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Genetics of Hearing Loss

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1. Introduction

The revolution in genetics in the past decades has enabled identification of many of the genes associated with human hereditary diseases, and hearing loss is no exception. These discoveries have a profound impact on knowledge about inner ear function and the pathology caused by mutations in these genes, which becomes clinically and socially relevant because a significant proportion of hearing loss is caused by hereditary mutations in genes. The identification of these genes and the proteins they encode allow for molecular diagnostics and genetic counseling for patients, and will help devise new ways to diagnose, treat, and prevent disorders of the auditory system.

1.1 Hearing Loss

Sound can be described in terms of frequency (pitch), measured in hertz (Hz), and in terms of intensity (loudness), measured in decibels (dB). Hearing is considered within the normal range if a person can process sound frequencies between 20 and 20,000 Hz and decibel levels from 0 to 140 dB. Hearing loss or hearing impairment occurs when all or a part of the normal range of hearing is lost (Newby 1992).

In brief, the mechanism of hearing is the transduction of sound, turning it into neural impulses and interpreting these impulses by the central nervous system. A defect at one or more of the levels in this system can lead to hearing loss. Occupying a wide spectrum of decreased hearing, "hearing loss" is defined as the reduced or absent ability to perceive or process auditory information. The World Health Organization (WHO) defines the term "deaf" only when referring to severe cases in which alleviation by hearing aids and cochlear implants are not optimal. For educational placement and integration purposes, children with hearing loss over 90 dB are considered deaf (National Dissemination Center for Children with Disabilities, Table 2.2).

Hearing loss is partitioned into several categories according to different factors. Hearing loss can affect either one or both ears, designated as unilateral hearing loss or bilateral hearing loss, respectively. Hearing loss can also be sorted

into stable hearing loss (permanent year after year), progressive, fluctuating (worsening and improving alternately, such in cases of inflammation of the tympanic membrane or fluid in the middle ear), or even transient (due to wax buildup in the ear canal, head injuries, ear infections, and reactions to medications, for example, aspirin). Based on the onset of hearing loss, it is possible to distinguish between congenital (present at birth), early-onset (commence at childhood), and late-onset loss (begins at adulthood). An additional discrimination relates to the development of language acquisition, as either prelingual or postlingual (before and after acquisition of language and speech, respectively).

Hearing loss severity is divided into five groups organized according to gradual deterioration of hearing: mild (thresholds of 21–40 dB), moderate (41–60 dB), moderately severe (61–80 dB), severe (81–100 dB), and profound (>100 dB). The range of hearing loss is generally grouped into three categories: low-frequency hearing loss (<500 Hz), middle-frequency hearing loss (501–2000 Hz), and high-frequency hearing loss (>2000 Hz).

Yet another classification subdivides hearing loss into four classic groups according to underlying pathologies. The first, sensorineural hearing loss, is due to malfunction of the inner ear or along the neuronal pathway between the inner ear and the brain. Such damage to the delicately correlated system of the transmission of sound waves from the hair cells to the supporting nervous tissue often causes hearing loss. In general, it is a permanent disturbance that cannot be cured by medical or surgical intervention.

The second group, conductive hearing loss, represents hearing obstructions present in the conduction canal leading to the inner ear, consisting of the external and middle ear. Common factors in this kind of hearing loss are wax in the ear canal, a perforation in the eardrum, infections, fluid in the middle ear, and fixation of the ossicles as occurs in otosclerosis, and erosion of the ossicles as in cholesteatomas, epithelial tumors of the middle ear that can be congenital or result from chronic infection. Conductive losses generally affect all frequencies and in many cases are surgically treatable. The third group, mixed background hearing loss, results from combined sensorineural and conductive factors. Finally, the fourth group, central auditory dysfunction, results from damage at the level of the eighth cranial nerve, auditory brain stem, or cerebral cortex.

1.2 Genetic Hearing Loss and Its Prevalence

A significant difference in the cause of hearing impairment is whether its origin is genetic or nongenetic. Genetic hearing losses are due to single or multiple lesions throughout the genome that may be expressed at birth or sometime later in life. Nongenetic “acquired” hearing loss, on the other hand, is a consequence of environmental factors that result in hearing impairment, with no regard to inheritance. Such factors might include infections such as meningitis and otitis media, traumatic injuries such as perforation of the eardrum, skull fractures and acoustic trauma (see Henderson et al., Chapter 7), and use of toxic drugs such as

aminoglycoside antibiotics or cisplatin (see Rybak et al., Chapter 8). However, even when speaking of environmental causes, genetic factors may be involved as modifying genes (Table 2.1) that may have an impact on onset, severity, and progressiveness of nongenetic hearing loss.

TABLE 2.1. Genetic definitions.

Genetic term	Definition
Recessive inheritance	Normal individual holds within its DNA two copies of each gene. A recessive characteristic or trait is apparent only when two copies of the gene encoding it are present. If a mutation in a gene is inherited in a recessive pattern, the mutated trait or characteristic will be phenotypically expressed only in a homozygous mode.
Dominant inheritance	A dominant pattern of inheritance of characteristic or trait in which one copy of an allele is sufficient to phenotypically confer this feature. In the case of a dominant mutation in a gene, it will be visually expressed in either a heterozygous or a homozygous condition. As a result, a dominant disorder is apparent when inherited from only one of the parents and will appear in each generation.
X-linked inheritance	Out of the 23 pairs of chromosome that a normal individual carries in his DNA, one pair of chromosomes is discriminated as the sex chromosomes (X and Y chromosomes) that contain genes that determine gender development and function, as well as other genes that are non related to sex configuration. X-linked is descriptive of an allele located on the X-chromosome.
Mitochondrial inheritance	Mitochondria are microscopic rod-like subcellular structures that are the principal energy source of the cell, by metabolizing nutrient molecules into available energy. A mutation in mitochondrial gene is maternally passed on to all of her children with no regards to their gender, while only her daughters will pass it to the next generations.
Expressed sequence tags (ESTs)	Expressed sequence tags are short DNA sequences from the expressed regions of a gene only (called 'exons'). These pieces of DNA (several hundred base pairs of length) can serve for rapid identification ('tagging') of the full length sequencing of the expressed genes (called cDNAs) and in developing DNA markers.
Penetrance	The likelihood or probability that a characteristic or a trait will be expressed as a phenotype as a result of a specific given phenotype.
Transcriptional regulators	Specific proteins that are required for the initiation of transcriptional process, thereby regulating it.
Heterozygous	A genotype that possesses two distinct copies of an allele of a certain characteristic or a trait.
Homozygous	A genotype that possesses two identical copies of an allele of a certain characteristic or a trait.

TABLE 2.1. (continued)

Genetic term	Definition
Bacterial artificial clones	In order to artificially transport fragments of DNA into cells, these fragments need to be introduced first into a molecule which is able to carry them into the host cell in vitro and facilitates their multiplication within the host. Bacterial artificial chromosomes are DNA vectors derived from genetically engineered bacteria *E. coli* chromosome, used to incorporate large fragments of DNA (100 to 300 kb).
Transgenes	DNA fragment or a gene which is artificially inserted into the germ line of another organism, which is then called a transgenic organism. These fragments of DNA can integrate into the host genome and alter its genotype.
In situ hybridization	An assay testing the hybridization ability of DNA/RNA probes which are applied on an intact tissue, in order to detect presence of the complementary DNA sequence
Hypomorphic allele	An allele that disrupts and diminishes the function of a gene, but does not absolutely abolish its activity.
Modifying genes	A gene that can influence the expression of another gene. Thus, modifying genes can alter the phenotype of a feature associated with a particular gene, and can result in multiple phenotypes for the same genotype.
Allele	One of the two or more alternative forms of a certain gene. A normal individual caries two alleles for each of his genes (one from each parent), which can either be two identical alleles (homozygous) or two distinct alleles (heterozygous).
Organogenesis	The process of organ formation during embryonic stages.
Monogenic	A monogenic disorder is a condition caused and controlled by a single gene.
Homologue	A gene or a locus from one species that shares high similarity in sequence to a gene or a locus of another organism. Homology between genes suggests a common origin and function of the gene in question.
Gene	A sequence of DNA that is the basic unit of inheritance. Most of the genes encode for proteins while their minority are non coding genes.
Phenotype	The observable or measurable expression of a gene coding for a certain characteristic. The phenotype can be the consequence of numerous factors including genotype, environmental factors, age, presence of modifier genes or interaction among several genes.
Genotype	The genetic identity of a certain gene of locus in the DNA. The genotype is not necessarily expressed visually.
Autosomal	Any of the chromosomes that are not the sex chromosomes (X and Y chromosomes).
Logarithmic odds (LOD)	A statistical estimate of whether two loci are likely to lie near each other on a chromosome and are therefore likely to be inherited together. A LOD score of three or more is generally taken to indicate that the two loci are close.

At least 50% of all hearing impairment are due to genetic factors (Skvorak-Giersch and Morton 1999; Nadol and Merchant 2001). About 3 out of 1000 newborns have a significant hearing impairment, and one half of the population older than 70 years of age develops some degree of hearing impairment. These numbers are turning hearing loss into one of the most common birth defects and into the most inherited sensory defect among adults worldwide (Hone and Smith 2003).

When relating to symptom manifestations, hearing loss is classically subdivided to syndromic hearing loss (SHL) and nonsyndromic hearing loss (NSHL). Hearing impairment accompanied by additional clinical symptoms is referred as SHL. Hearing dysfunction as the only observed phenotype is defined as NSHL. Worldwide, the incidence of nonsyndromic hearing loss (NSHL) is estimated to be responsible for 70% of genetic hearing loss. While the syndromic component (30% of all hearing loss) is almost purely of genetic etiology, NSHL can include nongenetic factors as well. Hereditary NSHL is most often autosomal recessive (75% to 80%; Table 2.1), with 18% to 20% attributed to autosomal dominant inheritance (Table 2.1) and the remaining percentage related to X-linked or mitochondrial inheritance (Table 2.1; Hertzano and Avraham 2005). Although the exact proportions can differ over time and place, current estimates ascribe greater than 50% of all hearing loss cases as originating from a mutation in a sole gene (a monogenic trait). As might be expected of a highly sophisticated and tightly coordinated mechanism such as the hearing apparatus, a large portion of the 30,000 genes in the human genome are dedicated to participate in this task, many of which were identified in the Human Genome Project (Collins and McKusick 2001). Sixty-five deafness-related genes have been already cloned (Hereditary Hearing Loss Homepage, Table 2.2), meaning that they were identified as hearing loss causative factors and were characterized. Moreover, 120 loci have been mapped for hearing loss so far, meaning that the region within the genome that contains the defective gene has been linked to hearing loss, though the specific gene has not yet been identified.

The first deafness locus to be mapped was *DFN3* (Brunner et al. 1988). The first autosomal dominant gene to be mapped was *DFNA1*. This form of NSHL was discovered in a Costa Rican kindred (Leon et al. 1992); the gene responsible was subsequently identified in 1997 as diaphanous (Lynch et al. 1997). No additional diaphanous mutations have been reported. The first autosomal recessive gene to be mapped was *DFNB1* (Guilford et al. 1994). Ironically, this locus has turned out to be responsible for the most prevalent form of NSHL, those associated with connexin 26*GJB2* mutations (Denoyelle et al. 1997; Kelsell et al. 1997; Zelante et al. 1997). Overall, the plethora of genes associated with NSHL has turned out to be fascinating, being involved in every aspect of auditory function. To add to the complexity, there are a number of genes that are associated with both NSHL and SHL, as well as both recessive and dominant hearing loss.

The comprehensive and diverse selection of cloned genes includes genes encoding a variety of different proteins with known functions. A few examples include genes that encode either components constituting the synaptic apparatus

TABLE 2.2. Web sites used for the genetics of hearing loss.

Web site	URL
National Dissemination Center for Children with Disabilities	http://www.nichcy.org/pubs/factshe/fs3txt.htm
Hereditary Hearing Loss Homepage	http://webhost.ua.ac.be/hhh/
Human Genome Project	http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml
UCSC Genome Browser	http://genome.ucsc.edu/
Genetic Linkage Analysis	http://linkage.rockefeller.edu/
The Connexin-Deafness Homepage	http://davinci.crg.es/deafness
The Jackson Laboratory Hereditary Hearing Impairment in Mice: Mouse Models of Human Hearing disorders	http://www.jax.org/hmr/models.html
National Institutes of Health Office of Human Subjects Research	http://ohsr.od.nih.gov/
The German Mouse ENU Project	http://www.gsf.de/isg/groups/enu-mouse.html
Harwell Mutagenesis Program	http://www.mut.har.mrc.ac.uk/
Epitope Prediction	http://www.sbc.su.se/~pierre/svmhc/new.cgi
Pubmed	www.pubmed.gov
Gene Expression Omnibus	http://www.ncbi.nlm.nih.gov/geo/
Unigene	http://www.ncbi.nlm.nih.gov/UniGene
SOURCE	http://source.stanford.edu

of the hair cells of the inner ear and the nerves (PMCA2 and otoferlin), cytoskeleton proteins (myosin VI, myosin VIIA, and myosin XVA), proteins implicated in structural integrity of the organ of Corti (α-tectorin, COL11A2, and COCH), and proteins significant for potassium recycling in the organ of Corti (connexin 26, connexin 31, KCNQ4, Pendrin, and Claudin 14) (Steel and Kros 2001; Tekin et al. 2001). Up to 50% of genetic hearing impairment is estimated to derive from mutations in the connexin 26 (*GJB2*) gene (Marazita et al. 1993; Steel and Kros 2001) that encodes the protein connexin responsible for the proper function of gap junctions between cells.

Chapter 3 by Wangemann complements the genetic information in this chapter by describing the consequences of several of the mutations in the context of cochlear homeostatic mechanisms.

2. Syndromic Hearing Impairment

Hearing impairment is denoted as an integral clinical phenotype in more than 400 genetic syndromes (Gorlin 1995; Steel and Kros 2001; Nance 2003). The presence of clinical features accompanying hearing impairment can vary on a wide scale, while hearing abnormalities are often mild, unstable, or a late-onset trait in these syndromes (Friedman et al. 2003; Nance 2003). Syndromic forms of hearing loss are estimated to be responsible for up to 30% of prelingual deafness, although in general, it endows only a small portion of the broad spectrum of

hearing loss. The prominent portion of these disorders are monogenic (Friedman et al. 2003), meaning that their hereditary component is derived from one mutated gene throughout the genome. A glance at some of the major SHL types that have been studied so far is given in the paragraphs that follow.

2.1 Waardenburg Syndrome

In 1947 P.J. Waardenburg, a Dutch eye physician, was the first to notice a link between retinal pigmentary differences, found in one of his patients, and congenital hearing loss. Four years later, after tracing other patients with similar symptoms, he described a new syndrome (Waardenburg 1951), involving congenital hearing loss; lateral displacement of the inner canthi, the inside corner of the eye (dystopia canthorum); and retinal pigmentary differences, referred to today as Waardenburg syndrome (WS) type 1 (WS1).

WS is mostly a genetic autosomal dominant disorder, evident at birth. It is considered to be the most frequent autosomal dominant form of syndromic hearing loss, constituting approximately 2% of all congenital hearing loss (Apaydin et al. 2004). The primary phenotypes observed in this syndrome may include irregular skin and eye pigmentations, seen as white lock patches of hair above the forehead, premature gray hair, light pigmented zones in the skin (leukoderma), two different colored segmented eyes (heterochromia irises), or as extraordinary brilliant blue eyes. In addition, one of the visible phenotypes present in WS patients is an unconventionally wide distance between the inner corners of the eyes and sometimes also confluent connected eyebrows and a high nasal root. The wide scale of symptoms and severities can vary greatly from one person to another and hearing loss in WS can fluctuate from moderate to profound among different WS patients.

Four different types of WS have been described, grouped by distinct physical characteristics. All four types share common sensorineural hearing loss and pigmentary abnormalities expressed at variable degrees. The majority of WS1 individuals display dystopia canthorum, a feature in which the inner corners of the eye are spaced farther apart than normal. WS type II (WS2) is not associated with this phenotype and is caused by mutations in a different gene. Type III and type IV (WS3 and WS4, respectively) are much rarer subforms of WS. WS3 is ascribed to malformations of the upper limbs. WS4 is associated with Hirschsprung disease, a digestive disorder typified by diminished motility in segments of the bowel caused by lack of nerve cells, and is therefore known as Waardenburg-Hirschsprung disease as well.

WS hearing and pigmentary deformities can both be caused due to a failure of proper melanocyte differentiation during embryonic development. Deficiency or lack of melanocytes can affect pigmentation in the skin, hair, and eyes and hearing as well. Melanocytes exist as intermediate cells of the stria vascularis in the organ of Corti, where they play a vital role in creating the endocochlear potential, positive voltage of 80–100 mV seen in the endolymphatic space of the cochlea, which is necessary for normal hair cell function.

All of WS1 and some of WS3 cases are associated with mutations in the *PAX3* gene. WS2 is caused by mutations in the transcription factors *MITF* and *SNAI2*. WS4 is due to alterations in either *EDNRB*, *EDN3*, or *SOX10* genes. *PAX3* is a member of the *PAX* paired-domain proteins family that function during embryonic development at the level of transcription. *PAX3* was found to strongly activate MITF protein expression, in synergy with *SOX10*, in vitro (Bondurand et al. 2000). Watanabe et al. (Watanabe et al. 1998) showed that *PAX3* regulates MITF expression through the MITF promoter. MITF was shown in 1996 (Tachibana et al. 1996) to act as a transactivator of the tyrosinase gene, a crucial key enzyme in melanocyte differentiation. MITF transactivates the *SNAI2* promoter (Sanchez-Martin et al. 2002). These interactions demonstrate that there is a common pathway that links several forms of this disease.

2.2 Branchiootorenal Syndrome

After WS, branchiootorenal syndrome (BOR) is the second most prevalent autosomal dominant syndromic type of hearing loss, responsible for approximately 2% of all profoundly deaf children (Steel and Kros 2001). The name branchiootorenal refers to three terms concerning its common symptoms: disturbances in the neck (*branchio*), ear disorders (*oto*), and irregular kidney formations (*renal*). The major clinical features of BOR syndrome are branchial cysts or fistulae (abnormal connections between organs/vessels); renal malformations ranging from asymptomatic hypoplasia (underdevelopment of a tissue) to entirely absence (agenesis; Melnick et al. 1976; Fraser et al. 1978); and deformities in formation of the external, middle and inner ear leading to either conductive, sensorineural, or mixed background hearing loss. Manifestations of BOR symptoms can differ in their presence and severity between individuals, and even within the same person, between the two body sides. A very mildly affected BOR parent can have a severely affected child and vice versa, a situation referred to as "variable expressivity." Although extremely variable, BOR symptoms are of high penetrance (Table 2.1).

Mutations in the *EYA1* gene were found in about 40% of families segregating BOR features. Mutations within the *Drosophila* fly gene *eyes absent* (*eya*), a known homologue of the human *EYA1* gene (Bonini et al. 1998), result in the complete absence of the fly eyes or a reduced eye phenotype. The *EYA1* particular mechanism of action has yet to be uncovered, although it is clear that this is a transcriptional regulator implicated in the development of numerous tissues and organs including the eye, ear, and the branchial arches (tissues that are involved in face and neck creation during early embryonic stages) formation.

A minority of BOR families were characterized as carrying a mutation in the *SIX1* gene (Ruf et al. 2004). EYA1 and SIX1 proteins are known to cooperate together with the *PAX* genes as transcriptional regulators (Table 2.1) in the organogenesis (Table 2.1) patterning (Ruf et al. 2004). Besides the two genes mentioned above, mutations in other genes, which have yet to be discovered, are likely to occur as well.

2.3 Usher Syndrome

Usher syndrome (USH) is the most frequent autosomal recessive syndromic form of hearing loss. Dual sensory defects, involving hearing and sight, are obvious in all USH-affected individuals. Sensorineural hearing loss is congenital, whereas vision is usually adversely affected after the first decade of life and worsens over time, as part of a retinitis pigmentosa disorder (an eye impairment leading to blindness). More than 50% of the deaf–blind community in the United States is affected by USH, making it the most prevalent condition of genetic hearing and vision deformities. In addition to auditory–vision symptoms, some patients suffer from balance problems, originating from internal ear defects in the vestibular apparatus, the center of equilibrium and balance. Cataract (cloudiness forming on the lens inside the eye that may cause blurred vision) can be an additional feature on top of those mentioned in each of the different types of USH.

The USH manifestations are variable and therefore are split into three distinct types of USH (USH1, USH2, and USH3), based on the degree of hearing and vestibular features. USH1 and USH2 subtypes are widespread, while USH3 account for 2% to 5% of USH cases. Each subtype of USH syndrome is heterogeneous, and is further subdivided according to their genetic cause.

USH1 individuals are born with severe to profound sensorineural hearing loss and suffer from improper vestibular function. Their balance and communication dysfunction comes to fruition in early infancy, as their motor capabilities (such as sitting and walking on their own) fall much behind normal. Prepubertal retinitis pigmentosa in USH1 commences with diminished sight at night and is rapidly followed by progressive reduction in the visual field that deteriorates until absolute blindness.

The hearing impairment characterizing USH2 is usually mild to severe, with progressive visual problems and no visible vestibular deficits. In contrast to USH1, USH2 patients can benefit from hearing aid amplification of sounds and their verbal communication is usually normal. Decreased eyesight first begins with blind spots appearing in the early teenage years and usually degenerates until complete loss of sight.

USH3 patients experience progressive hearing, vestibular, and vision deterioration. At birth, their hearing appears normal and their balance abilities are normal or near normal. Noticeable hearing and vision hypoactivity signs develop most often by the second decade of life, though they can appear at varying ages even between siblings of the same pedigree (Karjalainen et al. 1983; Pakarinen et al. 1995). Retinitis pigmentosa symptoms progress from night blindness that usually emerges along with puberty through loss of central visual acuity that begins after the age of 20, eventually resulting in blindness by mid-adulthood.

Twelve genetic loci comprising USH genes have been mapped, eight of which have been identified. Much of the genetic identification of USH genes relied on the study of mouse models. Despite the fact that the precise involvement in hearing and vision has yet to be elaborated, it is evident that mutations in USH genes result in anomalous inner ear hair cell function and in the retinal sensory

cells. Interestingly, a considerable portion of USH loci overlap with genetic loci identified in NSHL.

The *USH1B* gene isolation was achieved by the integration of a genetic study of human USH pedigrees and of the genetic mapping of the shaker 1 (*sh1*) mouse model. The *USH1B* locus was first defined as a result of research of numerous families from around the world. The identification of the myosin VIIa (*Myo7a*) gene as the causative mutation in the deaf and vestibular homozygous (Table 2.1) *sh1* mice rapidly led to the recognition that its corresponding human gene was the mutated gene in USH1B. The human orthologue, *MYO7A*, encodes for the myosin VIIa protein, which is a member of the myosin superfamily. Myosins are typified by the ability to move along actin filaments via hydrolysis of ATP molecules. Evidence gleaned from several myosin VIIa studies show that this protein is expressed in hair cell cytoplasm and stereocilia within the cochlea and that myosin VIIa may have a vital role in regulating the development and function of the hair bundle. When defective, this protein causes disturbances in the stereocilia distribution on top of the hair cells, which adversely impacts hearing perception. More than 80 *MYO7A* distinct mutations have been reported, some of which were found as the underlying genes in autosomal recessive NSHL DFNB2 and in autosomal dominant NSHL DFNA11.

The underlying gene of another form of USHI, named *USH1C*, codes for harmonin, a protein containing a PDZ domain, which specializes in protein–protein interactions (Verpy et al. 2000). Harmonin is probably an essential player in the gathering of the USH1 complex of proteins.

In 2000, mutations in the gene *CDH23*/*USH1D* encoding the cadherin-like protein were linked to the USH1D form of USH and were found a year later as the mutation responsible for the deaf mouse *waltzer* phenotype that exhibits disorganized stereocilia bundles. Cadherin proteins promote intercellular adhesion activities, from which they receive their name. Evidence of interaction between cadherin-like protein and harmonin connects these proteins to the evolving stereocilia bundles (Adato et al. 2005).

In addition to *CDH23*, an additional cadherin-like protein named protocadherin was revealed to be mutated in the USH1F subform (Bolz et al. 2001). The protocadherin gene, *PCDH15*, was also reported to affect hearing in the Ames *waltzer* deaf mouse model, which again displays hair bundle disorganization similar to those seen in the *waltzer* and *shaker1* mice. Autosomal recessive NSHL in the *DFNB23* locus resides in the *USH1F* interval. The transmembrane protocadherin 15 protein is localized to stereocilia as well as to the photoreceptor cells of the retina. It is thought to act as a significant mediator in the adhesion process related to stereocilia morphogenesis by means of protein–protein interactions.

The *USH1G* underlying mutation was detected in the *SANS* gene (Mustapha et al. 2002). The corresponding homologous gene in mice was found to be mutated in a deaf mutant mouse model named Jackson shaker (Kikkawa etal. 2003). The *Sans* gene codes fora scaffold-like protein that was shown to interact with harmonin. Similar to the other USH mice models, the hair bundles demonstrated abnormal arrangement of the stereocilia.

Two isoforms of a novel protein were isolated for USH2A, namely USH2 isoform a (previously called usherin) and USH2A isoform b (Reiners et al. 2006). The latter is a much larger isoform, which weighs more than three times that of isoform a. Isoform a carries several interesting domains: a transmembrane region enabling its anchoring to membranes, two laminin (glycoproteins found in the basal lamina) domains that may point to involvement in the construction of the lamina (the basement membrane, a glycoprotein sheet secreted by cells to form the extracellular matrix) and a PDZ-binding domain allowing protein–protein interconnections. Moreover, it shares a great similarity with extracellular matrix and cell–cell adhesion proteins. Both isoforms of USH2A localize to the basement membrane of the cochlea and retina; specifically USH2A is expressed in stereocilia and within the hair cell–nervous synaptic junctions and in the synaptic terminals of photoreceptor cells of the retina (Bhattacharya et al. 2002). These findings are providing evidence for the hypothesis that USH2A functions in the adhesion of pre- and postsynaptic membranes, which may fit into the USH network of proteins.

2.4 Pendred Syndrome

Recent estimations predict that 4% to 10% of prelingual genetic hearing losses are ascribed to a syndromic form of hearing loss named Pendred syndrome (Park et al. 2003). Reliant on these evaluations, Pendred syndrome is the far most common syndromic configuration of deafness. The two hallmarks characterizing this autosomal recessive syndrome are neurosensory deafness and enlargement of the thyroid gland, a feature defined as thyroid goiter.

The auditory failure in Pendred syndrome is usually in the form of severe to profound deafness, while an uncommon form of Pendred hearing loss may appear as mild to moderate. Hearing loss in Pendred syndrome is most frequently sensorineural, but in rare cases can manifest itself as mixed. In 40% of the cases, hearing loss is concomitant with vestibular hypofunction that can vary among patients. Auditory deformities are mainly associated with Mondini dysplasia, a situation of incomplete cochlear formation, whereby instead of 2.5 turns of a cochlear spiral, a diminished number of turns are present (usually 1.5 turns). Likewise, vestibular dysmorphologies in Pendred syndrome are often a direct consequence of a dilation of the vestibular aqueducts (very narrow channel connecting the inner ear “vestibule” and the skull) and their internal substances.

Malfunctions or blocks in thyroid hormone production, which are prominent characteristics of Pendred syndrome, usually lead to goiter of the thyroid. Extended size of the thyroid gland is not congenital and onset can range from early puberty to adulthood. Swelling in the front area of the neck can emanate from the goiter defects. Sometimes the thyroid gland goiter cannot be clinically diagnosed and thus remains inapparent.

Originally, Pendred syndrome was clinically documented in 1896 by the British physician Vaughan Pendred. A century of scientific research led to the isolation of the Pendred gene in 1997, named *PDS* or *SLC26A4*, encoding the

pendrin protein (Everett et al. 1997). Pendrin was defined as an anion transporter protein operating in the inner ear, thyroid, and kidney. It is considered to be the causative mutation in most Pendred syndrome cases, as well as the underlying gene in DFNB4 nonsyndromic deafness.

The phenotype–genotype correlation between the goiter and *PDS* function has been elucidated. Normal pendrin permits passage of iodide in the thyroid gland, which is then used for the assembly of the hormone thyroxine. When pendrin function is disrupted, a block or insufficient production can lead to the thyroid symptoms associated with Pendred syndrome. Little is known regarding the exact association of pendrin function and deafness. Based on the *Pds* null mouse model, it was postulated that pendrin upholds a function in the maintenance of the endocochlear potential in the intermediate cells of the stria vascularis (Royaux et al. 2003).

2.5 Alport Syndrome

Alport syndrome is a paradigm of SHL that can be inherited in either an X-linked mode (85% of the cases; Table 2.1), in an autosomal recessive mode (about 14% of the cases), or in autosomal dominant mode (less than 1%) (Endreffy et al. 2005). A triad of medical symptoms depicts Alport syndrome: sensorineural hearing loss, renal disorders, and visual problems. Hearing impairment usually begins in the second decade of life, while severity is progressive and can differ between patients. Kidney abnormalities are also progressive and ranges from glomerulonephritis (inflammation of the glomerulus in the kidney), through hematuria (presence of blood in the urine), to end-stage renal failure. Common ophthalmologic symptoms characterizing Alport's syndrome are lenticular abnormalities such as congenital cataract, anterior lenticonus (conical or spherical lens protrusion into the anterior chamber), and fleck retinopathy (thickening of the basement membrane).

The genetics in Alport syndrome is dictated by three genes: *COL4A3*, *COL4A4*, and *COL4A5*. As discussed, Alport syndrome can be the result of recessive, dominant or X-linked inheritance. Mutations in *COL4A3* and *COL4A4* accounting for recessively inherited Alport syndrome, mutations in *COL4A5* account for an X-linked mode of inheritance, and roughly 1% of Alport syndrome families have dominant mutations in *COL4A3* and *COL4A4* .

The *COL4* gene family encodes collagen IV, the primary structural protein constituent of the basal lamina (basement membrane). Type IV collagen protein is composed of three α chains encoded by three separate genes, which assemble to form a triple-helical molecule. The *COL4A3*, *COL4A4*, and *COL4A5* genes give rise to the α3, α4, or α5 chains of collagen IV, respectively. Collagen IV trimers that aggregate from the three α chains (α3–5) are constrained in their expression to the kidney, inner ear, and eye basal lamina, whereas α1 and α2 are universally expressed in body tissues (Zehnder et al. 2005).

In the kidney, and most probably in the cochlea as well, a change in the expression of collagen IV α subunits occurs during embryonic development from

α1 and α2 Type IV collagen in early development to α3–6 during maturity, called an "isotype switching" process (Zehnder et al. 2005). Alport syndrome renal pathologies are the result of glomerular basement membrane dysmorphologies in the kidney, bringing on a defective glomerular function that gradually induces hematuria or proteinuria (presence of an excess of protein in the urine), up to renal failure. The absence of α3–5 renal type IV collagen chains in most of Alport syndrome males implies that isotype switching does not take place in the patients' kidneys.

Recently, similar findings regarding the inner ear have been published (Zehnder et al. 2005). The first substantial finding was that α3 and α5 chains expression in the cochlea is selectively limited to the basilar membranes of the spiral ligament (the attachment of the basilar membrane to the outer bony wall in the cochlea) and spiral limbus (the thickened connective tissue membrane of the osseous spiral lamina at the attachment of Reisner's membrane), while α1 exists ubiquitously in the basilar membrane of the inner ear. The second finding was that patients with X-linked Alport syndrome α3 and α5 chains were completely missing while α1 sustained its regular expression. This suggested that the isotype switching process also does not appear to occur in the cochlea in Alport syndrome, further supporting the longstanding theory that Alport syndrome hearing loss is associated with functional disruption in cochlear micromechanics or the spiral ligament (Gratton et al. 2005). Deformities of the extracellular matrix due to alterations in the *COL4* genes, which lead to failure of the interaction between cells and their surroundings, are the main cause of Alport conditions in the kidney and the inner ear.

3. Nonsyndromic Hearing Loss

Thus far, more than 100 forms of NSHL have been discovered (Hereditary Hearing Loss Homepage). Most of these are still identified as loci: only the chromosomal location of the defective gene is known. These are designated as DFNA for autosomal dominant inheritance, DFNB for autosomal recessive inheritance, and DFNX for X-linked inheritance. Each locus represents a family or number of families with hearing loss inherited in the relevant mode. In some cases, the associated gene has been cloned and in others, the symbol only reserved. There are almost 100 loci for dominant and recessive forms and fewer than 10 for X-linked, though it appears that most of these are actually forms of SHL. The most recent additions are DFNY for Y-linked inheritance, DFNM for modifiers, OTSC for otosclerosis, and AUNA for auditory neuropathy. In these cases, only a handful of loci have been identified. The loci are numbered in the order in which they were discovered. The information is updated regularly on the Hereditary Hearing Loss Homepage (Table 2.2).

Genes for NSHL have been categorized by the function of the proteins each gene encodes. Several superfamilies are represented, including gap junctions,

myosins, adhesion proteins, and ion channels. The most prevalent protein families are discussed in the subsections that follow.

3.1 Gap Junction Proteins

A specialized intersection zone connecting the cytoplasms of two adjoining cells, which permit a free passage of ions and molecules, is defined as a gap junction. In vertebrates, the protein constructing unit of this junction is a connexin. Two connexons form hemichannel structures, each made of six subunits of connexins, resting within the plasma membrane of neighboring cells adjacent to each other to create the gap junction channel (Sabag et al. 2005). In the inner ear, a network of these junctions is situated between the ephithelial supporting cells.

Thus far, three connexin genes have been implicated in NSHL: *CX26*, *CX30*, and *CX31*, corresponding to the proteins connexin 26, connexin 30, and connexin 31, which are designated by their molecular mass (connexin 26, for example, has a mass of 26 kDa; Gerido and White 2004; Sabag et al. 2005). An additional connexin, *CX32,* is involved in a syndromic hearing loss condition named Charcot–Marie–Tooth syndrome.

3.1.1 Connexin 26

Connexin 26 is by far the foremost prominent hearing impairment gene. The discovery that *GJB2*, located at the *DFNB1* locus, is a causative gene for hearing impairment (Kelsell et al. 1997) led to the unexpected revelation that more than half of the recessive NSHL and approximately 30% to 50% of innate hearing loss are due to *GJB2* mutations (Denoyelle et al. 1997; Kelsell et al. 1997; Zelante et al. 1997; Kelley et al. 1998). Even more surprising was the finding that among the 101 mutations documented worldwide up to now (The Connexin-Deafness Homepage; Table 2.2), one particular mutation, 35delG, is responsible for 70% of all the *GJB2* mutations (Maw et al. 1995; Snoeckx et al. 2005). This finding made an enormous impact in the hearing loss field, as it facilitated diagnostic and genetic screening of NSHL patients. Another mutation imperative to note is 167delT, which is most frequent among Ashkenazi Jews and is also present in Palestinians (Sobe et al. 1999; Shahin et al. 2002).

The most accepted theory regarding the role of connexin 26 in the inner ear is that it propagates the recycling of potassium ions necessary for the transmission of the auditory signal within hair cells, through gap junctions situated between supporting cells. This way, rapid circulation of potassium ions back to the endolymph through the stria vascularis (Kikuchi et al. 1995; Forge et al. 1999), allows maintenance of a high cochlear potential. The above hypothesis is based on the expression profile of connexin 26 in the cochlea, which is markedly noticeable in the bulky gap junctions of supporting cells of the sensory hair cells of the cochlea, the spiral ligament and in the spiral limbus (Kikuchi et al. 1995; Lautermann et al. 1998).

3.1.2 Connexin 30

Connexin 30 is the protein product of the *GJB6* gene, which lies within the same genetic interval of *GJB2* gene, *DFNB1*. It presents a similar expression configuration in the inner ear as connexin 26. A 342-kb deletion in the *GJB6* gene is directly linked to recessive NSHL (Lerer et al. 2001; del Castillo et al. 2002). The deletion was initially identified when an unusually larger number of profoundly deaf individuals with only one *GJB2* mutation were documented, more than expected given the carrier rate of *GJB2* mutations in the general population. Linkage analysis suggested that the deafness was due to a defect from the same chromosomal region. Further investigation of the chromosomal region uncovered this large deletion, which is extremely rare in the homozygous form. The *GJB6* mutation is usually seen on one allele only, in heterozygous form and in conjugation with a heterozygous *GJB2* mutation, creating a recessive effect, which indicates that these two proteins act together and/or share similar redundant functions in the ear.

3.2 Myosins

A substantial number of members of this superfamily can bind to different types of molecules and transfer them across the actin filament network. As a direct consequence of the above activities, the myosin molecules participate in cell functions including muscle contraction, chemotaxis, cytokinesis, pinocytosis, targeted vesicle transport, membrane traffic, and signal transduction (Mermall et al. 1998; Frank et al. 2004).

The entire superfamily of myosins shares a few common domains reviewed in (Sellers 2000). In the N-terminal, there is the highly conserved head domain (also called the motor domain) which contains two subdomains, an actin binding domain and an ATP binding domain, both enabling the head domain to associate and dissociate from actin fibers, effecting movement along them. Next, there is the neck domain, which contains 1–6 IQ motifs (a consensus sequence serves as a binding site for calcium-binding domains). Following the neck region, at the C-terminus, comes the tail domain, the most diverse domain (in length and in sequence) in the myosin superfamily. The myosin tail domain binds cargo molecules and has been predicted to target particular myosins to specific cellular compartments, therefore determining the whole molecule function and specificity of each myosin type.

Numerous types of unconventional myosins have been identified as NSHL-causing genes: *MYO1A*, *MYO3A*, *MYO6*, *MYO7A*, and *MYO15A* (encoding myosin I, myosin IIIA, myosin VI, myosin VIIA, and myosin XVA proteins, respectively). The term "unconventional" myosins refers to all nonmuscle myosin subclasses (in contrast with the "conventional" myosins that include only the myosin II subclass). Several of these myosins are described in the paragraphs that follow.

3.2.1 Myosin VI

The myosin VI protein was first implicated in the hearing mechanism when it was found to be mutated in the spontaneously deaf mouse Snell's *waltzer* (*sv*) (Avraham et al. 1995), with a deletion in the myosin VI (*Myo6*) transcript. The deletion results in a truncated protein, leading to a frameshift and subsequent premature stop codon. Mutations in the corresponding human *MYO6* gene, which shares great similarities with the mouse gene, were also identified; a missense mutation (DFNA22) in the motor domain causing dominant NSHL (Melchionda et al. 2001), and three recessive mutations found in DFNB37, also related to profound congenital NSHL (Ahmed et al. 2003a). A form of cardio–auditory syndrome may also be due to a myosin VI mutation, as a dominant missense mutation was discovered in a family segregating both sensorineural deafness and familial hypertrophic cardiomyopathy (Mohiddin et al. 2004).

Snell's *waltzer* mice possess vestibular pathologies such as head tossing, circling, and hyperactivity (Deol and Green 1966). Likewise, their cochlear hair cell phenotype is abnormal; their hair cells appear normal after birth but become disorganized and fuse to form massive stereocilia by the 20th day after birth. Further study led to the revelation that the *sv* mice do not bear any detectable myosin VI in their hair cells (Avraham et al. 1995).

Two interesting facts suggest that myosin VI has a fundamental role in the inner ear; this is the only myosin known to move "backwards" toward the minus end of actin filaments (Wells et al. 1999), and in the inner ear it is expressed solely within the hair cells (Hasson et al. 1997). Within the hair cell, myosin VI is normally expressed in the cytoplasm, and mainly at the cuticular plate, the apical region of the hair cell at the base of the stereocilia, which serves to anchor the stereocilia to the intracellular cytoplasm, and at the pericuticular necklace (the intacellular zones surrounding the cuticular plate, from both sides) at the apical side of these cells (Avraham et al. 1997; Hasson et al. 1997).

The specific function of the myosin VI protein in the inner ear remains to be discovered. Suggestions of its potential roles came mainly from mouse and zebrafish myosin VI mutant models and from cultured cell lines. Among the more popular theories is that myosin VI functions as an anchoring protein that stabilizes the interface connection between the apical membrane to the actin mesh in the cuticular plate of hair cells (Hasson et al. 1997) or that myosin VI plays a role in endocytosis or membrane trafficking in hair cells, thereby involved in the development and maintenance of stereocilia structure.

3.2.2 Myosin VIIA

Hearing loss–related mutations in *MYO7A* were originally exposed in the *shaker1* (*sh1*) mouse, which was proposed as a model for Usher syndrome. Until now, 10 distinct mutations have been associated in different *sh1* alleles (Libby and Steel 2001), carrying vestibular defects pronounced as head tossing, circling, and hyperactivity (Gibson et al. 1995). The mutant cochlear phenotype is accompanied by progressive degeneration of the organ of Corti, resulting eventually

in profound deafness (Libby and Steel 2001). By virtue of using these models, mutations in the *MYO7A* human homologue were identified as the genetic cause for Usher syndrome type 1B (Weil et al. 1995, 1996) and Usher syndrome type 2A (Maubaret et al. 2005). Contiguous to this discovery, human *MYO7A* was also linked to NSHL in two distinctive loci: *DFNB2* (Weil et al. 1997) and *DFNA11* (Liu et al. 1997).

Myosin VIIa operates in a wide range of tissues but is highlighted in its appearance within the cochlea in hair cell epithelia, where it is expressed in the cytoplasm, the cuticular plate and along the length of the stereocilia (Hasson et al. 1997). A breakthrough in the elucidation of myosin VIIa function in the ear was achieved by identifying some of its protein interactors via yeast two hybrid screens. One possible destination for myosin VIIa in the inner ear is the anchoring of stereocilia crosslinks, in which an interactor transmembranal protein named vezatin can act as a mediator (Petit et al. 2001). Another possibility that myosin VIIa takes part in the development of the stereocilia bundle arose from observations that myosin VIIa binds to cadherin 23 and harmonin (Boeda et al. 2002).

3.2.3 Myosin XV

An interesting series of events led to the discovery of the *MYO15* gene as a causative deafness gene. Initially, a broad-scale mapping strategy in a Balinese community of 2200 peoples prompted the identification of the recessive NSHL locus, referred to as *DFNB3* on the long arm (p11.2) of chromosome 17 (Friedman et al. 1995; Liang et al. 1998). Based on homology of the *DFNB3* locus region to the mouse chromosomal region, the *shaker2* mouse mutant was a candidate mouse model. *Shaker2* is a profoundly deaf mutant presenting classical vestibular abnormal behavior with short hair cell stereocilia in the cochlear and vestibular systems (Probst et al. 1998; Beyer et al. 2000). A molecular genetic strategy was undertaken to prove a correlation between the mouse locus and its human corresponding locus. Bacterial artificial clones (BAC) (Table 2.1) containing candidate genes from the *DFNB3* interval were injected into *shaker2* embryos and their influence on the phenotype was assessed. One of these transgenes (Table 2.1) that managed to "rescue" the mutant *shaker2* contained the *Myo15a* gene (Probst et al. 1998). Sequencing of the *Myo15a* gene in the mutant *shaker2* mouse revealed a missense substitution of a highly conserved residue in the myosin XV head domain, clarifying that this is the deafness causing gene in these mice. *MYO15A* sequencing analysis in DFNB3 families confirmed that this unconventional myosin is implicated in human recessive NSHL (Wang et al. 1998; Liburd et al. 2001).

Myosin XVa was first documented to be expressed in inner and outer hair cells of the cochlea in an in situ hybridization analysis (Table 2.1; Lloyd et al. 2001). Further observations using an antibody raised against a portion of the myosin VIIa protein refined the localization of myosin XV within the hair cells, and proved that this protein is situated in the tip of each hair cell stereocilia (Belyantseva et al. 2003). Tip links, formed by microscopic filaments interconnected between

adjacent stereocilia, are speculated to be the position where the mechanically gated transduction channels are situated (Hudspeth and Corey 1977; Pickles et al. 1984). Therefore localization of a protein near this region implies its significance in hearing process.

One of the roles postulated for myosin XV is that it is a critical factor in the evolvement of the hair bundle and in the staircase formation of the bundle (Belyantseva et al. 2003), as the appearance of myosin XV expression coincides with the appearance of the graduated patterning of the stereocilia. Moreover, based on observations showing that myosin XV binds another deafness molecule, whirlin, at the tip link zone, it was suggested that the two molecules might control stereocilia elongation patterning during development and may be implicated in stabilizing connections between stereocilia (Delprat et al. 2005).

3.3 Adhesion Proteins

Adhesion proteins are a diverse group of distinct protein families including integrins, selectins, members of the immunoglobulin superclass family, and cadherins. They protrude upon the cell surface, where they facilitate cell to cell or cell–extracellular matrix interactions and binding. The common denominator for this set of molecules seems to be that they are all glycoproteins involved in adhesion, recognition, activation, and migration activities.

3.3.1 Cadherin 23

Cadherins are a subset of the adhesion molecule superfamily comprising cadherins, protocadherins, desmogleins, and desmocollins. They promote cell binding activities, and rely on the presence of calcium ions for their proper functions. Cadherin-specific calcium binding motifs are repetitively present along the molecule structure called EC domains (Suzuki 1996; Nollet et al. 2000). They are also predicted to share a common cytoplasmic motif enabling their connection to the cytoskeleton network (Gumbiner 1996).

As mentioned in Section 2.3, mutations in the *CDH23* gene, coding for the cadherin 23 protein, are associated with USH1D. The *USH1D* locus was found to coincide with the recessive NSHL locus *DFNB12*, which is associated with prelingual moderate to profound hearing loss. These data suggested that *DFNB12* and *USH1D* in fact are mutually derived from *CDH23* allelic mutations, both resulting in syndromic and nonsyndromic forms of hearing loss (Bork et al. 2001; Astuto et al. 2002).

Cadherin 23 appears as early as P0 in cochlear hair bundles and in Reissner's membrane, but later on, by P42, cadherin 23 expression is restricted to stereocilia tip links exclusively (Siemens et al. 2004), where it binds to myosin 1c, a predicted component of the mechano-transduction complex. Therefore, cadherin 23 was hypothesized to participate in the activation of this process. Interestingly, data from research in the Usher syndrome field has proven that cadherin 23 forms a complex with myosin VIIa and harmonin b, indicating that this

molecule is a vital member of a specific unit designated for maintaining stereocilia inter-cohesion (Boeda et al. 2002). The fact that mice cochlea deficient in *Cdh23* display disorganized stereociliary bundle patterns (Di Palma et al. 2001) emphasizes that this protein is a central factor in stereocilia maintenance.

3.3.2 Protocadherin 15

Another member of the cadherin superfamily of molecules is protocadherin 15, the protein product of the *PCDH15* gene. As discussed in the section dealing with Usher syndrome, the *PCDH15* gene was originally implicated as a hearing loss causing gene via the deaf and vestibular deformed Ames *waltzer* mouse mutant (Alagramam et al. 2001). Thereafter the human orthologous gene was cloned and identified as one of the genes responsible for Usher syndrome, USH1F (Ahmed et al. 2001). Only two years later, *PCDH15* was documented as a NSHL causing gene (Ahmed et al. 2003b). This case of a gene locus causing both NSHL and SHL leading to Usher syndrome is similar to the case of the *CDH23* gene.

An explanation of why certain mutations in *PCDH15* cause NSHL, while others bring on a set of additional symptoms and cause SHL, was suggested (Ahmed et al. 2003b). They demonstrated a genotype–phenotype correlation between distinct mutations and their derived phenotype, from which it was deduced that more acute *PCDH15* mutations result in USH1, whereas hypomorphic (Table 2.1) mutations render the phenotype into NSHL (DFNB23) only.

Clues regarding the function of protocadherin 15 in the hearing process emerged from the study of wild type and Ames *waltzer* mice. Normal expression of protocadherin 15 was seen at embryonic day 16, overlapping the period during which stereocilia begin their formation. On the other hand, the stereocilia of Ames *waltzer* were remarkably disoriented, and displayed degeneration. Clearly, this indicated that protocadherin 15 plays a vital role in the formation and maintenance of the stereocilia. Recent data support this theory, whereby protocadherin 15 binds to myosin VIIa protein in the hair bundle, where they seem to collaborate in the regulation of the bundle development (Senften et al. 2006). In addition, due to the expression pattern of protocadherin 15 seen along the length of the stereocilia, the cuticular plate and the cytoplasm of auditory hair cells, it was suggested that protocadherin 15 contributes to evolvement and maintenance of the stereocilia lateral links (Ahmed et al. 2003b).

3.4 Ion Channels

Opening or closing of ion channels regulates the diffusion of ions through membranes. In the inner ear, ion channels have a tremendous weight in the recycling and maintaining of endolymph ionic homeostasis, a crucial condition for normal auditory transduction. The mechano-transduction theory, in which ion channels at the stereocilia tip links open when the stereocilia bundle is deflected, thus permitting an ionic potassium current to alter the hair cell voltage,

emphasizes the imperative central role of ion channels in auditory hair cells. A wealth of evidence that has accumulated over the years has identified several different genes, both for syndromic and nonsyndromic hearing loss, which encode ion channels and transporters.

3.4.1 *KCNQ4*

The *KCNQ4* gene codes for one of the voltage-gated potassium channel proteins. Its ultrastructure and mechanism of action were precisely deciphered by X-ray three-dimensional modeling (Doyle et al. 1998); it is composed of four protein units forming a homo/heterotetramer. Six transmembrane domains and a one pore-loop segment in each of these proteins create the specific transformational structure of the whole channel.

In 1994, a new locus of dominant progressive NSHL, *DFNA2*, was defined (Coucke et al. 1994). The *KCNQ4* gene, which is located at the *DFNA2* interval, was a natural candidate as the causative gene for hearing loss in this locus. A few years later, this was indeed proven to be the case (Coucke et al. 1999; Kubisch et al. 1999). The *KCNQ4* mutations result in late adult-onset progressive hearing loss, commencing in the second decade of life, with a comparatively preserved lingual abilities (Coucke et al. 1999; Bom et al. 2001), while it deteriorates to profound hearing loss within 10 years.

Hypotheses regarding the role of the KCNQ4 protein in the inner ear are mostly derived from its cellular and subcellular pattern of expression in the auditory apparatus. The KCNQ4 protein is localized in the mouse cochlea in outer hair cells only, where it is bound to the basal membrane (Kharkovets et al. 2000), and appears also to express in a base to apex gradient in the spiral ganglion sensory neurons (Beisel et al. 2000). The gradient configuration of expression may be the underlying explanation of high-frequency hearing loss in DFNA2 families. This was the first ion channel noted to be specifically expressed in a sensory pathway, as KCNQ4 was demonstrated to be expressed in numerous nuclei of the central auditory pathway in the brain stem. Thus, KCNQ4 might stimulate a change in the electrical characterizations of outer hair cells, enable a path of exit for potassium ions out of the hair cells, and potentially operate in both functions (Kharkovets et al. 2000).

4. How Genes are Identified

Recombinant DNA and molecular biology techniques developed in the 1970s and 1980s made the discovery of human genetic disease genes possible. Several techniques revolutionized the way disease genes are found, including restriction fragment length polymorphism (RFLP) analysis using radioactive isotopes and Southern blotting, the polymerase chain reaction (PCR), and sequencing. For details on cloning techniques, refer to the *Cold Spring Harbor Laboratory Manual* (Sambrook and Russell 2001).

In brief, a family with inherited hearing loss is first ascertained by a medical geneticist or otolaryngologist. Close collaboration with audiologists is crucial for characterizing the auditory phenotype. A medical history is taken, with particular emphasis given to subjective degree of hearing loss, age at onset, evolution of hearing loss, symmetry of the hearing impairment, hearing aids, presence of tinnitus, medication, noise exposure, pathologic changes in the ear, and other relevant clinical manifestations. Blood samples for the extraction of genomic DNA are drawn from participating individuals after informed consent is obtained in accordance with guidelines of a Helsinki or Institutional Review Board (IRB) Committee (see National Institutes of Health Office of Human Subjects Research, Table 2.2).

Owing to the prevalence of connexin 26 and 30 mutations and the ease of screening, PCR is first performed on the proband to rule in or out connexin involvement. Several diagnostic algorithms have been suggested for clinical practitioners caring for the deaf when searching for the etiology of the hearing loss (Greinwald and Hartnick 2002; Brownstein et al. 2004). Unfortunately, the other genes are either less prevalent or cumbersome and costly to screen, making fast diagnostics impractical. Several approaches have been taken. When examining a particular ethnic group, one may examine for mutations already identified in this group. For example, in the case of Jewish Ashkenazi children diagnosed with profound congenital deafness, the R245X mutation in *PCDH15* is also examined (Brownstein et al. 2004). In the Palestinian population, mutations in several genes have already been identified, including *TMPRSS3* (Bonne-Tamir et al. 1996; Scott et al. 2001), *stereocilin* (Campbell et al. 1997; Verpy et al. 2001), *otoancorin* (Zwaenepoel et al. 2002), *whirlin* (Mustapha et al. 2002; Mburu et al. 2003), *DFNB33* (Medlej-Hashim et al. 2002), *pendrin* (Baldwin et al. 1995), *DFNB17* (Greinwald et al. 1998) and most recently, *TRIOBP* (Shahin et al. 2006). An alternative approach was taken to search for mutations in 156 Palestinian deaf probands and their families (Walsh et al. 2006). Initially, connexin 26 mutations were found in 17% of the group. Thereafter, a hearing-loss-targeted genome scan was performed on 10 families, using microsatelite markers flanking 36 loci associated with hearing loss. In the cases where linkage was found, the genes lying in the region were examined, leading to identification of *TMPRSS3*, *pendrin*, and *otoancorin* mutations.

When DNA from a large family can be obtained, the method of choice is to perform a whole genome scan, using evenly distributed microsatellite markers spanning the genome. Usually a set of 364 markers are used to identify a chromosomal location. To perform such a scan, multiply affected members (as well as unaffected members) are required from one extended family. The minimum number is approximately five for a family with recessive inheritance and 10 for a family with dominant inheritance; more individuals provide a greater chance of identifying a significant chromosomal location. Once such a region is defined by a logarithmic odds (LOD) score (Table 2.1) of at least three, the critical region is further defined by typing additional markers in the region. Statistical analyses are essential for human genetic mapping and programs are available on

the Internet (for examples, see Genetic Linkage Analysis, Table 2.2). Eventually, a map of the genes in the region of linkage is examined and mutation analysis by sequencing is performed. The most widely used resource for identifying the genes in the region is the UCSC Genome Browser (Table 2.2), which lists known annotated genes as well as expressed sequence tags (ESTs) (Table 2.1) that might represent additional splice forms. For example, while the *TRIOBP* gene was known to reside in the chromosome 22-linked region of the DFNB28 Palestinian families, no mutations were found. Additional ESTs revealed the presence of a longer isoform of TRIOBP; subsequent mutation analysis uncovered novel mutations in this novel isoform in the Indian and Pakistani population as well (Riazuddin et al. 2006; Shahin et al. 2006).

5. Animal Models to Study Human Genetic Hearing Loss

The study of the genetics of hearing loss in humans is fraught with difficulties, mostly due to the shortage of large affected families and heterogeneity of genetic NSHL in humans. On the other hand, utilization of the mouse as a research tool brings on several significant advantages that answer the needs of biological studies of genetic hearing loss: vestibular abnormalities are more apparent in mice and are often linked to deafness, making it easier to detect deaf mice. Moreover, the accessibility to the ear organs and the ability to introduce defined genetic changes through the generation of transgenic or knockout mouse models (Ahituv and Avraham 2002) have proved an exceptional tool for studying the mechanisms of genetic hearing loss.

The large reservoir of mouse models currently available are divided into three main groups: spontaneous mutants, transgenic or knockout mice, and radiation or chemically induced mutants. The main goal of inducing mutations by radiation or chemicals is to create random mutations throughout the genome, in order to produce new mouse mutants, which then could be screened according to their different mutant phenotypes. The *N*-ethyl-*N*-nitrosourea (ENU) mutagen is a potent alkylating reagent that primarily induces point mutations and mutagenizes mouse spermatogonial stem cells efficiently (Balling 2001). It produces single locus mutation frequencies of 6×10^{-3}–1.5×10^{-3}, which is equivalent to obtaining a mutation in a single gene of choice (Popp et al. 1983). The phenotype-driven screening approach is used to select the mutants one needs from G1 (first generation) offspring (Nolan et al. 2002). ENU mutagenesis is an efficient method for generating auditory and vestibular mouse mutants. First, in the context of the genetics of hearing research, several simple behavioral phenotypic tests are available that can easily indicate an auditory or a vestibular dysfunction, such as the Preyer reflex test (ear flick), which examines the response of the mouse to a calibrated sound burst (Balling 2001). Second, statistically, the ENU mutagen causes a point mutation in a single gene only, which simplifies the isolation of the mutated gene afterwards. Several examples demonstrate the discovery of mouse models for human deafness loci and syndromes, including Beethoven for

DFNA36 (Kurima et al. 2002; Vreugde et al. 2002), Headbanger for *DFNA11* (Rhodes et al. 2004; Street et al. 2004), and 9 mutant alleles mapping to mouse chromosome 4 for CHARGE syndrome (Bosman et al. 2005). Databases of ENU mutants with inner ear and other defects are available (The German Mouse ENU Project and The Harwell Mutagenesis Program, Table 2.2).

While using ENU or radiation-induced mutagenesis is a phenotype-driven approach (the phenotype is known, while the genotype has yet to be discovered), transgenesis or gene-targeted knockouts are part of a genotype-driven approach. By injecting a known gene into the single cell embryo (for producing transgenics) or gene-targeted embryonic stem cells into blastocysts (for generating knockouts), one discovers the role of the gene by observing the phenotype that arises from overexpressing the relevant gene or removing its function altogether from a mouse. The ability to create these mouse models has revolutionized the study of human genetic disease, since it opened up the way for creating valuable mouse models for disease. A knockout for the most prevalent form of deafness, connexin 26, was created by removing connexin 26 from specific portions of the inner ear only using an otogelin promoter (Cohen-Salmon et al. 2002); a gene knockout in mice is lethal due to species-specific differences between humans and mice (Gabriel et al. 1998). Other examples of mouse knockouts for human deafness loci are *DFNA2* and *KCNQ4* mutant mice (Kubisch et al. 1999; Kharkovets et al. 2006). An analysis of the mice demonstrated that *KCNQ4*-associated hearing loss is predominantly caused by a slow degeneration of outer hair cells (OHCs) resulting from chronic depolarization.

A comprehensive list of gene-targeted mice with inner ear defects is provided in a thorough review (Anagnostopoulos 2002) and several regularly updated web sites (Table 2.2).

6. Approaches to Understanding Protein Function

The gene "hunting" research commences with genotyping DNA derived from a family or families with hearing loss with microsatellite markers, followed by sequencing for mutations in the linked regions. This phase of the research is a time-consuming process and may take years. If a candidate gene is subsequently found to bear a mutation, the researcher is compelled to study the protein encoded by the deafness gene in order to understand how auditory function is compromised.

Expression studies make it possible to follow the localization of the gene and protein and often help provide a hypothesis regarding function based on the temporal and spatial expression. For expression of genes, PCR can be performed to determine whether the gene is expressed in the inner ear and at what stage. An example for the *Myo6* gene is shown in Fig. 2.1 (see Section 3.2.1). For evaluation of quantity, real-time reverse transcriptase (RT)-PCR should be performed. An example for the *Tmc1* gene is shown in Fig. 2.2. For examination of the temporal and spatial expression of a gene, mRNA in situ hybridization

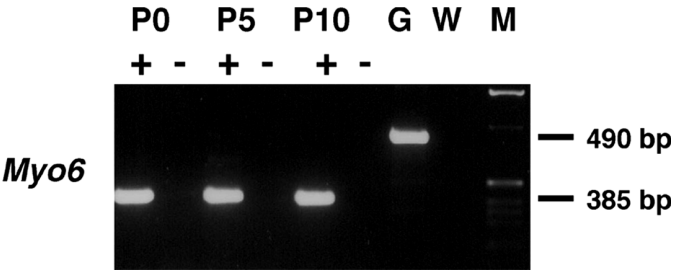

FIGURE 2.1. Expression of myosin VI (*Myo6*) in the mouse cochlea. RNA from postnatal (P) day stages P0, P5, and P10 was used to amplify the gene by RT-PCR. Amplifications were carried out with and without reverse transcriptase (+/–), by using cochlear RNA, genomic DNA (G), and a water control (W). (Modified from Walsh et al. 2002.)

is performed. An example for the *Ush3* gene is shown in Fig 2.3 (see Section 2.3). To follow the expression pattern of the protein, a suitable antibody directed against the protein is essential. This requires identifying an epitope that will be recognized uniquely by the protein, which can be determined using bioinformatic analyses (for example, Epitope Prediction, Table 2.2). The localization of Pou4f3, myosin VI and Lhx3 expression in the inner ear was revealed using specific antibodies against each protein, shown in Fig. 2.4. In many cases, the work has already been done by other investigators and a search through Pubmed (Table 2.2) and expression data sites will reveal where the gene and/or protein is expressed (for examples see NCBI sites: Gene Expression Omnibus, Unigene,

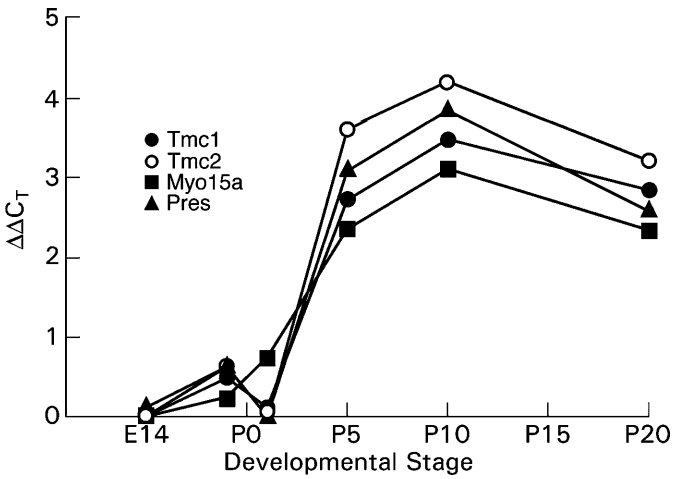

FIGURE 2.2. Real-time RT-PCR analysis of *Tmc1*, *Tmc2*, myosin XVa (*Myo15a*), and prestin (*Pres*) mRNA levels in mouse temporal bones at embryonic and postnatal stages. (From Kurima et al. 2002.)

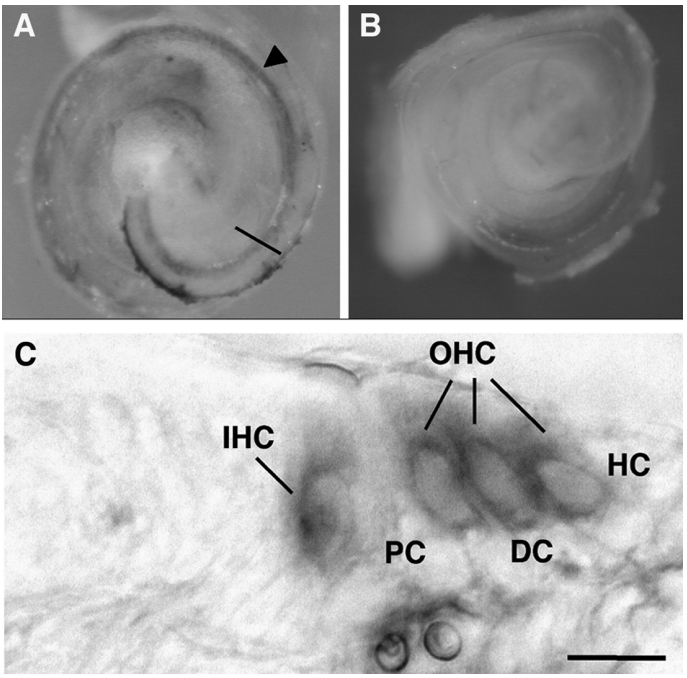

FIGURE 2.3. Detection of *Ush3a* mRNA expression by in situ hybridization. (**A**) Whole mount of a cochlea at embryonic (E) day 16, with staining along the region of the hair cells (*arrowhead*). (**B**) Sense probe demonstrates that staining in **A** is specific. (**C**) Sectioning through the cochleae (line in **A**) revealed specific hybridization in the inner (IHC) and outer hair cells (OHC) of the organ of Corti, but not in the Deiters cells (DC), pillar cells (PC), or the Hensen cells (HC). (Modified from Adato et al. 2002.)

and SOURCE, Table 2.2). There are fewer data about inner ear expression in these general expression databases.

Experiments using cell culture techniques may also reveal the function of a protein. One can overexpress the wild-type and mutant form of the gene that corresponds to the deafness phenotype. For example, to determine the mechanism for *POU4F3 DFNA15*-associated hearing loss in an Israeli kindred, the human gene was cloned into an expression vector and overexpressed in HEK293, COS-7, and the established cochlear cell line UB/OC-2 cells (Weiss et al. 2003). While the wild-type form of the gene was localized to the nucleus, as expected for a transcription factor, the mutant form was also expressed in the cytoplasm. Subsequent bioinformatics and experimental analysis revealed that a bipartite nuclear localization signal (NLS) was removed due to the truncation caused by the 8-bp deletion, leading to partial loss of nuclear localization (Fig. 2.5). To examine connexin mutations and proper localization of gap junction formation, the gene has been cloned in expression vectors and fused to a reporter, GFP, to enable localization of the wild-type and mutant proteins. In this way, many

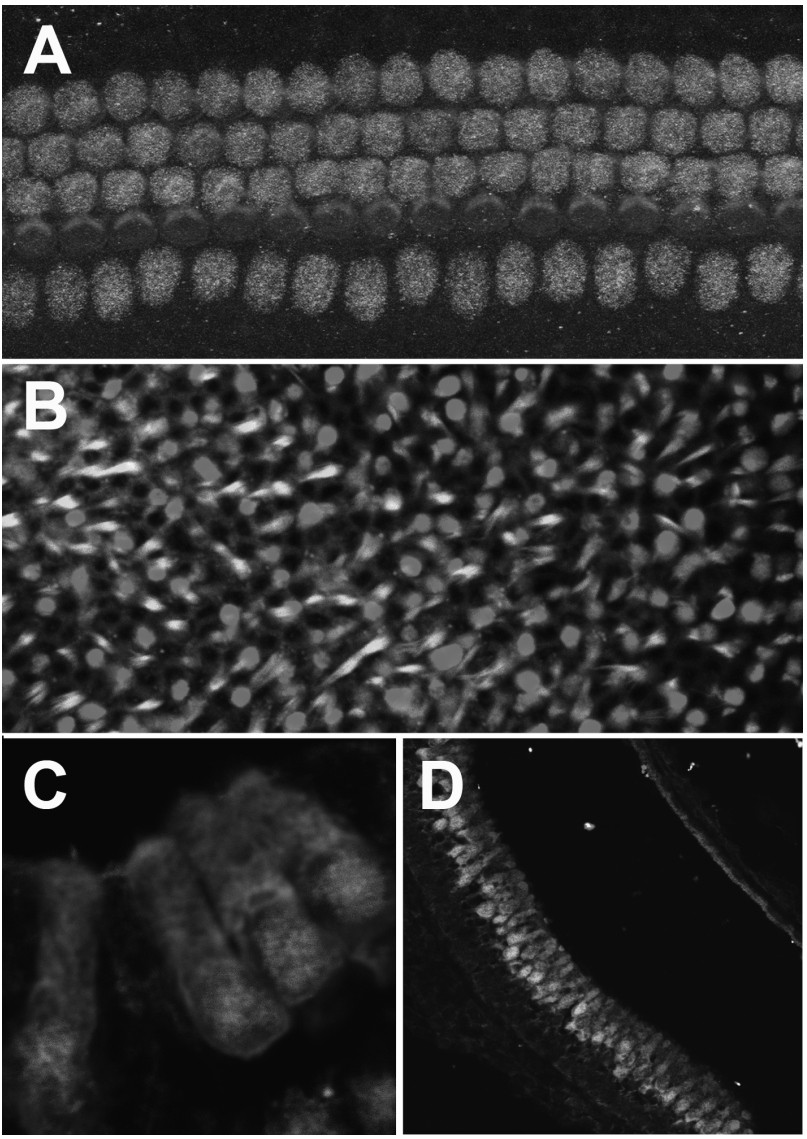

FIGURE 2.4. Expression of Pou4f3, myosin VI, and Lhx3 in the auditory and vestibular systems, demonstrated by immunohistochemistry with antibodies against each protein. (**A**)Whole-mount immunohistochemistry shows that Pou4f3 is expressed in the nuclei of inner and outer hair cells at E18.5 (green in online version). Actin can be visualized with phalloidin (red in online version). (Modified from Hertzano et al. 2004) (**B**) Whole-mount immunohistochemistry shows that the unconventional myosin VI (red in online version) is expressed in the cytoplasm of utricular hair cells at P10, while actin demonstrates the presence of stereocilia, stained with phalloidin (green in online version). (**C**) Cryosections shows the expression of Lhx3 (green in online version) and myosin VI (red in online version) in the cochleae and (**D**) the vestibular system neuroepithelial cells in the utricle from inner ears of E18.5 mice. Lhx3 is expressed in the nuclei of all hair cells. (**B**, **C**, and **D** provided by Amiel Dror and Karen Avraham; Hertzano et al., 2007.)

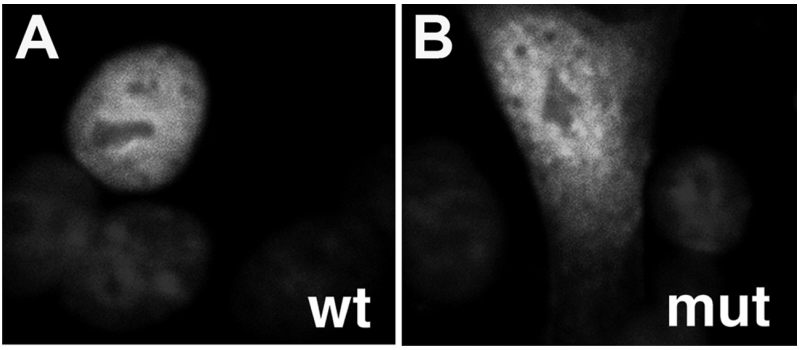

FIGURE 2.5. The dominant mutation in POU4F3 changes the subcellular localization of the protein in transfected COS-7 cells. (**A**) The wild-type form of the protein is localized to the nucleus. (**B**) The mutant form of the protein is localized to both the cytoplasm and nucleus. (Modified from Weiss et al. 2003.)

deafness causing connexin 26 mutations have been studied. For example, not only is the abnormality revealed by these experiments (Fig. 2.6), but determining whether the mutation is pathogenenic or not may be answered. For example, the M34T connexin 26 mutation has been the subject of debate for years. Though originally identified in a family with deafness (Kelsell et al. 1997), reports from other investigators revealed that this mutation exists in normal hearing individuals (Scott et al. 1998). One study suggests that the mutation is pathogenic, because

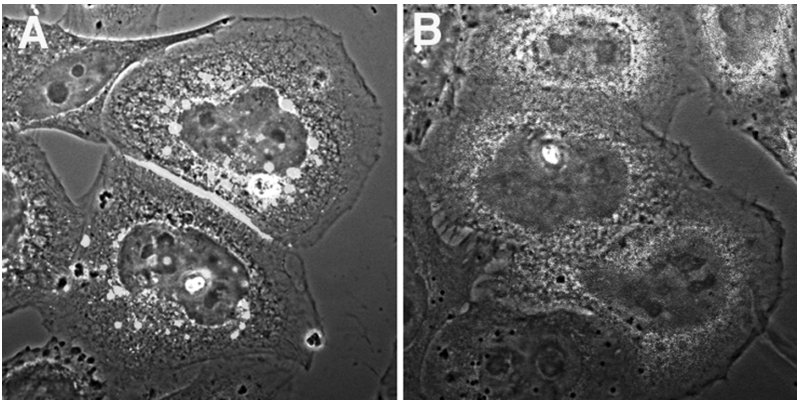

FIGURE 2.6. Connexin-GFP fused protein expressed in transfected HeLa cells. (**A**) Wild-type Cx26 is expressed in the plasma membrane, creating gap junction plaques between adjacent cells. (**B**) When expressing Cx26 carrying the deafness-causing mutation Ser139Asn in transfected cells, the protein fails to reach the plasma membrane and no gap junction plaques are formed. (Courtesy of Adi Sabag and Karen Avraham; Fleishman et al. 2006.)

although it leads to normal gap junction localization, it also leads to abnormal gating (Skerrett et al. 2004).

The espin actin-bundling proteins are involved in deafness in both *jerker* mice and DFNB36 (Zheng et al. 2000; Naz et al. 2004). A new function for this protein, the ability to assemble a large actin bundle when targeted to a specific subcellular location, was revealed by transfection into neuronal and other cells (Loomis et al. 2006). Most recently, siRNA has been developed, using the natural biological mechanism of RNA interference, where double-stranded RNA (dsRNA) induces gene silencing by targeting complementary mRNA for degradation, enabling silencing of genes in cells (Hannon and Rossi 2004). For example, RNAi reduced huntingtin mRNA and protein expression in cell culture and in the brain of a mouse model for Huntington's disease (Harper 2005). The silencing improved behavioral and neuropathological abnormalities associated with this disease. siRNA was successfully used in human HEK293 and mouse P19 cells to suppress a specific connexin 26 mutation (Maeda et al. 2005). Most important, the study demonstrated that a specific mutation could be suppressed in a transgenic mouse model, paving the way for future experiments of this sort for therapeutics.

There are numerous additional techniques available to determine gene and protein function, including microarray-based processes. DNA microarrays encompass a multifaceted approach to understanding complex interactions between genes (reviewed in Schulze and Downward 2001). Microarrays with the readily available genes from the genome can be screened to identify downstream targets for transcription factors, as was done by comparing RNA derived from Pou4f3-deficient inner ears to wild type ears (Hertzano et al. 2004). Alternatively, deafness genes can be identified by studying differential expression of genes within the cochlear using a custom mouse inner ear microarray (Morris et al. 2005). A review covering the uses of microarrays for inner ear research describes aspects of this experimentation (Chen and Corey 2002).

7. Summary: Implication of Discovery of Genes Associated with Hearing Loss

Why has the genetics of hearing loss become such a focus for researchers? First, from a biological perspective, the amount of information gained about the auditory and vestibular systems has been dramatic. Second, from a diagnostic aspect, clinicians are now able to discern the etiology of hearing loss a large number of patients by relatively simple genetic testing. Third, from a genetic counseling aspect, genetic counselors are able to predict with much greater certainty what the chances of another child being born with deafness in the family are. Fourth, from a therapeutic aspect, the discovery of genes may provide solutions for treatment and therapy for alleviating hearing loss (see Heller and Raphael, Chapter 11).

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