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Gene Therapy for the Inner Ear: Challenges and Promises

Allen F. Ryan^{a,b} and Stefan Dazert^c

^aDepartment of Surgery/Otolaryngology, UCSD School of Medicine and VA Medical Center, La Jolla, Calif., USA

^bDepartment of Neurosciences, UCSD School of Medicine and VA Medical Center, La Jolla, Calif., USA

^cDepartment of Otorhinolaryngology, School of Medicine, Ruhr University, Bochum, Germany

Abstract

Since the recognition of genes as the discrete units of heritability, and of DNA as their molecular substrate, the utilization of genes for therapeutic purposes has been recognized as a potential means of correcting genetic disorders. The tools of molecular biology, which allow the manipulation of DNA sequence, provided the means to put this concept into practice. However, progress in the implementation of these ideas has been slow. Here we review the history of the idea of gene therapy and the complexity of genetic disorders. We also discuss the requirements for sequence-based therapy to be accomplished for different types of inherited diseases, as well as the methods available for gene manipulation. The challenges that have limited the applications of gene therapy are reviewed, as are ethical concerns. Finally, we discuss the promise of gene therapy to address inherited and acquired disorders of the inner ear.

Even before DNA was discovered as the molecular basis of the genetic code in the 1950s, the idea of correcting inherited disorders had been voiced. Since the role of DNA, much less its manipulation, had yet to be achieved, this remained a matter of speculation [1]. However, even at very early stages, ethical issues were raised regarding the alteration of human genetics [2]. This was viewed as akin to the eugenics movement that began in the late 1800s.

As the genetic code and mechanisms of gene expression began to be understood in the 1960s, and the link between inherited disorders and mutations in individual genes became established, the concept of gene therapy took on a more concrete form. Initial ideas involved the transfer of normal DNA in vitro, into cells from individuals with a genetic defect. In an early study, Bensch and King [3] exposed bone marrow cells from sickle cell anemia patients to DNA from normal bone marrow cells. The sickle cell anemia cells began to express normal β -globin. However, attempts to similarly influence intact animals by the injection of DNA were unsuccessful, despite an early report of success [4] that could never be replicated.

However, it was not until tools of molecular biology were developed, allowing DNA sequencing [5] and synthesis of recombinant DNA [6] including individual genes, that identification of sequence mutations underlying genetic disorders and consideration of how gene therapy might be achieved began in earnest.

A key problem was the delivery of corrective DNA into cells. Early experiments with naked DNA were extremely inefficient. However, research on viruses had demonstrated their potential to transform mammalian cells. This led to suggestions that viruses might be used to deliver DNA to human cells for therapeutic purposes [7–9], and relatively quickly to the demonstration of viral DNA transfer to mammalian cells. In 1970, during research on polyoma virus, Osterman et al. [10] demonstrated the transfer of mammalian DNA into mouse cells via pseudovirions. These particles, with a viral coat but containing host DNA, are produced during the replication of the virus and can enter mammalian cells using the viral coat. The heart of this experiment was demonstrating the uncoating of pseudovirions within mouse cells, to release the encapsulated host-cell DNA, as verified by its sensitivity to DNases. However, the authors emphasized the implications of this for future DNA-based therapy. This discovery was followed by the demonstration that genetic disorders could be corrected by virally mediated gene transfer into cells, at least in culture [11], and the development of viral vectors that could incorporate specific DNA sequences for transfer into cells [12].

This research demonstrated the promise of gene therapy quite early in the development of molecular biology. Since that time, the identification of mutations that lead to inherited disease has undergone explosive growth, especially in the past two decades. This has vastly increased our understanding of the molecular substrates of many genetic diseases, and spurred the search for means of correcting these disorders via gene therapy. Indeed, sophisticated tools for gene delivery have been developed, and instances of successful gene therapy have been achieved. However, progress in therapeutic applications has been modest. Some early, and perhaps premature, gene therapy trials in humans were either unsuccessful or were subjected to substantial criticism [13], perhaps even setting the field back and leading to the formation of the NIH Recombinant DNA Advisory Committee to review all future human gene therapy trials.

However, a number of rigorous clinical trials using quite promising techniques have been performed. In the case of ex vivo gene therapy, in which cells are removed from the patient, treated and then returned, these trials have resulted in success. Patients with severe combined immunodeficiency disease (SCID) have been able to live productive lives for many years after ex vivo transfer of the *IL2RG* or *ADA* gene to T cells by retroviruses [14, 15]. This was not without side effects, since insertional mutagenesis affecting other genes has been noted in some such trials. However, these ex vivo trials were far more successful than any in vivo trials to date. Several trials attempting to transfer genes with nonretroviral vectors or liposomes have produced transient or very low level gene transfer, or in most cases lack of effect [16].

Thus while a few instances of successful human treatment have occurred, the promise of gene therapy remains more of a goal than an achievement, due in part to the many practical

difficulties involved in gene delivery. An additional reason for such slow progress has been the complexity of genetic diseases, which can profoundly influence strategies for gene therapy.

Disease-Causing Mutations Are Heterogeneous

Inherited diseases vary widely in both the mutations that cause them and the effect of the mutations, and this influences the manner in which gene therapy might be used to correct them. The most basic dichotomy in inherited disorders is between recessive and dominant inheritance. In the former two copies of the mutated gene, one from each parent, are required for the disease phenotype to be expressed. In a dominant disorder, inheritance of only one copy of the mutated gene from a single parent will produce the disease phenotype.

Recessive disease is typically caused by mutations that render a protein unable to perform its function. This is known as a loss-of-function mutation. In this case a single, normal copy of the gene produces enough normal protein to maintain function. Only when both copies of the gene carry a mutation is the protein absent.

Dominant disease typically occurs because the protein encoded by a mutated gene functions in an abnormal manner, and this aberrant function interferes with the action of the normal protein. Interference can be due to a protein that is active in the wrong time and place, such as an ion channel that is always open. This is known as a gain-of-function mutation. Alternatively, the mutant protein can suppress the function of the protein produced by a normal copy of the gene. This can occur when the protein in question normally binds to another protein to initiate function. If an inactive mutant protein binds with the protein partner, making it unavailable to the normal protein, decreased or no function can occur. This is known as a dominant negative mutation. Some forms of dominant deafness are caused by loss-of-function mutations, when the remaining single copy of the normal gene cannot produce enough normal protein to maintain function, a condition known as haploinsufficiency. In any of these cases, the presence of the mutated protein is deleterious, even though the normal protein is produced.

Other categories of genetic disease include mutations in mitochondrial DNA, which are passed only from mother to child. Mutations on the X chromosome produce X-linked traits. These are of course dominant in males. In females, they can be dominant or recessive depending upon the mutation. However, the random inactivation of the X chromosome that occurs in very early embryogenesis infrequently favors one X chromosome over the other by chance, resulting in the expression of an otherwise recessive mutation in a female. Mutations on the Y chromosome produce Y-linked traits that are passed from father to son. Because the Y chromosome is small and bears genes related primarily to fertility, most Y-linked mutations cause sterility.

Even more complex are multifactorial or polygenic diseases. These are caused by the combined effects of mutations or polymorphisms in several genes, often interacting with environmental factors to produce the disease phenotype. Polygenic disorders can 'run in families' but because of their complexity they are not inherited in a simple pattern. It is

thought that many forms of acquired hearing loss, such as noise-induced hearing loss, are polygenic.

Not only the inheritance but also the effects of mutations can vary widely. Congenital disorders are present prenatally or at birth. Late-onset disorders display a normal phenotype at birth, but become apparent later, often after many years. Many mutations vary in their penetrance. In fully penetrant mutations, 100% of those bearing the mutation express the phenotype. However, some mutations show variable penetrance, with only some carriers showing a detectable phenotype and/or a range of phenotypic severity. One mutation in a gene may result in a dominant phenotype, another may produce a recessive condition.

The prevalence of mutations can also differ. Some recessive and relatively benign mutations are present in a substantial percentage of the normal population. Other mutations have been discovered in only a single family. There may also be many different mutations for the same gene, each of which may cause a unique phenotype. One mutation in a gene may cause a severe phenotype and be fully penetrant. Another may cause a mild phenotype and only in some carriers.

The most common disease-causing mutations occur in the expressed sequence of genes, usually in the sequence that codes for protein. These can result in truncated proteins, or proteins in which one or more amino acids are changed to an alternate residue. However, coding sequences occupy less than 1% of the genome. The remaining genomic DNA encodes regulatory sequences or sequences of unknown function. Mutations in regulatory sequences can also produce disease by altering the extent, timing or location of gene expression.

A critical variable for gene therapy is the timing of the mutational effect. Especially during development, mutations may occur in genes that are expressed only for a short period of time. While the effects can be lifelong, the mutation exerts its effect over a relatively brief period. In other cases, as when a mutation involves a gene that is expressed throughout life, the mutation can similarly exert a lifelong effect.

These variations in inherited disorders have important implications for gene therapy. The type of mutation can have profound effects upon the nature of any potential genetic correction.

Different Mutations Will Require Different Therapeutic Approaches

Perhaps the simplest form of gene therapy would be to correct a recessive disorder caused by a loss-of-function mutation. A recessive disorder could be treated by supplying a normal copy of the gene, although even this simplest form of therapy must be delivered at the right time and in the appropriate cells. Most dominant disorders require a different approach, except for those caused by haploinsufficiency. In this special case, supplying an additional, normal copy of the gene should correct the disorder.

In contrast, for dominant disorders involving gain-of-function or dominant-negative mutations, supplying another normal copy of the gene would probably be ineffective since

the disorder is caused by the interfering effects of the abnormal protein. Silencing of the mutated gene would be required in order to allow the existing normal copy of the gene to be effective.

The timing of the effects of mutation also influences any potential gene therapy. A mutation in a gene that is expressed only developmentally would require only transient gene therapy. A gene expressed throughout life might require gene therapy that is effective indefinitely. Gene therapy for congenital disorders might need to be applied before birth, either in vitro or in utero. Late-onset disorders could be treated after birth.

The prevalence of mutations is another important factor. Gene therapy requires substantial effort and resources for the development even of experimental protocols. Given this cost in human and financial resources, common mutations would be much more likely to be targets for gene therapy than would rare disorders. This would be especially true for the commercial development of gene therapy. Given the very large development costs and regulatory hurdles, the likelihood that therapies would be developed for scarce mutations by a company is essentially nil.

DNA Manipulation for Gene Therapy

There are several different ways in which DNA can or might be manipulated to accomplish gene therapy. The most straightforward is to supply an additional copy of a gene. This would supplement a nonfunctional or weakly expressing gene responsible for a recessive disorder or a dominant disorder based on haploinsufficiency. This method has the great advantage that it would not need to be targeted to a particular locus in the genome. Supplying the normal gene could be accomplished in several ways. The gene could be delivered to cells using a DNA vector. By a process known as transfection, vectors derived from bacterial plasmids must be actively introduced into a cell often by packaging the plasmids in liposomes that fuse with the cell membrane. In contrast, vectors derived from viruses are altered to prevent replication but retain the capacity to enter cells on their own. Induction of gene expression with viral vectors is known as transduction. This process is typically more efficient than plasmid transfection. Either plasmid or viral vectors produce only transient expression of the target gene. However, viral vectors based on retroviruses can integrate into the genome of dividing cells, producing permanent expression of the inserted gene. Long-term transduction of nondividing cells can be accomplished by lentiviral vectors, although producing sufficiently high titers of this vector is very difficult and insertion into the genome can cause deleterious mutations.

Another strategy for gene therapy is gene replacement. This requires the simultaneous deletion of a mutant gene and insertion of a normal gene. This outcome would be ideal for many dominant mutations, since it deletes a mutant gene whose activity interferes with a normal copy. Gene replacement can be performed by homologous recombination. Recombination occurs as a normal event during cell division when segments of the two chromosomes 'cross-over' and are exchanged between the two chromosomes during DNA replication. If an extra DNA strand is provided, the ends of which correspond to parts of the

gene, it may be integrated into a chromosome during recombination instead of the DNA from the opposite chromosome.

However, while recombination occurs normally in dividing cells, recombination at any given location in the genome is an extremely low probability, occurring in only a few cells out of many thousands or millions. Given the low probability of recombination in a targeted region of the genome, this requires severe selection to remove the vast majority of nonrecombinant cells. This is useful for cells that can be expanded in culture, such as bone marrow or other adult stem cells. In experimental animals, embryonic stem cells are used to generate individual animals with recombined genes (e.g. knockout and knockin mice), although this is not practical in humans. However, recombination is more difficult to use in tissues, even if cells are dividing. This said, methods have been developed to increase the frequency of recombination, at least experimentally, using nucleases [17].

A related possibility is gene repair [18]. If a mutation involves a small amount of DNA sequence, as in single-base mutations, the gene could be repaired in situ. For example, chimeric proteins consisting of a sequence-specific DNA-binding domain targeting a specific area of the genome and an endonuclease domain capable of inducing double-stranded breaks in DNA allow small DNA oligonucleotides spanning the mutation and containing the correct base sequence to recombine and repair a mutation. Because the DNA sequences are short, the possibility of integration is increased when compared to recombination of large DNA fragments. This method has been used to correct the *IL2RG* gene in the cells of SCID patients with 5–17% efficiency [19].

Gene silencing is another method of therapy in dominant disorders [20]. The output of the abnormal gene can be reduced or eliminated allowing the normal copy of the gene to function unimpaired. Transient gene silencing can be accomplished using antisense DNA oligonucleotides which block the translation of mRNA into protein. However, RNA interference, in which naturally occurring or synthetic short RNAs interfere with translation and promoter mRNA instability, has proven to be more powerful. There are a variety of methods for RNA interference, but small interfering RNAs are the preferred technology. Gene silencing could also be accomplished by causing a second silencing mutation in the abnormal gene. This could be accomplished with DNA oligonucleotides as in gene repair.

The methods described above are based upon the introduction of DNA into the cells of the tissue affected by the mutation. However, another means of introducing genes into tissue is via cells. In this method, cells are transfected, virally transduced, or stably transformed via recombination to express the gene of interest. The cells are then introduced into the tissue where they produce the gene product. This method could be especially useful for secreted cell products, for which the position or identity of the secreting cell may not be critical. Gene products that are expressed at the cell surface and function via cell-to-cell interaction might also be delivered in this manner. Cells can be directly injected into a desired site. However, recently, stem cells that will cross the vascular barrier into a variety of tissues have been isolated from fetal aorta [21] or bone marrow [22]. For example, mesoangioblasts derived from fetal aorta can differentiate into most mesodermal tissues. In the special case of muscle, they have been shown to fuse with muscle cells and deliver genes that can correct a

genetic disorder [23]. While inner ear stem cells have yet to be identified with certainty, they are actively being sought in many laboratories [24].

Challenges for Gene Therapy

The discussion above suggests that there are many potential strategies for gene therapy that might be used to correct inherited genetic disorders, including genetic hearing loss. However, in fact there has been only limited success with gene therapy, even for cells and tissues that are far more accessible than the inner ear.

Why has progress in gene therapy been so slow? There are many technical hurdles that have prevented the rapid evolution of therapies based on gene sequence. A persistent problem has been the means of delivering gene sequences to the appropriate cells and tissues. Unless the cells can be extracted and grown in vitro, it is difficult to achieve high levels of gene delivery into a high proportion of targeted cells. An example is cystic fibrosis in which the gene defect has been known for many years [25]. The cells to be targeted, airway epithelial cells, are easily accessed and are natural targets of respiratory viruses. Yet gene therapy targeted toward these cells has achieved little or no success. The number of cells that can be effectively treated remains low, hindered by barriers to binding and uptake of viral vectors from the lumen of the airway [26]. The difficulties of accessing tissues that lie deep within the body can be even greater, as would also be the case for gene therapy in utero.

Once a cell has been successfully targeted, the degree and efficacy of gene expression is another potential hurdle. Many gene therapy attempts have failed due to low levels of induced gene expression in the target cells. The efficacy of induced gene expression is controlled by a number of factors. For example, in transient transfection or viral vector transduction, the number of gene copies that can be introduced into the cell helps to determine the level of transcription achieved. The inability to achieve high copy number has been a difficulty in many experimental and clinical gene therapy attempts. The efficiency of the promoter used to drive expression in the target cells is another determinant. Even 'constitutive' promoters that theoretically operate efficiently in all cell types produce unequal expression in different cells [27].

Another issue is targeting of gene expression to a desired time and place. Expression of a gene in the wrong cell types or at the wrong times can be ineffective or even harmful. This problem is particularly acute with complex tissues consisting of many cell types with different but critical functions, an apt description of the inner ear. Targeting may require a method for delivery of gene sequences that is restricted to a desired subset of cells, which can be achieved with viral vectors exhibiting different cellular tropisms [28]. Targeting within a desired time window can be achieved with promoters that can be regulated with a ligand [29]. Alternatively, specific and/or inducible promoters can be used to restrict gene expression to a desired spatiotemporal pattern [30].

Some methods used for gene delivery can be toxic. This is particularly true of viral vectors or lipid-based transfection reagents. This problem has been minimized with each generation of vector, but remains a potential concern if viral titers or transfectant concentrations are too high.

The transience of gene expression mediated by some viral vectors widely used for gene therapy investigation, such as adenovirus vectors, is also a limitation. Expression caused by a single viral vector introduction usually lasts only a few weeks. Because viral vectors are foreign entities, they elicit an immune response that typically prevents repeated administration.

Ethical Concerns

Regardless of the technical difficulties associated with gene therapy, there are ethical considerations that are of concern. In general, many of these address the issue of germ-line gene therapy, designed to permanently alter the DNA of an individual in a manner that would be inherited by their offspring. This type of gene therapy is seen, by some, as akin to genetic engineering as pioneered by the eugenics movement [31]. Even individuals who do not make this connection may believe that altering DNA is itself immoral on ethical grounds [32].

There is less concern regarding somatic gene therapy in which germ-line DNA is not altered. In the case of somatic gene therapy, only a few individuals argue that this is morally wrong in itself. Concerns are typically more focused on the possibility that transferred DNA might inadvertently be incorporated into gametes. However, as vectors have been perfected to virtually eliminate this possibility, these concerns have lessened. Still, a few individuals argue that even altering the DNA of nonreproductive cells is unacceptable, usually on religious grounds [33].

Even for germ-line gene therapy, fewer people would argue against gene therapy allowing children to be cured of terminal or debilitating illnesses that could be transmitted to their offspring. However, there is less agreement regarding therapy for disorders that are not life-threatening, such as deafness.

Genetic conditions that cause a disability, including deafness, are sensitive to additional ethical concerns. It is well known that many individuals in the deaf community consider deafness to be a culture not a disability. Such individuals consider gene therapy directed at reversing deafness to be unethical. At the extreme end of this issue is the potential for gene therapy to be used for the correction of conditions that are not pathological but may be undesirable. The use of gene therapy for improvement, rather than cure, is reminiscent of eugenics and is fraught with ethical considerations.

Another issue to consider is a potential hidden value of mutations. It is possible that a mutation, while deleterious in one system, may confer an advantage in another. Correction of the deficit may therefore have unintended consequences.

An issue that has generated considerable ethical debate is ownership and patenting of genetic information. Many people find the commercialization of human genetic information and traits to be morally wrong. However, the courts have generally upheld patents on genetic information and life forms.

Promise for the Future

While practical challenges and ethical issues have limited progress in gene therapy in general and in the inner ear in particular, it can be anticipated that many of the roadblocks that currently hinder gene therapy for hearing loss will eventually be overcome. There are many promising developments that presage the eventual application of gene therapy to hearing loss.

One such development is the ongoing identification of mutations that cause inherited hearing loss. There are currently more than 150 genetic loci that have been linked to deafness, and this number continues to grow. Up-to-date information on this fast-moving field can be obtained at the Deafness and Hereditary Hearing Loss webpage (<http://www.geneclinics.org/profiles/deafness-overview/details.html>). More than 60 deafness genes have been identified at these loci, providing the basic sequence substrates for gene therapy. Some of these defects are extremely common, as in mutations of the *Gjb2* gene encoding connexin 23, responsible for recessive (DFNB1) and more rarely for dominant (DFNA3) inherited deafness. In developed countries, more than 50% of inherited deafness is caused by mutations in this gene, and the carrier rate for the single most common mutation alone approaches 3% in Mediterranean populations [34], making it an extremely attractive target for gene therapy.

Perhaps more important, we are beginning to understand the normal and disordered functions of the proteins encoded by these genes. In most cases, it will take detailed knowledge of how and when proteins perform their functions, and in which inner ear cells they occur, before rationale gene therapy can be developed. For example, the large family of genes mutated in Usher syndrome is known to influence the development and function of hair cell stereocilia [35]. The proteins encoded by these genes operate at different times and perform quite different functions, from influencing the elongation and orientation of stereocilia to forming elements of the tip links that are required for hair cell transduction [36].

There have also been advances in the delivery of gene sequences to cochlear cells, using a variety of primarily viral vectors many of which are described in this issue. The evaluation of genes that are expressed in cochlear cells has also led to the identification of regulatory sequences that direct gene expression to particular cochlear cells and at particular times. This will facilitate the development of gene therapy vectors that are specific for a particular inner ear tissue and/or developmental stage.

There have also been advances in the identification and development of potential stem cell populations for the inner ear [37]. Since gene therapy has only been successful to date by the *ex vivo* treatment of cells that are later introduced into the patient, the development of stem cells that could integrate into the inner ear would be a very significant advance.

While this discussion has focused on inherited disorders, it should also be noted that gene therapy could be used to deliver treatments for acquired hearing loss. An example is hair cell regeneration. The delivery of the *atoh1* gene by adenoviral vectors has been shown to induce the formation of new sensory cells in animals that have experienced hair cell loss due to

ototoxicity [38]. This application has the advantage that no repair of an existing mutation is required. Moreover, transient expression of *atoh1* may be sufficient for effective therapy, reducing the potential for deleterious immune response to the virus. Viral vector delivery of growth factors has similarly been shown to enhance the survival of spiral ganglion neurons after hair cell loss [39]. This method could be used, for example, to protect these neurons from the trauma of cochlear implantation. This would also be consistent with transient transduction.

Conclusion

In summary, despite considerable theoretical and experimental support, gene therapy remains in its infancy, even in tissues that are far more accessible than the inner ear. Application of gene therapy to inner ear disorders will require advances in both the broader arena of gene therapy, and in its specific applications to the inner ear. This means that considerable research must yet be performed.

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