venture capitalists who fund much of the translational research in biotechnology. Therefore, the best line of attack, targeted gene correction, may not be the most practical. Given the accessibility of the skin, antisense oligonucleotides, triplex-forming molecules, or RNA interference using synthetic and stabilized nucleic acids is the most attractive approach. Success of these methods depends on the ability of pharmacologists and dermatologists to devise painless and consistent delivery methods for oligonucleotide medicines. This problem has been recognized for some time.

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