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**Positive selection of hearing loss candidate genes,
based on multiple microarray platforms experiments
and data mining**

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Positive selection of hearing loss candidate genes, based on multiple microarray platforms experiments and data mining

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1. Abstract

According to WHO estimates hearing impairment affects 278 million people worldwide. Approximately 1/1000 children are born with a significant hearing impairment. To date approximately 100 genetic loci involved in deafness have been described. Despite the fact that such a large number of genetic locations associated with deafness phenotypes are known, not all the genes involved have been identified yet. Using a traditional linkage approach, however, it is not always possible to map a locus to intervals short enough to be amenable for costly mutation analysis. So far no more than 40 deafness genes have been identified and these encode very heterogeneous proteins. The work presented in this thesis aims to identify a limited set of candidate genes with high potential to be involved in Non-Syndromic Hearing Loss using a combination of biological and bioinformatics approaches. The starting point of the analysis was the GJB2 gene. The GJB2 gene encodes for the gap junction protein Connexin26 and is responsible for more than half of the non-syndromic hearing loss cases. For this reason it has been proposed that this protein might play a wider role in the biology of the ear, beyond its mere channel function. I therefore performed whole genome expression profiles of HeLa cells transfected with the wild type form of the GJB2 gene and compared them to that of cells transfected with mutant forms of this gene to shed light on its function. Initially this experiment yielded a bewildering number of differentially expressed genes (4,984). Thus I devised an in silico strategy to narrow down this number, focusing on genes which were positionally linked to specific non-syndromic hereditary hearing loss conditions, as well as found within human ear cDNA libraries, thus potentially causative of the disease. This further analysis yielded 19 genes within 11 loci. In order to assess their relevance to hearing loss, the mouse homologs of these genes were identified for 5 of them and indeed they were all found to be expressed in the mouse organ of corti. These five genes were also validated by Real-time RT-PCR in the human cell line used for the microarray experiments. Segregation of mutations within the candidate genes identified will be the natural evolution of my PhD project.

2. Introduction

2.1 Anatomy and Physiology of the Ear.

Hearing is a complex process that consists of many steps. It begins when sound waves strike the outside of the ear. As we move through the environment we are continually receiving auditory information. Whether it is our own breathing, the pounding of waves, the playing of instruments, or the conversations whirling around us, the auditory system constantly detects the small, rapid fluctuations in air pressure that we call sound.

Fluctuations in air pressure are funneled by the outer ear, or pinna, into the ear canal, also called the external auditory meatus. At the end of the ear canal, the sound causes movement of the eardrum, which is connected to three small bones called ossicles: the *malleus*, the *incus*, and the *stapes*. As the eardrum vibrates the ossicles amplify the sound and send the sound waves to the inner ear and into the fluid-filled hearing organ (*cochlea*) through the oval window (Fig1).

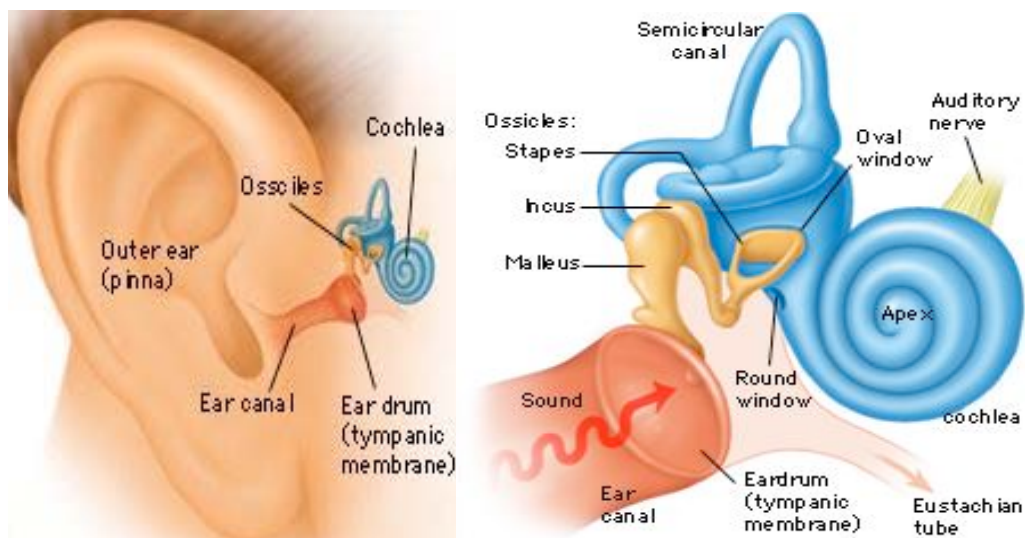
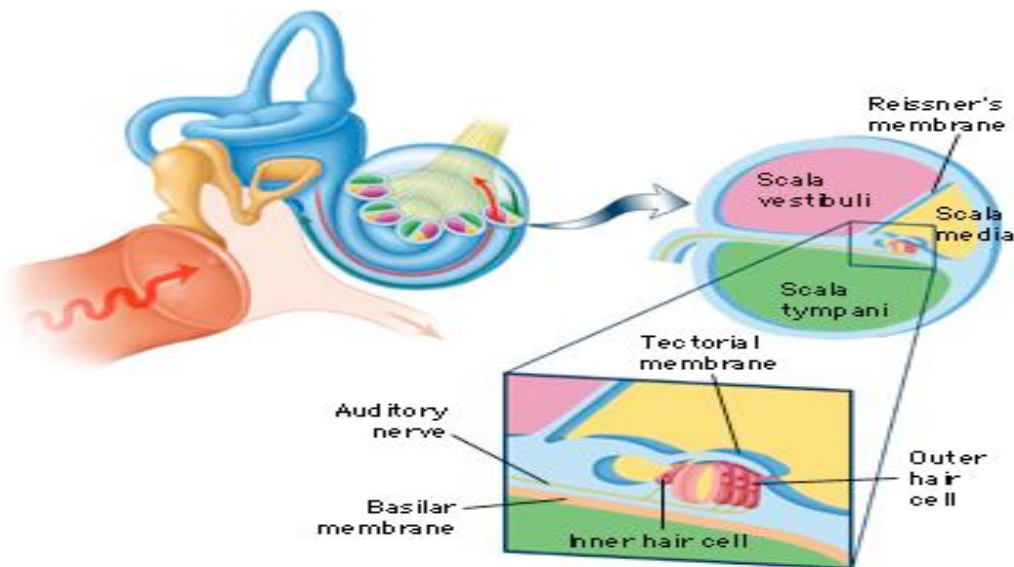


FIGURE (1). ANATOMICAL ORGANIZATION OF THE EAR. SCHEMATIC REPRESENTATION OF OUTER, MIDDLE AND INNER EAR . REPRESENTATION OF THE CONNECTION BETWEEN MIDDLE AND INNER EAR.

The *cochlea* is a bony spiral that makes roughly 2.5 revolutions in human. The number of cochlear turns in mammals varies from two in the mouse to four and a half in guinea pigs (Tian et al., 2006). The cochlea is divided along its length into three fluid-filled compartments: the *scala vestibuli*, the *scala media*, and the *scala tympani*. The *reissner's membrane* divides the *scala vestibuli* from the *scala media*. The *basilar membrane* divides the *scala media* from the *scala tympani*. The *scala tympani* and the *scala vestibuli* compartments are continuous at the *apex* (or tip of the spiral) through the *helicotrema*. The oval window is at the base of the *cochlea* in the *scala vestibuli*. The round window is at the base of the *cochlea* in the *scala tympani* (Fig.2).

Since fluids are basically incompressible, in order for the stapes to push the oval window in, and move the fluid inside the *cochlea*, the round window must bulge out. The *cochlea* spiral ducts are filled with fluids differing in their ionic contents: *perilymph* in the tympanic and vestibular ducts, *endolymph* in the cochlear duct. The endolymph composition, with a high potassium and a low sodium concentration, resembles the cytosol. In contrast, the perilymph composition is closer to the extracellular medium, with a low potassium but a high sodium content (Gan et al., 2007). The differing ionic composition results in a roughly 80 mV difference in potential between the endolymph and the perilymph.



FIGURE(2).INNER ORGANIZATION OF THE COCHLEA. REPRESENTATION OF HYPOTHETIC INNER EAR SECTION.

The endolymphatic potential (around 80 mV) depends on an active secretion of K^+ , which involves fibroblasts, other support cells as well as the *Stria vascularis* (i.e. the upper portion of the spiral ligament containing numerous capillary loops and small blood vessels). As the stapes move back and forth, responding to fluctuations in air pressure, the liquid inside the cochlea is also moving back and forth. The sensitive element in the inner ear is the *Organ of Corti* situated on the *basilar membrane*. It contains four rows of hair cells, which protrude from its surface. Above them there is the *tectorial membrane*, which can move in response to pressure variations in the fluid-filled tympanic and vestibular canals. (Fig.3).

Hair cells extend their cilia into the liquid in the scala media. The electric potential within the organ of Corti is thought to be generated by stereocilia displacement (Flock and Duvall, 1965; Hudspeth and Corey, 1977). Any movement of the stapes causes movement of the basilar membrane and the cilia, causing the hair cells to depolarize, initiating an action potential that moves up the auditory pathway via individual fibers of the auditory nerve. In vitro measurements from hemicochlea preparations show that at low stimulus frequencies the opening of

mechanically sensitive ion channels occurs when the organ of Corti is displaced towards the scala vestibuli (He et al., 2004). During sound-evoked vibration of the Organ of Corti, the gating of mechanically sensitive ion channels on the sensory cells produce rapidly alternating electric potentials. One type of sensory cell, the outer hair cell, responds to changes in membrane potential with alterations of cellular length and stiffness (Brownell et al., 1985; He and Dallos, 1999). Length and stiffness changes, in turn, depend on a specialized motor protein named Prestin (Zheng et al., 2000).

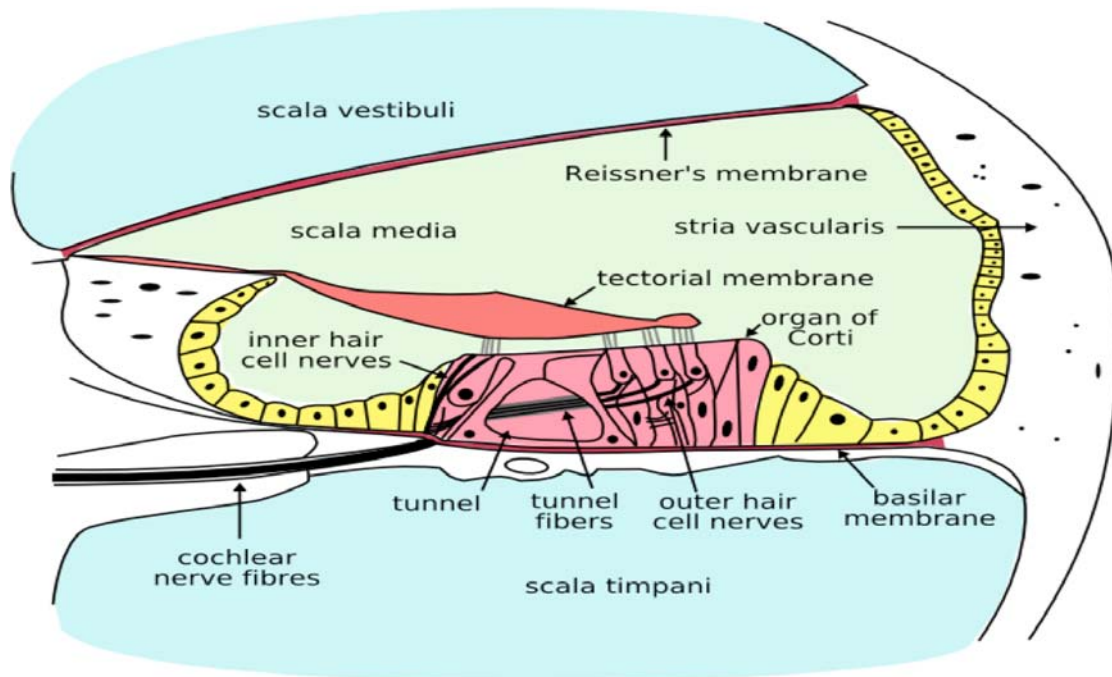


FIGURE (3). ORGAN OF CORTI ORGANIZATION.

The hair cells and the basilar membrane develop in such a way that high frequencies most strongly stimulate the hair cells closest to the oval window, while low frequencies most strongly stimulate the hair cells furthest from the oval window. To accomplish this, the basilar membrane is narrow and tight near the base, and wide and loose near the apex. In addition, hair cells are slightly longer at the apex than at the base. Thus, complex sounds are decomposed into the single frequencies of which they are made; auditory nerve fibers at one end of the cochlea, therefore, carry information about high frequencies while those at the other end carry information about low frequencies. These as well as other auditory nerve fibers synapse with neurons in the ipsilateral cochlear nucleus (Fuchs, 2005). From the cochlear nucleus, the pathway goes through the trapezoidal body and crosses to the contralateral side to synapse within the superior olivary nuclei. The superior olivary nuclei, including the lateral superior olivary nucleus and medial superior olivary nucleus, are the first nuclei to receive substantial input from both the ipsi and the contralateral sides (Batra and Yin, 2004)(Fig.4) .

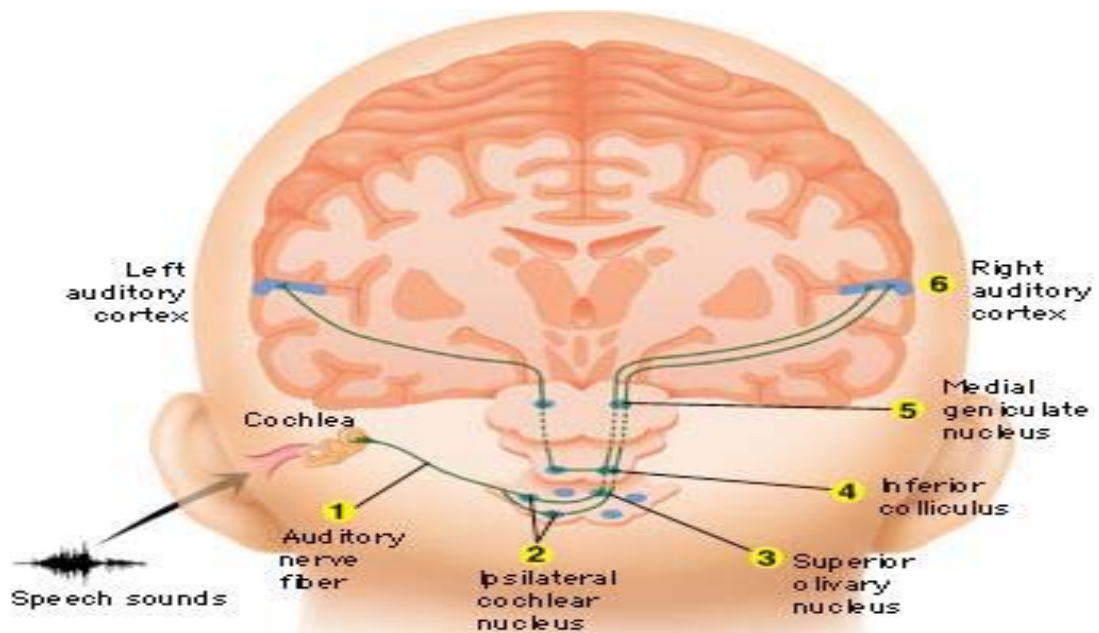


FIGURE (4). THE SOUND PATHWAY FROM EAR TO CORTEX.

Continuing up the auditory pathway, some fibers are connected to the *inferior colliculus* while others synapse at the lateral *lemniscal nuclei* before crossing to the other side and continuing up to the inferior colliculus. From the inferior colliculus, the pathway either crosses to the contralateral inferior colliculus or continues on to the medial geniculate body (on the ventral posterior portion of the thalamus). From the medial geniculate body, signals continue up the auditory pathway to the auditory cortex. In addition to the ascending pathway (from the cochlea to the cortex), there is a descending pathway (from the cortex to the cochlea). Many of these descending fibers end up synapsing to the outer hair cells as well as to afferent fibers from the inner hair cells (Tolomeo et al., 1996). Little is known about this pathway other than that it aids in the detection of sounds in a noisy background (Mulders and Robertson, 2005).

2.2 Hearing loss

A hearing impairment or hearing loss is a full or partial decrease in the ability to detect or understand sounds from one or both ears. Thus, deafness may be viewed as a condition that prevents an individual from receiving sound in all or most of its forms (NICHCY, January 2004). Hearing loss is caused by a wide range of biological and environmental factors: it may be inherited, caused by complications at birth, or by certain infectious diseases (such as meningitis), use of ototoxic drugs, and exposure to excessive noise. According to 2005 estimates by the World Health Organization (W.H.O., 2007), 278 million people worldwide have moderate to profound hearing loss in both ears and the number of people worldwide with all levels of hearing impairment is rising mainly due to a growing global population and longer life expectancies. According to the same estimate, more than 50% of deafness and hearing impairments are avoidable through prevention, early diagnosis, and disease management. Overall estimates suggest that approximately 1-6 children per 1,000 newborns have congenital hearing loss (Adams et al., 1999; Cunningham et al., 2005; Kemper and Downs, 2000). From a clinical point of view hearing loss is further categorized on the basis of three main attributes: type of hearing loss, degree of hearing loss, and the age of onset.

2.2.1 Types of Hearing Loss

Hearing loss is classified into three main types according to which part of the auditory system is damaged: *conductive hearing loss*, *sensorineural hearing loss* and *mixed hearing loss*. Hearing loss that occurs prior to the oval window is named "*conductive hearing loss*", owing to complications in the conductance of the sound through the outer ear canal to the eardrum and the tiny bones (*ossicles*) of the middle ear. Doctors can test for conductive losses using the "bone conduction" test. When a vibrating object, such as a tuning fork, is touched to the head, vibrations conduct directly to the cochlea through the bone. A person with only a conductive hearing loss will be able detect these sounds as well as someone with normal hearing. A hearing problem within the cochlea is called a "*sensorineural loss*". A *sensorineural loss* occurs when the mechanical energy is correctly conducted to the oval window of the cochlea, but it is not appropriately converted to a signal to be carried by the nerves. It can occur when there is damage to the inner ear (cochlea) or to the nerve pathways from the inner ear (retrocochlear) to the brain, and can be also related to genetic causes. The great majority of human sensorineural hearing loss is caused by abnormalities in the hair cells of the organ of Corti in the cochlea. There are also very unusual sensorineural hearing impairments that involve the VIIIth cranial nerve (the

Vestibulocochlear nerve) or the auditory portions of the brain. In the rarest of these sorts of hearing losses, only the auditory centers of the brain are affected. In this situation, named central hearing loss, sounds may be heard at normal thresholds, but the quality of the sound perceived is so poor that speech can not be understood. Sensorineural hearing loss cannot be medically or surgically corrected, thus it is a permanent loss. Sensorineural hearing loss not only involves a reduction in sound level, or ability to hear faint sounds, but also affects speech understanding, or ability to hear clearly. Sometimes a conductive hearing loss occurs in combination with a sensorineural hearing loss. In other words, there may be damage in the outer or middle ear and in the inner ear or auditory nerve. When this occurs, the hearing loss is referred to as a *mixed hearing loss*.

2.2.2 Degree of hearing loss

The sound is measured by its loudness or intensity (measured in decibels, dB) and its frequency or pitch (measured in hertz, Hz). Impairments in hearing can occur in either or both areas, and may exist in only one ear or in both ears. Hearing loss is generally described as mild, moderate, severe, or profound, depending upon how well a person can hear the intensities or frequencies most greatly associated with speech. It is quite common for someone to have more than one degree of hearing loss (i.e. mild sloping to severe) at different frequency. For this reason is better to refers to a configuration or shape into a plot of Frequency respect Intensity called Audiogram to obtain the overall picture of hearing.

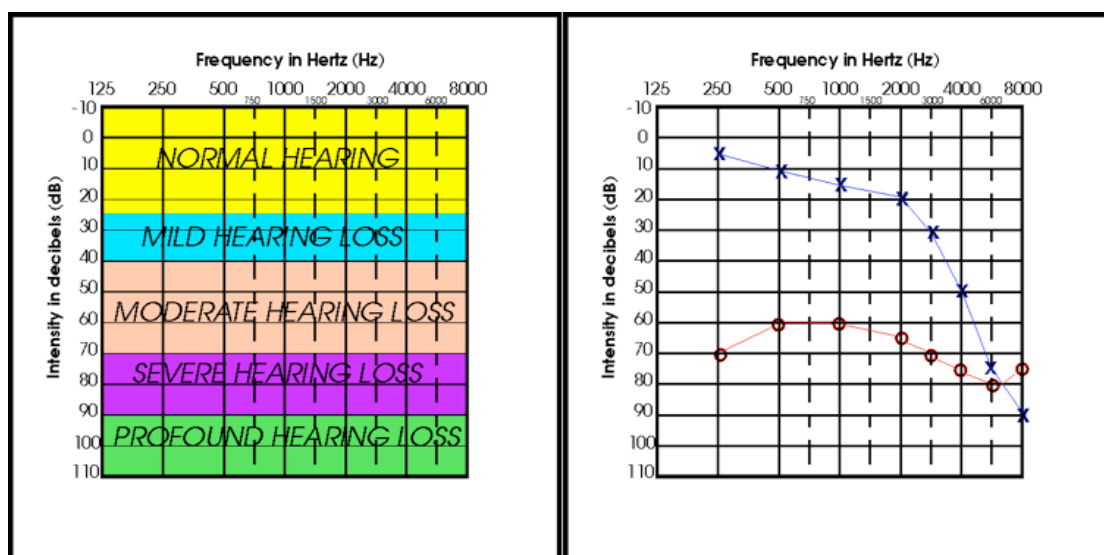


FIGURE (5).AUDIOGRAM STRUCTURE. AUDIOGRAM OF DIFFERENT DEGREES OF HEARING LOSS AND AN EXAMPLE OF SEVERE HIGH FREQUENCY HEARING LOSS IN THE LEFT EAR (BLUE LINE) AND A MODERATE TO SEVERE HEARING LOSS IN THE RIGHT EAR (RED LINE).

For example, a hearing loss that only affects the high frequencies would be described as a high-frequency loss. Its configuration would show good hearing in the low frequencies and poor

hearing in the high frequencies. Some hearing loss configurations are flat, indicating the same amount of hearing loss for low and high tones (Fig5). Some people may merely find it difficult to differentiate between words that begin with consonantal sounds such as the fricatives or sibilants, z, or th, or the plosives d, t, b, or p. They may be unable to hear thin, high-pitched or metallic noises, such as birds chirping or singing, clocks ticking, etc. Often, they are able to hear and understand men's voices better than women's.

2.2.3 Age of onset

Two persons with the same severity of hearing loss will experience it quite differently if it occurs early or late in life. The age at which the hearing impairment develops is crucial to spoken language acquisition. Post-lingual hearing impairments are far more common than pre-lingual impairments. Prelingual hearing impairment exists when the impairment is congenital or otherwise acquired before the individual has acquired speech and language, thus rendering the disadvantages more difficult to treat because the child is unable to access audible /spoken communication from the outset. It is important to note that those children born into signing families have no delay in language development and communication. Most pre-lingual hearing impairment is due to an acquired condition, usually either disease or trauma; therefore, families commonly have no prior knowledge of deafness. Post-lingual hearing impairment where hearing loss is adventitious after the acquisition of speech and language, It may develop due to disease, trauma, or as a side-effect of a medicine. Typically, hearing loss is gradual, and often detected by family and friends of the people so affected long before the patients themselves will acknowledge the disability. If the hearing loss occurs at a young age, interference with the acquisition of spoken language and social skills may occur. Hearing aids, which amplify the incoming sound, may alleviate some of the problems caused by hearing impairment, but are often insufficient. Cochlear implants artificially stimulate the VIIIth Nerve by providing an electric impulse substitution for the firing of hair cells. People who have hearing impairments, especially those who develop a hearing problem in childhood or old age, require support and technical adaptations as part of the rehabilitation process.

2.3 Genetic of Deafness

The study of the genetic bases of deafness is unique among inherited disorders for several reasons and is important because it explain clearly various concepts in human genetics. Notably, there is incomparable genetic heterogeneity with over 90% of mating among the deaf, resulting in all hearing offspring. This reflects mating among the deaf with mutations in different genes as well as mating of couples in which one individual is deaf due to a genetic mechanism and the other due to an environmental etiology. Mating among the deaf are well recognized and, with the exception of assortative mating for stature, may represent one of the most common genetic traits on which an altered mating structure occurs in human populations. Furthermore, the hearing offspring of deaf couples who themselves can be native signers are more likely than random members of the population to have a partner who is deaf due to a shared language and culture. Both genetic heterogeneity and assortative mating confound gene discovery using traditional methods of genetic linkage analysis. Hundreds of syndromic forms of deafness have been described (Guy Van Camp, 2007) and the underlying genetic mutation identified for many of the more common forms, but despite this only 30% of genetic cases are estimated to be part of a heritable syndrome. Thus, the vast majority of genetic deafness is designated as non-syndromic deafness (Morton, 2002). Progress in identifying genes involved in deafness has been remarkable over the past few years. At the end of 1996, no non-syndromic deafness genes had been cloned. In the 11 years since then, 37 new genes involved in non-syndromic deafness have been identified (Fig6), together with an even larger number of genes implicated in syndromic deafness. Whereas single-gene defects probably account for over half of the cases of childhood deafness, the nature of the genetic contribution to progressive hearing loss has not yet been clearly defined. (Petit, 1996). Traditional methods for mapping disease genes, such as genetic linkage analysis, have a less than totally optimal use in gene discovery efforts for hearing disorders, mainly because of the complex genetic nature of deafness. Successful use of genetic linkage for mapping hearing disorders, especially for autosomal recessive non-syndromic loci, has been restricted largely to consanguineous kindreds or populations in which there has been limited immigration. Even in families in which a heritable hearing disorder is successfully mapped, there are often an insufficient number of recombination events to narrow a chromosomal interval, resulting in a candidate region consisting of megabases of genomic DNA. Positional cloning has been productive for a modest number of human deafness genes including NDP (Berger et al., 1992; Chen et al., 1992), TCOF1(Dixon, 1996), DDP (Jin et al., 1996), SLC26A4 (Everett et al.,

1997), USH2A (Eudy et al., 1998) and DFNA5 (Van Laer et al., 1998). Positional candidate genes from human (e.g. COL4A5 (Barker et al., 1990),TECTA (Verhoeven et al., 1998), COCH (Robertson et al., 1998), COL4A and 4A4 (Mochizuki et al., 1994), GJB2 (Denoyelle et al., 1998), GJB3 (Xia et al., 1998)) and mouse (e.g. PAX3 (Tassabehji et al., 1992), MITF (Tassabehji et al., 1994), OTOF (Yasunaga et al., 1999), USH1C (Verpy et al., 2000), STRC (Verpy et al., 2001)) among others have been the primary method for gene identification. So far, 120 loci involved in non-syndromic deafness have been reported, and over 52 distinct syndromes including hearing impairment are listed in Online Mendelian Inheritance in Man.

Gene_Name	description	Homologus
MYH14	Myosin-14 (Myosin heavy chain, nonmuscle IIc) (Nonmuscle myosin heavy chain IIc) (NMHC II-C). [Source:Uniprot/SWISSPROT;Acc:Q7Z406]	<NULL>
WFS1	Wolframin. [Source:Uniprot/SWISSPROT;Acc:O76024]	ortholog_one2one
ACTG1	Actin, cytoplasmic 2 (Gamma-actin). [Source:Uniprot/SWISSPROT;Acc:P63261]	<NULL>
TFCP2L3	Grainyhead-like protein 2 homolog (Brother of mammalian grainyhead) (Transcription factor CP2-like 3). [Source:Uniprot/SWISSPROT;Acc:Q6IS83]	<NULL>
TMC1	Transmembrane cochlear-expressed protein 1. [Source:Uniprot/SWISSPROT;Acc:Q8TDI8]	<NULL>
MYO1A	Myosin Ia (Brush border myosin I) (BBM-I) (BBMI) (Myosin I heavy chain) (MIHC). [Source:Uniprot/SWISSPROT;Acc:Q9UBC5]	<NULL>
GJB2	Gap junction beta-2 protein (Connexin-26) (Cx26). [Source:Uniprot/SWISSPROT;Acc:P29033]	<NULL>
MYO7A	Myosin-7A (Myosin VIIa). [Source:Uniprot/SWISSPROT;Acc:Q13402]	<NULL>
MYO15A	Myosin-15 (Myosin XV) (Unconventional myosin-15). [Source:Uniprot/SWISSPROT;Acc:Q9UKN7]	ortholog_one2one
TMIE	Transmembrane inner ear expressed protein precursor. [Source:Uniprot/SWISSPROT;Acc:Q8NEW7]	apparent_ortholog_one2one
TMPRSS3	Transmembrane protease, serine 3 (EC 3.4.21.-) (Serine protease TADG- 12) (Tumor-associated differentially-expressed gene 12 protein). [...]	<NULL>
OTOF	Otoferlin (Fer-1-like protein 2). [Source:Uniprot/SWISSPROT;Acc:Q9HC10]	<NULL>
CDH23	Cadherin-23 precursor (Otocadherin). [Source:Uniprot/SWISSPROT;Acc:Q9H251]	ortholog_one2one
STRC	Stereocilin precursor. [Source:Uniprot/SWISSPROT;Acc:Q7RTU9]	<NULL>
USH1C	Harmonin (Usher syndrome type-1C protein) (Autoimmune enteropathy- related antigen AIE-75) (Antigen NY-CO-38/NY-CO-37) (PDZ-73 p...	<NULL>
TECTA	Alpha-tectorin precursor. [Source:Uniprot/SWISSPROT;Acc:O75443]	ortholog_one2one
OTOA	Otoancorin precursor. [Source:Uniprot/SWISSPROT;Acc:Q7RTW8]	<NULL>
PCDH15	Protocadherin-15 precursor. [Source:Uniprot/SWISSPROT;Acc:Q96QU1]	apparent_ortholog_one2one
TRIOBP	TRIO and F-actin-binding protein (Protein Tara) (Trio-associated repeat on actin). [Source:Uniprot/SWISSPROT;Acc:Q9H2D6]	apparent_ortholog_one2one
CLDN14	Claudin-14. [Source:Uniprot/SWISSPROT;Acc:O95500]	<NULL>
MYO3A	Myosin IIIA (EC 2.7.11.1). [Source:Uniprot/SWISSPROT;Acc:Q8NEV4]	<NULL>
WHRN_Human	Whirlin (Autosomal recessive deafness type 31 protein). [Source:Uniprot/SWISSPROT;Acc:Q9P202]	<NULL>
ESPN	espin [Source:RefSeq_peptide;Acc:NP_113663]	<NULL>
MYO6	Myosin-6 (Myosin VI). [Source:Uniprot/SWISSPROT;Acc:Q9UM54]	ortholog_one2one
COL11A2	Collagen alpha-2(XI) chain precursor. [Source:Uniprot/SWISSPROT;Acc:P13942]	<NULL>
PJVK_HUMAN	Pejvakin (Autosomal recessive deafness type 59 protein). [Source:Uniprot/SWISSPROT;Acc:Q0ZLH3]	<NULL>
LHFPL5	lipoma HMGIC fusion partner-like 5 [Source:RefSeq_peptide;Acc:NP_872354]	<NULL>
DIAPH1	Protein diaphanous homolog 1 (Diaphanous-related formin-1) (DRF1). [Source:Uniprot/SWISSPROT;Acc:O60610]	<NULL>
GJB3	Gap junction beta-3 protein (Connexin-31) (Cx31). [Source:Uniprot/SWISSPROT;Acc:O75712]	<NULL>
KCNQ4	Potassium voltage-gated channel subfamily KQT member 4 (Voltage-gated potassium channel subunit Kv7.4) (Potassium channel subunit al...	<NULL>
GJB6	Gap junction beta-6 protein (Connexin-30) (Cx30). [Source:Uniprot/SWISSPROT;Acc:O95452]	<NULL>
COCH	Cochlin precursor (COCH-5B2). [Source:Uniprot/SWISSPROT;Acc:O43405]	<NULL>
EYA4	Eyes absent homolog 4 (EC 3.1.3.48). [Source:Uniprot/SWISSPROT;Acc:O95677]	<NULL>
POU4F3	POU domain, class 4, transcription factor 3 (Brain-specific homeobox/POU domain protein 3C) (Brn-3C). [Source:Uniprot/SWISSPROT;Acc:Q15319]	<NULL>
MYH9	Myosin-9 (Myosin heavy chain, nonmuscle IIa) (Nonmuscle myosin heavy chain IIa) (NMMHC II-a) (NMMHC-IIA) (Cellular myosin heavy chain, ...	<NULL>
SLC26A4	Pendrin (Sodium-independent chloride/iodide transporter) (Solute carrier familv 26 member 4). [Source:Uniprot/SWISSPROT;Acc:O43511]	<NULL>

FIGURE (6).TABLE OF GENES INVOLVED IN NON-SYNDROMIC DEAFNESS

2.3.1 The Gap Junction Proteins into Deafness.

Gap junctions mediate one method of intercellular communication by allowing for the passage of ions, second messengers, metabolites, and other small molecules. By enabling direct communication between cells, gap junctions are thought to be important for cell proliferation, differentiation, and maintenance of tissue homeostasis (Loewenstein, 1981). Gap junction plaques are composed of a few to hundreds of gap junction channels clustered together. Each individual channel is made up of two hemichannels; one hemichannel (or connexon) is contributed from each of two adjoining cells (Goodenough et al., 1996). In turn, each connexon consists of six oligomerized polypeptides called connexins (Bruzzone et al., 1996a). Homomeric connexons contain six identical connexins, and heteromeric connexons are composed of two or more types of connexins (Fig7). The discovery of genetic deafness diseases associated with mutations in 3 distinct connexin genes (Gasparini et al., 1997; Grifa et al., 1999; Kelsell et al., 1997; Richard et al., 2000) has validated the view that gap junction-dependent intercellular signaling fulfils a crucial role in coordinating several aspects of tissue homeostasis included the sound trasduction. It is well established that the Connexin family of genes encode the polypeptide subunits that make up gap junctions, and genomic study highlighted the common features of the 19 mouse and 20 human Cx genes (Willecke et al., 2002). Most tissues express more than one member of the Cx family (Goldberg et al., 1999). This is particularly important as connexons composed of different members of the Cx family exhibit selective permeability to endogenous metabolites (Bevans et al., 1998; Elfgang et al., 1995; Nicholson et al., 2000; Niessen et al., 2000). Altering the subset of Cxs spatially and temporally expressed may allow cells within a tissue to respond differentially to cell signals, leading to specific changes in differentiation.

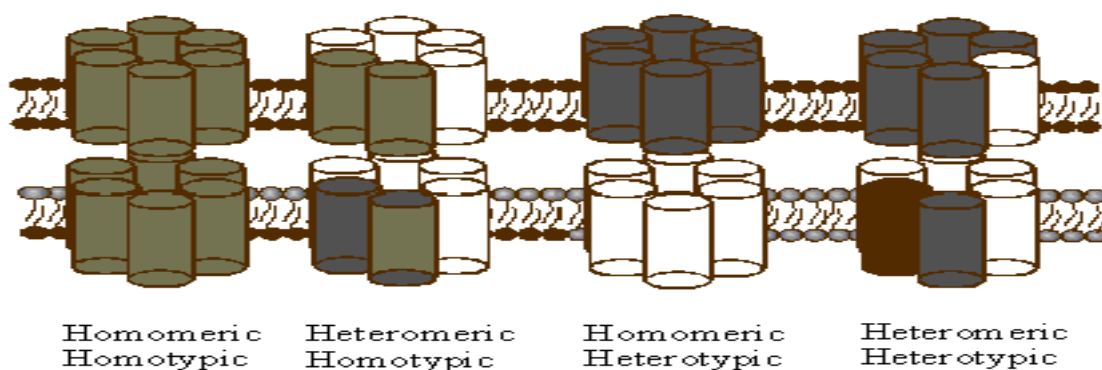


FIGURE (7). POSSIBLE ARRANGEMENTS OF CONNEXONS TO FORM GAP JUNCTION CHANNELS.

2.3.1.1 Locus DFNB1 and DFNA3 Connexin-26

Connexin-26 (Cx26) is a 26-kDa gap junction protein originally purified from the liver (Zhang and Nicholson, 1989). Cx26 is widely distributed in the body and has been shown to mediate the metabolic and electrical couplings in many tissues (Bruzzone et al., 1996b; Kumar and Gilula, 1996). Even if Cx26 is not expressed in hair cells, Cx26 expression in the inner ear is believed to play a major role in the formation of gap junction system. Indeed Cx26 protein is found in non-sensory epithelial cells surrounding the hair cells, which include supporting cells of the organ of Corti, inner sulcus cells, outer sulcus cells, and interdental cells of the spiral limbus. Cx26 is also expressed in connective tissue cells of the cochlea including strial basal cells, strial intermediate cells, mesenchymal cells lining the scala vestibuli, and fibrocytes in the spiral limbus and spiral ligament (Fig8). The Cx26 gap junction system is believed to serve as the structural basis for recycling endolymphatic K⁺ ions . Upon sound stimulation, inner hair cells of the cochlea release K⁺ ions and neurotransmitter glutamate into the perilymph. K⁺ ions are recirculated back to endolymph through the Cx26 gap junction system. Extracellular glutamate is removed by the glutamate transporter GLAST expressed in supporting cells surrounding inner hair cells (Hakuba et al. 2000). It is hypothesized that when extracellular K⁺ concentration level is high, resulting from the absence of functional Cx26 gap junctions, GLAST glutamate transporter could function in a reverse mode, leading to extracellular accumulation of glutamate and the subsequent apoptosis of cochlear epithelial cells and outer hair cells (Teubner et al., 2003). Therefore, Cx26 mutations that cause a functional loss of cochlear gap junctions could impair the auditory transduction. The physiological importance of Cx26 gap junctions in normal auditory function, is further supported by the finding that mutations in the coding region of Cx26 gene are linked to autosomal recessive and dominant non-syndromic deafness (Denoyelle et al., 1998; Rabionet et al., 2000). Among the deafness loci of recessive non-syndromic deafness, it has been reported that as many as 50% of families affected with a recessive hearing impairment are linked to DFNB1 (Estivill et al., 1998; Scott et al., 1998). Direct DNA sequencing studies demonstrated that Cx26 mutations are associated with the autosomal recessive DFNB1 form of hearing loss (Carrasquillo et al., 1997; Kelsell et al., 1997) (Zelante et al., 1997). In addition to the most common deletion mutations 35delG , point mutations within the coding region of Cx26 gene are also found in families affected with recessive DFNB1 sensorineural deafness (Estivill, 2008).

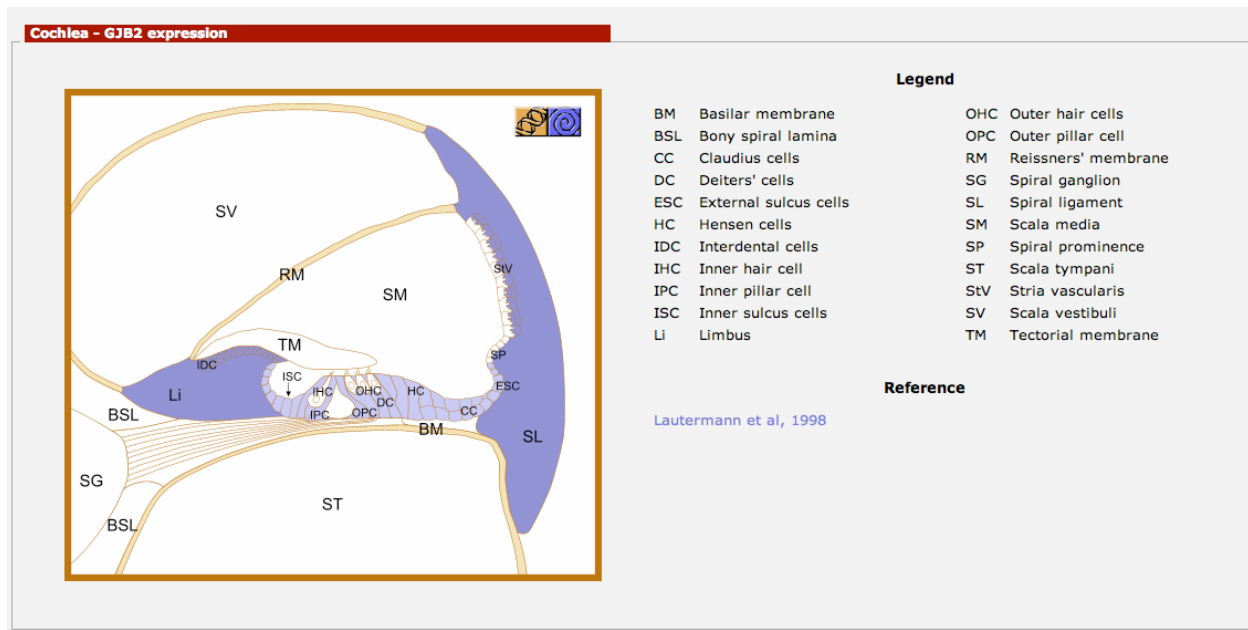


FIGURE (8). Cx26 EXPRESSION IN THE INNER EAR.

2.3.1.2 DFNA2 Connexin-31 and DFNA3 Connexin-30

Connexin-30 (Cx30) and Connexin-31 (Cx31) are the other two protein correlated with non-syndromic deafness. Human connexin 26 and connexin 30 are expressed in the same cells (Fig9) of the cochlea and share 77% identity in amino acid (Kelley et al., 1999), leading to the potential formation of heteromeric hemichannels and heterotypic gap junction channels as demonstrated from invitro assays (Yum et al., 2007) and in vivo co-localize to the same gap junction plaques. The Connexin-30 gene (GJB6) mutations has been reported to cause deafness in the homozygous state or in combination with heterozygous mutations in Cx26 (Frei et al., 2004; Lerer et al., 2001). Most families with this form of hearing loss are missing a large section of the DNA that includes one copy of their Cx30 gene. Usually, these families actually have a single mutation in Cx26 as well as the deletion of one of their copies of Cx30. However, some families have also been found with two copies of the Cx30 deletion and no Cx26 mutations (Grifa et al., 1999).

Connexin-31 gene (Cx31), has also been reported to cause deafness. Mutations in Cx31 can lead to both recessive and dominant forms of non-syndromic deafness (Liu et al., 2000; Lopez-Bigas et al., 2001) but no digenic mutation between Cx26 gene and Cx31 gene has been reported so far (Gao et al., 2004) even if Cx31 appears to form functional heterotypic channels with Cx26 and Cx30 in invitro assay (Abrams et al., 2006). Experimental evidence underline a potential distinct disease mechanism of Cx31 mutation regarding the involvement of wild-type and mutant Cx31 in neurite outgrowth (Unsworth et al., 2007).

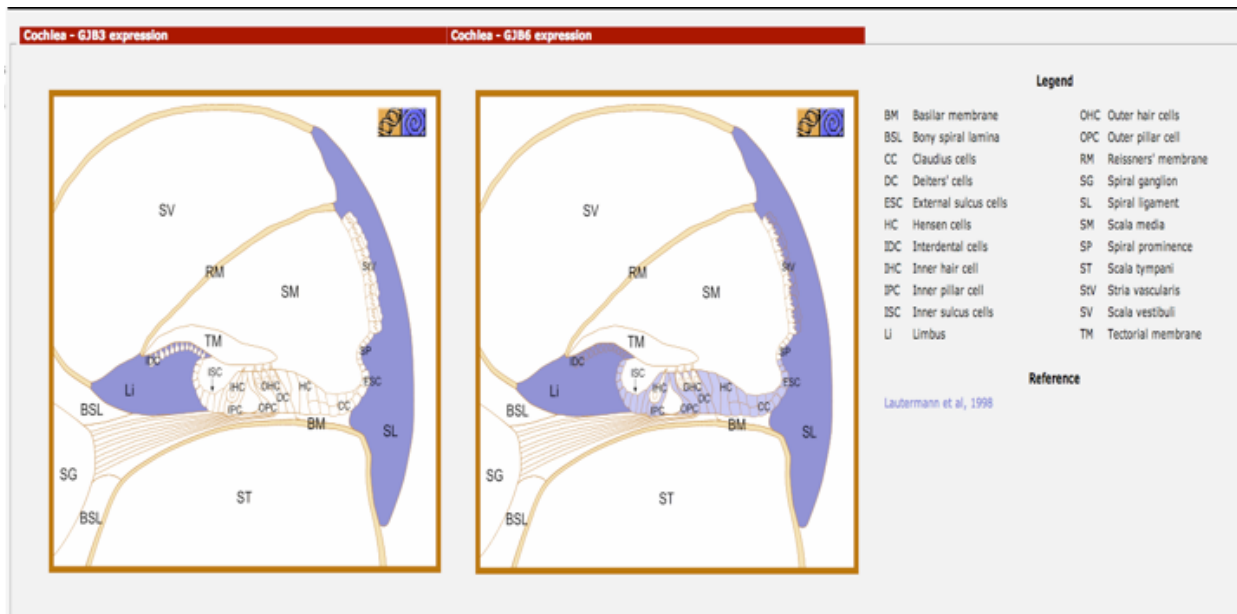


FIGURE (9). Cx30 AND Cx31 EXPRESSION IN THE INNER EAR.

2.3.1.3 Molecular Screening for Hearing Loss.

Thanks to the identified genes many molecular screening are now available as standard tests into genetics laboratories. Molecular screening tests require a small sample of child's or parents blood to extract the DNA, which is used for sequencing one or more of the following conditions: Connexin 26, Connexin 30, Pendred syndrome. Screening results typically are reported within 48 hours of specimen receipt. Screening for these specific causes of deafness provide an affordable method to detect the most common causes of congenital and delayed-onset pre-lingual deafness. Families in which the probable cause for the hearing loss is identified should seek professional advice from primary care physicians, geneticists, otolaryngologists, or experts in infectious disease, if appropriate, for a full evaluation and counseling about the significance of test results.

2.3.2 Mouse deafness models.

Without an animal model for hereditary deafness, it is quite difficult to determine the anatomical, biochemical, and cellular basis for the phenotype, or to use gene rescue to prove unequivocally that the disease-causing gene has been identified. For this reason during the last years different species has been used as model: Chickens for the inner hair cell (Levic et al., 2007; Savin, 1959; Stone and Cotanche, 2007) Guinea pigs for cochlear implant and drug treatments (Maruyama et al., 2007; Rejali et al., 2007; Sly et al., 2007; Versnel et al., 2007), but is using mouse deafness models that the genetics results has been primarily investigated. Rapid progress has been made in identifying deafness genes in the mouse. Over 90 different genes have now been identified that affect inner-ear development or function, with many more loci known to be involved in deafness but not yet identified. Relatively few of the identified mouse deafness genes, however, have been shown to be involved in human deafness. (Steel, 2001) Only few of the mouse deafness genes found provide non-lethal models for non-syndromic deafness in humans: *Myo7a* (Gibson et al., 1995), *Myo15* (Probst et al., 1998), *Pou3f4* (Phippard et al., 1999), *Col11a2* (McGuirt et al., 1999), *Tecta* (Legan et al., 2000), *Cdh23* (Di Palma et al., 2001) and *Pds* (Everett et al., 2001). The limited overlap in deafness genes identified in mice and humans indicates that there are still many deafness genes that have yet to be found in both species. Mouse allows also the use of an invitro model of the Organ of corti. It's indeed possible to dissected , from postnatal mouse pups, segments of the cochlea and to isolate from the Scala media an Scala Tympani a narrow strip of cells that can be cultured in vitro (Rastel et al., 1993). The cell cultured include all the corti organ even the hair cell and are still functional to trigger the neuronal signal on stimulation (Zheng and Gao, 1996). Because mice are born with an immature hearing organ, and their cochleae continue to develop postnatally, this model is often used also for developmental studies (Zhang et al., 2005).

2.4 Microarrays state of art.

Microarray technology enables analysis of relative abundances of typically 30,000-50,000 different nucleic acid molecules simultaneously. The basic concept of the DNA microarray is to take advantage of that DNA or RNA molecules with complementary sequences bind to each other, hybridize. The DNA molecules to be measured are immobilized onto a solid support in an organized pattern. Analysis can be performed by hybridizing DNA or RNA in the sample to complementary DNA on the array and then measure the amount. The immobilized DNA is called probe and the molecules binding to it, target. To enable measurements, the target is labelled with molecules making it possible to detect with fluorescence or radioactive radiation, how much has bound. Depending on probe lengths, concentrations and melting temperatures, different specificities and sensitivities can be obtained. Originally, quantification of a specific DNA molecule in a complex mix by hybridization, was introduced already in 1975 by Southern, but it was not until the 90's high-throughput platforms were launched. During the past ten years microarrays have had a revolutionary impact on biomedical research. The parallelization, miniaturization, and automation opportunities offered by chip technology have resulted in that methods examining one gene at a time in many cases have been exchanged by microarrays as the standard tool, generating vast amounts of information with few experiments. The DNA microarray has so far mainly been used for large-scale gene expression analysis, but plays a central role also in other fields, such as genotyping, epigenetics and promoter analysis (Gresham et al., 2006; Kaller et al., 2005; Matsuzaki et al., 2004; Wang et al., 2005). To obtain reliable data, techniques for microarray production, sample preparation and data handling are continuously being developed and improved.

2.4.1 Spotted Microarrays

Spotted microarrays refer to chips where pre-synthesized probes are applied to the array surface by robotic printing. The spot size is typically 100-200 μm . There are mainly three types of probes for gene expression analysis; full length cDNAs, shorter PCR amplified gene specific tags, and single stranded oligonucleotides. The efforts made to discover new genes by sequencing of cDNA clones in the 1990's, generated a large amount of cDNA libraries. Sequencing data of these libraries for different organisms and tissues provided a first glance into specific transcriptomes. Expressed sequence tags (ESTs) contribute to 69% of human gene sequences in the GenBank database (Benson et al., 2006). By amplifying normalized cDNA

libraries with PCR, probes suitable for global microarray gene expression analysis were obtained. In 1995 construction of the first microarray was published consisting of 1000 *Arabidopsis thaliana* genes (Schena et al., 1995). The first whole genome array was made for *Sacharomyces cerviseae* and was used in the landmark cell cycle study of Spellman and colleagues (DeRisi et al., 1997; Spellman et al., 1998).

2.4.2 Oligo microarrays

A less labour intensive strategy to obtain specific probes is to synthesize an oligonucleotide for each probe (Call et al., 2001; Kane et al., 2000; Zhao et al., 2001). Commercial 40-70mer oligonucleotide sets are available from several companies. The shorter hybridising sequence introduces however an increased susceptibility to polymorphisms. Besides the commercially available oligo probe sets, there have been numerous developments of methods for optimized probe design, which allows for probes adapted to individual requirements (Emrich et al., 2003; Mrowka et al., 2002; Wang et al., 2005; Wernersson and Nielsen, 2005). The choice of oligo length is not evident and depends on the application. Shorter sequences are cheaper to synthesize and are sometimes necessary to achieve specificity for certain transcripts, while longer ones can ameliorate signal intensities as well as specificity remarkably (Ramdas et al., 2004). Important features in construction of spotted microarrays besides probe design are spot concentration and morphology, as well as the background intensity. These are affected by the printing buffer used, humidity and temperature, robotic delivery and surface chemistry (Wrobel et al., 2003).

Different case is the one of Agilent's microarrays that is non-based on contact. These microarrays are fabricated with Agilent's SurePrint technology, based on non-contact industrial inkjet printing process that uniformly deposits oligo monomers onto specially prepared glass slides. Agilent makes real time quality control to verify chemical deposition at each step in the process to minimize premature truncation of the oligonucleotide probe. With this process oligonucleotides of 60 bases in length can be synthesized. Probes were selected using Agilent's own Design algorithms that minimize the self and cross hybridization and design a single probe for each gene.

2.4.3 In Situ Synthesized Arrays

Affymetrix is the most widespread in situ synthesis technique, commercialized by Affymetrix in the mid 90's, is based on photolithography technology from the semiconductor industry to direct DNA synthesis on glass slides producing high density oligonucleotide microarrays (GeneChip) (Fodor et al., 1993; Fodor et al., 1991; Lockhart et al., 1996). The procedure is based on synthetic linkers with photoprotected groups attached to the array in a narrow pattern. The probe sequences are elongated stepwise by cyclic addition of dNTPs. To obtain different sequences on distinct positions, probes are alternating being protected and deprotected by light using different photolithographic masks for every step. Probes are designed to give optimal hybridization conditions considering parameters such as melting temperature and sequence. Unspecific and repetitive regions are avoided. Since the technique only allows for synthesis of maximum 25 nucleotides, development of methods to compensate for this shortcoming has been forced. To increase signal to noise ratio as many as 11-20 different probes are synthesized for each mRNA. The redundancy increases the dynamic range, lower cross-hybridization and reduces the number of false positives. The probes are designed to match sequences through out the whole transcript but with emphasis on the 3' end. An additional method to filter out false positives is offered by also synthesizing an almost identical set of replicate probes. All probes which perfectly matched sequences (PM) have a partner probe which only has one mismatch (MM). These MM probes allow discrimination between real signals and signals due to cross reactivity and other artefacts. In 2002 NimbleGen Systems launched a new method for high density in situ oligosynthesis; the Maskless Array Synthesizer (MAS) (Nuwaysir et al., 2002). The principle is to create virtual photolithographic masks by using a digital light processor, directing the light to specific alternating probe positions with around 800,000 individually addressable mirrors. Advantages with this new technique were the reduced cost, increased flexibility, longer maximum probe length (90 nt), and the possibility to synthesize the oligonucleotide in both 3' to 5' and 5' to 3' indirection. The last point enables elongation with DNA polymerases after target hybridization to the probe. Although the methods described above have shown good results with robust and standardized laboratory management, efforts are being made to develop alternative strategies with increased sensitivity and specificity. In particular during the last 10 years a new bead based technology is wide spreading among laboratories.

2.4.4 Bead Based Arrays

A bead based array system introduced by Michael and colleagues in 1998 have been used as basis for further developments by Illumina which now has a platform which allows for analysis of 46,000 transcripts with more than 6.5 million features using a randomly ordered array assembly followed by a decoding procedure (Gunderson et al., 2004; Kuhn et al., 2004; Michael et al., 1998). Illumina's BeadArray Technology is based on 3-micron silica beads that self assemble in microwells on either of two substrates: fiber optic bundles or planar silica slides. When randomly assembled on one of these two substrates, the beads have a uniform spacing of ~5.7 microns. Each bead is covered with hundreds of thousands of copies of a specific oligonucleotide that act as the capture sequences in one of Illumina's assays. Individual bead types are created in manufacturing lots of several million. Oligos are subjected to extensive QC before being coupled to beads. QC includes; OD260 monitoring, MALDI Mass Spec/UV capillary electrophoresis, Real-time monitoring of every chemical step. In every base addition on every oligo Full-length oligos are coupled to beads through an amine linkage, creating a "bead type" for each bead + oligo combination.



Figure (10). Hypothetical zoom in of BeadChip surface.

BeadChip formats, uniform pits are etched into the surface of each substrate (to a depth of approximately 3 microns) prior to assembly. The BeadChip format uses a planar silica substrate in a slide format coated with a photo-resistant substance before plasma etching, followed by cleaning, to reveal uniformly etched wells. Beads are then randomly assembled and held in these micro-wells by Van der Waals forces and hydrostatic interactions with the walls of the well. The oligos attached to beads in Illumina Gene Expression arrays contain a 29-base address (used in the manufacturing/decoding process) and a 50-base gene-specific probe. The randomly assembled array is sequentially hybridized to 16 "decoder oligonucleotides", each of which is a perfect match for one of the assay oligos bound to a particular bead type. The array is hybridized, imaged, and stripped to the first set of 16 decoder oligos, labeled as described above. The second hybridization includes the same 16 decoder oligos, labeled in a different order with fluorescent

dyes. Following the second round of hybridization, an image is taken and it is simple to precisely identify the exact bead type in each position on the array (Walt, 2000). Each address and probe sequence combination has been carefully selected bioinformatically, followed by a functional screen in the laboratory to ensure that no cross-hybridization is observed. Gene-specific probe design is accomplished through a rigorous multi-step bioinformatics algorithm that scores potential probes by considering; the uniqueness compared with other genes, the sequence complexity, the self-complementarity for hairpin structure prediction, the melting temperature for hybridization uniformity and the distance from 3' end of the transcript. Probe design also takes into account exon structure, enabling design of probes that target specific splice isoforms or identify all known isoforms.

FIGURE (11) PUBLICATION AND CITATION TRENDS RESPECT DIFFER COMMERCIAL PLATFORMS'

2.4.5 Conversions Between Microarray Platforms

The last few years numerous platform comparisons have been published (Barnes et al., 2005; Kuo et al., 2002; Mah et al., 2004; Wang et al., 2005). Surprisingly these showed that the various platforms do not give concordant results in many cases.

One explanation is the ambiguity of comparing sequences from different parts of the transcript. To be able to combine data from different platforms, it is of high importance that the probe annotations are in agreement. In the beginning GeneChip users had the annotation provided by Affymetrix to rely on since probe sequences were not made public. In 2003 the sequences were released together with an annotation software allowing customers to do more thorough inspections of their expression data (Liu et al., 2003a).

Recent studies have indicated that as much as 20% of the Affymetrix probe sequences lack a match in RefSeq db and almost 40% were wrongly (Harbig et al., 2005; Mecham et al., 2004). The accelerated curation of sequences in the databases improves however the robustness of probe design. An advantage with cDNA arrays has so far been that the sequences are based on actual mRNA transcripts. A drawback has been that up to 30 % of the probes in some cases have been misidentified (Watson et al., 1998). Resequencing of the printing plates followed by reannotation by blasting eliminates this problem. The most reliable strategy to do conversions between probe sets is by sequence-matching (not by annotation) (Mecham et al., 2004; Wang et al., 2005). Other reasons to differences in results are inconsistencies in experimental procedures and data analysis (Shi et al., 2006). The largest platform comparison so far with standardized protocols is the recent study by the MicroArray Quality Control (MAQC) project. Illumina Human-6 Expression BeadChips, 48k v1.0, Agilent Whole Human Genome Oligo Microarrays, Affymetrix HG-U133 Plus 2.0 Arrays reached in this evaluation similar results regarding accuracy and sensitivity (Shi et al., 2006).

2.4.6 RNA Quality Assessment

The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. Using intact RNA is a key element for successful microarray or RT-PCR analyses. The Microarray Quality Control Consortium (Shi et al., 2006) uses a RNA Integrity Number (RIN) >8.0 , 28S/18S ratio > 0.9 as quality criteria. Using electrophoretic separation on microfabricated chips, RNA samples are separated and subsequently detected via laser induced fluorescence detection. The bioanalyzer software generates an electropherogram and gel-like image and displays results such as sample concentration and the so-called ribosomal ratio. The electropherogram provides a detailed visual assessment of the quality of an RNA sample.

However, methods that rely on human visual interpretation of data are intrinsically flawed. The RNA Integrity Number (RIN), was developed to remove individual interpretation in RNA quality control. The analysis takes the entire electrophoretic trace into account and the RIN software algorithm allows for the classification of riboeukaryotic total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. In this way, interpretation of an electropherogram is facilitated, comparison of samples is enabled and repeatability of experiments is ensured. RIN has been designed to provide unambiguous assessment of RNA integrity.

2.4.7 Target Labelling and Hybridization

Labelling efficiency of the target molecules is a main issue for transfer of the transcript abundances to detectable reliable signals. In the standardized method for many commercial platforms (as Illumina, Agilent and Affymetrix) biotin labelled cRNA is in vitro transcribed from double stranded cDNA. In 1990 Eberwine and colleagues introduced the technique of amplifying mRNA by in vitro transcription maintaining relative transcript abundances for low-throughput gene expression analysis in single neurones (Van Gelder et al., 1990). Numerous variations of this technique have been applied and evaluated for microarray analysis and are today a standard procedure for RNA amplification in many labs (Baugh et al., 2001; Dafforn et al., 2004; Dumur et al., 2004a; Dumur et al., 2004b; Eberwine, 1996; Freeman et al., 1999; Lindberg et al., 2006; Luzzi et al., 2003; Pabon et al., 2001; Scherer et al., 2003; Stoyanova et al., 2004; Wadenback et al., 2005). The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter using a reverse transcriptase (RT) engineered to produce higher yields of first strand cDNA than wild type enzymes. The cDNA then undergoes second strand synthesis and clean up to become a template for in vitro transcription with T7 RNA Polymerase. Since the transcriptase generates RNA with the same sequence as the strand containing the promoter, the RNA produced will be antisense (aRNA) (t Hoen et al., 2003). One can also add a promoter sequence of another transcriptase to the primer for the second strand synthesis, and then either sense or antisense RNA can be transcribed depending on which viral enzyme is used (Brazma et al., 2001). The three most common transcriptases employed are T7, T3 and SP6, named after which bacteriophages they are cloned from.

With the only exception of Illumina that use the whole cRNA, prior to hybridization, the cRNA is fragmented to reduce cross hybridization. After the hybridization, streptavidin labelled with phycoerythrin is applied in two stains enabling detection, with a biotinylated anti-streptavidin

antibody staining in between. For dual channel microarrays the most common fluorophores are cyanines 3 and 5 and Alexa Fluors. These are either introduced by direct or indirect labelling corresponding to using labelled nucleotides in the reverse transcription or using aminoallyl labeled nucleotides in which case the fluorophores are attached after cDNA synthesis by a diestrification reaction. The latter method is more time consuming but produces higher amounts of cDNA and causes less dye effects. Signal enhancement may be an alternative when there are limiting amounts of RNA available, the main two strategies being tyramide signal amplification and dendrimer technology. These have proven to give reproducible reliable signals with up to 100-fold amplification (Karsten et al., 2002; Manduchi et al., 2002).

2.4.8 Data analysis

A main issue in microarray studies is how to retrieve valuable information from the enormous amount of generated data. Since the field is so new, methods are being developed continuously to meet the demands of biological researchers. The main processes in the data analysis are extraction of spot/feature signals, filtering, normalization, assessment of differential expression, clustering and classification, and placing the study into context of other sources of information, such as biological data bases, clinical features and other microarray experiments. There are several reviews which summarize these procedures (Brazma, 2001; Quackenbush, 2006). The rapid development of methods for data handling puts high requirements on the software development. Besides a large amount of bioinformatic tools designed to make specific operations there are several commercial (e.g. Genespring, ArrayAssist and Kensington Discovery Environment) and open-source software (Mev 4, Bioconductor) providing complete solutions. Commercial company often link their own products with specific dedicated software, Illumina as example provide the BeadStudio software as tool for analyzing the data generated from their BeadArray Reader. In the last few years many companies has started to integrate their software with the open-source ones (Technologies). The R language for statistical computing has been increasingly popular much due to that a large number of statistical tools are available open-source and that many new methods for microarray data are written in R (Gentleman et al., 2004; Gunderson et al., 2004).

2.4.9 Handling Microarrays Experiments Data

Although many significant results have been derived from microarray studies, one limitation has been the lack of standards for presenting and exchanging such data. In the 2001 the European Bioinformatics Institute published a work called “About a Microarray Experiment (MIAME)” as part of the Microarray Gene Expression Data Societ (MGED). The ultimate goal of this work was to establish a standard for recording and reporting microarray-based gene expression data, which will in turn facilitate the establishment of databases and public repositories and enable the development of data analysis tools describing the minimum information required to ensure that microarray data can be easily interpreted and that results derived from its analysis can be independently verified (Brazma et al., 2001).

The six most critical elements contributing towards MIAME are:

1. The raw data for each hybridisation .
2. The final processed (normalised) data for the set of hybridisations in the experiment (study).
3. The essential sample annotation including experimental factors and their values.
4. The experimental design including sample data relationships.
5. Sufficient annotation of the array.
6. The essential laboratory and data processing protocols.

MIAME does not specify any particular terminology, however for automated data exchange the use of standard controlled vocabularies and ontologies are desirable and MGED recommends the use of MGED Ontology (Whetzel et al., 2006) for the description of the key experimental concepts, and where possible ontologies developed by the respective community for describing terms such as anatomy, disease and chemical compounds.

Thanks to this project, public database of microarray gene expression data started to be more effective using MIAME requirements as mandatory for the data submission (Ball et al., 2005; Brazma et al., 2003; Ikeo et al., 2003; Killion et al., 2003; Rocca-Serra et al., 2003; Tang et al., 2005; Webb et al., 2004; Wheeler et al., 2001) even the U.S. Food and Drug Administration National Center adopted it as standards for microarrays toxicogenomic research (Ball et al., 2005; Tong et al., 2003; Tong et al., 2004). In addition more of 50 well-known journals requiring MIAME compliant data as a condition for publishing microarray based papers. As direct effect many commercial and open MIAME compliant software has been developed (Brazma et al., 2006; Draghici et al., 2007; Saal et al., 2002).

2.4.10 Microarrays into Deafness

Use of Microarrays into ear research has started from the end of the ninety's , when Lomax et al. used home made slides of cDNA spotted Arrays to investigate differential gene expression after noise trauma in the chick ear (Lomax et al., 2000). In the following years similar approach has been made for investigate differential expression into mouse models for deafness; induced by noise traum, drugs, viral and bacterial infections (Dillman et al., 2006; Lin et al., 2002; Sabourin et al., 2004). Big efforts have been also made to delineate the specific gene expression inside the inner ear and those differentially expressed during the development of the cochlea (Hawkins et al., 2007; Lee et al., 2004; Lin et al., 2003; Toyama et al., 2005).

A milestone for the field has also been the Morton et al. work, that starting form the construction of a human fetal cochlear cDNA library (Robertson et al., 1994) and the characterization of additional cochlear-expressed sequences (Morton, 2004; Resendes et al., 2002) leads to the production of cDNA spotted Arrays enriched of cochlear-expressed genes that is now constantly used as tools or as comparison for the experiment regarding human inner ear expressed genes (Hertzano et al., 2007; Sajan et al., 2007).

Even if so far comparison has been made between human deafness locus and mouse orthologues of expressed genes, this approach has not been used as selective method for locus investigation or used to distinguish different gene expression due to mutant Connexin gene expression.

3. AIMS OF THE STUDY

Hearing loss, acquired or genetic, is a major worldwide public health concern. Numerous loci have been linked to hearing disorders through genetic studies. So far these studies have been performed in large families with multiple affected members using microsatellite markers and other DNA polymorphisms. It is not always possible to use these methods to map a gene within a genetic interval that is amenable for mutation analysis since the mutation analysis of all genes encoded within a large genomic interval is extremely labor-intensive. The work presented in this thesis aims to identify candidate genes potentially involved in Non-Syndromic Hearing Loss using an innovative approach merging biological and bioinformatics approaches.

More specifically the study aims to achieve the following goals:

- investigate the expression profile of the wild type form of the *GJB2* gene in transfected HeLa cells with respect to the transfection of clinically relevant *GJB2* mutants comparing two different microarray platforms for a genome-wide perspective;
- Verify the genomic location of genes differentially expressed in the above experiment to identify candidates which are found within non-syndromic deafness loci with unidentified causative genes;
- Further narrow down the selection of genes using publicly available expression data to select genes expressed within human tissues pertaining the sound pathway;
- Identify the mouse orthologs of the candidate genes to further verify their expression in the mouse Organ of Corti;
- Validate the final candidate gene list by Real-Time PCR within the cell models used;
- Use final validated genes as potential candidates for mutational screening.

4. MATERIALS AND METHODS

4.1 Cloning of the Human Cx26 Gene

Genomic DNA was extracted from peripheral blood and used as a template for gene amplification because it presents the whole open reading into one exon. The coding region of Human Connexin 26 (GenBank accession no. NM 004004) was amplified by polymerase chain reaction (PCR). Briefly primers were designed using Primer 3 software and HindIII and BamHI sequence was added at the flanking region to allow the correct insertion during the cloning. Amplification was performed using a reaction mix composed of 10X reaction buffer, 250 ng of oligonucleotide primer sense 5'CAAGCTTGCAATTCGTCTTTCCAGAGC3', 250 ng of oligonucleotide primer antisense 5'CGGATCC TTGTGGCATCTGGAGTTCA3', 50 ng of DNA, 2.5mM of dNTP mix, 2.5 U of TAQ gold DNA polymerase (Roche) and H₂O RNase-free DEPC water to volume using the following PCR conditions : 8 minutes at 95°C , 30 seconds at 95°C followed by 18 cycles of 30 seconds at 95°C, 1 minute at the annealing temperature of 57°C and 5 minutes at 68°C. DNA sequencing of the PCR product was performed with an BigDye Terminator v3.0 Cycle Sequencing Standard Kit and loaded to a 3130 ABI genetic analyzer. The full open reading frame has been cloned into pcDNA3 expression vector (Invitrogen). Briefly 100ng of HindIII and BamHI (New England Biolabs) digested pcDNA3 vector and 30ng of HindIII and BamHI digested PCR product were mixed for ligation for 5 minutes at room temperature using 1 unit of fast T4 ligase (Roche) with 10 X Fast ligation buffer (Roche) that includes ATP in a total volume of 20 µ. The ligation product has been used to transform 50 µl of DH5- α the competent cells and plated. I picked a single colony from each freshly streaked selective plate and inoculate a starter culture of 5 ml LB medium containing ampicillin. I incubated the starter culture for 12 h at 37°C with vigorous shaking. The day after I diluted the starter culture into 100 ml of selective LB medium and let them grow at 37°C for 12–16 h. I checked the culture cell density of approximately 3–4 x 10⁹ cells per ml the day after and I extracted the pcDNA3_Connexin26 plasmid using the Quiagen EndoFree Plasmid Maxi Kit according to the manufacturer's protocol. The obtained plasmid has been redissolved into TE buffer, pH 8.0 and stored at -20°C.

4.2 Site-Directed Mutagenesis.

Tryptophan to Serine point mutation and guanine deletion in the connexin gene were introduced by site-directed mutagenesis using the Stratagen QuickChange Kit. For every gene mutation two oligonucleotides were synthesized according to the Stratagene Primer design Guidelines.

Gene Name	Forward Primer	Reverse Primer
Connexin26 mutant 35delG	5'CTGCAGACGATCCTGGGGGGTGTGA ACAAACT3'	5'AGTGTTCACACCCCCAGGATCGTCT GCAG3'
Connexin26 mutant W44S	5'TGGCTGCAAAGGAGGTGcGGGAGA TGAGCAGGC3'	5'GCCTGCTCATCTCCCgACACCTCC TTTGCAGCCA3'

Table 1. Oligonucleotides used for *Site-Directed Mutagenesis*.

Briefly amplification was performed using a reaction mix composed of 10X reaction buffer, 250 ng/ μ l of oligonucleotide primer sense, 250 ng/ μ l of oligonucleotide primer antisense, 50 ng of dsDNA pcDNA3_GJB2 template, 2,5mM of dNTP mix, 2.5 U of PfuTurbo DNA polymerase (Stratagene) and H₂O RNase-free DHPC water to volume using the following PCR conditions : 30 seconds at 95°C followed by 18 cycles of 30 seconds at 95°C, 1 minute at the annealing temperature of 55°C and 4 minutes at 68°C. After the PCR amplification I placed the reaction into ice for 2 minutes and checked by electrophoresis 10 μ l of the product on a 1% agarose gel. If a band may be visualized at this stage, I proceed to adding 1 μ l 10 U/ μ l of the Dpn I restriction enzyme (New England Biolabs) directly to each amplification reaction and placed it to 37°C for 1 hour to digest the parental supercoiled dsDNA. I thawed E.Coli DH5- α competent cells on ice and, for each control and sample reaction to be transformed, I aliquoted 50 μ l of the competent cells to a pre-chilled 14-ml polypropylene round-bottom tube. I transferred 1 μ l of the Dpn I-treated DNA from each control and sample reaction to separate aliquots of the prepared competent cells. I heat pulsed the transformation reactions for 45 seconds at 42°C and then place the reactions on ice. I added 0.5 ml of S.O.C medium broth preheated to 42°C and incubate the transformation reactions at 37°C for 1 hour. I plated 50 μ l of each transformation reaction on a LB Ampicillin agar plates and I incubated the transformation plates at 37°C for >16 hours. Ten colonies of each plate have been collected and the plasmid extracted using the Quiagen QIAprep Spin Miniprep Kit according to the manufacturer's protocol. All the obtained plasmids have been sequenced with an 3130 ABI genetic analyzer to confirm the mutagenesis. The confirmed plasmid has been used to transform DH5- α competent cells and grewed to obtain the required amount of cells to perform a Quiagen EndoFree Plasmid Maxi extraction according to the manufacturer's protocol.

4.3 HeLa cells Transfection

The day before the transfection, I've seeded 8×10^5 HeLa cells (ATCC) per 60 mm dish in 5 ml of DMEM 10% FBS for a total of 14 different dishes. All the dishes included a cover glass for the further immunofluorescence analysis. I've Incubated the cells at 37°C and 5% CO₂ in an incubator. The following day I selected 9 dishes that were at 80% of confluence . I dilute 3 µg of each DNA (i.e pcDNA3 Connexin26 Wild type, pcDNA3 Connexin26 deletion 35delG and pcDNA3 Connexin26 point mutation W44S), DNA purified with the EndoFree Plasmid Kit and dissolved in TE buffer pH 8, with cell growth medium containing no serum, proteins, or antibiotics to a total volume of 150 µl. For each transfection I added 25 µl of PolyFect Transfection Reagent (Quiagen) to the DNA solution and incubated the samples for 5–10 min at room temperature (20–25°C) to allow the complex formation. I've aspirate the growth medium from the dish and washed cells once with 4 ml PBS, and add 3 ml of fresh cell growth medium. I added 1 ml of cell growth medium (containing serum and antibiotics) to the reaction tube containing the transfection complexes, mixed by pipetting, and immediately transferred the total volume to the cells in the 60 mm dishes. Finally I incubated cells with the complexes at 37°C and 5% CO₂. I harvest cells after 14h of incubation time.

4.4 RNA Isolation and integrity control.

To extract the RNA from the transfected cells I aspirated the medium, and washed the cells with PBS. Then I removed the PBS, and add 0.1% trypsin in PBS to detach cells from the dish, I added a complete medium (containing serum to inactivate the trypsin),and transferred the cells to an RNase-free polypropylene centrifuge tube and centrifuged at 300 x g for 5 min to obtain a cell pellet. I removed the supernatant, and loosen the cell pellet thoroughly by flicking the tube. The RNA extraction has been performed using Quiagen RNeasy Mini Kit and ,to avoid DNA contamination, using the Quiagen DNase I treatment according to the manufacturer's protocol. The purified RNA has been eluted into 50 µl RNase-free DEPC water.

To check the integrity of each eluted, I read 1ul of RNA using the nanodrop Spectrophotometer and loaded 50ng of RNA into RNA 6000 nano assay Chip (Agilent). Briefly I prepared the Agilent Gel-Dye Mix adding the matrix to the supplied spin filter and microcentrifuge and spin at 1.500 g for 10 minutes. I added 1 µl of RNA dye concentrate into 65 µl of filtered RNA gel matrix , vortex and centrifuge 3.000 g for 10 min at room temperature. I prepared a RNA 6000

nano assay Chip filling it with the prepared matrix and dye using the Chip Priming station and loading 5ul of internal marker into each loading well. Finally I loaded 1µl of heat denatured RNA 6000 ladder into the well ladder and after I loaded all the heat-denature RNA samples. Every chip assay was run in a 2100 Bioanalyzer (Agilent) for about 30 minute. The RNA Integrity Numbers were calculated using the 2100 Expert software (Agilent). Only the samples with Rna Integrity Number (RIN) above 8 are considered worthy for the further laboratory steps.

4.5 Western blot

I placed cells dishes on ice and washed cells with ice cold PBS to remove media. I added 1 ml of RIPA buffer (150 mM NaCl ,10 mM Tris, pH 7.2,0.1% SDS ,1.0% Triton X-100 ,1% Deoxycholate ,5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 2 µg/ml leupeptin 100 µM sodium orthovanadate, 10 mM p-nitrophenylphosphate) with 100 µl of 10X Protease Inhibitor Cocktail (Roche) to 60 mm plate. I scraped cells into RIPA buffer and transferred to small centrifuge tubes. I let stand on ice for 10 min, vortexing every few minute to dissolve material. The lysates has also been passed through a 22 g needle to aid solubilization. I centrifuged at 14,000 rpm for 10 min and removed the supernatant for protein assays and discard the pellet. For each supernatant 3ul has been read using the Biorad Bradford Protein assay. To each sample of protein solution containing 50 ng of the target protein, I added an equal volume of 2x SDS-PAGE sample buffer (Biorad) with 2-mercaptoethanol. I denatured the proteins by heating the sample to 95 °C for 5 min and loaded the sample onto an SDS-polyacrylamide gel 12% and run the gel under standard conditions. I transfer the proteins to pre-wet methanol PVDF membrane using Criterion Blotter (Biorad). This device use wet transfer method using the transfer solution (25 mM Tris,192 mM glycine 20% v/v methanol, pH 8.3) .The efficiency of transfer has been determined by staining the membrane 10 s in Ponceau stain. The stain has been removed by washing in TBST (10 mM Tris-HCl, pH 8.0 150 mM NaCl,0.05% Tween-20). I blocked the membrane with a blocking buffer containing dried milk 5% w/v nonfat dry milk TBST. Incubated overnight at 4°C with agitation. I diluted the primary antibody Mouse anti-Connexin 26 (Zymed) in blocking buffer to 1:100 and incubating 1ml of primary antibody solution blot in a sealed bag overnight at 4 °C with agitation. I rinsed the blot TBST and then washed twice for 5 min each and twice for 15 min at room temperature. I diluted the horseradish peroxidase (HRP) labeled secondary antibody Rabbit Anti-Mouse IgG (Zymed) at 1/5000 in blocking buffer. I rinsed the blot in TBST and then wash twice for 5 min each and twice for 15 min at room temperature. I used Pierce SuperSignal® West Pico Chemiluminescent Substrate

mixing the two substrate components at a 1:1 ratio and I incubated the blot 5 min in the mixed solution. I drained the excess reagent and covered the blot with clear plastic wrap before expose it to X-ray film.

4.6 RT-PCR

For all RT-PCR, two oligonucleotides were synthesized, one in mRNA sense and one in antisense orientation and used for reverse transcription. The oligonucleotides, used to amplify the sequence, were in two successive exons of the gene in order to distinguish it from amplification of contaminant genomic DNA. Always a Dnase treatment was used before RT-PCR analyses.

Gene Name	Forward Primer	Reverse Primer
<i>β-Actin</i>	5'AAGGCCAACCGCGAGAAGATGA3'	5'TGGATAGCAACGTACATGGCTG3'
<i>GJB2</i>	5'TCTGGCTCACTGTCCTCTTC3'	5'ATGATCAGCTGCAGAGCCCA3'
<i>GAPD</i>	5'CCCTCAACGACCACTTTGTCA3'	5'GGTCCACCACCCTGTTGCT3'
<i>FoxG1</i>	5'GAACGGCAAGTACGAGAAGC3'	5'TCACGAAGCACTTGTGAGG3'
<i>DR1</i>	5'GTGCCGTTTAGGTTTCGGTTA3'	5'GTGCAGAAACCAGGAAGGAG3'
<i>Slc22a5</i>	5'ACAGTATCCCGTTGGAGACG3'	5'AACAAGGAGGTGGTGAGTGG3'
<i>Hdac2</i>	5'TGGAGGAGGCTACACAATCC3'	5'TTTGAACACCAGGTGCATGT3'
<i>Polr3h</i>	5'CCAGGGCCTCTTTCATGTTA3'	5'ATCACACCGCCACTAGGTTC3'

Table 2. Oligonucleotides used for RT-PCR

Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen) and sequence specific antisense primer. Briefly reverse Transcription was performed in 10 µl final volume containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM MgCl₂, 0.25 mM dNTPs, 4 units of RNase Inhibitor, 2 units of Superscript II reverse transcriptase, 15 pmoles of antisense primer and 5ug of RNA. The reaction proceeded at 42°C for 60 minutes. Amplification was performed by adding 40 µl of 10 mM Tris-HCl, pH 8.3, 55 mM KCl, 15 pmoles of upstream primer, 1.2 units of Taq DNA Polymerase. Samples solutions were denaturated 3 minutes at 95°C, subjected to five cycles of 1 minute at 95°C, 1 minute at the annealing temperature and 1 minute at 72°C and followed by 35 of cycles of 30 seconds at 95°C, 30 seconds annealing temperature and 30 seconds at 72°C. I checked by electrophoresis 10 µl of the product on a 8% acrilamide gel.

4.7 Immuno fluorescence

To confirm the HeLa cells transfection I checked the human connexin 26 expression with immunofluorescence analysis respect to the non transfected HeLa cells that not express connexin 26 at all. Moreover to confirm that the organ of corti dissection still contain the inner and outer hair cells, I checked the expression of Myosin VIIa with Immunofluorescence on the obtained organotypic culture. Briefly I recover a round slide from each transfected dish or cultured organ of corti dissection and washed each of them with PBS 1X (Sigma). Cells were fixed with 4% paraformaldehyde (Merck) for 20 min at Room temperature and permeabilized for 4 min with PBST (0.1% Triton X-100 in PBS 1X) and washed three times with PBS 1X. Non-specific antibody binding sites were blocked with blocking buffer PBST containing 2% Fetal Calf Serum and 1% BSA (Sigma) for 10 min. Subsequently HeLa cells were labelled using a 0.25 mg/ml solution in 10 mM phosphate buffered saline PBS of Mouse anti-Human Connexin 26 primary antibodies (Kumar and Gilula, 1996) (Zymed) diluted 1:150 in blocking buffer for 1 hour. Mouse organotypic Corti culture were labeled using a 200 µg IgG Goat anti-Human Myosin VIIA primary antibodies (Tuxworth et al., 2001; Weil et al., 1995) (Santa Cruz Biotechnology) in 1.0 ml of PBS containing 0.1% sodium azide and 0.2% gelatin diluted 1:100 in blocking buffer for 2 hours. Cells were then washed three times with PBST and incubated 1 hour at room temperature with Rhodamine (TRITC)-conjugated AffiniPure Rabbit Anti-Mouse IgG secondary antibodies (Jackson Laboratories) diluted 1:1000 in blocking buffer or in the case of Myosin VIIA with Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Goat IgG secondary antibodies (Jackson Laboratories) diluted 1:1000 in blocking buffer. Cells were washed three times with PBST and once with distilled water and mounted in MOWIOL (Calbiochem, Bad Soden, Germany) supplemented with 6-diamidino-2'-phenylidole dihydrochloride. Images were recorded on a cooled CCD camera with the OpenLab Software (Improvision, Coventry, UK) attached to an inverted Zeiss microscope (Oberkochen, Germany)

4.8 Labeling Agilent

I've labeled each of the retrieved RNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent).

Briefly to a 1.5 mL microcentrifuge tube I added 500 ng of total RNA in a volume of 10.3 μL . In the same tube I added 1.2 μL of T7 Promoter Primer and I denatured the primer and the template by incubating the reaction at 65°C in a heating block for 10 minutes. I vortexed the diluted Two-Color RNA Spike-In solution (Agilent) and heated it at 37°C for 5 minutes. I added 2 μL of the heated solution to each sample. After that I placed the reactions on ice for 5 minutes while I pre-warmed the 5X First Strand Buffer by incubating the vial in a 65°C waterbath for 4 minutes. Using room warmed reagent I assembled the cDNA Master mix (4.0 μL 5X First Strand Buffer, 2.0 μL 0.1 M DTT, 1.0 μL 10 mM dNTP mix, 1.0 μL MMLV RT, 0.5 μL RNaseOUT). To each sample tube I added 8.5 μL cDNA Master Mix and I incubated the samples at 40°C in a circulating water bath for 2 hours and to 65°C for 15 minutes and finally to ice for 5 minutes. For both Cyanine 3-dCTP, and Cyanine 5-dCTP, I thawed a 1 mM stock and place on ice. I pipetted 12.5 μL of cyanine stock solution into a microcentrifuge tube. To each sample tube, I added 2.4 μL cyanine 3-CTP (10 mM) or 2.4 μL cyanine 5-CTP (10 mM). I pre-warm the 50% PEG solution in a 40°C waterbath for 1 minute and I kept it at room temperature until use. Using room warmed reagent I assembled the Transcription Master Mix (15.3 μL Nuclease-free water, 20 μL 4X Transcription Buffer, 6.0 μL 0.1 M DTT, 8.0 μL NTP Mix, 6.4 μL 50% PEG, 0.5 μL RNaseOUT, 0.6 μL Inorganic Pyrophosphatase, 0.8 μL T7 RNA Polymerase). To each sample tube I added 57.6 μL of Transcription Master Mix and I incubated the samples in a circulating water bath at 40°C for 2 hours. After the incubation I added 20 μL of nuclease free-water to each cRNA sample, to obtain a total volume of 100 μL and to purify the cRNA from the enzymes and buffers I added 350 μL of Buffer RLT and mix thoroughly, added 250 μL of ethanol 100% and mixed thoroughly by pipetting. I transferred all the sample to an RNeasy mini column (Quiagen) placed in a 2 mL collection tube and centrifuged the sample for 30 seconds at 13,000 rpm. After I transferred the RNeasy column to a new collection tube. To clean the filter I added 500 μL of buffer RPE to the column and I centrifuged the sample for 30 seconds at 13,000 rpm. I repeated the cleaning step twice. I eluted the cleaned cRNA sample by transferring the RNeasy column to a new 1.5 mL collection tube, adding 30 μL RNase-free water directly onto the RNeasy filter membrane, waiting 60 seconds and centrifuging for 30 seconds at 13,000 rpm twice. The total final flow through volume has be approximately 60 μL and has been store at -80°C until the use. I used 1 μL of the amplified cRNA for Spectrophotometric quantification using Nanodrop ND-100.

4.9 Labeling Illumina

I've labeled each of the retrieved RNA using the Illumina® TotalPrep RNA Amplification Kit (Ambion).

Briefly I placed 500 ng of total RNA in a volume of 11 µL and at room temperature, I've prepared the Reverse Transcription Master Mix (1 µl T7 Oligo(dT) Primer, 2 µl 10X First Strand Buffer, 4 µl dNTP Mix, 1 µl RNase Inhibitor, 1 µl ArrayScript) in a nuclease-free tube. I transferred 9 µl of Reverse Transcription Master Mix to each RNA sample, mixed thoroughly by pipetting up and centrifuged. I placed the samples in a 42°C incubator for 2 hours. On ice I prepared the Second Strand Master Mix in a nuclease-free tube (63 µl Nuclease-free Water, 10 µl 10X Second Strand Buffer, 4 µl dNTP Mix, 2 µl DNA Polymerase, 1 µl RNase H). I transferred 80 µl of the Second Strand Master Mix to each sample and mixed thoroughly by pipetting. I placed the tubes in a 16°C thermal cycler for 2 hours. I passed to the cDNA Purification, adding 250 µl of cDNA Binding Buffer to each sample and passing the mixture through a cDNA Filter Cartridge (Ambion) seated in its wash tube. I washed the Cartridge adding 500 µl of Wash Buffer and centrifuged for 1 min at 10,000 X g. Finally I discarded the flow-through and transferred the cDNA Filter Cartridge to a cDNA Elution Tube where I put 10 µl of preheated Nuclease-free Water and left at room temperature for 2 min. Then I centrifuged for 1.5 min at 10,000 X g and applied a second aliquot of 9 µl preheated Nuclease-free Water and centrifuged another time for 2 min. At room temperature I prepared an IVT Master Mix (2.5 µl T7 10X Reaction Buffer, 2.5 µl T7 Enzyme Mix, 2.5 µl Biotin-NTP Mix) and I transferred 7.5 µl of IVT Master Mix to each cDNA sample, I placed the tubes at 37°C for 14 hours. I stopped the reaction by adding 75 µl Nuclease-free Water to each cRNA sample to bring the final volume to 100 µl. For each sample, I placed a cRNA Filter Cartridge into a cRNA Collection Tube. I added 350 µl of cRNA Binding Buffer to each cRNA sample and after 250 µl of ACS reagent grade 100% ethanol. The whole solution has been pipetted onto the center of the filter in the cRNA Filter Cartridge and centrifuged at 10,000 X g for 1 minute. I discarded the flow-through and replaced the cRNA Filter Cartridge back into the cRNA Collection Tube washing the cartridge adding 650 µl of Wash Buffer and centrifuging at 10,000 X g for 1 minute. The Filter Cartridge was transferred to a fresh cRNA Collection Tube and cRNA eluted with 100 µl of preheated Nuclease-free Water. I used 1 µl of the amplified cRNA for Spectrophotometric quantification using Nanodrop ND-100.

4.10 Hybridization and Scanning Agilent

I performed the Hybridization on 44K Whole Human Genome Oligo Microarray (Agilent) according with the decided experimental design. Briefly I added 500 μL of nuclease-free water to the vial containing lyophilized 10X Blocking Agent supplied with the Agilent Gene Expression Hybridization Kit and heated the mix for 5 minutes at 37°C . For each microarray, I added 90 μL of 8.3 ng/ μL cyanine 3-labeled, linearly amplified cRNA, 90 μL of 8.3 ng/ μL of cyanine 5-labeled, linearly amplified cRNA, 50 μL of 10 X Blocking Agent, 10 μL of 25 X Fragmentation Buffer. I incubated at 60°C for 30 minutes to fragment RNA each samples. Finally I added 250 μL of 2 X Hybridization Buffer to the samples for a final volume of 500 μL . I mixed well by careful pipetting and placed each sample on ice before load it onto the array. To load each array I dispensed the volume onto the gasket slide (loaded into the Agilent SureHyb chamber) and than covering it with the array “active side”. I Hand-tightened the clamp onto the chamber and rotate the assembled chamber to wet the gasket and assess the mobility of the bubbles. I assembled each slide chamber in rotisserie in a hybridization oven set to 65°C with rotator at 4 rpm and hybridized at 65°C for 17 hours. I dispensed 1000 mL of Gene Expression Wash Buffer 2 directly into a sterile 1000-mL bottle. I tightly capped the 1000-mL bottle and place in a 37°C water bath the night before washing arrays. I Completely fill a slide-staining dish with Gene Expression Wash Buffer 1 at room temperature and place a slide rack into second slide-staining dish also full of Gene Expression Wash Buffer. I placed also a third empty dish on the stir plate and add a magnetic stir bar. I removed one hybridization chamber from incubator and disassembled the hybridization chamber. I submerged the sandwich (array and array-gasket) into the Gene Expression Wash Buffer 1 and removed the array-gasket. From the first dish I removed the “active side” microarray slides and place them into the slide rack in the second dish containing Gene Expression Wash Buffer 1 at room temperature for 1 minute. During this time I filled the third dish with Gene Expression Wash Buffer 2 from the 37°C water bath. I transferred the slide rack to slide dish containing the Gene Expression Wash Buffer 2 at 37°C and waited for 1 minute. After that time I slowly removed the slides from the solution and assembled slide into holders of the scanner carousel and cheked the scanner settings and read them using the Agilent DNA Microarray Scanner.

Scan region	Scan Area (61 x 21.6 mm)
Scan resolution (μm)	10
Dye channel	Red&Green
Green PMT	100%
Red PMT 100%	100%

Table 3. Agilent Scanner settings.

4.11 Hybridization Illumina

I performed the Hybridization on Illumina Human Whole-Genome 6-Sample BeadChip according with the decided experimental design. Briefly for each sample I added 10 μl of RNase-free water to 1.5 μg of Biotinilated cRNA .I warmed the GEX-HYB and GEX-HCB (Illumina supplied solutions) at 58°C for 10 minutes ,to dissolve any salts that may have precipitated in storage, and added 20 μl to each cRNA sample.I placed Illumina Hyb Chamber Gaskets into BeadChip Hyb Chamber and dispensed 200 μL of GEX-HCB solution into each of the two humidifying buffer reservoirs in each Hyb Chamber and placed each BeadChips into the Hyb Chamber. I preheat the assay sample at 65°C for 5 minutes and than allowed the sample to cool to room temperature before dispense 30 μL of cRNA sample onto the large sample port of each array, close the Hyb Chamber and incubate for 16 hours at 58°C with rocker speed at 5. The day after I warmed 500ml of 10 X High-Temp Wash buffer at 55°C and prepared the two solutiouons; E1BC solution (3 mL of E1BC buffer into 1 L of RNase-free water) and Block E1 buffer with streptavidin-Cy3 (Illumina supplied). I removed the Hyb Chamber from the oven, disassembled the Chamber and removed the coverseal from the BeadChip. I submerged each chip into a dish containing 250 mL Wash E1BC solution and than using a slide rack handle, I transferd the rack into the High-Temp Wash buffer and waited for 10 minutes. After the 10 minute of High-Temp Wash buffer incubation I transferred the slide rack into the a dish containing fresh E1BC buffer and leaved the slides for 5 minutes. After that I washed the slides with 250 ml of absolute Ethanol for 10 minutes and repeated these washes for a total of 2 times.I blocked each slide with 4 ml of Block E1 buffer into a Wash Tray incubating 10 minutes. After that the slides has been stained using 10 minutes incubation with 2 mL of Block E1 buffer with streptavidin-Cy3 and washed with 250 mL of Wash E1BC solution. Before the scanning each slide has been dried centrifuging 4 minutes at 275 rcf. I read the slides using an Illumina BeadArray Reader with the default settings. Thanks to the BeadStudio software package, gene expression data from images collected from the Illumina BeadArray Reader has been extracted.

4.12 Analysis Agilent

The scan data were analysed with Agilent Feature Extraction Software, which performs spots localization (Find Spot Algorithm), outlier pixels rejection based on the Interquartile Range method (Cookie Cutter Algorithm), flagging of saturated features (a feature is considered saturated when more than 50% of its pixels had an intensity above 65502). The local background value, calculated by the software through the radius method, was subtracted from every feature intensity signal and only features positive and significantly above background with a confidence level of 99% were selected for subsequent analysis. For dye normalization, the LOWESS (locally weighted linear regression) method (Wang et al., 2004) was applied, using the Rank Consistency Filter method, which select a set of features falling within the central tendency of the data, which are assumed to be unchanged, as the normalization set. For the processed data a log ratio of red and green channels signal is calculated. An error associated with each signal calculation, both for green and red channel, is also determined by the software. Then the software calculate a significance value (P-value), based on Universal Error Model, developed by Rosetta for the ResolverTM microarray data management system.

4.13 Analysis Illumina Beadstudio

I performed differential Gene expression Analysis on arrays grouped according the type of the different transfection using the BeadStudio expression module. Each group has included 4 biological replicates. I've normalized the samples using the Rank Invariant algorithm or the quantile . Briefly this method uses a rank invariant set of probes between a given sample and a virtual sample. The rank invariant set is found as follows: illumina starts by considering probes with intensities ranked between LowRank = 50th percentile and HighRank = 90th percentile. If the probe's relative rank changes $[(rx-rv)/rv] < 0.05$ the probe is considered to be rank invariant. If less than 2% of all probes in the region are identified as rank invariant, LowRank is gradually decreased until it reaches the 25th percentile. Rank invariant normalization operates under the assumption that probes with similar ranking between samples have similar expression levels. This method minimizes the effects of additive and multiplicative factors. Normalization coefficients are computed using iteratively re-weighted least squares. This method is applied after a background normalization that remove signal due to nonspecific hybridization. After the normalization the algorithm used to compare the tree group of samples is the Illumina Custom supplied from Illumina. This model assumes that target signal intensity (**I**) is normally distributed among replicates corresponding to some biological condition. The variation has three

components: sequence specific biological variation (σ_{bio}), nonspecific biological variation (σ_{neg}), and technical error (σ_{tech}). Variation of nonspecific signal (σ_{neg}) is estimated from the signal of negative control sequences (using median absolute deviation). For (σ_{tech}) illumina estimates two sets of parameters **A_ref B_ref** and **A_cond B_cond** for reference and condition groups respectively. Illumina estimate (σ_{tech}) using iterative robust least squares fit, which reduces the influence of highly variable genes. This implicitly assumes that the majority of genes do not have high biological variation among replicates. Due to the presence of biological replicas inside each group Illumina produces p-values using the following approach:

S_ref= $(\max(s_{\text{ref}} * \mathbf{A}_{\text{ref}} + \mathbf{B}_{\text{ref}} * \mathbf{I}_{\text{ref}}))$ and **S_cond**= $(\max(s_{\text{cond}} * \mathbf{A}_{\text{cond}} + \mathbf{B}_{\text{cond}} * \mathbf{I}_{\text{cond}}))$ Where **S_ref** and **S_cond** are respectively the standard deviations of probe signals into reference and condition groups while **I_ref** and **I_cond** are respectively the target signal intensity into reference and condition group respectively. Thanks to the standard deviations is possible to produce a p-value using Z two-sided tail probability of standard normal distribution.

$N = \sqrt{(\mathbf{S}_{\text{ref}}^2 * N_{\text{cond}} + \mathbf{S}_{\text{ref_neg}}^2 * N_{\text{cond}} + \mathbf{S}_{\text{cond}}^2 * N_{\text{ref}} + \mathbf{S}_{\text{cond_neg}}^2 * N_{\text{ref}}) / (N_{\text{cond}} * N_{\text{ref}})}$
and $p = z [(|\mathbf{I}_{\text{cond}} - \mathbf{I}_{\text{ref}}|) / N]$ where **S_ref_neg** and **S_cond_neg** are standard deviations of negative probe signals in the reference and condition groups while **N_cond** and **N_ref** denote the number of samples in the reference and condition groups respectively. Finally a Diff Score for a probe is computed as **DiffScore**= $(10 \text{sgn}(\mathbf{I}_{\text{cond}} - \mathbf{I}_{\text{ref}}) \log_{10}(p))$. While for the gene, Diff Scores of corresponding probes are averaged.

4.14 Analysis of Illumina and Agilent data using open source software.

Assessment of differential expression levels among different group has been performed using R version 2.6.0, Bioconductor packages Limma version 2.12.0, Lumi version 1.2.0, Annotate version 1.16.0, Biobase version 1.16.0, Ebam version 1.9.27, Siggenes version 1.12.0 and compared with the result obtained with the commercial analysis software. Agilent and Illumina raw data has been loaded into R session using respectively the function read.maimages and LumiR with the supported parameter, and background subtracted and lowess normalized using backgroundCorrect and normalizeWithinArrays function included into the Limma package or quantile normalized using the Limma normalizeBetweenArrays. Limma provides the ability to analyse comparisons between many RNA targets simultaneously (Wettenhall and Smyth, 2004). A targets file, which lists the RNA target hybridized to each channel of each array in tab-delimited format, has been loaded into the R session and used as parameter for the modelMatrix

function that computes a design matrix for input to lmFit function either for Agilent that for Illumina data. The lmFit function fits multiple linear models using the produced design matrix, and the command makeContrasts has been used to make comparisons of interest. The fit and the contrast matrix are used by contrast.fit function to compute fold changes and t-statistics for the contrasts of interest (Wettenhall et al., 2006). A multi-class analysis using a Benjamini and Hochberg statistic has been performed using the Sam function of siggenes package using the same starting raw data (Benjamini et al., 2001; Keselman et al., 2002; Reiner et al., 2003). The FDR value for the contrasts of interest is estimated by analyzing permutations of the analyzed data, where case and control labels are randomized to achieve an estimated distribution of test statistics for non differentially expressed genes (Jeffery et al., 2006).

4.15 Databases

A local Database has been built using Mysql 5.0 and populated with the most current information and identified loci for the various non-syndromic hearing loss and syndromic forms. The data was obtained from the Hereditary Hearing Loss Homepage (Guy Van Camp, 2007) and from the survey of latest literature. Into the database has been included also the set of information was obtained from fetal cochlear cDNA library and EST database of the Morton Hearing Research Group (Morton, 2002, 2004). To survey the change into the field, the list of deafness loci with unknown specific genes for the autosomal dominant, autosomal recessive, and syndromic forms was compiled from the same web based source. Using a Perl-based script I created a subroutine that check every week the web site and update the changes eventually published. Briefly I used the LWP::Simple and HTML::TableExtract Perl packages retrieved from CPAN repository. The script works connecting with the Hearing Loss Homepage and seek among the pages Locus, Markers_Name ,Chromosome ,Marker_Start , Marker_End. All the retrieved data are then compared with that belonging from the local database and, If difference can be found, a subroutine procedure using the package Net::SMTP send an e-mail advise before update the Database. Internal Perl-based subroutine , that works using the recovered genome position of the unknown loci, retrieve all the genes included among the markers and update the database table using the Ensamble API into the fields Description, Gene_Symbol, Homologus_Gene. A Base2 database with web interface has been built on a server (<http://biodev.cbm.fvg.it/base2/>) to load all the produced and retrieved data from gene expression experiment. Thanks to the Base2 the structure, all data has been loaded according to the MIAME standards and Autodetecting importer has been settled as plugin.

4.16 Cochlear dissection and Culture.

All the mice were purchased from Charles River Laboratories and were killed before the beginning of the experiments. Before each sacrifice small round glass of the culture have been prepared. Briefly under hood silice circular glass must be insert into 35 mm Petri. An aliquot of Cell Tack DB (Becton Dickinson Labware) solution has been diluted 1:16 into NaHCO_3 ph 8 0,1M and 20ul of the diluited solution has been i putted without bubble into the glass and left dry for 5 minute under hood. I've sacrificed the animal cutting the head near the shoulder. Without the use of dissection solution the head has been be skinned. Briefly taking the head from the nose of the mouse I comped the muzzle ,just under the whiskers , and the end of jaw too before skinn the head. After that I divide the head following the middle vein on the skull. Using cooled Hank's balanced salt solution (Invitrogen) as dissection solution to cover each semi-head I remove the brain and locate inside the skull a vain near the Vestibuli and the palate bone must be localized to correctly locate the cochlea. Using the two localized point I cut all around and turned the head back. I remove the tissues into the corresponding zone of the vein until highlight the cartilaginous ring that fix the eardrum. I remove the eardrum and the ring and highlighted the otic capsule. At this point the otic capsule was dissected using well shaped forceps and the stria vascularis and spiral limbus were discarded, while the epithelia containing the organ of Corti and basilar membrane were dissected into two part using a forceps. One part has been stored for RNA extraction and the other has been transferred to the Cell tack treated 35 mm Petri filled with 1 mL of DMEM-F12 (1:1, Invitrogen), 20% fetal bovine serum (FBS, Hyclone), 10 mM sodium pyruvate 2 mM glutamine (Invitrogen), 10% streptomycin and penicillin, and incubated at 37°C in a 5% CO_2 incubator. During a night of incubation the *reissner* membrane vanished and the cell grewed spreading into the glass.

4.17 Real Time PCR

For quantitative RT-PCR, TaqMan probes has been purchased from Applied Biosystems, they were all conjugated to the fluorochrome FAM, these assays come in a 20x reaction mix, span an exon-exon junction, and are optimized to give close to 100% efficiency.

Gene Name	TaqMan Gene Expression Assay
Polr3h	Hs00386079_m1
Hdac2	Hs00231032_m1
Slc22a5	Hs00929869_m1
Gapdh	Hs00266705_g1
DR1	Hs00172424_m1
FoxG1	Hs01850784_s1
β -Actin	Hs03023943-g1

Table 4.List of TaqMan Gene Expression Assay

Briefly 5 μ g of the same RNA used for the hybridization was used as a template for the reverse transcription using First-Strand Synthesis kit (Invitrogen) with random hexamer primers according to the manufacturer's protocol. The produced cDNA has been used as template for the Real-time pcr after a 1:20 dilution of the reaction. All the samples to be compared were processed in parallel. The reactions were performed using TaqMan Universal PCR Master Mix (Applied Biosystems), probes, primers and template according to the instruction manuals, 5 μ l volume of the templates were used in 20 μ l reaction volume into 96 well plates covered with optical adhesive covers (Applied Biosystems) on a Applied Biosystems StepOne Plus Real-time PCR Systems. The cycle conditions for real-time PCR were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. In order to measure reaction efficiency a standard curve was generated using 10-fold serial dilutions made from the same amount of starting RNA converted to cDNA. For each reaction, the cycle threshold was determined as the cycle number at which the fluorescence value reached a threshold level. The threshold level was set above the background fluorescence in the exponential phase of the real-time curves. Gene expression was normalized to Gapdh or β -Actin and calculated using the delta-delta cycle threshold ($\Delta\Delta$ CT) method on the StepOne Software V2.0 (Applied Biosystems). The $\Delta\Delta$ CT method is based on the subtraction of the cycle threshold of housekeeping (CT_h) gene from the cycle threshold of the gene target (CT_T) into a normal (ΔCT_r) respect the on study (ΔCT_q) condition and finally the subtraction of the two obtained Delta (Dussault and Pouliot, 2006).

$$\Delta CT_r = CT_{Tr} - CT_{hr} \quad \Delta CT_q = CT_{Tq} - CT_{hq} \quad \Delta\Delta CT = \Delta CT_r - \Delta CT_q$$

From this formula, a positive result would indicate an increase in the expression of the gene of interest in the investigated condition, whereas a negative result would indicate a decreased expression. In order to transform the results into fold variation the following relation form can be used: $\text{Fold Change} = (2 * \text{Reaction Efficiency})^{\Delta\Delta CT}$, in the case of efficiency close to 1 approximate fold variation measurements are usually done using the simplify formula $\text{Fold Change} = 2^{\Delta\Delta CT}$.

5. Results

5.1 Cloning and Trasfection of Wild Type Human Connexin26 Gene and Mutants

Due to the crucial importance of gap junctions in normal auditory function, the starting point of the project was to clone the full-length ORF of the human GJB2 gene, encoding the Connexin26 protein, into an expression vector. Since the GJB2 open reading frame consists of only one exon, genomic DNA was used as a template for gene amplification. Wild type GJB2 was cloned into pcDNA3 expression vector. It was then further manipulated by site-directed mutagenesis in order to obtain the two major known connexin mutants: the missense mutant W44S, which is associated with the dominant form of the disease, and the frameshift mutant 35delG, which causes autosomal recessive deafness. The integrity of all clones was confirmed by sequencing and the constructs were used to transiently transfect Hela cell lines. The transfection experiments were optimized to ensure similar percentage of transfected cells. In order to verify protein expression and membrane localization, round disc glasses were inserted into the dishes before seeding and were recovered 12h after the transfection. They were then used for Immunofluorescence staining with Mouse anti-Human Connexin 26 primary antibodies, which are able to identify wild type and mutant forms (Fig12). Thus we were able to obtain Hela Cells (which normally do not express Connexin protein) showing significant accumulation of Connexin protein within the plasma membrane. The percentage of cells positive to the Immunofluorescence has been taken into account in order to select comparable amounts of positive trasfected cells among wild type and mutant transfection replicas.

Figure (12). Connexin26 Immunofluorescence within Hela cells. After transfection of Connexin26 the protein start to accumulate at the edge of two adjacent trasfected cells.

The corresponding Petri dish has been used to retrieve the cells and extract the RNA. In order to verify the presence of Connexin26 mRNA, I performed RT-PCR on the extracted RNA (Fig13).

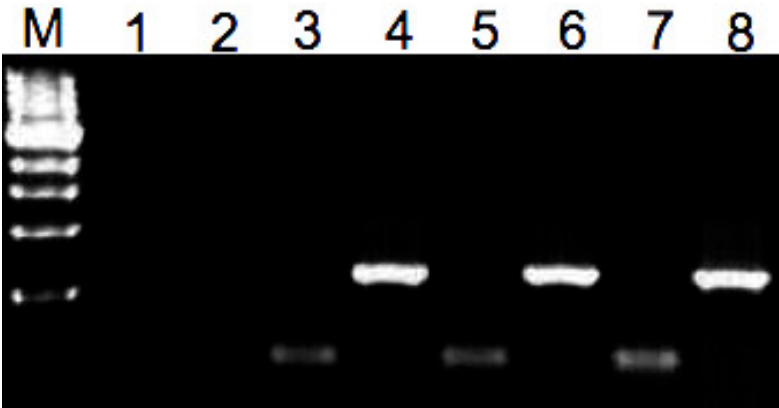


Figure (13). Connexin26 RT-PCR. Electrophoresis run using 10 ul of the product on acrylamide gel. Lane 10 contains the 1kb marker. Lanes 1 and 2 contain blank control and dna negative control, respectively. lane 3,5,6 contain GAPDH from 3 different replicas and lanes 4,6,8 contain Connexin26 from 3 different transfections

To further verify the effective expression of Connexin 26 and of the missense mutant W444S after transfection I performed a Western blot revelation of the protein 12h later (Fig14).

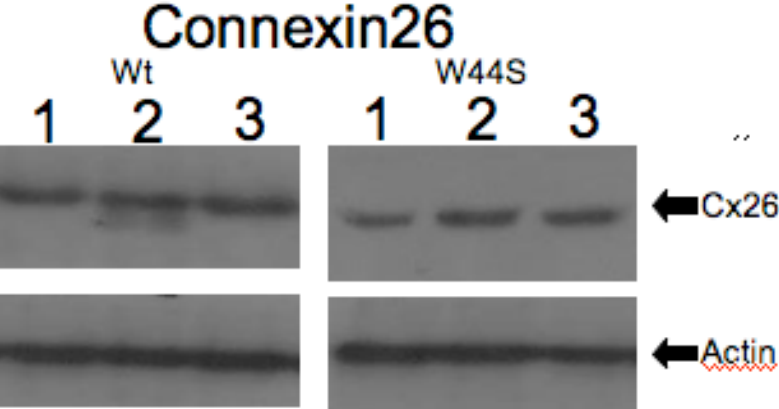


Figure (14). Western Blot of Connexin 26 wild type and Connexin 26 W44S. Connexin 26 protein revelation has been performed for all the selected trasfected dishes. As can seen the level of protein expression is similar among the three replicas.

5.2 Expression profiling of wild type vs. mutant forms of Human Connexin26

5.2.1 RNA Quality Control

The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. Since methods that rely on human visual interpretation of data are intrinsically flawed, the RNA Integrity Number (RIN) was developed to remove individual interpretation in RNA quality control. Calculated using the 2100 Expert software (Agilent), RIN is considered acceptable if it is >8.0 and the 28S/18S ratio is >0.9 as suggested by the Microarray Quality Control Consortium (Shi et al., 2006). To evaluate the quality of RNAs extracted from transfected cells, therefore, all the samples were analyzed using the RNA 6000 nano assay Chip using a 2100 Bioanalyzer (Agilent). All the samples which did not meet a quality threshold sufficient for reliable microarray analysis, were thus discarded (Fig15).

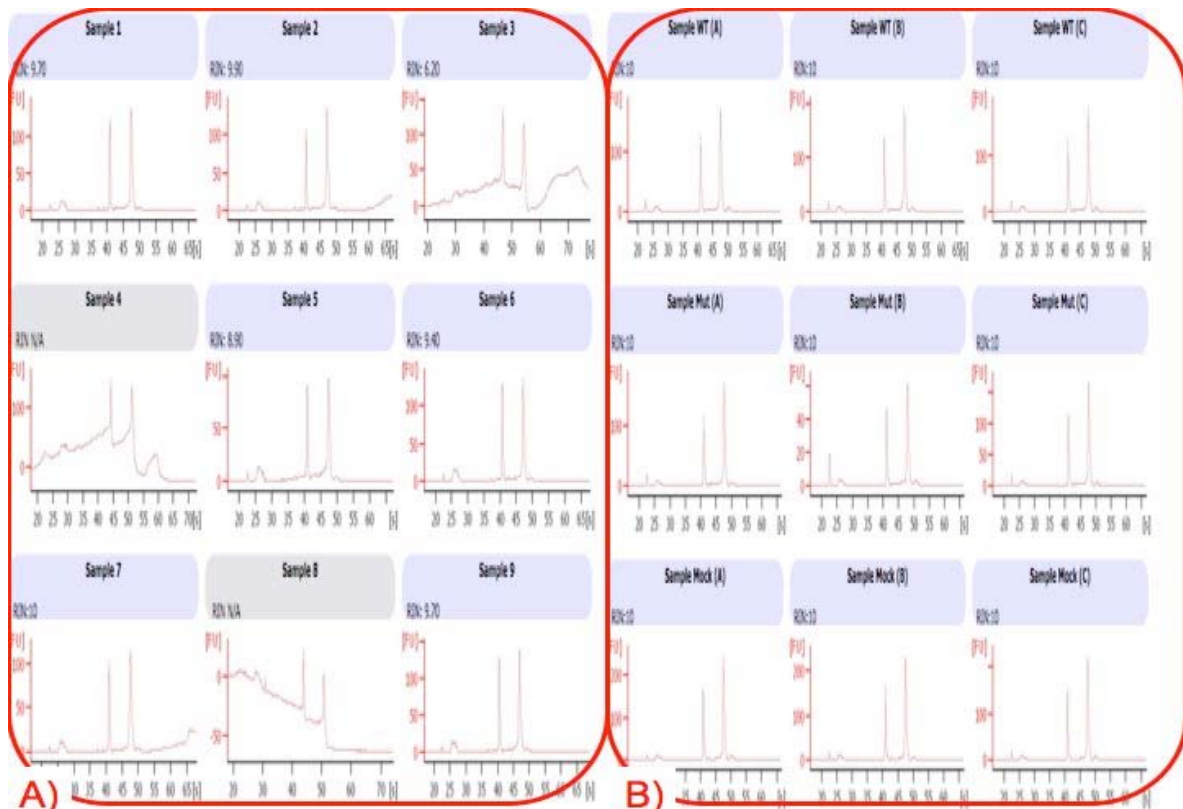


FIGURE (15). RNA QUALITY CHECK. ELECTROPHEROGAM OF RNAs FROM TRANSFECTED HELa CELLS BEFORE (A) AND AFTER CHERRY PICKING OF THE BEST EXTRACTIONS (B). AS CAN BE SEEN FROM THE LABEL ON THE TOP OF EACH ELECTROPHEROGAM THE RNA INTEGRITY NUMBER IS ALWAYS ABOVE 9.

5.2.2 Whole Genome Microarray experiment and Data analysis using Agilent Platform

All the RNA samples that passed the quality control were analyzed utilizing 12 44K Whole Human Genome Oligo Microarray (Agilent). Due to the competitive hybridization protocol required in this platform, samples were hybridized in pairs as reported in Table 5.

cRNA Cy3	cRNA Cy5	Array Number
Hela Connexin26 Wild Type	Hela Connexin26 mutant W44S	251239121880_S01_44k
Hela Connexin26 mutant 35delG	Hela Connexin26 Wild Type	251239121881_S01_44k
Hela Connexin26 mutant W44S	Hela Connexin26 Wild Type	251239121882_S01_44k
Hela Connexin26 Wild Type	Hela Connexin26 mutant 35delG	251239124962_S01_44k
Hela Connexin26 Wild Type	Hela Connexin26 mutant 35delG	251239124963_S01_44k
Hela Connexin26 mutant 35delG	Hela Connexin26 Wild Type	251239124964_S01_44k
Hela Connexin26 Wild Type	Hela Connexin26 mutant W44S	251239124965_S01_44k
Hela Connexin26 mutant W44S	Hela Connexin26 Wild Type	251239124966_S01_44k

Table 5. Lists of the cRNA targets hybridized to each channel of each array

The high definition images obtained from the scanner were analyzed with the Agilent Feature Extraction Software that performed, local background subtraction and LOWESS normalized before calculating the differentially expressed genes (Fig16).

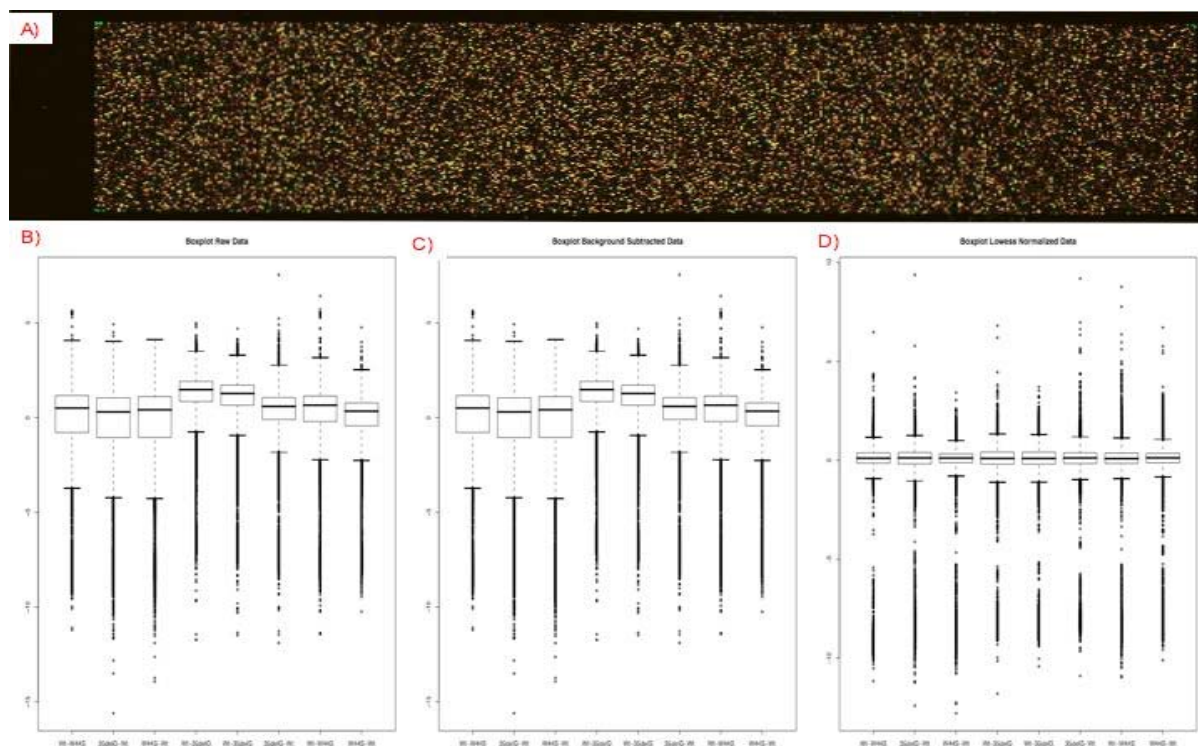


FIGURE (16). OUTPUT OBTAINED FROM AGILENT SCANNER. AN EXAMPLE OF COMPETITIVE HYBRIDIZATION OUTPUT OBTAINED FROM AGILENT SCANNER (A). BOX PLOT OF SIGNAL DISTRIBUTION OF RAW FIG(B) BACKGROUND BACKGROUND SUBTRACTED (C) AND LOWESS NORMALIZED FIG(D) DATA.

Normalization has been performed as an attempt to correct systematic bias in the data and to remove their impact on biologically significant features of the data. The labeling and detection

efficiencies are often systematic bias sources that cannot be eliminated using two-colour Hybridization. As can be seen from the density and M vs A (i.e $\text{Log}_2[\text{Cy}5/\text{Cy}3]$ vs $0.5\text{Log}_2[\text{Cy}3\text{Cy}5]$) diagnostic plots (Fig17), the Lowess normalization reduces this source of bias present in the Data Set.

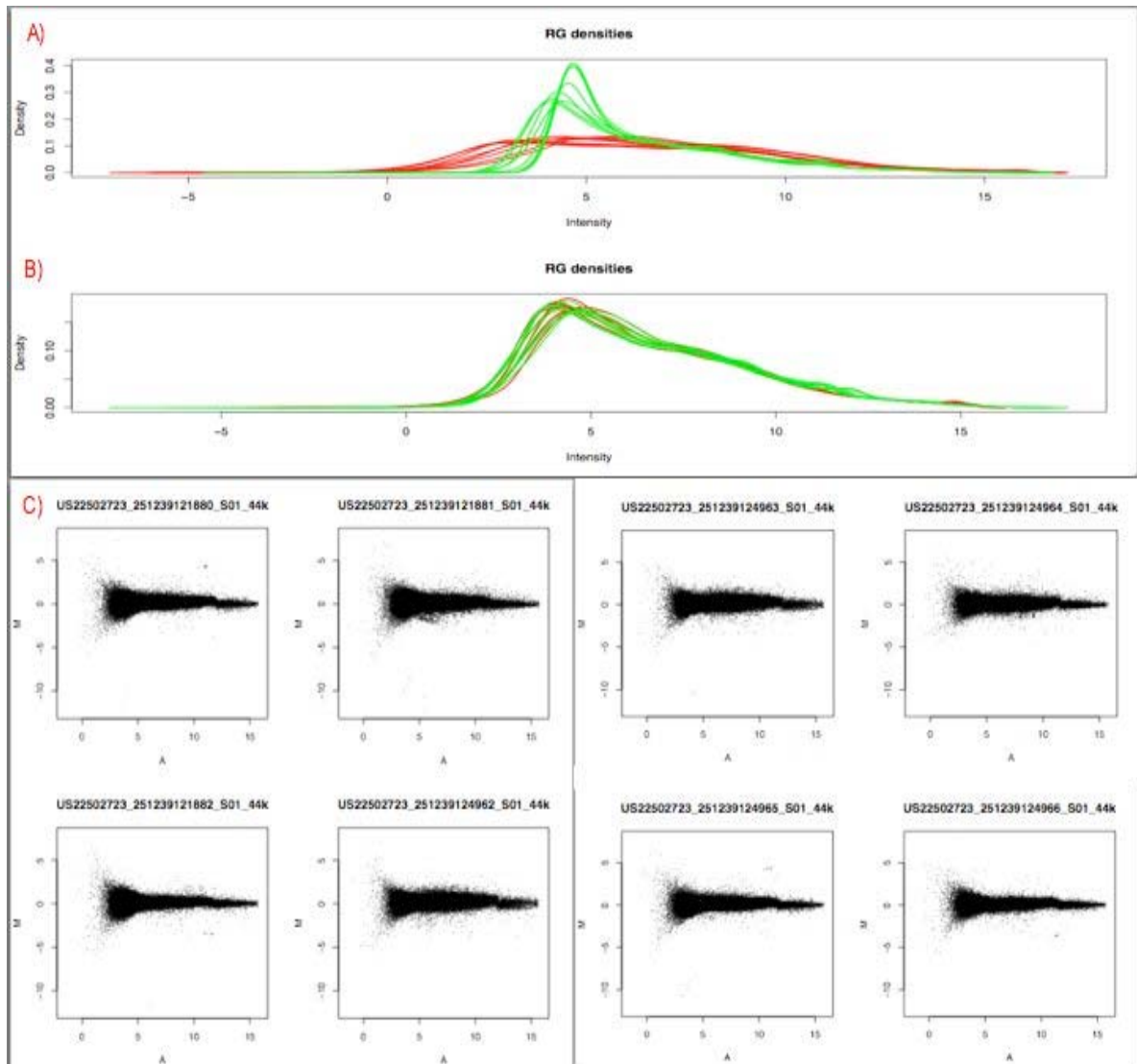


FIGURE (17).DIAGNOSTIC PLOTS OF INTENSITY DATA. DENSITY DISTRIBUTION OF RED AND GREEN CHANNELS BEFORE AN AFTER LOWESS NORMALIZATION(A). THE $\text{Log}_2(\text{Cy}5/\text{Cy}3)$ vs $0.5\text{Log}_2(\text{Cy}3\text{Cy}5)$ PLOT OF LOWESS NORMALIZED DATA (C).

The differentially expressed genes were calculated using the Universal Error Model, found within the Rosetta Resolver™ microarray data management system on the Lowess normalized data. The system reported a good correlation between the swap and replicas with a weighted correlation value of common signature always greater than 0.9 (Fig.18).

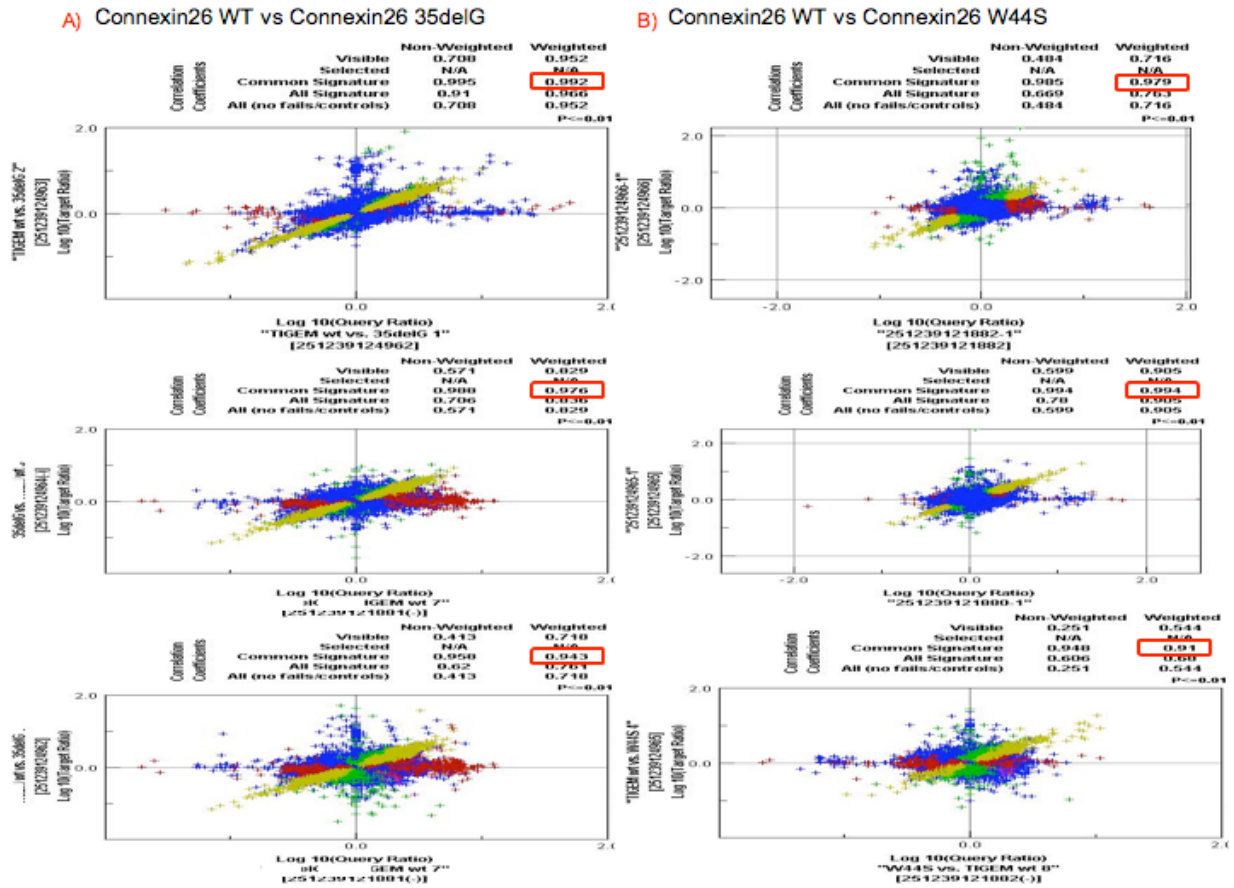


FIGURE (18). Scatter plots of intensity data. The figure reports all possible hybridization comparisons between the first replicate, the second replicate and the swap for two experiments: A) HeLa Connexin 26 Wild type vs. HeLa Connexin 26 mutant 35delG B) HeLa Connexin 26 Wild Type vs Connexin mutant W44S.

The analysis identified 6,259 genes differentially expressed within cells transfected with W44S with respect to those expressing Wild Type with a p-value < 0.001. Comparison between cells expressing 35delG vs. Wild Type detected 4,474 genes differentially expressed (Fig19).

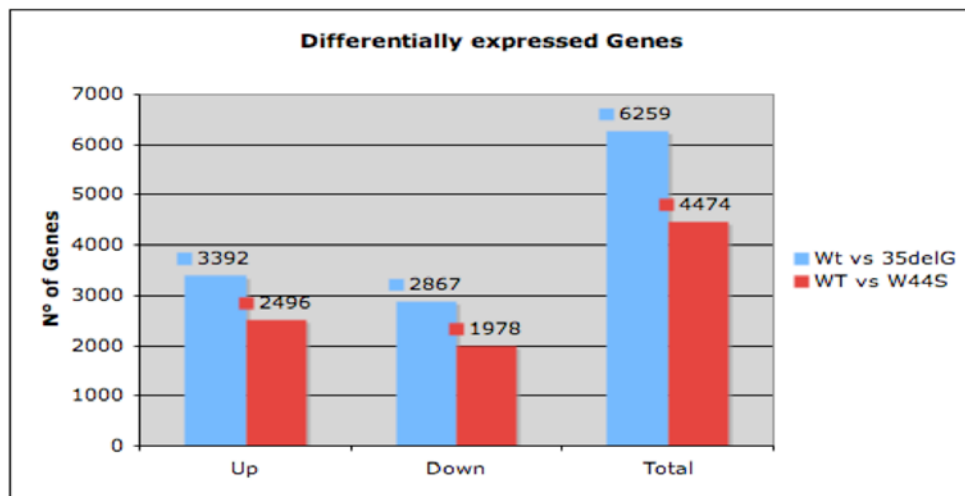


Figure (19). Number of differentially expressed genes between wild type connexin transfected cells and mutant connexin transfected cells obtained by expression profiling based on the Agilent microarrays.

The same raw data produced from the Agilent Feature Extraction Software has been analyzed using the R Limma package. The analysis identified 4,868 genes differentially expressed within cells transfected with W44S with respect to those expressing Wild Type with a p-value < 0.01. The comparison between cells expressing 35delG vs. Wild Type detected 8,613 genes differentially expressed. An overlap analysis between all the experiments performed indicates that 530 genes are found to be differentially expressed in all of them (WT vs 35DelG, WT vs W44S and W44S vs 35DelG), and that over 4,000 other genes are not specific to any of the experiments. The genes which are differentially expressed in a specific manner for the W44S mutant as compared to the WT are 1,043 and those which are specific for the 35DelG mutant are 3,991 (see Fig.19 for a Venn diagram of the complete overlap analysis).

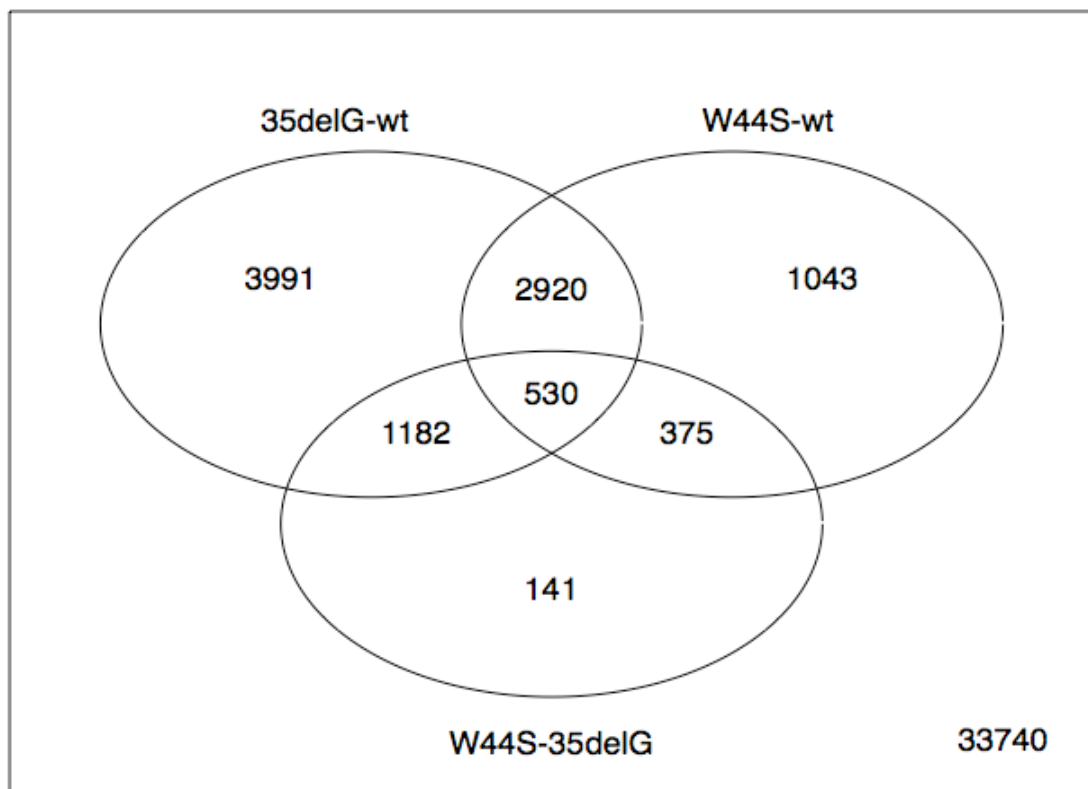


FIGURE (19). VENN DIAGRAM OF DIFFERENTIALLY EXPRESSED GENES. The numbers within the circles indicate the genes found to be differentially expressed ($p < 0.01$) in the following comparisons: 35delG vs. Wild type comparison presents 8,613 genes, of which 3991 are specific only to this experiment. In the W44S vs. Wild Type comparison 4,868 are differentially expressed, 1043 of which are specific. The W44S vs 35delG circle shows that 2,228 genes are differentially expressed between 35delG and W44S mutants (of which only 141 are specific).

5.2.3 Whole Genome Microarray experiment and Data analysis using Illumina Platform

All the RNA samples used with the Agilent Platform were also analyzed utilizing the Human Whole-Genome 6-Sample BeadChip V2.0 (Illumina). As opposed to the Agilent Arrays, Illumina technology uses single channel arrays, which means that only one dye (Cy3) is used during the hybridization and no competition for the probes is present during the annealing step of the hybridization. Moreover a single Illumina Human-6 Chip contains 6 whole genome microarrays that must be used for each experiment. Due to this properties samples were hybridized as reported in Table 6.

Array Number	Array Position	cRNA
1814647021	A	Hela Connexin26 Wild Type
1814647021	B	Hela Connexin26 Wild Type
1814647021	C	Hela Connexin26 mutant 35delG
1814647021	D	Hela Connexin26 mutant W44S
1814647021	E	Hela Connexin26 mutant W44S
1814647021	F	Hela Connexin26 Wild Type
1888857164	A	Hela Connexin26 mutant W44S
1888857164	B	Hela Connexin26 mutant W44S
1888857164	C	Hela Connexin26 mutant 35delG
1888857164	D	Hela Connexin26 mutant 35delG
1888857164	E	Hela Connexin26 mutant 35delG
1888857164	F	Hela Connexin26 Wild Type

Table 6. List of the cRNA targets hybridized to Human Whole-Genome 6-Sample BeadChip.

The high definition images obtained from the BeadArray Reader (Illumina) (Fig20) were analyzed with the Bead Studio Gene Expression Module Version 3.0 that performed local background subtraction and background quantile normalization before calculating the differentially expressed genes using the Illumina Custom algorithm.

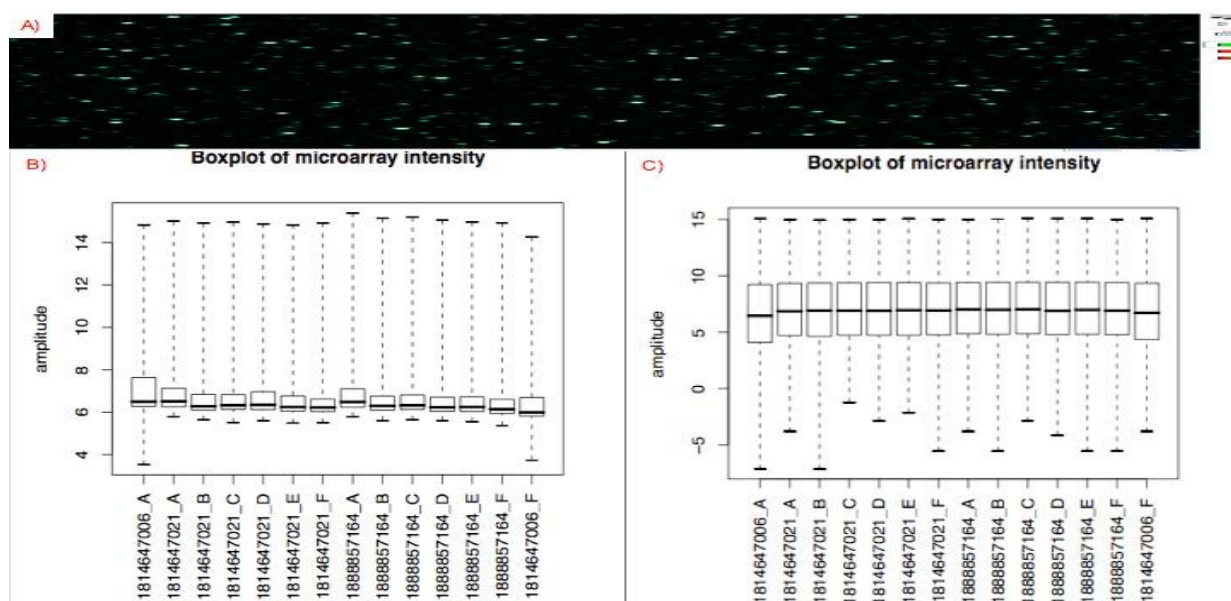


FIGURE (20) OUTPUT OBTAINED FROM BEADARRAY READER. AN EXAMPLE OF ONE CHANNEL HYBRIDIZATION OUTPUT OBTAINED FROM BEADARRAY READER (A) BOX PLOT OF SIGNAL DISTRIBUTION OF RAW (B) AND BACKGROUND QUANTILE NORMALIZED DATA (C).

Normalization was also performed for Illumina arrays. Within the Illumina BeadChip, 3 different probes are designed for each gene and each probe is randomly distributed for an average of 40 times within the microarray slide (Fig21E). Given the number of probe replicas present within the Illumina system, the normalization is particularly useful to reduce variability among probe replicas (see comparison between Fig21C and D), rather than in the overall signal (see comparison between Fig.21 A and B) as was the case for Agilent hybridizations.

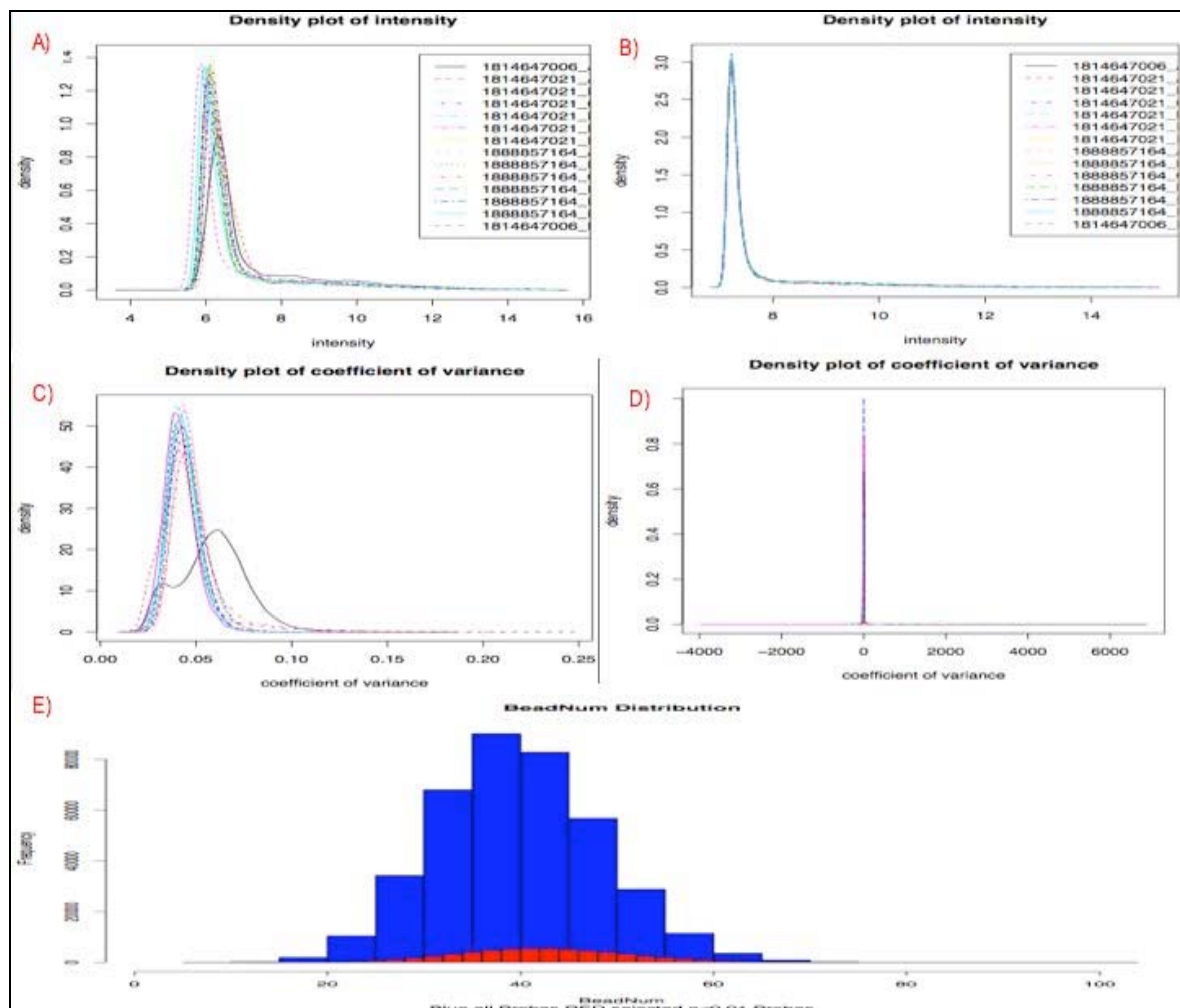


FIGURE (21) DIAGNOSTIC PLOTS OF INTENSITY AND VARIANCE DATA. DENSITY DISTRIBUTION OF SINGLE CHANNEL BEFORE (A) AND AFTER NORMALIZATION (B). DENSITY DISTRIBUTION OF COEFFICIENT OF VARIANCE BEFORE (C) AND AFTER NORMALIZATION (D). PROBES_TYPE DISTRIBUTION INTO THE USED ARRAYS. AS CAN BE SEEN FROM THE FREQUENCY, THE PROBES TYPES FOLLOW A NORMAL DISTRIBUTION FOR THE NUMBER OF REPLICAS PRESENT ON THE CHIP.

The differentially expressed genes were calculated using the Illumina Custom algorithm. The analysis indicated 1,055 genes differentially expressed between Hela Connexin26 mutant W44S group vs. Hela Connexin26 wild type with a $p_value < 0.05$. In the Hela Connexin26 mutant 35delG vs Wild Type group the genes differentially expressed were 2745 (Fig22).

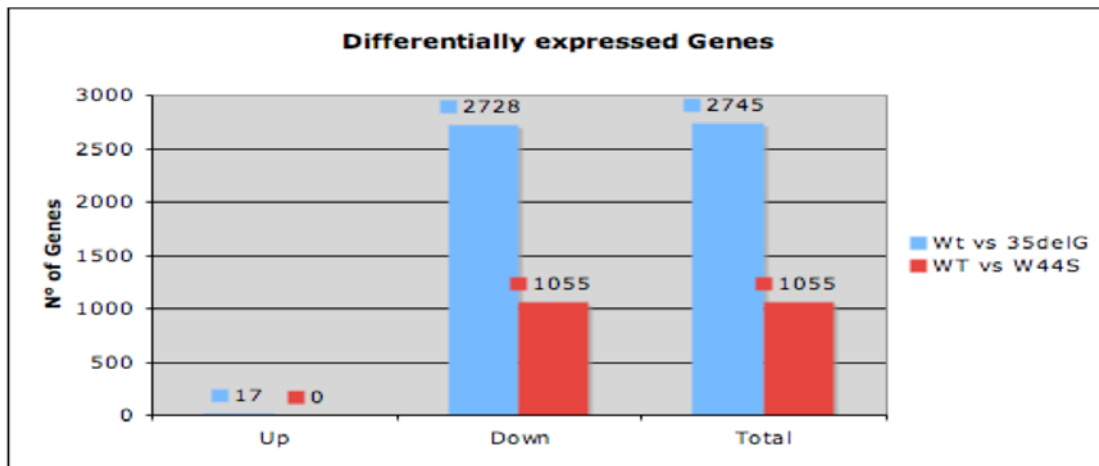


FIGURE (22). NUMBER OF DIFFERENTIALLY EXPRESSED GENES BETWEEN WILD TYPE CONNEXIN TRANSFECTED CELLS AND MUTANT CONNEXIN TRANSFECTED CELLS OBTAINED BY EXPRESSION PROFILING BASED ON THE ILLUMINA MICROARRAY PLATFORM. BOTH MUTANTS YIELDED MOSTLY DOWNREGULATED GENES (1,055 FOR W44S AND 2,728 FOR 35DELG), ONLY 17 GENES RESULTED AS UPREGULATED IN THE 35DELG MUTANT VS WILD TYPE.

As was done for Agilent data the raw data from the Illumina experiment was normalized, subsequently a linear model was fitted using a design matrix and finally moderated using a Bayesian model. The analysis identified 1,245 genes differentially expressed within cells transfected with W44S with respect to those expressing Wild Type with a p-value < 0.05. The comparison between cells expressing 35delG vs. Wild Type detected 2,364 genes differentially expressed. In this analysis the number of genes differentially expressed between mutants was very small (only 14). (Fig23).

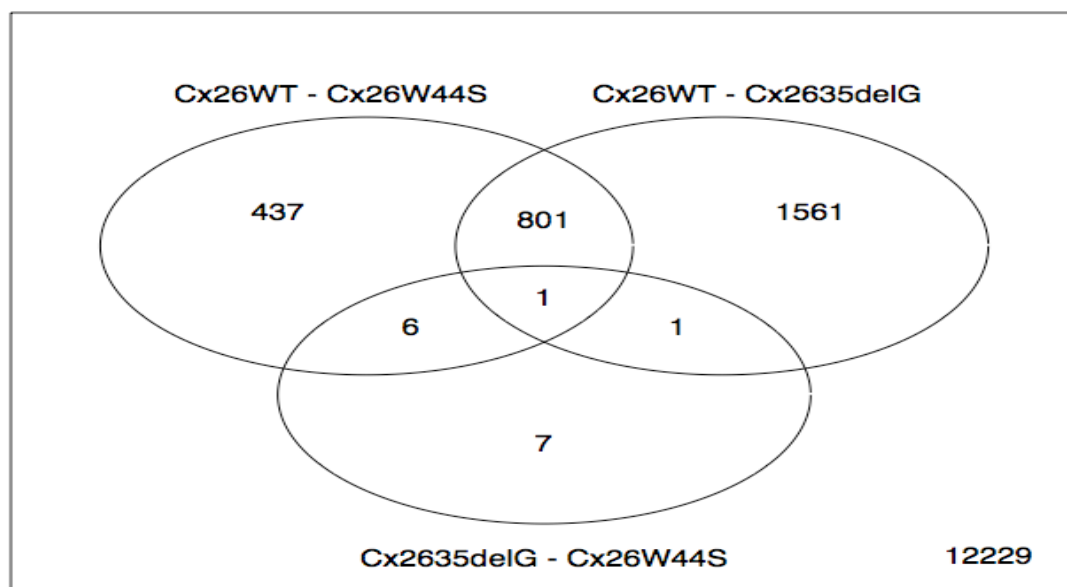


Figure (23) Venn diagram of differentially expressed genes.The circles take count of the genes resulting differentially expressed in the following comparison: 35delG vs. Wild type comparison presents 1245 genes with a p-value<0.05 801 of them are specific. In the W44S vs. Wild Type comparison 2364 are differentially expressed with a p-value<0.05 1561 of which specific. The last circle contains only 15 genes belonging from the comparison between 35delG and W44S mutants.

For the remaining part of the project I focused only on the W44S mutant owing to the technical limitations in handling the 35DelG mutant which produces a very short peptide.

5.3 Locus survey and Data Mining

In order to narrow down the results obtained with my microarray experiments I decided to use a bioinformatics approach that would assist in the selection of genes either expressed in the ear or located within known genetic loci for deafness. Thus I created a database to store all the genetic information published related to known deafness loci. The database has been built using Mysql 5.0 and populated with the most current information and identified loci for the various non-syndromic hearing loss forms. The database also includes information obtained from the Human fetal cochlear cDNA library and EST databases of the Morton Hearing Research Group (Fig24).

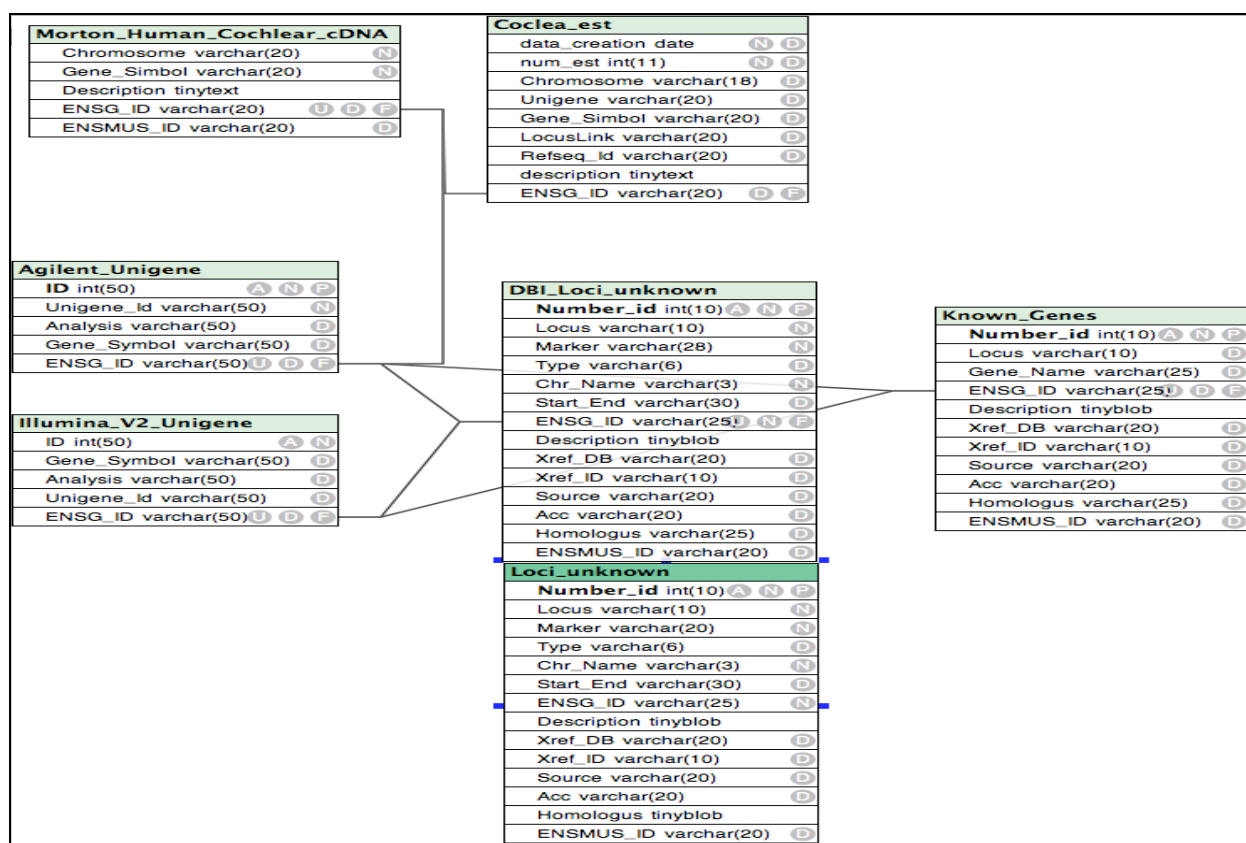


FIGURE (24).TABLE STRUCTURE. STRUCTURE OF THE DATABASE HOLDING GENETIC AND EXPRESSION INFORMATION FOR GENES UNDER INVESTIGATION WITHIN THE PROJECT.

The database is kept up to date on a weekly basis synchronizing information from the external databases utilized. A Base2 (Saal et al., 2002) database with a web interface has been developed (<http://biodev.cbm.fvg.it/base2/>, access available on request) to load all the data produced from the gene expression experiments within this project. Thanks to the Base2 the structure, all the expression data has been loaded according to the MIAME standards (Fig.25).

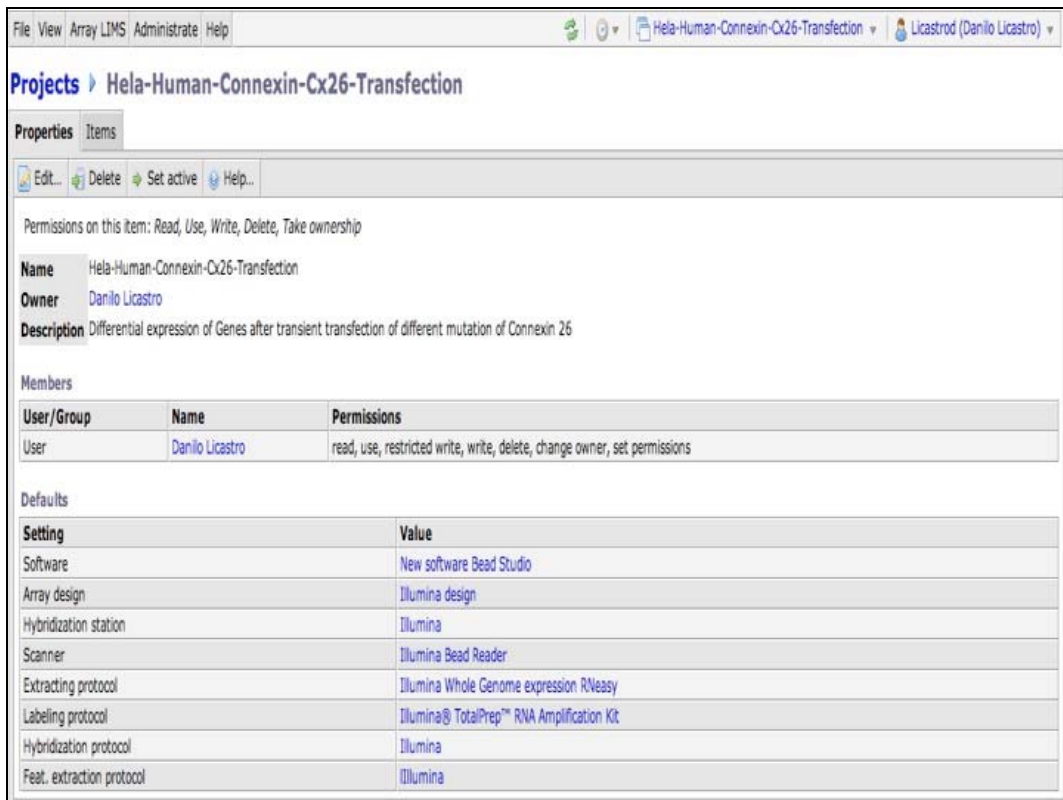


FIGURE (25).BASE2 INTERFACE. EXAMPLE OF A WEBPAGE SHOWING THE PROJECT STRUCTURE FOR THE ILLUMINA HELA ARRAY EXPERIMENT.

I also proceeded to compare the Illumina and Agilent results of the W44S mutants, in which only downregulated genes resulted to be differentially expressed. Several methods are utilized to compare gene lists across platforms. As can be seen in Fig.26 I mapped the genes obtained in both platforms to IDs in common databases, such as Unigene, HGNC and Ensembl. Unigene was the database which gave the highest number of IDs and therefore the highest potential of comparison. I proceeded, therefore, to map all genes obtained to Unigene IDs to identify unique genes common to the two experiments. This comparison downsized the number of genes found to be significantly downregulated in the W44S mutant in both platforms to 57 genes (Fig26).

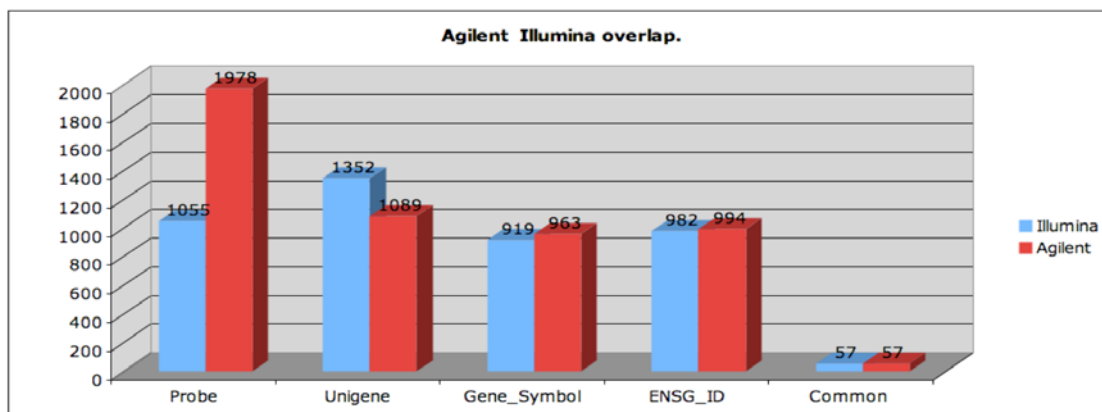


FIGURE (26).AGILENT ILLUMINA COMPARISON. COMPARISON OF AGILENT AND ILLUMINA DIFFERENTIALLY EXPRESSED GENES. THE DIFFERENT COLUMNS REPRESENT THE NUMBER OF IDs TO WHICH THE GENES WERE MAPPED WITHIN EXTERNAL DATABASE S SUCH AS UNIGENE, HGNC GENE SYMBOLS AND ENSEMBL GENE ID (ENSG_ID) . THE LAST COLUMN REPRESENTS UNIQUE GENES COMMON AMONG THE TWO DATASETS.

The integration of all the data produced during my project with external data such as genetic and expression information allowed me to further analyze high potential candidates. Due to the limited number of genes obtained from the comparison of the data obtained from the two platforms, I decided to analyze both datasets independently. Thus I compared the original subsets of 1,055 Illumina and 1,978 Agilent genes with known deafness loci without an identified causative gene. The comparison narrowed the number of genes to 189 for the Illumina set and to 144 for the Agilent set, with only 4 genes in common between the two datasets. Furthermore I selected among the 189 and 144 genes only those present within the human cochlea cDNA library and the Human cochlea EST dataset and the number was reduced to 12 and 8 genes respectively (of which one overlapping), which localized within 11 different deafness loci (Fig27).

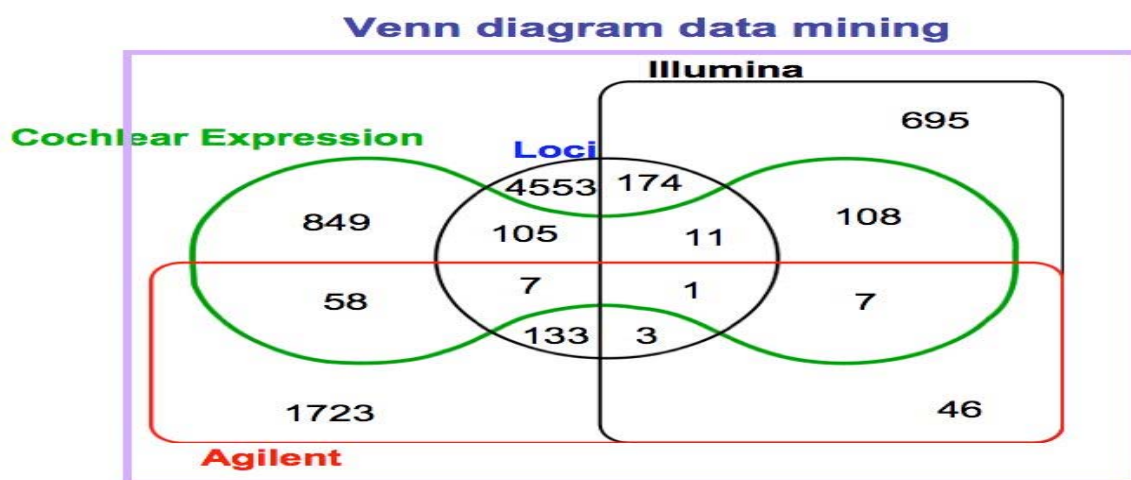


Figure (27).Edwards' Venn diagram of data mining. Comparison of genes which resulted as candidates from 4 different analysis: Illumina expression profiling of W44S mutant vs WT (Black), Agilent expression profiling of W44S mutant vs WT (Red), genes located within genetic loci for deafness (Black), genes identified in cochlear cDNA and EST libraries (Green). The figure shows that only 1 gene overlaps all 4 datasets, and 18 more genes overlap the expression and genetic datasets as well as one of the two expression profiling experiments.

5.4 Mouse Corti expression of mouse orthologs of selected candidate genes

Taking into account that the cochlear cDNA and EST libraries utilized are derived from large sections of the ear, it was important to verify the expression of the candidate genes selected above in the organ of Corti, in order to validate their presence in the parts directly involved in sound transduction. Since RNA from human inner ear is not easily available, I decided to identify the mouse orthologous genes of the candidate genes selected. Only 5 of the 19 genes selected as candidates had a clear mouse ortholog, thus I proceeded with those 5. Furthermore, it was important to obtain Corti organotypic cultures to verify specifically expression of candidate genes within this organ. Thus inner ear dissections containing the organ of Corti were surgically isolated using micro-mechanical dissection technique under a stereo-microscope. Subsequently half of the dissected material was put in culture and verified by immuno fluorescence for the presence of hair cells using MyoVIIA as a marker (Fig28). Once immunofluorescence was confirmed the other half was used for RNA extraction.

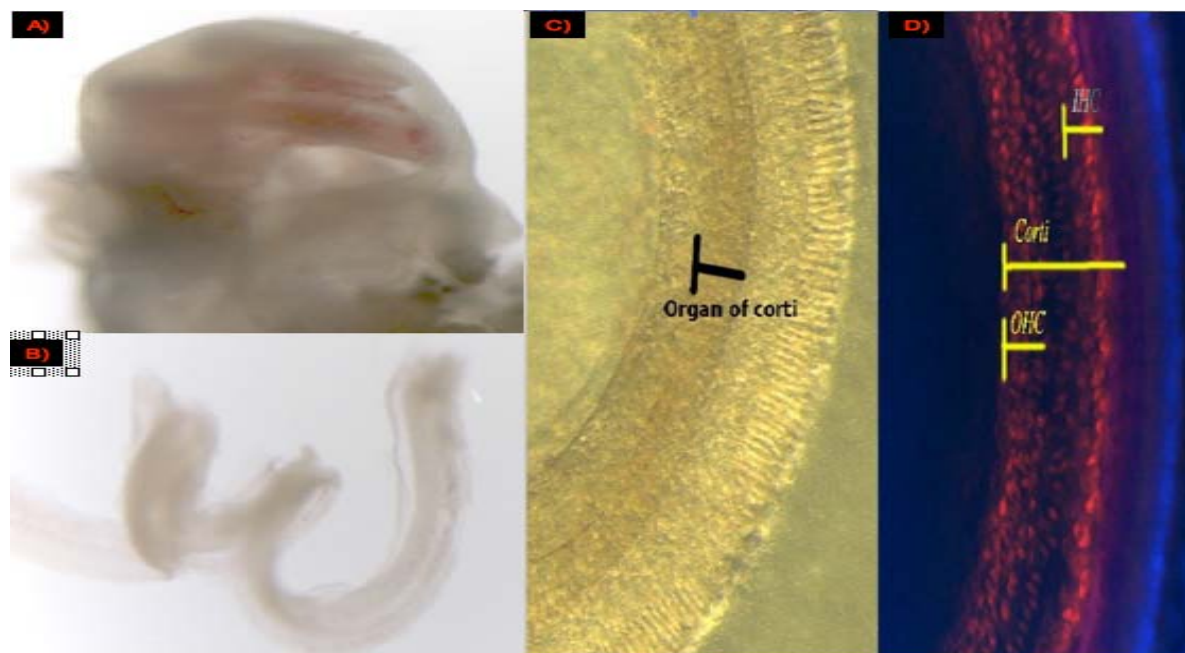


FIGURE (28).DIFFERENT PHASES OF COCHLEAR DISSECTION AND ORGANOTYPIC CULTURE. A. OPENING OF THE OPTIC CAPSULE B. STRIA VASCULARIS SEPARATED FROM THE EPITHELIA CONTAINING THE ORGAN OF CORTI AND BASILAR MEMBRANE. C. ORGANOTYPIC CULTURE D. HAIR CELLS REVEALED BY IMMUNOFLORESCENCE WITH ANTIBODY AGAINST MYOVIIA

Two Corti dissections obtained from the same pups were used for RNA extraction. RT-PCR and sequencing of bands extracted from the gel confirmed the expression of all selected genes within the Organ of Corti (Fig29).

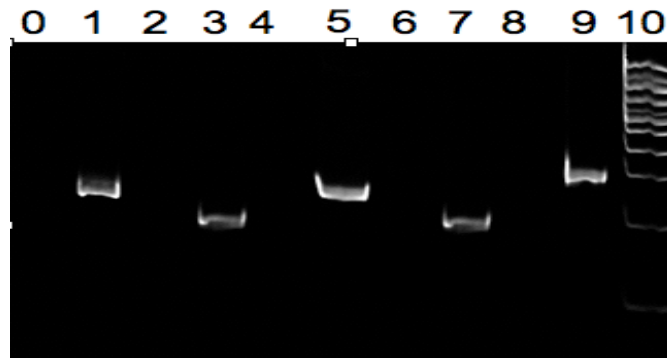


Figure (29). RT-PCR from Mouse dissected Cochlea. Electrophoresis run using 10 ul of RT-PCR product on 8% acrilamide gel. Lane 10 contains 100bp marker. Odd Lanes (1,3,5,7,9) contain respectively Slc22a5 250bp, FoxG1 200 bp , Hdac2 240 bp, DR1 190bp and Polr3h 290bp Rt-PCR + amplification while the pair Lanes (0,2,4,6,8) the respective RT-PCR - controls.

5.5 Real-time validation of microarray data

In order to validate the data obtained from the microarrays experiments, the 5 candidates gene expressed in the mouse Corti organ were analyzed by real-time RT-PCR. The obtained value of $\Delta\Delta CT$ was used to obtain fold variations. The data obtained confirmed that the genes DR1 and Slc22a5 are indeed down regulated after transfection with the construct containing the W44S mutant but not with the wild type and 35delG mutant forms. The Hdac2 gene showed a slight down regulation in the 35delG mutant transfection and a significant downregulation in the W44S mutant transfection. The genes FoxG1 and Polr3h did not present any variation of expression when comoparing the mutants with respect to the wild type forms.

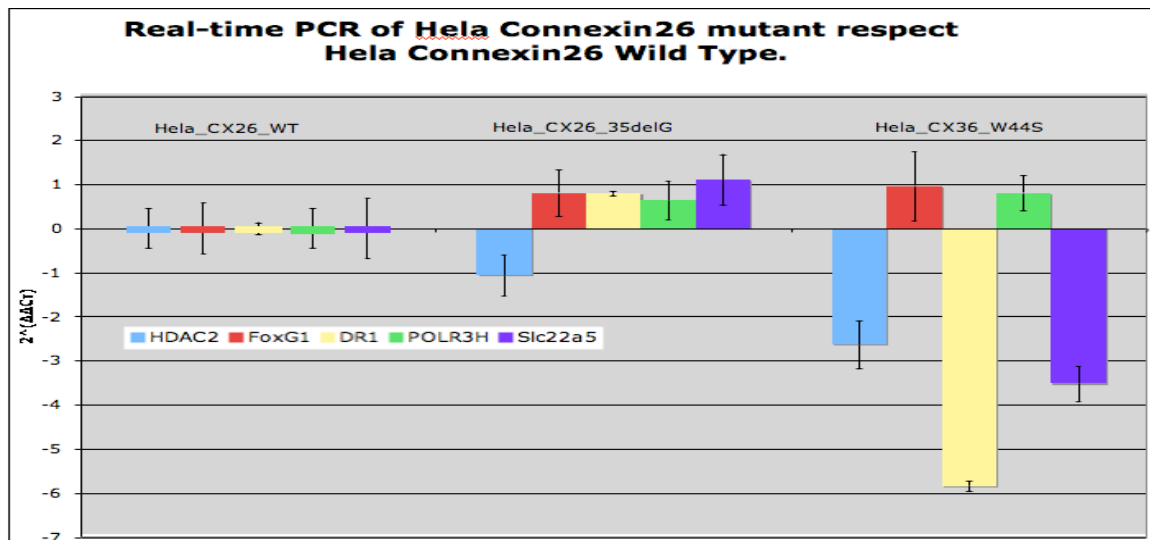


FIGURE (30). FOLD CHANGE RESULTS OF REAL-TIME PCR EXPERIMENT. GRAPH SHOWING THE FOLD CHANGE DATA OF VALIDATION REAL-TIME RT-PCR EXPERIMENTS COMPARING THE LEVELS OF EXPRESSION OF FIVE CANDIDATE GENES (HDAC2, FOXG1, DR1, POLR3H AND SLC22A5) BETWEEN HELA CELLS TRANSFECTED WITH WT CONNEXIN26 VS CELLS TRANSFECTED WITH 35DELG AND W44S MUTANTS OF THE SAME GENE. FOLD CHANGE RESULTS SHOW SIGNIFICANT DOWNREGULATION OF GENES DR1 AND SLC22A5 IN THE W44S MUTANT TRANSFECTION, WHILE THE LEVELS OF EXPRESSION OF FOXG1 AND POLR3H GENES DO NOT SEEM TO BE AFFECTED. THE HDAC2 GENE IS SIGNIFICANTLY DOWNREGULATED IN THE W44S MUTANT TRASFECTON, AND ONLY SLIGHTLY DOWNREGULATED IN THE 35DELG MUTANT.

6. Discussion

6.1 Wild Type and Mutants of Human Connexin26 as a starting point for the project

During the last 12 years the central role of connexin proteins in the auditory function has been broadly demonstrated, as well as their role (including the principal role of connexin 26) in genetic deafness diseases. (Carrasquillo et al., 1997; Estivill, 2008; Gasparini et al., 1997; Grifa et al., 1999; Kelsell et al., 1997; Richard et al., 2000; Zelante et al., 1997). Thus we decided to verify if the transient transfection of human connexin26 mutant protein could induce changes in gene expression that might indicate other important actors in the biology and pathology of hearing. This approach was based on the assumption (not so far demonstrated) that the activity of the mutant form of connexin26 could disrupt, as an indirect effect, the expression of genes potentially involved in the biology of hearing and thus perhaps in deafness. We chose to focus on two specific mutants, Connexin26 W44S and 35delG based on their relevance in the dominant and recessive forms of the disease.

In all the experiments within this project the transfections performed were made in the transient form, raising a potential problem with regards to the proportion of cells successfully transfected. Variations in transfection efficiency would among the experimental replicates could lead to differences which would be due not to biologically relevant phenomena, but to merely to changes in transfection levels. For this reason transfections I performed several transfections and chose only three that would show comparable efficiency. However, I was only able to verify efficiency appropriately in the Connexin26 W44S mutant because it expresses the full-length protein. The Connexin26 35delG mutant, on the other hand, produces a short peptide which cannot be easily detected neither at the RNA level nor at the protein level. Thus, although transfections were performed in a similar manner for all three constructs, the HeLa cells transfected with the 35delG mutant, could not be verified and were therefore not taken into account for later stages of the project aimed at selecting candidate genes.

6.2 Whole genome microarray experiments.

The data produced on two different microarray platforms showed clearly that the transfection of mutant forms of the Connexin26 gene have a significant effect on transcription, as compared to the transfection with the wild type form of Connexin 26. It is well known that microarray outputs are subject to substantial variability even under relatively well controlled experimental conditions (Lee et al., 2000). Moreover different analysis algorithms (such as the commercial

Rosetta Resolver algorithms and the open source R Bioconductor LIMMA package used in this project) lead to different results in terms of differentially expressed genes (Shi et al., 2006). This could explain the different number of genes resulting to be differentially expressed in the Bioconductor analysis with respect to the analysis performed with commercial software. It is important to note that the analyses performed used different normalization, different algorithms and different p-value cut-offs between the Agilent and Illumina microarrays (local background and Lowess regression with $p\text{-value} < 0.01$ for the Agilent data; background subtraction and quantile normalization with $p\text{-value} < 0.05$ for the Illumina ones). Moreover the two platforms rely on different types of chemistry (i.e one color for the Illumina platform and two color for the Agilent one), which also impacts the final differences observed in the results of the microarray experiments.

6.3 Platform comparison.

Existing literature about comparisons of data obtained from different platforms showed that different microarray technologies do not give concordant results in many cases (Barnes et al., 2005; Kuo et al., 2002; Mah et al., 2004; Wang et al., 2005). Currently the platforms used are both considered reliable, thus a robust approach has to be used to compare results across them. For this reason I compared the two datasets using the Unigene cluster ID, as representation of unique genes for both platforms. As a consequence, this process lowered the number of the analyzable genes from Agilent, due to the presence of multiple probes for the same unigene cluster, whereas it raised the number of Illumina genes due to the fact that Illumina usually provides multiple probes for the same gene often mapping to more than one Unigene cluster.

This is probably a good procedure but not the best. Different approaches have been used during the years and due to the characteristics of both platforms (i.e probes of 50-60bps) a better result could be obtained by mapping the probe sequences on the human genome and taking into account only the overlapping ones (Cheadle et al., 2007), or taking into account overlap with genome annotation (such as Ensembl genes). For these reasons, major commercial vendors now release the exact sequence of each of their probes and the database release version used for the probe design.

An important bioconductor resource (NuID) has been released (Du et al., 2007) to face the cross-platform comparisons. NuID is a tool available for Illumina probes, that creates a non-degenerate encoding scheme that can be used as a universal representation to identify an oligonucleotide across manufacturers based on the raw sequence of the oligonucleotide for the true definition of identity for a probe. Unfortunately, though, this has not yet been implemented for Agilent

probes. I'm now trying to implement this latest approach for better cross-platform comparisons for future application of this project.

6.4 Data mining and locus survey.

Given the low amount of overlap between the two datasets, I decided to use both of the original datasets independently for further bioinformatics analysis. I used an *in silico* strategy to assemble a list of candidate genes that are positionally linked to, and could be causing, specific non-syndromic hereditary hearing loss conditions. As presented in the results, the positionally linked genes helped me to narrow a subsets of 1,055 Illumina and 1,978 Agilent genes to a list of 189 genes for the Illumina platform and 144 for Agilent. The filter for the presence of a candidate gene within the cDNA library dataset and the Human Cochlear EST dataset further narrowed the list of genes to 19 candidate genes within 11 loci.

It warrants mention that the potential of the approach presented here will be better harnessed as more information becomes available about inner ear transcripts and as soon as more microarrays experiments, regarding known deafness genes, become public available. The importance of integrating information about candidate deafness genes that are positionally linked has been showed, by an article published in the end of 2006 (Alsaber et al., 2006). In this methodological article, utilizing a similar approach, they integrated protein-protein interaction data, cochlear gene expression data and genetic locus data to select candidates for non-syndromic hereditary hearing loss. However, even if they further narrowed the candidate genes list thanks to the knowledge of protein-protein interactions, they did not test any of the candidates experimentally. Moreover the resource was never published publicly. Previous attempts to integrate existing microarray data with genes within candidate genetic loci had also been made in 2002 for the Leigh syndrome, French-Canadian type (Giallourakis et al., 2005). In contrast with the approach used here they analyzed four unrelated diseases utilizing public RNA expression data sets together with proteomics datasets and intersecting this information with the genes of a single relevant locus, identifying a single candidate gene and finally identifying two mutations in two independent haplotypes.

6.5 Mouse Corti expression of selected genes

To confirm the expression of the selected genes not in the whole inner ear but in the part directly involved in sound transduction, we decided to perform RT-PCR to verify the presence of these genes in the organ of Corti. It would have been ideal to verify expression in human cochlear RNA, but unfortunately this was not very feasible. Firstly it is difficult to obtain RNA from human inner ear (i.e. it is not routinely taken as sample during surgery). Moreover the bony labyrinthine channels located in the temporal bone and the ossification of the otic capsule makes difficult to dissect the cochlear duct without disrupting the hair cells. This problem practically limits the collection only to Human fetal inner ear, as has been done in the past (Morton, 2002, 2004). Moreover the hair cells are highly sensible to neuronal stimuli. In mouse models it has been shown that direct somatic injury (laser or mechanical) inflicted on hair cells does not necessarily cause their death. By contrast, ablation of their afferent spiral ganglion neurons causes a most spectacular degeneration of sensory cells within 18 hours after the insult (Sobkowitz et al., 1999, 2003). This explains the difficulties to obtain good Hair cells from autopsies.

To avoid all these problems we decided to use an animal model to analyze the candidate genes. Among the various species normally used for deafness, the mouse model has been one of the better investigated. Moreover during the last years, deafness mouse models enabled the identification of many deafness genes (Steel, 2001). Even if this approach reduced the investigation to only those genes that showed one to one corresponding homologous between mouse and human, the possibility to use mouse pups for cochlear dissection as starting material, provided a good physiological model and the capability to narrow and identify the cells that could be used. Indeed to be sure that the extraction has been performed correctly, half of the dissection has been used for organotypic culture of cochlea and checked by immunofluorescence. The experimental data confirmed the expression of the selected orthologous genes into the Corti organ strengthening the idea of their value as potential candidate genes.

6.6 Microarray Data validation

Even if during the last years microarray platforms have successfully evolved to a new state of accepted technological maturity with regards to reliability and consistency, an independent validation of the results of a microarray experiment is still necessary especially when the p-value threshold used has not been strict. Experimental validations are essential because microarray data is inherently noisy. The large numbers of gene expression measurements obtained in a typical microarray experiment, by virtue of their sheer numbers, can often yield significant numbers of false positive and negative results. Such artifacts can arise due to numerous experimental and technical reasons, including spurious signals caused by microarray probes, cross-hybridizing to related transcripts of similar sequence, artifacts induced by the sample preparation technique ecc. For all these reasons it is essential to have the resources to perform at least a measure of independent validation. The Real Time Quantitative PCR (qPCR) procedure is an extremely powerful technique that is fast becoming the accepted ‘gold standard’ in the field, for validation of microarray results. Among the 5 selected genes verified by qPCR, the genes *FoxG1* and *Polr3h* are clearly false positive and not differentially expressed between the mutant and wild type form. On the contrary the genes *Slc22a5*, *Hdac2* and *DR1* result to be effectively downregulated in Hela Connexin26 W44S mutants with respect to Hela Connexin26 wild type. In particular the fold change is lower with respect to the one obtained by qPCR according to some literature data (Hwang et al., 2002; Morey et al., 2006). Standard criteria to determine acceptable validation of microarray results is not available, for example Rajeevan et al. (Rajeevan et al., 2001) considered a result valid if the fold change measured by both qPCR and microarray were greater than or equal to 2-fold while Svaren et al. (Svaren et al., 2000) takes into account the magnitude of difference between the measurements. In my specific case using both the approach esthe *Slc22a5*, and *DR1* genes result to be validated.

6.7 Future prospects

The mutation analysis and confirmation of the segregation within families will be the natural evolution of my PhD project. To proceed with the mutation analysis, collaboration with groups of clinical researchers that identified the loci and are in posses of the DNA samples and clinical data of the patients will be required. In particular the candidate gene *DR1* obtained for the locus DFNB32 overlap also with DFNA37 locus and seems particularly interesting. The locus has been identified in 2003 on 10 individuals from a large consanguineous Tunisian family with congenital profound autosomal recessive deafness (Masmoudi et al., 2003). Many genes inside the region have been screened without finding mutations, while mine obtained candidate gene has never been screened.

The same applies to the candidate gene *Slc22a5* included in the locus DFNB74 (data unpublished). This gene is included in the large “Solute carrier family” (Hediger et al., 2004) that includes more than 290 transporter genes (including *Slc26a4* and *Slc26a5* which had already been identified as deafness genes, within the locus DFNB4) (Li et al., 1998; Liu et al., 2003b). It is also interesting to note that another solute carrier gene, *Slc38a6*, has been selected trough this integrated analysis, for the autosomal dominant locus DFNA23 (Salam et al., 2000), even though it has not been validated jet.

7. Conclusion

The work presented in this thesis was aimed to narrow the candidate genes potentially involved in Non-Syndromic Hearing Loss using a combination of biological and bioinformatics approach that can reduce the candidate genes to a manageable number for mutation analysis. From the biological point of view I focused my attention on the GJB2 gene. The GJB2 gene encodes for the gap junction protein Connexin26 and is responsible for more than half of the non-syndromic hearing loss cases. For this reason it has been proposed that this protein plays a wider role than its mere channel function. During my PhD project I performed whole genome expression profiles in cells transfected with the wild type form of the GJB2 gene as compared to cells transfected with mutant forms to shed light on his function. In order to narrow down the results obtained with my microarray experiments I have used an in silico strategy to assemble a list of candidate genes that are positionally linked to and could be causing specific non-syndromic hereditary hearing loss conditions. As presented in this thesis, a list of 4,984 genes mapping to deafness genomic intervals have been narrowed down to 19 genes as candidates for 11 loci. Among those 19 genes, 5 candidates have been tested for the expression of their homologs in the mouse organ of corti and further verified by Real-time PCR. In particular two interesting candidate genes for the loci DFNB32 and DFNB72 have been related with the connexin26 W44S mutant transfection and confirmed by the expression profiling experiment. The mutation analysis and confirmation of the segregation within a family for the candidate genes will be the natural evolution of my PhD project to identify new Non-Syndromic Hearing Loss Genes. In order to perform the mutation screening, collaboration with clinical researchers should be established to collect the DNA samples that have been used to map the original loci.

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