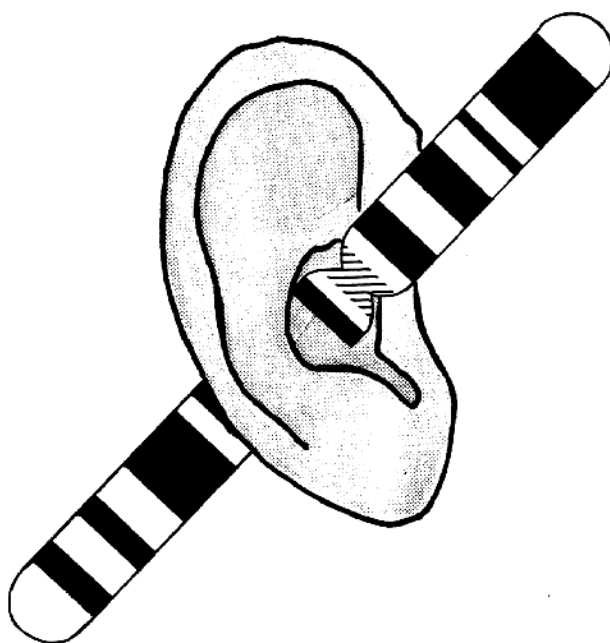


HEREDITARY DEAFNESS NEWSLETTER



April 2001

No. 18



Defeating Deafness

THE HEARING RESEARCH TRUST

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Helping researchers defeat deafness

330/332 Gray's Inn Road, LONDON, WC1X 8EE
Telephone: +44 171 833 1733 Fax: +44 171 278 0404

HEREDITARY DEAFNESS

NEWSLETTER

April 2001

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Editorial

This is the last issue of the Hereditary Deafness Newsletter. The field is moving so fast, and web-based access to information so much easier to obtain that a paper newsletter seems redundant. I would like to thank the many scientists and clinicians who have contributed items to the newsletter over the years, my assistants in compiling the newsletter (most recently Charlotte Rhodes) and the sponsors (presently Defeating Deafness, formerly RNID) for their continued support.

Happy Surfing!

Karen Steel
Editor

ANN ARBOR

University of Michigan¹
 Otolaryngology – HNS
 1500 East Medical Center Drive
 Ann Arbor
 Michigan 48105
 USA

DJ Brown¹
 I Bespalova¹
 MM Lesperance¹

Candidate gene analysis of DFNA25, a novel locus responsible for autosomal dominant, high frequency, nonsyndromic hearing loss

DFNA25 is a novel locus responsible for autosomal dominant, delayed onset, progressive, high frequency, non-syndromic hearing loss in a large family of Bohemian descent. Haplotype analysis revealed recombination events between *D12S327* and *D12S1051* in 4 affected individuals and a recombination event between *D12S1030* and *D12S84* in one affected individual. The DFNA25 interval is thus defined by *D12S327* (centromeric) and *D12S84* (telomeric), a 20cM region of chromosome 12q21-24. The maximum two-point LOD score was 6.82 at capital Theta, Greek = 0.041 for *D12S1030*.

Candidate genes lying within the DFNA25 interval include *UBE3B*, *ATP2A2*, *ATP2B1*, *zFOCI*, *LTA4H*, and *PAH*. BAC contigs lying within the DFNA25 genetic interval were identified (Baylor HGSC). Polymorphic markers mapping to BACs containing candidate genes of interest were genotyped in the family members to assess for recombination events that would allow exclusion of the BAC from the candidate region. The marker *RK341/301* colocalizes to the same BAC in 12q24 as the *UBE3B* gene and was informative in the DFNA25 family. *UBE3B* is a member of the E3 ubiquitin ligase family involved in cellular homeostasis and apoptosis (Ciechanover et al. 1991) and upregulation of its mRNA has been observed after noise exposure in the chick (Lomax et al 2000).

ATP2A2 is a calcium channel gene related to *Atp2b2*, which is known to be mutated in deafwaddler (*dfw*) mice (Street et al. 1998). Mutations in *ATP2A2* cause Darier-White disease, which is involved in intercellular communication between epidermal cells, similar to the role of connexin 26 (*GJB2*) and connexin 31 (*GJB3*). *D12S2398* was confirmed by BLAST to map to the same BAC as *ATP2A2*, and this marker was genotyped in the DFNA25 family.

For BACs lacking described polymorphic markers, novel polymorphic markers were developed by screening the sequence for short tandem repeats (STRs), designing PCR primers, amplifying the STR sequence in genomic DNA of unrelated controls, and assessing the heterozygosity of the marker using SSCP.

ANN ARBOR

University of Michigan¹
 Room 4303
 MSRB 3
 Ann Arbor

F Probst¹
 Y Raphael²
 K Bromfield¹
 D Martin

MI 48109
USA

University of Michigan²
Kresge Hearing Research Institute 5032
Ann Arbor
Michigan
48109-0506
USA

NIDCD³
Room 2A-19,
5 Research Court,
Rockville,
MD 20850
USA

J Karolyi
L Beyer²
H Odeh
G Dootz²
D Dolan²
D Kohrman²
K Avraham
R Fridell
T Friedman³
S Camper¹

Roles of Myo6, Myo7a, and pirouette are distinct from Myo15

The shaker-2 (*Myo15sh2/sh2*) mouse is a model for human nonsyndromic deafness DFNB3 (Probst et al 1998; Wang et al 1998). DFNB3 and shaker-2 encode an unconventional myosin (*myosin XV*) that is similar to two other known deafness genes, namely *Myo6*, which is mutated in the Snell's waltzer mouse (Avraham et al 1995), and *Myo7a*, which is mutated in the shaker-1 mouse (Gibson et al 1995). In addition, the presence of similar abnormal actin bundles in the inner hair cells and vestibular hair cells of the deaf pirouette (*pi/pi*) and *Myo15sh2/sh2* mice suggest that *Myo15* may also be functionally related to the gene responsible for the pirouette phenotype (Kohrman et al 1998). *Myo15* transcripts are readily detectable only in the inner ear and the pituitary gland (Liang et al. 1999, in press), while *Myo6* and *Myo7a* are expressed broadly, including in the pituitary gland. In order to investigate possible interactions between these genes, we crossed *Myo15sh2/sh2* mice to *Myo6sv/sv*, *Myo7a4626SB/4626SB*, and *pi/pi*. We observed no obvious hearing loss in double heterozygotes from any of the three crosses. Viable double mutants were detected in each cross and no endocrine defects were obvious. Each single mutant has unique features that characterize the apical domain of the hair cells in the organ of Corti. The hair cells of the double mutants displayed the combined features of each single mutant. These results suggest that *Myo6*, *Myo7a*, and *pirouette* each play critical roles in the development and function of the inner ear that do not overlap with the role of *Myo15*.

ANN ARBOR

University of Michigan¹
Kresge Hearing Research Institute
1150 West Medical Center Drive
Rm 9200 MSRB 111
Ann Arbor
Michigan 48109-0648
USA

LA Beyer¹
S Kanzaki²
HM Odeh¹
FJ Probst³
SA Camper³
DC Kohrman¹
Y Raphael¹

University of Michigan²
 Department of Otolaryngology
 1301 East Ann Street
 Ann Arbor
 Michigan 48109-0648
 USA

University of Michigan³
 Department of Human Genetics
 3816 Med Sci 11
 Ann Arbor
 Michigan 48109-0648
 USA

Inner and vestibular hair cells in the Pirouette and Shaker 2 mutants are attached to the basement membrane

In the early development of the mammalian inner ear sensory epithelium, all cells of the otocyst are attached to the basilar membrane. As auditory and vestibular hair cells differentiate, they detach from the basement membrane. As a result, the epithelium becomes pseudo-stratified. The shaker 2 (*sh2*) and pirouette (*pi*) mouse mutants display severe inner ear dysfunction that involves both auditory and vestibular manifestation. The inner and vestibular hair cells display a pathological actin bundle. The apical (proximal) end of the bundle is near the cuticular plate and the basal (distal) end extends from the basal domain of the cells towards the basement membrane. We have previously named this bundle cyto-caud (cell-tail). We now report on light and transmission electron microscopy observations that helped us to further characterize the cyto-caud. Using light microscope analysis of phalloidin-stained specimens, these cyto-cauds could first be detected on postnatal day 3 of *pi* mice. In mature cochleae, inner and type I vestibular hair cells contain cyto-cauds that extend towards the basement membrane and make contact with the basal lamina. The cyto-cauds contain cytoplasm, mitochondria, a prominent actin bundle and a surrounding cell membrane. At the site of contact with the basal lamina there are specialized electron-dense anchors. The data suggest that the cyto-cauds are associated with a failure of hair cells to differentiate and detach from the basement membrane. It is not clear at present how the mutation in *myo15* (in *sh2*) or the *pi* gene is associated with cyto-caud formation and the continued contact between hair cells and the basement membrane.

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BALTIMORE

Johns Hopkins University¹
 Oto-HNS Johns Hopkins University²
 601 N.
 JHOC 6th Floor
 Baltimore

H Francis¹
 L Lustig²
 C Street³
 M Gorelikow⁴
 C Limb⁵

MD 21287
USA

M Lee⁶
A Feinberg⁶

Baltimore³
MD 21287-0910
USA

Johns Hopkins University⁴
Johns Hopkins Medical Institution
720 Rutland Avenue
813 Ross
Baltimore
MD 21205
USA

Johns Hopkins University⁵
Johns Hopkins Medical Institution
720 Rutland Avenue
510 Traylor
Baltimore
MD 21205
USA

Johns Hopkins University⁶
Johns Hopkins Medical Institution
720 Rutland Ave.
1064 Ross
Baltimore
MD 21205
USA

Otopathology of the KVLQT-1 knockout mouse: A new murine model of deafness

KVLQT-1 is a potassium channel expressed by cells in the stria vascularis where it is thought to play an important role in the homeostasis of endolymph. A homozygous mutation of the *KVLQT-1* gene is associated with the Jervell and Lange-Nielsen syndrome in humans. The syndrome is characterised by congenital bilateral sensorineural hearing loss and cardiac conduction abnormalities leading to syncope and sudden death (Neyroud et al., 1997). This study evaluates the hearing and inner ear histology of a newly created knockout mouse lacking the *KVLQT-1* gene. The homozygous knockout mouse (n=5) exhibited no evoked auditory brain stem responses (ABRs) to clicks (>95 dB SPL), whereas heterozygous littermates (n=4) and CBA/J mice (n=4) demonstrated normal ABR thresholds. Compared to the histologically normal heterozygous mouse, a 4 month-old *KVLQT-1* mutant (-/-) mouse showed severe abnormalities of the cochlear duct including stria atrophy, complete absence of the organ of Corti, and collapse of Reissner's membrane onto the tectorial membrane. These effects were all more severe in basal compared to apical cochlear regions. In addition, the endolymphatic compartments of the vestibule and semicircular canals were markedly reduced in the knockout mouse, and there was separation of the otolith membrane from the maculae and a marked decline in hair cell

density. These abnormalities are consistent with the idea of a critical role for the *KVLQT-1* gene product in the development or maintenance of the inner ear. The data suggest that strial atrophy leading to contraction of the endolymphatic compartment may be the first of a series of degenerative changes occurring in the inner ears of these mutant mice.

BAR HARBOR

The Jackson Laboratory¹
600 Main Street
Bar Harbor
Maine 04609
USA

QY Zheng¹
LS Bross²
JF Willott³
K Johnson¹

Northern Illinois University²
Department of Psychology
De Kalb
Illinois 60115
USA

Department of Psychology³
BEH 339
4202 East Fowler Avenue
Tampa
Florida 33620
USA

Hearing impairment and inner abnormalities of coloboma mutant mice

Mice that are heterozygous for the coloboma (*Cm/+*) mutation show eye defects and abnormal posture, head shaking or bobbing, circling, and hyperactivity. This mutation occurred among offspring of an irradiated (C3H/HeH x 101/H) F1 male and was mapped to Chromosome 2 near the agouti locus. Here we demonstrate that coloboma heterozygotes also exhibit hearing impairment and inner abnormalities.

Auditory brainstem response (ABR) thresholds were obtained for clicks and tone pips of 8, 16, and 32 kHz. Coloboma heterozygotes aged 44 days had high thresholds for all stimuli (70-90 dB SPL). Threshold elevations were similar at ages 82 days to 334 days, and worsened during the second year of life.

Surface-mounted cytochleograms from several mice suggested that the basilar membrane was abnormally short in coloboma mutants; the alignment of outer and inner hair cell rows was disrupted toward the cochlear apex, and there was some loss of outer and inner hair cells.

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BAR HARBOR

The Jackson Laboratory¹
600 Main Street
Bar Harbor
Maine 04609
USA

QY Zheng¹
KR Johnson¹

Hearing loss associated with modifier of deaf waddler (mdfw) alleles corresponds with age-related hearing loss (Ahl) in 13 inbred strains of mice

The modifier of deaf waddler (*mdfw*) and age-related hearing loss (*Ahl*) loci were both discovered as inbred strain polymorphisms that affect hearing loss in mice. Both loci map to the same position on Chr 10. The *mdfw* locus interacts epistatically with the deaf waddler (*dfw*) mutation on Chr 6 and the *Ahl* locus is a major contributor to age-related hearing loss in several inbred strains. To investigate the possibility of allelism, we examined the correspondence of *mdfw* and *Ahl* phenotypes among 13 inbred mouse strains. The effects of strain-specific *mdfw* alleles on hearing loss were assessed in *dfw*/+ F1 hybrids produced from mating BALB-*dfw*2J mice with mice from each of 13 inbred strains. F1 hybrids were then assessed for hearing by ABR threshold analysis and classified as *dfw*2J/+ or +/+ by PCR typing. Heterozygosity for *dfw* accelerated hearing loss in F1 hybrids derived from all strains tested, except those produced with the B6.CAST + *Ahl* congenic strain. *dfw*/+ F1 hybrids derived from parental strains 129P1/ReJ, A/J, BUB/BnJ, C57BR/cdJ, C57L/J, DBA/2J, NOD/LtJ, SKH2/J, and STOCK760 exhibited a severe hearing loss by 12 weeks of age. Those derived from strains 129T2/SvEms/J, C3H/HeJ, CBA/CaJ, and NON/LtJ exhibited only a slight to intermediate hearing loss at that age. We conclude that the hearing loss associated with these strain-specific *mdfw* alleles corresponds with previously determined *Ahl* allele effects in these strains, providing additional evidence that *mdfw* and *Ahl* are manifestations of the same gene. A functional relationship may therefore exist between the Ca²⁺ transporting activity of the *dfw* gene and age-related hearing loss.

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BAR HARBOR

The Jackson Laboratory¹
600 Main Street
Bar Harbor
Maine 04609
USA

KR Johnson¹
QY Zheng¹
Y Bykhovskaya²
O Spiria²
N Fischel-
Ghodsian²

The Cedars-Sinai Medical Center²
Department of Pediatrics
8700 Beverly Blvd
Los Angeles
CA 90048
USA

Heritable effects of a strain-specific mitochondrial DNA polymorphism on hearing impairment in mice

The A/J inbred mouse strain exhibits age-related hearing loss by three months of age (Zheng et al. 1999, *Hear. Res.* 130, 94-107). Backcross linkage analysis with the normal hearing CAST/Ei strain has shown that much of this recessively inherited hearing loss can be attributed to a single locus on Chr 10, *Ahl* (Johnson et al. 2000, *Genomics*, in press). Here we show, by matrilineage analysis of reciprocal backcrosses, that mitochondrial DNA (mtDNA) derived from the A/J strain accelerates the hearing loss of *Ahl/Ahl* mice. Backcross matings were set up using males and females from both types of reciprocal F1 hybrids, and maternal strain origins in N2 backcross mice were noted. All backcross mice were tested for ABR thresholds successively at 3 and 6 months of age. ABR threshold averages for N2 mice with A/J matrilineage were significantly higher than age-matched averages for N2 mice with CAST/Ei matrilineage. The maternal effects on average ABR thresholds were highly significant in backcross mice that inherited two copies of the A/J *Ahl* allele; whereas, a single copy of the CAST/Ei *Ahl* allele prevented AHL regardless of maternal origin (TABLE). These results clearly show an interaction between the effects of the nuclear *Ahl* gene and maternal origin in determining hearing impairment in (A/J x CAST/Ei) x A/J backcross mice. This mtDNA effect was not seen in two other backcrosses that were similarly analyzed: (NOD/LtJ x CAST/Ei) x NOD/LtJ and (SKH2/J x CAST/Ei) x SKH2/J. Sequencing of the mitochondrial genomes in the parental inbred strains of these backcrosses revealed a single nucleotide insertion in the *tRNA-Arg* gene as the likely mediator of the mitochondrial effect. This is the first mouse model with a mtDNA mutation affecting a clinical phenotype, and provides an experimental model to dissect the pathophysiological processes connecting mtDNA mutations to hearing loss.

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BETHESDA

NIDCD¹
Bldg 36
Room 5D15
36 Convent Drive
Bethesda
Maryland 20892
USA

IA Belyantseva¹
HJ Adler¹
JP Inda¹
CJ Davies²
GI Frolenkov¹
B Kachar¹

NIDCD²
9000 Rockville Pike
Bethesda
Maryland 20892
USA

Expression and localization of prestin and the sugar transporter GLUT-5 during development of electromotility in cochlear outer hair cells

Electromotility, i.e. the ability of cochlear outer hair cells (OHCs) to contract and elongate at acoustic frequencies, is presumed to depend on the voltage-driven conformational changes of motor proteins present in the OHC lateral plasma membrane. Recently, two membrane proteins have been proposed as candidates for the OHC motor. A sugar transporter, GLUT-5, was proposed based on its localization in the OHCs and on the observation that sugar transport alters the voltage sensitivity of the OHC motor mechanism (Nakazawa et al., 1995; Geleoc et al., 1999). Another candidate, prestin, was identified from a subtracted OHC cDNA library and shown to impart voltage driven shape changes to transfected cultured cells (Zheng et al., 2000). Using confocal immunofluorescence and immunoelectron microscopy techniques with antibodies specific to these proteins, we show that both GLUT-5 and prestin are highly expressed in the lateral plasma membrane of rat OHCs. In contrast to GLUT-5, no labelling was detected in inner hair cells or supporting cells with anti-prestin antibodies. The labelling of the lateral membrane of OHCs extended uniformly from the region just below the level of the cuticular plate to the level of the nucleus. No labelling was detected in the subnuclear region. We also compared the postnatal expression patterns of these proteins with the development of electromotility in OHCs of the apical turn of the rat organ of Corti. The patch-clamp recording of transient charge movement associated with electromotility indicates that half of the maximal expression of the motor protein occurs at postnatal day 9. Prestin incorporation in the plasma membrane begins from postnatal day 0 and increases progressively in a time course coinciding with that of electromotility. GLUT-5 is not incorporated into the lateral plasma membrane of apical OHCs until postnatal day 15. Thus, we provide the first direct evidence for the localization of prestin in the lateral plasma membrane of OHCs. Furthermore, our results suggest that while GLUT-5 may be involved in the control of electromotility, prestin is likely to be a fundamental component of the OHC membrane motor mechanism.

BOSTON

Mass. Eye & Ear Infirmary
243 Charles Street
Boston
MA 02114
USA

J Adams

Immunolocalization of connexin 31 in the cochlea

Gap junctions play critical roles in cochlear function and nearly all non-sensory cells are part of one of two gap junctional systems. Gap junctions permit movement of ions and small compounds between cells. They are comprised of connexin molecules and most gap junctions contain more than one class of connexin molecule. At least some gap junctions found among all cochlear cells appear to contain connexin 26 (Kikuchi et al, 1995, Anat. Embryol. 191:101-118) and there are numerous reports that mutations of the connexin 26 gene are leading causes of hereditary sensorineural hearing loss. At recent report by Xia et al. described families with defects in the connexin 31 gene who had hereditary hearing loss. In the present report the sites positive for connexin 31 within the cochlea were localized by immunostaining. Most observations were made in mouse, but were confirmed in rat,

monkey, and human. In contrast to connexin 26, connexin 31 was found to be associated with a limited number of cell classes and these were all non-epithelial cells. Three sites showed positive staining. Within the spiral ligament, type 2 fibrocytes were immunopositive. This included those located near the spiral prominence as well as those located apical to the stria vascularis. The remaining positive cells were connective tissue cells within the spiral limbus. The organization of gap junctional systems, in the context of numerous other cell specializations and functional studies, led Kikuchi et al. to conclude that gap junctions play an essential role in recycling K⁺ ions from hair cells back to the stria vascularis. If mutations of connexin 31 incapacitate type 2 fibrocytes' gap junctions this would be expected to impede or halt K⁺ recycling and thereby produce a hearing loss by eliminating the K⁺ ion supply of the scala media.

BRIGHTON

School of Biological Sciences¹
University of Sussex
Brighton
BN1 9QG
UK

K Legan¹
R Goodyear¹
V Lukashkina¹
I Russell¹
G Richardson¹

Transgenic mice with a deletion in the entactin domain of alpha-tectorin have detached tectorial membranes lacking striated sheet matrix and are deaf

Mutations in the human alpha-tectorin gene lead to both recessive and dominant forms of non-syndromic deafness (Verhoeven et al, 1997, Alloisio et al, 1999, Mustapha et al, 1999). It is not known how these mutations cause deafness, nor how alpha and beta-tectorin interact to form the non-collagenous, striated-sheet matrix of the tectorial membrane. The alpha-tectorin gene encodes a large (239 kDa), inner-ear specific protein containing an N-terminal entactin-like domain, 3 full and 2 partial vWF type D repeats and a C-terminal zona pellucida domain. The beta-tectorin gene encodes a small (36 kDa) protein with a single zona pellucida domain. To investigate how the tectorins interact and how the tectorial membrane influences frequency tuning in the cochlea we made a transgenic mouse with a 96 amino acid deletion in the entactin-like domain of alpha-tectorin using homologous recombination in ES cells. Homozygous mutant (-/-) mice are viable and have no obvious behavioural defects. RT-PCR shows alpha-tectorin mRNA is expressed in the inner ears of early postnatal -/- mice. However, alpha-tectorin precursor protein cannot be detected in -/- mice by immunoblotting suggesting it is rapidly degraded. In 3 week postnatal -/- mice the tectorial membrane is completely detached from the spiral limbus and the organ of Corti. Electron microscopy shows that the tectorial membrane of -/- mice consists solely of collagen fibrils. Hair cells appear normal and have well organised stereocilia bundles. In the utricle and saccule the otolithic membrane is severely reduced and a few scattered, abnormally large otoconia lie directly on the apical surface of the sensory epithelia. The cupulae are normal in -/- mice. Two-tone otoacoustic distortion products cannot be detected from the ears of -/- mice suggesting they are deaf (see abstract by Russell, Lukashkina, Legan, Goodyear & Richardson). These results indicate alpha-tectorin is a major component of the striated-sheet matrix, that beta-tectorin is unlikely to form homomeric

filaments, and that attachment of the tectorial membrane to the epithelial surface is likely to be mediated via its non-collagenous components.

CHICAGO

Northwestern University Medical School¹
Institute for Neuroscience²
Cell and Molecular Biology
303 East Chicago Avenue
Chicago
IL 60611
USA

L Zheng¹
G Sekerkova²
B Changyaleket¹
E Mugnaini²
JR Bartles²

Impact of the jerker deafness mutation on the espin actin-bundling proteins of hair cell stereocilia

The espins are actin-bundling proteins discovered originally in the parallel actin bundles of Sertoli cell-spermatid junctions and brush border microvilli (reviewed by Bartles, 2000, *Curr. Opin. Cell Biol.* 12, 72-78). We have recently demonstrated that espins are also present in hair cell stereocilia and have linked the espin gene to jerker, an autosomal recessive mutation that results in hair cell degeneration, deafness and vestibular dysfunction (Zheng et al., 2000, *Cell* 102, 377-385). The coding portion of the espin gene of jerker mice contains a single point mutation, a frameshift mutation that affects the 116-amino acid C-terminal actin-bundling module shared among known espin isoforms. The espin gene of jerker mice is predicted to encode truncated espins that are 24 amino acids shorter than the corresponding wild-type proteins and include a novel C-terminal peptide with a high net positive charge (+14 vs. +3 for the wild type), a large number of serine residues (7 vs. 1 for the wild type), a dramatically reduced propensity to form an alpha-helix and coiled coil, and a single cysteine near the C-terminus. The jerker mutation evidently destabilizes espin proteins in all cells that ordinarily express the proteins, because mutated espin proteins cannot be detected in multiple tissues of homozygous jerker mice even though mRNAs are present at normal levels. Comparisons of wild-type and jerker espin constructs using transient transfection and in-vitro actin-bundling assays reveal that the jerker mutation alters the localization and actin-bundling activity of the espins in unexpected ways.

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CINCINNATI

University of Cincinnati¹
Biological Sciences
Mail location #6
Cincinnati
Ohio 45221
USA

LC Erway¹
KR Johnson²
QY Zheng²

The Jackson Laboratory²
 600 Main Street
 Bar Harbor
 Maine 04609
 USA

A Major Gene Affecting Age-related Hearing Loss Is Common to at Least Ten Inbred Strains of Mice

Inbred strains of mice provide models for understanding genetic basis of human presbycusis, or age-related hearing loss (AHL). We previously mapped a major gene affecting late-onset AHL (by 18 months) to Chr.10 in C57BL/6J mice. We now show that the same Chr.10 gene (*Ahl*) is a major contributor to early onset AHL from 3-6 months of age in nine other inbred strains: 129P1/ReJ, A/J, BALB/cByJ, BUB/BnJ, C57BR/cdJ, DBA/2J, NOD/LtJ, SKH2/J, and STOCK760. Each of these strains was crossed to normal hearing CAST/Ei (+*Ahl*/+*Ahl*) mice, yielding F1 progeny (+*Ahl*) with normal hearing thresholds, indicating that inheritance of AHL is recessive (*Ahl/Ahl*). The backcross progenies segregated approximately ½ with normal hearing and ½ with AHL from three to six months of age. Analyzing the ABR thresholds quantitatively for more than 1,500 N2 mice indicated linkage associations with Chr.10 markers. Highly significant linkage was found in all nine strain backcrosses, with the highest probability (LOD>70) near the marker *D10Mit112*. This map position for *Ahl* is near the waltzer mutation (*v*) and the modifier of the deaf waddler (*mdfw*) locus, suggesting the possibility of allelism for a single gene. Results from an intercross of C57BL/6J and NOD/LtJ mice indicate that the 6-10 month difference in AHL onset between these two strains is not due to allelic heterogeneity of the *Ahl* gene. Experiments are underway to identify genetic basis for differences in age of onset of AHL among these strains. The *D10Mit130/158** and *D10Mit299/185** alleles are closely linked with +*Ahl* in four normally hearing strains. By contrast, the *10Mit130/150** and *D10Mit299/188** alleles are closely linked with *Ahl* in the nine AHL strains. Such close linkage, with only two possible recombinants, supports common ancestry for the *Ahl* allele. (*Allelic numbers of nucleotides distinguish Mit-alleles; Supported by: NIH/NIDCD Contract: DC6218)

CINCINNATI

University of Cincinnati School of Medicine
 Molecular Genetics, Biochemistry and Molecular Biology¹
 Department of Biological Sciences²
 University of Cincinnati
 OH 45267
 USA

PJ Kozel¹
 RR Davis³
 ER Krieg⁴
 GE Shull¹
 LC Erway²

Mailstop C27³
 4676 Columbia Parkway
 Cincinnati
 Ohio 45226

USA

National Institute for Occupational Safety and Health⁴
Monitoring Research Statistics Activity
C-22 4676 Columbia Parkway
Cincinnati
Ohio 45267
USA

Genetic deficiency in PMCA2 confers susceptibility to noise-induced hearing loss

The genetic and molecular basis for susceptibility to noise-induced hearing loss (NIHL) are not well understood. Targeted deletion of the gene coding plasma membrane calcium ATPase isoform 2 (*PMCA2*) results in deaf homozygous mutant mice. Given *PMCA2*'s location on the outer hair cell stereocilia and the observation that OHC cytoplasmic calcium concentration rises following acoustic over stimulation, we hypothesized that *PMCA2* heterozygous mice were more susceptible to NIHL. As ABR thresholds of *PMCA2*^{+/-} siblings vary with strain background, *PMCA2*^{+/-} were outcrossed to normal hearing CAST/Ei mice. Both *PMCA2*^{+/+} and *PMCA2*^{+/-} F1 offspring had normal hearing. These mice were exposed to 113 dB broadband noise for 8 hrs. Permanent threshold shifts were significantly greater (15-25 dB, p<0.003) in *PMCA2*^{+/-} mice than in *PMCA2*^{+/+} siblings. This is the first demonstration of a gene with a known function that confers susceptibility to permanent noise-induced hearing loss. "

HANNOVER

Association for Research in Otolaryngology¹
Institut für Pharmakologie²
Institut für Pharmakologie³
Institut für Zoologie⁴
Tierärztliche Hochschule
Hannover Bunteweg 17
D-30559
Hannover
Germany

A Kaiser¹
M Fedrowitz²
W Löscher³
E Zimmermann⁴
HJ Hedrich⁵

Medizinische Hochschule⁵
Zentrales Tierlaboratorium
Hannover
Germany

New deafness mutant in the Lewis rat: the circling (ci2)-rat

Deaf mutants of laboratory rodents are ideal subjects for investigating hereditary hearing loss. In the shaker-waltzer mouse and the pirouette mouse, deafness is combined with movement disorders which result in circling behaviour and hyperactivity. It has been suggested that these mutant phenotypes may result from a malfunction of the vestibular and auditory periphery or from central disorders.

Recently, similar phenotypic features were described in the stargazer (*stg*) rat (Truett et al. Lab An Sci 44:595-599, 1994). We report a new mutant from the Lewis rat strain (LEW-Ztm) with inherited deafness and movement disorder, *ci2/ci2*. The autosomal recessive mode of inheritance allows a comparison of normal and mutant phenotypes within the same litter (Löscher et al., Neurosci 74:1135-1142, 1996). Homozygous *ci2/ci2* rats can easily be distinguished from their heterozygous siblings (*ci2/+*) by their hyperactivity and circling behaviour which are triggered by mild disturbances of the animal. Both groups of *ci2* rats were tested for the presence of brainstem auditory evoked potentials (BAEP) as a response to pure tones (0.5 to 60.0 kHz; up to 100 dB SPL). In addition, the cochleae, the vestibular organs and the brains were processed for histological examination. Either surface preparations for staining of actin filaments with phalloidin-TRITC or semithin toluidine stained sections were obtained from the hair cell organs. Nissl stained brain sections were analyzed for differences in gross cell morphology within the cochlear and vestibular nuclei. While *ci2/+* rats had quite normal audiograms and hair cell organs, no BAEP's could be measured from *ci2/ci2* animals. In the latter, hair cells were absent in the cochlea and the vestibular organs. A comparison of cell morphology of CN neurons revealed a significantly rounder shape with fewer cell processes and a higher number of cells in *ci2/ci2* rats suggesting a lesser differentiation of those neurons. Our findings raise several questions: 1) Are the phenotypic disorders of the *ci2/ci2* rat of a mono- or multigenetic origin? 2) Are similar phenotypic defects between *ci2/ci2* and *stg* rats correlated with mutations of the same genes?

HELSINKI

University of Helsinki¹
 Institute of Biotechnology
 P.O. Box 56
 00014 Helsinki
 Finland

Imperial Cancer Research Fund²
 London
 WC2A 3PX
 UK

Creighton University³
 Dept. Biomed Sciences
 Omaha, Nebraska
 68178
 USA

U Pirvola¹
 B Spencer-Dene²
 XQ Liang¹
 B Fritsch³
 C Dickson²
 J Ylikoski¹

FGF/FGFR2 signaling in the developing inner ear

Fibroblast growth factors comprise a large gene family that regulates cellular proliferation, migration and differentiation by activating members of a family of tyrosine kinase receptors (FGFRs 1-4). In the case of FGFRs 1-3, alternative exon usage gives rise to 2 functional isoforms, the IIIb and IIIc isoforms. The receptor variants have differential expression patterns and ligand specificity. Available data

show that the FGF pathways underlying morphogenesis are evolutionary conserved and are used repeatedly to make different organs. We have studied the expression of *FGF10* as well as its specific receptors, *FGFR2 IIIb* and *FGFR3 IIIb* in the developing cochlea. Expression of *FGF10* mRNA is found in the otic vesicle. Also *FGFR2 IIIb* mRNA is expressed in the early inner ear in a non-overlapping pattern as compared to *FGF10* mRNA. During mid- and late-embryogenesis, *FGF10* mRNA is expressed in the greater epithelial ridge and in the cochlear ganglion neurons. At these stages, *FGFR2 IIIb* mRNA is found in the nonsensory epithelia of the cochlear duct. *FGFR3 IIIb* mRNA is expressed in the sensory epithelium of the late-embryonic cochlea. To explore the functions of *FGF/FGFR2 IIIb* interaction in inner ear development, we have analyzed the phenotype of *FGFR2 IIIb* null mutant mice. The null mutation inhibits cochlear development at early stages. Also the vestibular part of the inner ear is affected, consistent with the prominent expression of *FGFR2* and its ligands there. Our data suggest that interaction between *FGFR2 IIIb* and its ligands, one of which appears to be *FGF10*, regulates inner ear formation

HELSINKI

University of Helsinki¹
 Inst. Of Biotechnology and Dept. of ORL
 PO Box 56 (Viikinkaari 9)
 Helsinki 00014
 Finland

U Pirvola¹
 XQ Liang¹
 N Trokovic¹
 J Ylikoski¹
 J Partanen¹

FGFR signaling is required for the formation of the auditory sensory epithelium

The mammalian auditory sensory epithelium, the organ of Corti, comprises the inner and outer hair cells (IHCs and OHCs) and the supporting cells, the pillar and Deiters' cells. Differentiation follows medial-to-lateral (IHCs show first signs of differentiation) and base-to-apex gradients. Based on the strict developmental gradients, differentiation might be stimulated by signals emanating from the neighboring, previously determined cells, this being analogous to the process seen in the developing compound eye and chordotonal sense organs of *Drosophila* in which interactions between diffusible ligands and receptor tyrosine kinases trigger differentiation. In the organ of Corti, fibroblast growth factor receptor (*FGFR*) 3 has been shown to be essential for pillar cell differentiation (Colvin et al., Nature Gen., 1996). Our in situ hybridizations show that, in addition to *FGFR3* mRNA, *FGFR1* mRNA is expressed in the presumptive organ of Corti. We have studied the inner ear phenotype of mice in which loss-of-function mutations have been introduced by gene targeting into the *FGFR1* locus. We have also studied functional significance of the IIIb and IIIc isoforms of *FGFR1*. Reduced *FGFR1* signalling caused distinct patterning defects in the auditory sensory epithelium, affecting both hair cell and supporting cell development. The presence of OHCs was linked to the presence of IHCs, these data supporting the hypothesis that secreted factors produced by IHCs influence development of the rest of the sensory epithelium (Pirvola et al., ARO abstract, 1998). Since generation of IHCs, the first-forming cells of the sensory epithelium, as well as more global growth of the cochlear duct were also affected in *FGFR1* loss-of-function mutant mice, we conclude that *FGFR1* signalling might regulate both growth and patterning of the developing cochlea and that these

processes are closely linked to each other. Together, these data provide direct evidence that *FGFR1* signalling is required for generation of the organ of Corti.”

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ILLINOIS

Northwestern University
Auditory Research Laboratory¹
Auditory Physiology Laboratory²
Frances Searle Building⁵
2299 North Campus Drive
Evanston
Illinois 60208
USA

J Zheng¹
D He²
K Long³
LD Madison⁴
P Dallos⁵

Northwestern University
Medical School
Department of Endocrinology³
Center for Endocrinology and Metabolic Diseases⁴
Tarry 15-703 303 E
Chicago Avenue
Chicago
Illinois
60611
USA

Isolation of a Novel Gene from Gerbil Outer Hair Cells

In order to identify genes that are associated with outer hair cell (OHC) function, we created an OHC-subtracted plasmid library using a combination of suppression subtractive hybridization and differential screening strategies. In the present study, a unique and apparently abundant unknown gene, designated as *OHC3*, has been cloned. Although its low overall homology with any known gene suggests that *OHC3* is novel, some regions of the gene show significant homology to pendrin (*PDS*) and sulfate transport genes. Sequence data and gene expression patterns suggest that *OHC3* is not *PDS*, but a novel gene expressed in outer hair cells. To assess the tissue specificity of *OHC3* expression, a Northern blot panel of 8 gerbil tissue RNAs was probed with *OHC3*, and no signal was observed in liver, brain, ovary, spleen, muscle, heart, lung, and kidney. Virtual Northern dot blot experiments demonstrate that *OHC3* mRNA is only expressed in adult OHC and adult organ of Corti tissue. No expression was detected in newborn organ of Corti, the inner hair cells, vestibular system and thyroid tissue, suggesting that *OHC3* is specifically expressed in outer hair cells. The ontogeny of *OHC3* expression was examined by PCR with cDNA derived from basilar membrane and associated organ of Corti of various post-natal ages, and it was determined that expression increases with gerbil growth (from 0 to 20 DAB). In contrast, expression of *PDS* was observed at all stages (0 to 20 DAB), with no significant increase or decrease in pattern evident (Long et al., ARO Abstracts, 2000). Using an *OHC3* DNA fragment as a probe, we isolated the full-length *OHC3* cDNA clone from the lgt11 gerbil cochlea library. The *OHC3*

cDNA is approximately 4.1 kb in length and features an open reading frame of 2232 bp encoding a putative protein of 744 amino acids. Since the *OHC3* gene is abundantly expressed in outer hair cells and its expression is developmentally controlled, the role of *OHC3* may relate to the unique amplifier function of outer hair cells.

IOWA

University of Iowa
200 Hawkins Drive¹
3107 Medical Laboratories⁵
Iowa City
Iowa 52242
USA

Brigham and Women's Hospital and Harvard Medical School²
Pathology, Obstetrics, Gynecology and Reproductive Biology
Boston
MA 02115
USA

University of Antwerp³
Medical Genetics
Antwerp
Belgium

UCSD and VA Medical Center⁴
Surgery/Otolaryngology and Neurosciences
La Jolla
CA 92093
USA

University of California⁶
Neurosciences Div. of Otolaryngology
9500 Gilman Dr
La Jolla
California 92093-0666
USA

S Wayne¹
NG Robertson²
F DeClau³
N Chen⁴
K Verhoeven³
S Prasad⁵
CC Morton²
AF Ryan⁶
G Van Camp³
RJ Smith¹

Mutations in the transcriptional activator EYA4 cause late-onset deafness at the DFNA10 locus

Progressive late-onset sensorineural hearing loss, or presbycusis, is a major handicap for the elderly, and is thought to be caused by a complex interaction between environmental and genetic factors. The characterization of genes associated with presbycusis may foster the development of new habilitation options. DFNA10, a locus associated with autosomal dominant, progressive, late-onset sensorineural hearing loss is a potential gene involved in presbycusis. It was previously mapped to a 17 centimorgan (cM) interval on chromosome 6q22-23. In this work, we identified

EYAA (Eyes absent 4), a member of the vertebrate Eya family of transcriptional activators, as the causative gene of hearing loss at the DFNA10 locus.

EYAA was an excellent candidate gene for DFNA10 in view of the association of syndromic hearing loss with mutations in another Eya gene, *EYAI*, in branchio-otorenal syndrome. Two unrelated families from Belgium and America with DFNA10 hearing loss were screened for mutations in *EYAA*. We found different mutations in *EYAA* segregating with the hearing loss in each family. Both mutations create premature stop codons, and are predicted to disrupt the conserved region of the protein. Cochlear *Eya4* expression was examined by in situ hybridization, as well as by an analysis of alternative splicing in the cochlea. *Eya4* is expressed in the neuroepithelium of the developing cochlea, and in cochlea-associated tissues post-natally. Developmentally regulated alternative splicing of *Eya4* was detected in the cochlea.

Although EYA proteins interact with members of the PAX, SIX and DACH protein families in a conserved network that regulates early embryonic development, our findings show that *EYAA* also is important post-developmentally for continued function of the mature organ of Corti.

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KUMASI, GHANA

Department of EENT¹
SMS- KNUST
Kumasi
Ghana
West Africa

GK Amedofu¹

Non syndromic Genetic Hearing Loss in Ghana: An update

We do know that the majority of children with bilateral sensorineural hearing impairment are believed to be affected congenitally (Fraser, 1976). It is also known that most congenital sensorineural hearing loss are attributable to dominant, recessive or X-linked genes or infections acquired *in utero*. To be true, non-syndromic genetic hearing loss is the most common group of hereditary deafness. Indeed, approximately 70% of genetically determined deafness are said to occur in non-syndromic forms (Kontig Mark and Gotlin, 1976). Newtons work (1985) demonstrated that 43% of childhood deafness is due to pre-natal causes (29 genetic and 14 non-genetic). Paparella (1981) noted that 50% of all profound bilateral sensorineural hearing loss have a genetic aetiology even though these are classified as unknown. Holborow (1986) observed that genetic hearing loss is a problem in the Indian sub continent and parts of East Africa and that in West Africa genetic hearing loss is uncommon. We think the assertion on the situation in West Africa is a mere speculation as revealed by recent chromosomal studies in Ghana. Since 1995, we have been doing genetic studies in Ghana to determine the genetic causes of deafness, in view of the huge percentage of causes of congenital deafness (about 40%) classified as of unknown aetiology (Amedofu et al 1997).

Our first genetic study was at Adanarobe village in Ghana about 40 kilometers from Accra, the capital. A high prevalence of deafness was discovered in 1961 during an Africa survey in 1961 (David et al 1971). In view of the high prevalence of deafness in the village, a study was conducted (Brobbly et al 1997) to determine the genetic cause of deafness in the area. It was found that 35 subjects from the 11 families studied had the same connexin 26 mutation (R143W) in amino acid position. A linkage analysis was performed in 6 families and 29 subjects revealed a lod score of 4:5 at marker position *D135175* corresponding to the DFNB/DFNA3 locus on chromosome 13q11. Sequencing of the connexin 26 coding region in 21 hearing impaired subjects from 11 families revealed in all of them a homozygous C>T mutation in the first position of codon 143 resulting in the exchange of an arginine to a tryptophan residue. A disease haplotype comparison indicated that the mutation arose approximately 60 generations ago.

Later, in 1998 368 apparently unrelated individuals with non-syndromic congenital deafness from 10 schools for the deaf were studied all over Ghana (Hamelman, Amedofu et al 2001). This study revealed that 16% of cases seen were caused by connexin 26 mutation, and 90% of these were caused by R143W.

All mutations except one which was known from Europe had not been seen before. In the case of the R143W, there were 7 novel mutations, 6 of which were located in the 'anterior' position of the molecule and all the Ghanaian mutations caused single amino-acid exchanges which did not affect the structure of the mutation.

Further genetic studies currently going on are revealing and colleague would be informed at the appropriate time. These studies suggest that genetic factors would be considered greatly in analysing the causes of congenital deafness in Ghana. The above studies are a collaborative effort of the department of Ear, nose and throat, School of Medical Studies, Ghana and the department of molecular medicine Bernhard Nocht Institute for Tropical Medicine, Hamburg with the full support of Professor R D Horstmann.

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LA JOLLA

The Salk Institute for Biological Studies
 Molecular Neurobiology Lab-H¹
 Peptide Biology Lab²
 10010 North Torrey Pines Road
 La Jolla
 California 92037
 USA

DE Vetter¹
 C Li²
 M Charles Liberman
 L Zhao²
 G Smith²
 S Heinemann¹
 W Vale²
 K-F Lee²

Urocortin expression and function in the cochlea

The mammalian olivocochlear (OC) system is neurochemically heterogeneous, using both acetylcholine and GABA as conventional neurotransmitters and various peptides as neuromodulators to convey neural signals to the cochlea. Although numerous peptides, including enkephalins, dynorphin and CGRP, are localized within some OC terminals, the role of peptidergic systems in auditory function is obscure. Recently, another peptide, urocortin (*Ucn*), has been identified immunohistochemically in lateral OC neurons and the organ of Corti (Vetter et al. ARO abstract 1999). *Ucn* is a member of the corticotropin releasing factor (CRF) family, known to function in the pituitary-adrenal axis in stress-related responses.

In adult mice, antibodies against *Ucn* reveal localization in OC terminals in the IHC region (but not at OHCs). In situ hybridization analysis reveals cochlear expression pattern of CRF receptors 1 and 2, both of which bind *Ucn*, predominantly in lateral support cells such as Hensen's cells. The developmental expression patterns of these receptors are currently being examined.

Homologous recombination was used to delete the *Ucn* gene in mice. Cochlear function was evaluated, via auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs), in homozygous knockout and wildtype littermate controls at 3 and 6 months of age. Cochlear histopathology was evaluated at the light microscopic level, revealing no significant hair cell loss or other clearcut pathology in the knockout ears. Although the degree of cochlear dysfunction in *Ucn* knockouts was variable, mean ABR threshold elevation at 3 months was 13 dB at 4 ñ 16 kHz, growing to 25 dB at 32 kHz. Hearing loss in *Ucn* knockouts continued to deteriorate, and by 6 months mean thresholds were elevated by 20 dB at frequencies up to 22.6kHz. DPOAE data showed similar trends; however between-group differences were less dramatic, suggesting that the *Ucn* knockout may cause dysfunction in both OHC and IHC areas.

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LAUSANNE

Institute De Physiologie De Lausanne¹
 7 Rue Du Bugnon
 Lausanne
 Switzerland
 100

A Zine¹
 A Aubert¹
 J Qiu¹
 S Therianos¹
 F Deribaupierre¹

The role of bHLH genes *Hes1* and *Hes5* during the development of the mammalian cochlea.

The cellular and molecular mechanisms regulating hair cell development during mammalian cochlea morphogenesis have just begun to be defined. Recent evidences indicate that *Math1*, a member of a bHLH family of transcription factors is required for the genesis of hair cells (Bermingham et al., 1999) and that *Notch1* signalling regulate their pattern of differentiation through cell-cell interactions (Lanford et al., 1999; Zine et al., 2000). Other bHLH genes, like *Hes1* and *Hes5* act downstream of *Notch* signalling to regulate the transcription of positive-bHLH genes, such as *Math1*, in the embryonic CNS. Previous studies have shown that mice lacking *Hes1* display severe neural tube defects and did not survive beyond 1 day after birth. In this study, we have looked at the developing cochleae of embryonic and neonatal mice lacking *Hes1* and *Hes5* genes. Cochleae of *Hes1*^{-/-} and of *Hes5*^{-/-} contain significantly greater numbers of hair cells as compared to their wild-type and heterozygote littermates. In *Hes1*^{-/-}, cochleae displayed a nearly complete second row of inner hair cell rather than one and also regions with four rows of outer hair cells rather than three. Cochleae of *Hes5*^{-/-} had similar anomalies, but duplication of inner hair cells was discontinuous. Moreover, these mutants developed extra hair cells outside the sensory epithelium within the greater epithelial ridge region. Extra hair cells that developed in the cochleae of either *Hes1*^{-/-} and *Hes5*^{-/-} expressed *Math1*, *myosin VIIa* and acquired immature stereociliary bundles. Quantitative analysis of (*Hes1*^{-/-}; *Hes5*^{+/-}) cochleae indicates an enhanced effect on the number of extra hair cells within the sensory epithelium suggesting a functional redundancy of the two *Hes* genes. RT-PCR analysis of microdissected sensory epithelia and in situ hybridization on embryonic cochleae indicated that *Hes1*, *Hes5* and *Math1* genes are expressed in the normal cochlea during initial steps of hair cell differentiation. Together, our results suggest that hair cell determination and differentiation in the developing sensory epithelium of the mammalian cochlea is regulated by the pathway *Notch1/Hes/Math1*.

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LONDON

University College London¹
 Institute of Laryngology and Otology
 330-332 Gray's Inn Road
 London
 WC1X 8EE
 University College London²
 Anatomy & Developmental Biology
 Gower Street
 London
 WC1E 8TT

J Edwards¹
 N Marziano¹
 D Becker²
 S Casalotti¹
 G Nevill¹
 A Forge¹

Co-localisation of connexin b2 (26) and connexin b6 (30) in cochlear gap junctions

Individual gap junctions can be comprised of more than one connexin isoform. There may be heteromeric channels, heterotypic junctions, or separate islands of homomeric-homotypic junctions of differing connexins within the same gap junction plaque. Immunohistochemical studies have revealed that connexin (*cx*) *b2* and *cx**b6* are both highly expressed in the cochlea. We sought to determine the exact location of these connexins within the cochleae of mice and guinea pigs by immunogold labelling of thin sections with antibodies shown to be specific to *b2* and *b6*. Two labelling protocols were carried out. In the first, adjacent thin sections were separately labelled with anti-*b2* and anti-*b6*. In the second, opposite sides of an individual section were labelled with one of the two primary antibodies and then with a secondary conjugated to gold particles of different size. Stereomicroscopy confirmed the confinement of the larger gold particles to one side of the section, and the smaller particles to the other.

The results obtained following both procedures were identical. Each antibody labelled at plasma membranes exclusively at sites of contact between adjacent cells. The sizes and sites of the labelled junction profiles matched those of gap junction plaques revealed by freeze-fracture. Every junction labelled with anti-*b2* also labelled with anti-*b6*. These junctions were located in the inner sulcus, supporting cells in the organ of Corti, Hensen's cells, the outer sulcus and spiral ligament fibrocytes. The labelling for each connexin was evenly distributed along the junction profiles, with no indication of differential labelling, suggesting that the two connexins are 'mixed' within the junctions, compatible with a heteromeric configuration or a random mixture of homomeric connexons. 'Annular' gap junctions within the cells were also evenly labelled with both antibodies. These structures are thought to be entire gap junction plaques removed from the membrane and destined for destruction. The widespread identification of these structures suggests rapid gap junction turnover. This must represent a considerable metabolic investment by the non-sensory cells of the cochlea."

LONDON

Vertebrate Development Lab
Imperial Cancer Research Fund
London

M Eddison

Delta-Notch signalling and cell differentiation in the chick inner ear

The sensory patches of the inner ear consist of two types of cell: sensory hair cells and supporting cells. The pattern is such that supporting cells surround each hair cell and no two hair cells touch each other. Our lab is trying to uncover the genetic mechanisms that control both the differentiation and patterning of these two cell types.

The alternating pattern of hair cells and support cells has led to the suggestion that their differentiation is co-ordinately regulated by cell-cell interactions involving the *Notch* signalling pathway. The key players in this pathway are *Delta*, a ligand, and *Notch*, its receptor, mediating a process known as lateral inhibition - a mechanism which forces neighbouring cells of an initially equivalent group to become different.

The hypothesis that *Notch* signalling regulates cell differentiation within the ear is corroborated by evidence from gene expression patterns and mutant phenotypes. In fact two *Notch* ligands *Delta1* and *Serrate2* are expressed in the nascent hair cells and are thought to deliver lateral inhibition to their neighbours, which become supporting cells. Intriguingly, the supporting cells themselves also express a *Notch* ligand, *Serrate1*.

To test the function of *Delta1* in the chick ear we over expressed it using the replication-competent RCAS retrovirus. The original hypothesis predicts that overexpression of *Delta* in a patch of sensory cells would inhibit the production of hair cells in that patch. Surprisingly, we were unable to see any effect on hair cell number. We have found a possible explanation for this lack of phenotype- the protein *Numb*. *Numb* binds to the intracellular domain of *Notch* and blocks *Notch* signalling. At early stages, *Numb* is present in the sensory epithelium, and is asymmetrically localised to the basolateral surface of the cells. At later stages we see *Numb* expressed selectively in mature hair cells and here it is evenly distributed throughout each cell. This suggests that the unrestricted distribution of *Numb* in the hair cells makes them immune to *Notch* signalling and that asymmetric localisation of *Numb* may act in conjunction with *Delta-Notch* signalling to dictate the choice between hair cell fate and supporting cell fate.

In complementary experiments, we have over expressed a dominant negative form of *Delta1*, which blocks *Notch* signalling. This caused a down regulation of expression of *Serrate1*, implying that *Serrate1*, in contrast with *Delta1*, is positively regulated by *Notch* activation. Remarkably, hair cell numbers were not increased. A possible interpretation is that more than one *Notch* is operating in the ear: and one *Notch* family member maybe sensitive to dominant negative *Delta* and regulate *Serrate1*, while another maybe insensitive to dominant negative *Delta* and regulate commitment to the hair cell fate. Another possible interpretation is that infected cells may have differentiated prematurely and died, or may have delaminated to become part of the cochleo-vestibular ganglion.

LOS ANGELES

House Ear Institute¹
2100 W. 3rd Street
Fifth Floor
Los Angeles
California 90057
USA

P Chen¹
F Zindy²
M Roussel²
N Segil¹

St. Jude Children's Research Hospital²
Department of Tumor Biology
St. Jude Children's Reserach Hospital
Memphis
TN 38105
USA

Synergistic action of Ink and Cip/Kip CDK inhibitors in the developing organ of Corti.

Coordinated regulation of cell proliferation and differentiation is required for the normal morphogenesis of multicellular organs, such as the organ of Corti. This is partly achieved through the developmentally regulated appearance of cell cycle inhibitors leading to timely withdrawal from the cell cycle. Previously, we reported that the expression of a member of *Cip/Kip* family of cyclin-dependent-kinase (CDK) inhibitors, *p27Kip1*, is up-regulated in the developing organ of Corti as cells exit the cell cycle. During development of this organ, mitotic activity is prolonged when *p27Kip1* is inactivated, leading to the production of supernumerary sensory hair cells and supporting cells. These results indicate that *p27Kip1* plays an important role in developmental timing of cell cycle withdrawal in the developing organ of Corti. However, the inactivation of *p27Kip1* does not result in runaway growth implying the presence of compensatory pathways for *p27Kip1* mediated cell cycle withdrawal. Here we report that the inactivation of a member of the *Ink4* family of CDK 4/6 inhibitors, when combined with the inactivation of *p27Kip1*, has a dramatic effect on the morphogenesis of the organ of Corti. While mitotic activity is only present in the region of pillar cells and Hensen's cells in postnatal *p27Kip1* null mice, it is also detected in the hair cell region of double null animals after birth. This increased mitotic activity was associated with multiple layers of cells recognized by the hair cell specific marker *Myosin VIIa*. When *p19Ink4d* alone is inactivated, the morphology of the organ of Corti in neonatal animals appears normal, however, a low level of ongoing postnatal proliferation is observed and by P10 we note a partial loss of hair cells from the innermost row of outer hair cells. These preliminary data suggest that *p19Ink4d* may be part of the cell cycle machinery responsible for cell cycle exit during development of the sensory epithelia of the inner ear, and may partially substitute for the loss of *p27Kip1* function in this tissue.

MARYLAND

Howard Hughes Medical Institute
4000 Jones Bridge Road
Chevy Chase
MD 20815-6789

AB Elgoyhen

Cloning and functional properties of hair cell nAChRs

The neurotransmitter acetylcholine (ACh) mediates efferent inhibition of outer hair cells (OHCs) of the cochlea. While both muscarinic and nicotinic acetylcholine receptors appear to be involved in OHC function, pharmacological and electrophysiological data suggest a central role for an atypical, nicotinic subtype of AChR in the inhibition of all vertebrate hair cells. The properties of the native OHC receptor are most similar to those described for receptors assembled in vitro from the small alpha, *Greek9* subunit gene (Elgoyhen et al., Cell, 79: 715, 1994). Moreover, the fact that a strain of mice with a null mutation in the small alpha, *Greek9* gene are functionally de-efferented, supports the central role of small alpha, *Greek9*-containing nAChRs in mediating the effects of the olivocochlear system (Vetter et al., Neuron, 23: 93, 1999). We have recently isolated and characterized small alpha, *Greek10*, a novel nAChR subunit. GenBank database searches revealed several human expressed sequence tags with sequence similarity to the rat small alpha, *Greek9* subunit. Using

this information we isolated a clone encoding a protein of 441 residues from a rat cochlear cDNA library. Sequence analysis revealed that the small alpha, *Greek10* subunit is most closely related to small alpha, *Greek9* (80% at the amino acid level). small alpha, *Greek10* transcripts are co-expressed with small alpha, *Greek9* in rat cochlear OHCs, vestibular crista and both saccular and utricular macular hair cells. When expressed in *Xenopus laevis* oocytes, the small alpha, *Greek10* subunit does not form functional homomeric receptors. However, the heteromeric assembly of small alpha, *Greek9* and small alpha, *Greek10* yields a functional receptor that responds to ACh with robust ionic currents. The pharmacological profile of the small alpha, *Greek9* small alpha, *Greek10* receptor does not differ significantly from that of small alpha, *Greek9*. However, small alpha, *Greek9* small alpha, *Greek10* displays a desensitization pattern, a biphasic response to extracellular Ca²⁺ and a current-voltage relationship that differ from those described for the homomeric small alpha, *Greek9* receptor, but resemble those reported for the native OHC receptor. We suggest that the OHC cholinergic receptor is composed of both small alpha, *Greek9* and small alpha, *Greek10* subunits, with small alpha, *Greek10* being a major determinant of channel properties.

MELBOURNE

Murdoch Childrens Research Institute¹
Royal Children's Hospital
Parkville, 3052
Australia
Tel: (03) 8341 6238
Fax: (03) 9348 1391

Paediatric Hearing Loss Investigation Clinic²
Children's Program and Department of ENT, Head and Neck
Surgery
Monash Medical Centre
Southern HealthCare Network
& Monash University
Department of Paediatrics
Monash Medical Centre
Clayton, 3168
Australia

Department of Audiology³
Monash Medical Centre
Southern HealthCare Network
Clayton, 3168
Australia

Department of Otolaryngology⁴
University of Melbourne
Parkville, 3052
Australia

M Kamarinos¹
SA Wilcox¹
K Saunders²
AH Osborn¹
A Arnold¹
J Wunderlich³
T Kelly¹
V Collins¹
LJ Wilcox¹
RJMCK Gardner¹
B Cone-Wesson⁴
R Williamson¹
H-HM. Dahl¹

High frequency hearing loss correlated with mutations in the GJB2 gene

Genetic hearing impairment affects approximately 1/2000 live births. Mutations in one gene, *GJB2*, coding for connexin 26 causes 10-20% of all genetic hearing loss. Our study aimed to characterise connexin 26 mutations in hearing impaired children in Victoria. Families who attended the Paediatric Hearing Loss Investigation Clinic (PHLIC) at the Monash Medical Centre, Melbourne, between the years of 1994-1998 were invited to participate in our study. We focused on a subgroup of 74 families (80 children) in which a diagnosis of recessive non-syndromic hearing loss had been established or could not be excluded. DNA sequence analysis was performed on the entire coding region of the *GJB2* gene in these families along with audiological assessment.

Seven mutations were detected, four of which (35delG, M34T, L90P and 167delT) have been previously reported. In our study the M34T mutation was found in 5 families and was shown to act in a recessive manner. A novel change resulting in the amino acid substitution R184W within the coding region was also found. Two previously reported sequence variants, V27I and V37I, were also detected. The V37I change has been previously reported as a sequence variant but in our study suggests that it is a pathological mutation. We also screened 1026 individuals for the 35delG mutation using a PCR based assay and found the carrier frequency to be 1/100 in the Australian population.

Connexin 26 mutations were found in 26% of our cohort with the severity of hearing loss ranging from mild to profound. Of this group 57% had an identifiable mutation in both alleles and the remaining 43% had a mutation in only one allele.

Children with mutations in both *GJB2* alleles showed a sloping or flat audiogram with hearing loss predominantly at high frequencies. Children with no mutations in the *GJB2* coding region had more varied audiogram shapes with little change in pure-tone air conduction across all frequencies. Perhaps the most intriguing group is that consisting of children with only one *GJB2* mutation. This group showed audiological patterns identical to those observed for children with two mutant alleles. These audiological findings suggest that the hearing loss is related to *GJB2* yet only one mutation was detected. The exact explanation for these findings is unclear at this stage but it opens up some intriguing questions about how *GJB2* might cause hearing loss in these people.

MISSOURI

University of Missouri-Columbia¹
 Division of Otolaryngology
 205 Allton building DC 375.00
 Columbia
 Missouri 65212
 USA

SM Jones¹
 LC Erway²
 G Subramanian¹
 KR Johnson³
 TA Jones¹

University of Cincinnati²
 Biological Sciences
 Mail location #6

Cincinnati
Ohio 45221
USA

The Jackson Laboratory³
600 Main Street
Bar Harbor
Maine 04609
USA

Vestibular function in shaker1 and lethal milk mouse mutants

Putative vestibular functional deficits have been inferred largely from behavior and inner ear morphology of mutant strains of mice. We have initiated a program of research to test vestibular function directly in mutant strains known or suspected to have vestibular abnormalities. The present report describes results for two mutant strains with known vestibular structural deficits. First is the shaker1 (*sh1*) mouse which has degenerative changes in inner ear hair cells, ganglion cells and vestibular nuclei (n=6, 1 to 3 months old). Second is the lethal milk (*lm*) mouse which has absent utricular otoconia and variable loss of saccular otoconia (n=8, 3 months old). General behavior and swimming ability were noted prior to functional measures. Linear vestibular evoked potentials (VsEPs) were recorded using techniques described elsewhere (1999, Hear. Res. 136, 75-85). Peripheral response components were measured and VsEP thresholds, latencies, and amplitudes were compared with age matched controls. Temporal bones were cleared for otoconial measurement. *Sh1* mutants displayed typical circling behaviors and VsEPs were virtually absent for 3 mice at the maximum stimulus intensity (+6dBre:1.0g/ms). In 3 others, an onset response was discernible at approximately 3ms or later. Otoconia were found to be absent either in the utricle, saccule or both. *Lm* mice displayed variable swimming abilities. Most were able to keep their nose above water with the body in a vertical orientation. In comparison to control mice, *lm* mutants displayed VsEP thresholds that were significantly higher (-8.8±4.1dB vs. -13.3±2dBre:1.0g/ms) and P1/N1 amplitudes that were significantly smaller (1.5±0.8µV vs. 3.0±0.4µV). Among *lm* mutants, however, VsEPs varied considerably. Thresholds ranged from -4.5 to -13.5dBre:1.0g/ms and P1/N1 amplitudes ranged from 0.6 to 2.7µV. Saccular otoconia ranged from near absence to normal. Saccular deficits were correlated with functional loss. In conclusion, *sh1* mutants clearly manifest a profound macular functional deficit and tests of *lm* mutants confirm degraded macular function that is correlated with the degree of otoconial loss.

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MISSOURI

Washington University School of Medicine¹
Department of Otolaryngology
517 South Euclid Avenue
Saint Louis
Missouri 63110

J Sun¹
A Van Alphen²
B Bohne³
B Tempel⁴
Q yin Zheng⁵

USA

C De Zeeuw²

Erasmus University Rotterdam²
The Netherlands

Washington University³
4566 Scott Avenue
Saint Louis
Missouri 63110
USA

The V.M. Bloedel Hearing Research Center⁴
Box 357923
CHDD Building
Room CD-040C
University of Washington School of Medicine
Seattle
Washington 98195-7923
USA

600 Main Street⁵
Bar Harbor
Maine 04609
USA

Functional vestibular testing of deafwaddler mutant mice

Deafwaddler (*dfw*) mutant mice are deaf and display a wobbly gait. The gene encoding a plasma membrane Ca²⁺-ATPase type 2 pump (*PMCA2*) has been found to be defective in the deafwaddler mice. Histologically, otoconia are present in their utricles but not in their saccules. Our goal was to determine the effect of their otoconia deficit on vestibular function. We investigated compensatory eye movements during passive sinusoidal body roll under two conditions: 1) rotation about an earth-horizontal axis (nose forward) and 2) rotation about an earth-vertical axis (nose up). In the earth-horizontal axis rotation, the vestibular-ocular response (VOR) is modulated by both semicircular canals and otolith organs. In the earth-vertical axis rotation or rotation orthogonal to gravity, the VOR is modulated by the semicircular canals only. Using these two paradigms, we determined the otoconia contribution to the compensatory eye-movement response of homozygous deafwaddlers and heterozygous control littermates. The gain and phase values of the VOR were measured with the scleral search coil method modified for mice (Koekkoek et al., 1997 Genes and Function; De Zeeuw et al., 1998 Neuron). Whole body rotations were tested over a range of amplitudes (5 to 30 degrees) and frequencies (0.2 - 1 Hz). The VOR gain values of the controls were higher during rotation about the earth-horizontal axis than during rotation about the earth-vertical axis at all frequencies tested. However, the gain value of the deafwaddler during rotation about the earth-horizontal axis was not significantly different from that during rotation about the earth-vertical axis, which demonstrates their lack of otolith input. In addition, at low frequency rotation about an earth-horizontal axis, the deafwaddler showed a prominent phase lead that was significantly greater than that of

their control littermates. Therefore, the present study indicates that the otolith deficit in the deafwaddler mice hampers their ability to obtain an optimal compensatory eye-movement response during natural vestibular stimulation.

NEBRASKA

Boys Town National Research Hospital¹
Biophysics
555 North 30th Street
Omaha
Nebraska 68131
USA

D He¹
M Pearce²
MA Cheatham³
DE Vetter⁴

Northwestern University
Auditory Physiology Laboratory²
Communication Sciences and Disorders³
2-240 Frances Searle Building
2299 N Campus Drive
Evanston
Illinois 60208
USA

The Salk Institute for Biological Studies⁴
Molecular Neurobiology Lab-H
10010 North Torrey Pines Road
La Jolla
California 92037
USA

Alpha-9 knockout mice show OHC electromotility

In adult mammals, outer hair cells (OHC) are innervated predominantly by efferents, which originate in the brainstem. Efferent fibers form chemical synapses at the bases of OHCs, with acetylcholine (ACh) being their principal neurotransmitter. The OHC ACh receptors, which have unusual pharmacology, have been cloned and identified as a new subunit of the nicotinic ACh-receptor family, known as alpha 9. The effect of efferent action, mediated by the release of ACh and the activation ACh receptors on OHCs, is generally known to be inhibitory. In this investigation, we attempt to examine the possible role(s) of the alpha 9 subunit in regulating OHC function by examining OHC electromotility and compound action potentials (CAP) in mice carrying a null mutation for the *alpha 9* gene.

Mice of either sex were tested between 4 and 9 weeks of age. In the in vivo experiments, cochlear sensitivity was measured using CAPs in animals anesthetized with either urethane or pentobarbital. After tracheotomy, the bulla was exposed and a round window electrode positioned. Results indicate that these mice, with the 129/SvEv genetic background, have age-related hearing loss at high frequencies above 35 kHz. Cochlear sensitivity, however, was similar for homozygous (-/-), heterozygous (+/-) and wild type (+/+) mice. In separate in vitro experiments, OHCs were isolated from the cochleae of homozygous animals as well as heterozygous and

wild-type controls. In order to avoid the influence of age-related hearing loss, OHCs were not obtained from the very base of the cochlea where the electrophysiology indicated threshold shifts in the CAP. Electromotility was assessed with a photodiode-based system using the microchamber technique. Results indicate that electromotility was present in all OHCs, independent of whether the alpha 9 subunit was present or absent. This observation suggests that efferent action mediated by alpha 9 subunit is not required for the development and/or maintenance of OHC motor function. Supported by NIH grants DC00089 and DC00708 to P. Dallos; DC02764 to L. Madison, and DC02871 to S.F. Heinemann.

NEW YORK

Albert Einstein College of Medicine¹
1925 Eastchester Road
Apartment 2E
Bronx
New York
10461
USA

M Bove¹
Y Suda¹
D Frenz²
T Van De Water¹

1410 Pelham Parkway²
South Yeshiva University
Bronx
New York 10461

*Functional equivalency between *Otx1* and *Otx2* in development of the inner ear*

Otx1 and *Otx2*, two bicoid-class homeobox genes, are thought to play critical roles in the processes of head development. In the developing mouse embryo, both *Otx* genes are expressed in the rostral head region as well as in sense organs such as the inner ear. To explore the relative contribution of each *Otx* gene to the morphogenesis of the inner ear, we compared, at embryonic day 14, the ears of *Otx1* homozygous knock-out mice with those of knock-in mice in which the *Otx2* gene was replaced by an *Otx1* gene. Our results show that in the pars inferior of both knock-in and knock-out mice, the macula utriculi was displaced medially and fused with the macula sacculi. This phenotype was more severe in the knock-in mice. The cochlea in the knock-in mice displayed two consistent abnormalities: (1) the position of the developing cochlear apparatus was posteriorly displaced relative to its wild type position, placing it directly medial, instead of anteromedial, to the developing vestibule; and (2) the cochlear duct was abnormally rotated such that, on axial section, the broad axis of the distal cochlea lay perpendicular in orientation to that of the basilar cochlea. In the pars superior, the lateral semicircular canal, ampulla, and crista were absent in all *Otx1* knock-out mice and in the knock-in mice with the most severe rostral deficit. The auricles of the knock-in mice were low set. Their external auditory canals communicated with those from the contralateral side. The pharyngotympanic tube was truncated and the tubotympanic recess was absent. Although Meckel's cartilage was present in all knock-in embryos, the individual ossicular primordia were not formed. These results suggest the importance of the *Otx* genes both for the specification of inner ear structures such as the lateral canal, and for the

ability of the rostral neurectoderm and Meckel's cartilage to form their respective adult derivatives.

NEW YORK

Apartment 25F¹
1935 Eastchester Road
Bronx
New York
10461
USA

E Chan¹
S Baron²
W Wang³
T Lufkin³
T Van De Water²

Albert Einstein College of Medicine²
1925 Eastchester Road
Apartment 2E
Bronx
New York
10461
USA

Mount Sinai School of Medicine³
Mt Sinai Hospital
New York
10001
USA

Vestibular defects in mice lacking the Hmx2 homeobox gene: embryonic day 14.5 to birth

Hmx2 and *Hmx3* are two *Hmx* homeobox genes expressed in the developing mouse inner ear. *Hmx2* and *Hmx3* expression are essentially confined to the vestibular apparatus, suggesting overlapping functions in inner ear development for these two genes (Rinkwitz-Brandt et al., Hearing Research 99:133). Disruption of *Hmx3* causes a reduction in saccular and utricular sensory epithelia, partial fusion of the utricle and saccule, and an absence of the lateral crista and ampulla (Wang et al., Development 125:623). The purpose of our study was to analyze the histologic abnormalities in the inner ears of mice lacking the *Hmx2* gene. Morphologic examination has revealed partial fusion of the saccular and utricular chambers with only a single common macula evident at E14.5. The posterior, superior, and lateral semicircular ducts were all absent. The three primordial diverticuli were unable to develop into the semicircular ducts because the absorption foci of the diverticuli failed to form, resulting in a common cavity formed by these diverticuli and the fused utriculosaccular chamber. The lateral crista, lateral ampulla, and superior ampulla were all absent. However, the superior crista, posterior crista, and its ampulla were present. Microscopic measurements demonstrated a 50% decrease in the macular area of the utricle and a > 30% decrease in the average thickness of the cristae at E14.5, with increasing percentages of losses as the embryo approached birth. Histologic analysis revealed a 100% reduction in utricular hair cells as early as E14.5 and a corresponding increase of > 20% in the number of saccular hair cells as compared to wild-type mice. Neuron counts showed a progressive reduction in vestibular ganglion

neurons from E16.5 to birth but no corresponding reduction in spiral ganglion neurons. This study illustrates that the *Hmx2* homeobox gene plays an important role in the normal embryonic development of the vestibular system, including the morphogenesis of all three semicircular ducts, the integrity of the utricular and saccular chambers, the formation of separate sensory receptor epithelia, and the survival of vestibular ganglion neurons.

NOTTINGHAM

MRC Institute of Hearing Research¹
University Park
Nottingham
NG7 2RD
UK

Phone: +44 1159 223431
Fax: +44 1159 518509

Department of Human Genetics and Molecular Medicine²,
Sackler School of Medicine,
Tel Aviv University,
Tel Aviv,
Israel

Phone: 972-3-640-7030
Fax: 972-3-640-9900

GSF Research Center for Environment and Health³,
Institute of Experimental Genetics, Neuherberg, Germany

GSF National Research Center for Environment and Health⁴
Institute of Experimental Genetics
Ingolstaedter Landstr. 1
D-85764
Neuherberg
Germany

Phone: 089 3187 3302
Fax: 089 3187 3500

MRC Mammalian Genetics Unit⁵
Harwell
Didcot
Oxfordshire
OX11 0RD
UK

Phone +44 1235 824541

Institute Pasteur⁶

KP Steel¹
AE Kiernan¹
A Erven¹
C Rhodes¹
H Tsai⁵
RE Hardisty⁵
P Nolan⁷
J Peters⁷
SDM Brown⁵
J Hunter⁷
N Ahituv²
M Zalzman²
R Hertzano²
O Ben-David²
S Vreugde²
KB Avraham²
H Fuchs³
R Balling³
M Hrabé De Angelis⁴
JL Guénet⁶

25 Rue du Dr. Roux
Paris
France 75015

Department of Neuroscience⁷
Smithkline Beecham Pharmaceuticals
New Frontiers Science Park
Harlow
Essex
UK

New mouse models for hearing and balance defects from the European mutagenesis programmes

Mouse mutants with ear defects are valuable as models for human hereditary deafness, as well as giving us tools for understanding normal cochlear development and function. We have generated 50 new dominantly-inherited mutants affecting hearing and/or balance by mutagenising males with *N*-ethyl-*N*-nitrosourea (ENU), then mating with normal females. The F1 offspring are screened for behaviour indicative of a balance defect or for the lack of a Preyer reflex in response to a 20kHz, 90dB SPL toneburst. A total of 53,000 F1 offspring were screened to yield 50 new mutants, all confirmed to carry heritable defects. 15 of these new mutations have been mapped to a chromosomal region. We found that backcrossing to a different mouse strain altered the phenotype in five of these new mutants, emphasising the importance of genetic background in expression.

Six new mutants map to proximal chromosome 4 and show lateral semicircular canal truncations; these may be new alleles of the *Wheels* locus. Two mutants (*Headturner* and *Slalom*) have been identified as *Jag1* mutations, and show truncation of the posterior and anterior semicircular canals as well as pattern defects in the organ of Corti, a unique phenotype. Three mutants have middle ear defects: *Doarad* on chromosome 13 has misshapen ossicles, *Pardon* on chromosome 19 has malformed ossicles combined with supernumary hair cells in the organ of Corti, and *Jeff* shows a predisposition to middle ear inflammation, the mutation mapping to chromosome 17. Two mutants have abnormal development of stereocilia bundles, *Tailchaser* on chromosome 2 and *Headbanger* on chromosome 7. *Beethoven* on chromosome 19 shows progressive postnatal degeneration of inner hair cells while outer hair cells remain intact. *Dearisch* mutants have progressive hearing loss but the mutation is not yet mapped, and finally *Spin cycle* has a balance defect and maps to 15.

Eight of these mutants show novel phenotypes, suggesting that the mutagenesis programmes will continue to be a rich resource for investigating auditory function and development.

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NOTTINGHAM

MRC Institute of Hearing Research¹
University Park
Nottingham
NG7 2RD
UK

Phone: +44 1159 223431
Fax: +44 1159 518509

Department of Human Genetics and
Molecular Medicine²,
Sackler School of Medicine,
Tel Aviv University,
Tel Aviv,
Israel

Phone: 972-3-640-7030
Fax: 972-3-640-9900

GSF Research Center for Environment and
Health³, Institute of Experimental
Genetics, Neuherberg, Germany

GSF National Research Center for
Environment and Health⁴
Institute of Experimental Genetics
Ingolstaedter Landstr. 1
D-85764
Neuherberg
Germany

Phone: 089 3187 3302
Fax. 089 3187 3500

MRC Mammalian Genetics Unit⁵
Harwell
Didcot
Oxfordshire
OX11 0RD
UK

Phone +44 1235 824541
FAX: +44 1235 824542

CR Rhodes¹
AE Kiernan¹
A Erven¹
R Hertzano²
KB Avraham²
H Fuchs³
M Hrabéde Angelis⁴
R Balling³
H Tsai⁵
RE Hardisty⁵
SDM Brown⁵
KP Steel¹

ENU Induced Mutations causing Vestibular and Hearing Dysfunction in Mice

In order to gain a better understanding of human deafness, the generation and analysis of mouse models for the 100 or more known human deafness loci will

provide us with a clearer understanding of the developmental and functional pathways of the auditory system. To address this, a phenotypic approach has been adopted in the mouse to identify molecules involved in ear development and function. Mutant mice were obtained using *N*-ethyl-*N*-nitrosourea mutagenesis and were screened for dominant mutations that affect hearing and/or balance. Three of these mutants, Headbanger, Pardon and Slalom are presented.

Headbanger (*Hdb*) mutants display classic behavioural symptoms of a vestibular dysfunction, including hyperactivity and head shaking. Headbanger mice have a strong Preyer reflex. However, surface analysis of the organ of Corti by scanning electron microscopy (SEM) has revealed defects within the stereocilia bundles of both inner and outer hair cells. In the apex, the mutant outer hair cell bundles form an O-shape rather than the usual V-shape. The mutation has been mapped to chromosome 7.

Slalom (*Slm*) mutants show subtle head weaving and shaking indicative of a vestibular defect. Paint-filling of the inner ear has shown truncations of the posterior and the superior semi-circular canals. Analysis of the organ of Corti by SEM has shown a decrease in OHC numbers and the presence of atypical cells within the IHC row. We have identified the mutation in the *Jagged1* gene on chromosome 2.

Pardon (*Par*) mutants are identified by a complete lack of a Preyer reflex but have no obvious balance defects. Gross dissection of the middle ear has revealed morphological defects in the middle ear ossicles. In addition, SEM of the organ of Corti shows regions of extra inner and outer hair cells. The mutation has been mapped to chromosome 19.

These mutants may contribute to the identification of new genes involved in hearing and balance mechanisms.

Supported by the MRC, EC contract CT97-2715 and Defeating Deafness.

NOTTINGHAM

MRC Institute of Hearing Research¹
University Park
Nottingham
NG7 2RD
UK

Phone: +44 1159 223431
Fax: +44 1159 518509

GSF Research Center for Environment and Health²,
Institute of Experimental Genetics, Neuherberg,
Germany

GSF National Research Center for Environment and
Health³
Institute of Experimental Genetics

A Erven¹
M Hrabé de Angelis³
H Fuchs³
R Balling³
JL Guénet⁷
KB Avraham⁴
Nadav Ahituv⁴
S Vreugde⁴
P Nolan⁶
J Peters⁶
M Skynner⁶
N Allen⁶
S Brown⁵
KP Steel¹

Ingolstaedter Landstr. 1
D-85764
Neuherberg
Germany

Phone: 089 3187 3302

Fax: 089 3187 3500

Department of Human Genetics and Molecular
Medicine⁴,
Sackler School of Medicine,
Tel Aviv University,
Tel Aviv,
Israel

Phone: 972-3-640-7030

Fax: 972-3-640-9900

MRC Mammalian Genetics Unit⁵,
Harwell,
Didcot,
Oxfordshire,
OX11 0RD,
UK

Phone +44 1235 824541

Fax: +44 1235 824542

GlaxoSmithKline Pharmaceuticals⁶,
New Frontiers Science Park,
Harlow,
Essex,
CM19 5AW,
UK

Institute Pasteur⁷
25 Rue du Dr. Roux
Paris
France 75015

Phenotypic analysis of new mouse models for inherited deafness.

Phenotypic and genotypic analysis of deaf and/or vestibular mouse mutants has helped greatly with the identification of genes involved in hearing impairment and has contributed to the understanding of mechanisms underlying hearing. For phenotypic analysis of human deafness these mouse models are essential although there are a great number of human deafness loci for which homologous mouse models

are not yet known. This project attempts to address this deficiency in mouse models for deafness and vestibular mutations by generating and analysing new mutants.

Four mutations were analysed which were derived using various mutagenesis methods. One mutant, Head-shaker circler (*Hsc*), arose by X-ray mutagenesis. This dominant mutation results in deafness, circling and head shaking behaviour, associated with thin semi-circular canals and abnormal otolithic membranes. The mutant Tasmanian devil (*tde*) is a transgenic insertional mutation that results in deafness and circling behaviour. This recessive mutation leads to thin and disorganised stereocilia in both types of hair cells of the cochlea and vestibular system. Two mutants were created by ENU mutagenesis. These two dominant mutants, Doarad (*Dor*) and Beethoven (*Bth*), are both deaf but show two distinctly different phenotypes. One mutant has abnormal middle ear ossicles while in the other mutant the inner hair cells of the cochlea degenerate, leaving outer hair cells intact.

These mutants will help with the unravelling of the genetics of deafness and the understanding of the development of the mammalian ear.

This project is supported by the MRC, Defeating Deafness and the EC (contract number: CT97-2715).

NOTTINGHAM

MRC Institute of Hearing Research¹
University Park
Nottingham
NG7 2RD
UK

Tim Self¹
Steve D M Brown²
Karen P Steel¹

Phone: +44 1159 223431
Fax: +44 1159 518509

MRC Mammalian Genetics Unit²
Harwell
Didcot
Oxfordshire
OX11 0RD
UK

Phone +44 1235 824541
FAX: +44 1235 824542

Mutations in Myosin VIIA and Hair Cell Development

The deaf mouse, shaker1, has a recessive mutation in the gene encoding myosin VIIA (*Myo7a*). We have looked at sensory hair cells in the cochlea of some of these mice, two with null mutations and two with mutations in the tail of *myosin VIIA*, to uncover the role of this unconventional myosin in their development. The mutants and littermate controls were examined at 3 days after birth. In the normal

mouse, the microvilli which cover the upper surface of the hair cells start to elongate and form an ordered V-shaped array of tall stereocilia from around 16 days of gestation onwards. In the mutants by three days after birth, the stereocilia have become disordered in their arrangement on the apical surface of the hair cells. The hair cells from the null mutants have stereocilia which are frequently splayed as well as being disorganised when compared to the controls. The tail mutants did not show any splaying of stereocilia, but did have disorganised arrays, often arranged in several clusters. The finding of stereocilia splaying in the null alleles supports the suggestion that *myosin VIIA* may be involved in cross-linking adjacent stereocilia, while the observation of disorganisation of the stereocilia bundle in all four alleles suggests that *myosin VIIA* is required to maintain the correct arrangement of stereocilia at the top of the hair cell. Supported by the MRC.

NOTTINGHAM

MRC Institute of Hearing Research¹
University Park
Nottingham
NG7 2RD
UK

Phone: +44 1159 223431
Fax: +44 1159 518509

AE Kiernan¹
N Ahituv²
H Fuchs³
C Rhodes¹
R Balling³
KB Avraham²
M Hrabé de Angelis⁴
KP Steel¹

Department of Human Genetics and Molecular
Medicine²,
Sackler School of Medicine,
Tel Aviv University,
Tel Aviv,
Israel

Phone: 972-3-640-7030
Fax: 972-3-640-9900

GSF Research Center for Environment and
Health³, Institute of Experimental Genetics,
Neuherberg, Germany

GSF National Research Center for Environment
and Health⁴
Institute of Experimental Genetics
Ingolstaedter Landstr. 1
D-85764
Neuherberg
Germany

Phone: 089 3187 3302
Fax. 089 3187 3500

The Notch ligand Jagged1 is required for inner ear sensory development

Using an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis approach, we have identified a new mouse mutant Headturner (*Htu*) that showed dominant head-shaking behaviour indicative of a vestibular defect. Investigation of the gross structure of the inner ear by paintfilling demonstrated that the posterior and sometimes anterior ampullae, the structures that house the sensory cristae, were absent and those canals were truncated. In addition, scanning electron microscopy in the organ of Corti of *Htu*/*+* mutants showed that there were often only two rows of outer hair cells, rather than the normal three rows, leading to a 33% decrease in the numbers of outer hair cells. Interestingly, the numbers of inner hair cells was increased slightly.

Htu was mapped to a 6 centimorgan region on mouse chromosome 2, between markers D2Mit399 and D2Mit280. The Notch ligand *Jagged1* (*Jag1*), was located in this region and was considered a good candidate gene because it is expressed throughout the prospective sensory regions of the inner ear. Sequence analysis of the *Jag1* gene in *Htu* mutants revealed a missense mutation leading to a non-conservative amino acid change in the second EGF-like repeat of the JAG1 protein. Furthermore, analysis of *Htu* homozygote embryos revealed that they were dying around embryonic day 11.5 due to vascular defects in the yolk sac and embryo, similar to other *Jag1* loss-of-function mutants.

The *Notch* signalling pathway has been implicated in mediating lateral inhibition within the ear and mutations in several genes in this pathway lead to an increase in the numbers of hair cells as the model would predict. However, since *Htu*/*+* mutants show an overall decrease in the numbers of hair cells in the organ of Corti and loss of several vestibular sensory organs, our findings suggest a new and perhaps earlier role for *Notch* signalling within the ear.

This work is supported by the MRC, Defeating Deafness, a grant from the EU (contract number BMH4-CT97-2715) and from the German Human Genome Project (DHGP).

PARIS

Institute Pasteur¹
25 Rue du Dr. Roux
Paris
France 75015

E Verpy¹
M Leibovici¹
I Zwaenepoel¹
C Petit¹

From the construction of a subtracted cDNA library to deafness genes : identification of harmonin, the protein defective in Usher syndrome type 1C

Genes specifically or preferentially expressed in inner ear sensory hair cells represent good candidates for deafness. Efforts to identify such genes have been hampered by the small number of these cells. We constructed a subtracted mouse cDNA library derived from the sensory areas of the vestibule. Sequence analysis of this library revealed that half of the clones appeared to correspond to transcripts known to be specifically or preferentially expressed in the sensory epithelia of the

inner ear. Approximately 200 other sequences have been characterized. Several of them identified genes which, based on their chromosomal localization, their expression pattern and the nature of the protein they encode, represent very good candidates for isolated or syndromic deafness. As an example, we will report the identification of the gene (*USH1C*) underlying Usher syndrome type 1C. This autosomal recessive sensory defect involves congenital profound sensorineural deafness, vestibular dysfunction and blindness due to progressive retinitis pigmentosa. *USH1C* encodes a PDZ domain-containing protein, harmonin, expressed in the inner ear sensory hair cells. Several *Ush1c* transcripts were identified in mouse inner ear, predicting harmonin isoforms containing 2 or 3 PDZ domains and 1 or 2 coiled-coil domains, some of them also containing an additional proline- and serine-rich region. Since several of these transcripts were absent from the eye, we propose that *USH1C* also underlies the DFNB18 form of isolated deafness which maps to a chromosome interval encompassing *USH1C*.

PARIS

Institute Pasteur¹
25 Rue du Dr. Roux
Paris
France 75015

Zoologisches Institute
Johannes-Gutenberg-Universitat Mainz
Mainz
Germany 55099²

A El-Amraoui¹
P Kussel-
Andermann¹
S Safieddine¹
I Perfettini¹
M Lecuit¹
P Cossart¹
U Wolfrum²
C Petit¹

Vezeatin, a novel transmembrane protein, bridges the unconventional myosin VIIA to the cadherin/catenins complex

Defects in *myosin VIIA* are responsible for deafness in human and mouse. To get an insight into the cellular function of the unconventional *myosin VIIA*, we sought proteins interacting with its tail region, using the yeast two-hybrid system. One of the five isolated candidates encodes for the type Ia regulatory subunit (RIa) of protein kinase A, which led us to suggest that *myosin VIIA* is a novel A-kinase anchoring protein, that targets protein kinase A to definite subcellular sites of the inner ear sensory cells. The second ligand, named *vezatin*, is a novel transmembrane protein, an ubiquitous protein of adherens cell-cell junctions where it interacts both with myosin VIIA and the cadherin/catenins complex. Its recruitment to adherens junctions implicates the C-terminal region of alpha-catenin. Taken together, these data suggest that myosin VIIA, anchored by vezatin, creates a tension force between adherens junctions and the actin cytoskeleton which is expected to strengthen cell-cell adhesion. In the inner ear sensory hair cells, vezatin is, in addition, concentrated at another membrane-membrane interaction site, namely at the fibrillar links interconnecting the bases of adjacent stereocilia. The activity of vezatin-myosin VIIA complex at both sites can account for the splaying out of hair cell stereocilia in *myosin VIIA* defective mutants.

PORTLAND

Oregon Health Science University¹
OHRC/Vollum Institute
3181 Sam Jackson Park Road
Portland
Oregon 97201
USA

PG Gillespie¹
J Holt²
DP Corey³
K Shokat⁴
JA Mercer⁵

Harvard University
Wellman 414²
50 Blossom Street³
Massachusetts General Hospital
Boston
Massachusetts 02114
USA

University of California⁴
San Francisco
Cellular & Molecular Pharmacology
513 Parnassus
San Francisco
California 94143
USA

McLaughlin Research Institute⁵
1520 23rd Street South
Great Falls
MT 59405
USA

Selective inhibition of Y61G myosin I-beta by N6-modified nucleotide analogs

At least three myosin isozymes, VI, VIIA, and XV, are required for hair-cell function; a fourth, myosin I-beta, has been implicated as the adaptation motor. Because knock-outs or natural mutations in these myosin isozymes only reveal the first essential cellular function that the myosin participates in, we have developed a mutant/inhibitor strategy that allows us to test the role of a myosin isozyme rapidly and at any time point. We mutate myosin in a manner that maintains normal ATP hydrolytic and chemomechanical activity, yet allows specific binding of an inhibitor to the mutant myosin but not the wild-type myosin from which it was derived.

We have designed such a mutation in the ATP-binding pocket of *myosin I-beta*, Y61G, which sensitized the mutant protein to N6-modified ADP analogs, such as N6(2-methylbutyl) ADP, when expressed in the baculovirus system and assayed for ATPase activity in vitro. By contrast, the mutant protein retained nearly normal hydrolysis of ATP in the absence of N6-modified ADP analogs. Modeling the effects of Y61G on adaptation, we predict that a relatively small fraction of mutant myosin, when inhibited by N6(2-methylbutyl) ADP, will slow adaptation mediated by a myosin I-beta ensemble. This conclusion was supported by in vitro motility experiments, which showed that N6(2-methylbutyl) ADP arrests actin-filament movement on mixtures of wild-type and *Y61G myosin I-beta* that contained less than

30% of the mutant protein. We have therefore expressed *Y61G myosin I-beta* as a transgene in mice, using 6 kb of 5' genomic sequence to allow control of expression by the natural *myosin I-beta*. Although expression of the transgene is considerably lower than the wild-type myosin, we predict that N6 (2-methylbutyl) ADP will slow adaptation in transgenic mouse hair cells.

ROTONDO

IRCCS-Hospital "CSS"¹
 Medical Genetics Service
 San Giovanni
 Rotondo I-71013
 Italy

Hospital Duran I Reynals²
 Medical and Molecular Medicine Center
 Barcelona
 Spain

Department of Human Genetics and Molecular Medicine³,
 Sackler School of Medicine,
 Tel Aviv University,
 Tel Aviv,
 Israel

Phone: 972-3-640-7030
 Fax: 972-3-640-9900

P Gasparini¹
 X Estivill²
 N Ahituv³
 M Arbones²
 L Bisceglia¹
 S Melchionda¹
 A Notarangelo¹
 R Rabionet²
 T Sobe³
 L Zelante²
 KB Avraham³

A mutation in MYO6, the human homologue of the gene causing deafness in Snell's waltzer mice, is associated with hearing loss in an Italian family

Mutations in unconventional myosins have been identified in syndromic and nonsyndromic deafness in humans and mice. Unconventional myosins comprise a group of 14 nonfilament-forming myosins, defined by a conserved motor domain and different tail domains. An intragenic deletion in the unconventional myosin, *myosin VI*, was found to be the cause of deafness and abnormal vestibular function in Snell's waltzer mice. The human homologue *MYO6* maps to chromosome 6q13. A genome wide scan of an Italian family presenting with autosomal dominant progressive hearing loss localized the mutant locus to an 11 cM region on chromosome 6. The human *MYO6* gene maps within the candidate region and was thus an excellent candidate based on its chromosomal location, cochlear expression and function. Mutation analysis of the exons and exon-intron junctions revealed a missense mutation in the coding region of this gene. The mutation segregates with all affected individuals in the Italian family and was not identified in 200 control individuals, fulfilling the criteria for a disease-causing mutation. We constructed a model of human *myosin VI* (based on the known structure of *myosin II*), enabling us to predict the mechanism of this mutation in affecting the normal function of this protein.

SAN FRANCISCO

Department of Otolaryngology¹
Head and Surgery, A730,
400 Parnassus Avenue
San Francisco
CA 94143-0342
USA

AK Lalwani¹
JA Goldstein¹
MJ Kelly²
W Luxford³
AN Mhatre¹

Duke University Medical Center²
Internal Medicine
Hematology/Oncology (111G)
508 Fulton Street
Durham
NC 27705
USA

House Ear Clinic³
Otolaryngology
2100 West Third Street
Los Angeles
CA 90057
USA

Human nonsyndromic hereditary deafness DFNA17 is due to a mutation in nonmuscle myosin MYH9

We had previously reported mapping of a new locus for nonsyndromic hereditary hearing impairment DFNA17, to chromosome 22q12.2-q13.3, spanning a 17 to 23 cM region. *MYH9*, a non-muscle myosin heavy chain gene, is located within the linked region. Because of the importance of myosins in hearing, *MYH9* was tested as a candidate gene for DFNA17. Expression of *MYH9* in the rat cochlea was confirmed using RT-PCR and immunohistochemistry. *MYH9* was immunolocalised in the organ of Corti, the sub-central region of the spiral ligament and the Reissner's membrane. Sequence analysis of *MYH9* in the DFNA17 family identified a G to A transition at nucleotide 2114 that co-segregated with the inherited autosomal dominant hearing impairment. This missense mutation changes codon 705 from an invariant arginine (R) to histidine (H), R705H, within a highly conserved SH1 linker region. Previous studies have shown that modification of amino acid residues within SH1 helix causes dysfunction of the ATPase activity of the motor domain in *myosin II*. The precise role of *MYH9* in the cochlea and mechanism by which R705H mutation leads to the DFNA17 phenotype of progressive hearing impairment and cochleosaccular degeneration remains to be elucidated.

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SENDAI

Tohoku University School of Medicine¹
 Department of Otolaryngology
 1-1 Seiryomachi
 980-77 Sendai
 Japan

K Ikeda¹
 T Kikuchi²
 AP Xia³
 Y Katori³
 T Oshima⁴
 T Takasaka¹

Association for Research in Otolaryngology²
 1-1 Seiryomachi
 Aoba-ku
 980-857 Sendai
 Japan

Tohoku University School of Medicine³
 1-1 Seiryomachi
 Aoba-ku
 Sendai
 Japan

Tohoku University⁴
 1-1 Seiryomachi
 1-2 Aoba-ku
 Sendai 980-8574
 Japan

Progressive hearing loss in female heterozygotes of a mouse model of DFN3

Recently, we reported that homozygous males and females of a mouse model of DFN3 nonsyndromic deafness showed profound deafness due to severe alterations in cochlear spiral ligament fibrocytes from 11 weeks of age, whereas no hearing loss was recognized in female heterozygotes (Minowa et al., Science 285:1408-1411, 1999). In human, DFN3 is known to show progressive hearing loss. Moreover, a part of obligate female carriers showed moderate hearing loss (Cremers et al., Arch Otolaryngol 111:249-254, 1985). Histological analysis of the inner ear structure in *Brn-4* mutant males and homozygous mutant females at the age of 1 year revealed that abnormalities in Reissner's membrane attachment and type 2 fibrocytes in the suprastrial zone became evident under light microscope. These mutant animals were also found to have a weak expression in immunostaining of *connexin 26*, *connexin 31* and *Na, K-ATPase* in the spiral ligament fibrocytes, but not of Na-K-Cl cotransporter. Interestingly, one fourth of heterozygous female mice showed profound deafness after 1 year, which was accompanied by morphological abnormalities and low levels of immunostaining of the aforementioned proteins in the spiral ligament fibrocytes. Elucidation of the mechanism underlying this late onset phenotype in heterozygous mutants may explain the progressive hearing loss observed in human female heterozygotes.

TEL AVIV

Department of Human Genetics and Molecular
 Medicine¹,
 Sackler School of Medicine,

S Weiss¹
 SL Khare²
 M Xiang²

Tel Aviv University,
Tel Aviv,
Israel

SJ Dawson³
KB Avraham¹

Phone:972-3-640-7030
Fax: 972-3-640-9900

UMDNJ-Robert Wood Johnson Medical School²
Center for Advanced Biotechnology & Medicine
Department of Pediatrics
Piscataway
NJ USA

University College London Medical School³
Department of Molecular Pathology
London

Abnormal cellular expression of mutant POU4F3 associated with DFNA15 autosomal dominant nonsyndromic hearing loss

An 884del8 mutation in the *POU4F3* gene is responsible for DNFA15 (MIM 602459), autosomal dominant nonsyndromic hearing loss, in an Israeli family, Family H. Having identified the causative mutation, we are now trying to understand the exact mechanism by which the mutation causes this phenotype. We speculated that the progressive hearing loss is due to a dominant-negative effect. In vivo binding assays revealed hardly any binding of the protein product of the 884del8 mutation to a *Pou4f* consensus DNA binding site. Moreover, this mutant product does not appear to interfere with binding of *POU4F3* and *POU4F2* to the consensus site. We cloned the wild type and mutant *POU4F3* into expression vectors and transfected them into HEK293, cos-7, and cochlear cells. The 884del8 mutation presumably leads to a truncated protein product. To examine whether this protein is translated, we performed Western blot analysis. Both the wild type and truncated *POU4F3* were found to be stably translated. However, the truncated *POU4F3* protein was more abundant when compared to the wild type product. To examine cellular localization of mutant *POU4F3*, we performed *in situ* immunofluorescent localization. Dramatically, most of the truncated *POU4F3* protein is localized in the cytoplasm, while the wild type form is in the nucleus. In order to estimate the transcriptional activity of the mutant product and to check whether the mutation acts in a dominant-negative fashion, we used the luciferase reporter gene assay. The truncated transcription factor is less active than the wild type in regulating the SNAP25 promoter. Co-transfection of the wild type and mutant constructs demonstrates additive activity. Finally, the transfected *POU4F3* mutant cells become aberrant in their morphology over time as compared to the cells transfected with the wild type *POU4F3*. In summary, our results suggest that the *POU4F3* mutation in Family H is not acting in a dominant-negative fashion and that another unknown mechanism is involved in causing hearing loss in Family H.

WASHINGTON

R Gu¹

Association for Research in Otolaryngology
4010 Stone Way
North Suite 120
Seattle
Washington 98103
USA

YD Zhao
T Hasson
H Lowenheim
ML Fero

Otogene USA, Inc.,
4010 Stone Way
North Suite 120
Seattle
Washington 98013
USA

Biology Department 0368 9500
Gilman Drive
2129 Bonner Hall
San Diego
California 92093-0368
USA

Department of Otorhinolaryngology
University of Tübingen
Silcherstrasse 5
Tübingen 72076
Germany

Department of Basic Sciences
Fred Hutchinson Cancer Research Center
1124 Columbia Street
Seattle WA 98104
USA

Inhibition of p27Kip1 Induces Hair Cell Regeneration in the Organ of Corti

The development of the organ of Corti is highly dependent on *p27Kip1*, an inhibitor of cyclin/cyclin-dependent kinase complexes. Disruption of this gene allows the normally terminally mitotic supporting cells to proliferate in postnatal and adult life. Here we described the ongoing production of supporting cells, hair cells (HC) and HC regeneration in *p27Kip1* $-/-$ and $+/-$ mice. *p27Kip1* $-/-$, $+/-$, wt (P10-12) received systemic injections of bromodeoxyuridine (BrdU), which is incorporated into proliferating cells during S phase. Mice were recovered for two weeks. BrdU positive HCs were identified with antibodies against BrdU and HC specific proteins (myosin-VI and VIIa). In *p27Kip1* $-/-$, BrdU/myosin-VIIa positive HCs were observed in IHCs and OHCs. *p27Kip1* $+/-$ contained low levels of BrdU positive cells and no labeled HCs. Wt organ of Corti had no BrdU positive cells. The number of IHCs in *p27Kip1* $-/-$ was 20% greater than that in *p27Kip1* $+/-$ and wt. HCs were lesioned using systemic injections of amikacin (P7-P12) followed by BrdU (P10-P12). Mice were sacrificed either 2 d or 2 wks after the last injection. In *p27Kip1* $-/-$ and $+/-$, the number of BrdU positive cells increased following amikacin/BrdU vs BrdU. In

p27Kip1 ^{-/-}, the number of BrdU labeled cells increased, however not all cochlea displayed BrdU positive cells. A greater number of BrdU positive HCs were observed following amikacin lesioning in *p27Kip1* ^{-/-} and ^{+/-}. The majority of the BrdU positive HCs appeared in regions where the amikacin had injured or killed HCs (in the basal half). No BrdU positive cells were observed in wt after amikacin/BrdU or BrdU injections. Single cochlear extracts were serially diluted and run on polyacrylamide gels to determine p27 Kip1 and myosin-VI and VIIa protein levels. Western blotting showed that myosin-VI and VIIa levels appeared equal across *p27Kip1* ^{-/-}, ^{+/-} and wt. *p27Kip1* ^{+/-} contained about 50% of the *p27Kip1* found in wt, indicating a reduction to 50% of normal can allow supporting cell proliferation and some hair cell regeneration to occur.

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DEFEATING DEAFNESS

This issue of the Hereditary Deafness Newsletter is sponsored and distributed by Defeating Deafness (The Hearing Research Trust), the UK's medical research charity for deaf and hard of hearing people. The charity is committed to promoting research leading to the prevention and alleviation of hearing loss and associated conditions such as tinnitus.

It supports UK-based basic and clinical research, and grants are currently concentrated on, but not restricted to, a number of priority areas: basic research into the nature and causes of hearing loss; early detection of deafness; OME (glue ear); the genetics of deafness; improved performance of hearing aids; cochlear implants and tinnitus.

Grants are made in the following categories:-

- **Project Grants (£30,000 and over).** Normally for two to three years in support of one precisely formulated line of research.
- **Small Grants (less than £30,000).** So that Defeating Deafness can help researchers meet urgent requirements, or to conduct pilot studies.
- **Equipment Grants**
- **Postgraduate Studentships**
- **Fellowships**
- **Travel Grants.** To enable Defeating Deafness grant-holders and others to attend conferences or training courses, in the UK or abroad, within our priority areas.
- **Grants for Infrastructure.** Occasionally, grants may be made to provide facilities (equipment, accommodation, and/or support staff for a research group rather than for a named project).
- **Meetings.** To promote discussion in areas where it is felt important to stimulate research, review future directions or improve communication between researchers.

Full details and application forms are available from:-

Vivienne Michael
Defeating Deafness
330/332 Gray's Inn Road
London
WC1X 8EE

Tel: +44 171 833 1733
Fax: +44 171 278 0404