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Diverse deafness mechanisms of connexin mutations revealed by studies using *in vitro* approaches and mouse models

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Abstract

Mutations in connexins (Cxs), the constitutive protein subunits of gap junction (GJ) intercellular channels, are one of the most common human genetic defects that cause severe prelingual non-syndromic hearing impairments. Many subtypes of Cxs (e.g., Cxs 26, 29, 30, 31, 43) and pannexins (Panxs) are expressed in the cochlea where they contribute to the formation of a GJ-based intercellular communication network. Cx26 and Cx30 are the predominant cochlear Cxs and they co-assemble in most GJ plaques to form hybrid GJs. The cellular localization of specific Cx subtypes provides a basis for understanding the molecular structure of GJs and hemichannels in the cochlea. Information about the interactions among the various co-assembled Cx partners is critical to appreciate the functional consequences of various types of genetic mutations. *In vitro* studies of reconstituted GJs in cell lines have yielded surprisingly heterogeneous mechanisms of dysfunction caused by various Cx mutations. Availability of multiple lines of Cx-mutant mouse models has provided some insight into the pathogenesis processes in the cochlea of deaf mice. Here we summarize recent advances in understanding the structure and function of cochlear GJs and give a critical review of current findings obtained from both *in vitro* studies and mouse models on the mechanisms of Cx mutations that lead to cell death in the cochlea and hearing loss.

Keywords

inherited deafness; gap junction function; connexin mutations; review; deafness mechanism

1. Introduction

Gap junctions (GJs) are intercellular membrane channels that possess the unique feature of directly connecting the cytoplasm of neighboring cells. GJs connect cells electrically when they are open, acting like opened ion channels to generate high conductance pathways, a phenomenon at the basis of electrical synapses (Bennett and Zukin, 2004). Unique to GJs is their ability to allow small molecules (cut-off molecular weight at ~1,000 Daltons), such as

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second messengers (e.g., cAMP, IP_3) and intracellular metabolites (e.g., glucose, ATP), to diffuse down their concentration gradients (Evans and Martin, 2002). GJs are formed by the juxtaposition of two hexameric structures called hemichannels (or connexons) at the GJ plaques, where a large number of GJs cluster at the cell-cell contact points. Before two hemichannels are aligned to form a whole GJ, they may perform functions independent of those carried out by GJs (Goodenough and Paul, 2003).

GJs are found in both invertebrates (Cruciani and Mikalsen, 2007) and vertebrates (Cruciani and Mikalsen, 2006; Evans and Martin, 2002). Invertebrate GJs are assembled from innexins (Phelan et al., 1998). Vertebrate GJs are formed by the assembly of six compatible connexin (Cx) subunits (Willecke et al., 2002). All Cx subtypes share a common topology that includes four transmembrane domains, two extracellular and one intracellular loop. Both amino and carboxyl termini of all Cxs are located on the cytoplasmic side of the membrane. Innexins and Cxs generally share little sequence similarity. However, another group of GJ subunits with homologies to the innexin family, called pannexins, are also found in the vertebrates (Baranova et al., 2004; Panchin et al., 2000). By allowing electrochemical as well as biochemical coupling between cells, GJs generally function to maintain tissue homeostasis and to allow fast intercellular electrical communication. Many fundamental biological processes require GJs (Lo, 1996) and the importance of these unique intercellular channels are demonstrated by the linkage of their mutations to a wide spectrum of human diseases, such as peripheral neuropathies (e.g. the X–linked Charcot-Marie-Tooth disease) (Bergoffen et al., 1993), various skin disorders (Richard, 2000), cataracts (White, 2002), oculodental dysplasia (Paznekas et al., 2003) and deafness (Chang et al., 2003; Rabionet et al., 2002).

In the cochlea, GJs were first revealed in the 1970s by ultrastructural observations (Forge, 1984; Iurato et al., 1977; Jahnke, 1975; Laciano et al., 1977) that suggested the existence of a “functional syncytium” among cochlear supporting cells. Intercellular electrical communication consistent with the existence of GJs was later demonstrated by patch-clamp recordings (Santos-Sacchi and Dallos, 1983; Zhao and Santos-Sacchi, 2000). Immunolabeling studies identified various types of Cxs in the cochlea as the molecular building blocks of GJs (Kikuchi et al., 1995; Lautermann et al., 1998; Tang et al., 2006; Xia et al., 2000). The essential role of GJs in the hearing process has been highlighted by a large number of genetic studies linking mutations (supplemental Table 1) in Cx genes to inherited deafness (Ballana et al., http://davinci.crg.es/deafness/). More than half of congenital deafness cases are caused by genetic mutations (Petit, 2006; Smith et al., 2005). Currently, at least 46 genes are known to cause hearing impairments in humans (Hilgert et al., 2008) and many more are suggested by animal studies. Strikingly, mutations in a single gene (*GJB2*, which codes for Cx26) account for a large proportion (up to 50%) of inherited prelingual non-syndromic deafness cases in almost all ethnic populations studied (supplemental Table 1). It is established that mutations in Cx genes are one of the most common forms of human genetic defects resulting in hearing losses in millions of patients with either autosomal dominant or recessive deafness (Chang et al., 2003; Denoyelle et al., 1997; Estivill et al., 1998; Kelsell et al., 1997). Carrier rate of various disease-causing Cx26 mutations is estimated to be 1–4% in many populations, which makes the *GJB2* one of the most common disease-linked genes in humans (supplemental Table 1). In addition to *GJB2*, mutations in *GJB6* (coding for Cx30) (Grifa et al., 1999) and *GJB3* (coding for Cx31) (Liu et al., 2000; Xia et al., 1998) are known to cause hereditary deafness in humans. Other deafness-linked Cx candidates include *GJB1* (coding for Cx32) (Bergoffen et al., 1993), *GJE1* (Cx29) (Yang et al., 2007) and *GJA1* (Cx43) (Liu et al., 2001).

Many of the Cx subtypes in the ~20 mammalian Cx genes (Sohl and Willecke, 2004) are expressed in the cochlea. Cx26 and Cx30 are the two predominant cochlear Cx subtypes in terms of their cellular distributions and reported mutational effects for human hearing. General reviews on GJ nomenclature (Sohl and Willecke, 2003), structure (Sosinsky and Nicholson,

2005; Yeager and Harris, 2007) and function (Evans and Martin, 2002; Laird, 2006; Nicholson, 2003)}, regulations of expressions (Laird, 2006; Oyamada et al., 2005; Saez et al., 2003)}, biophysical properties (Alexander and Goldberg, 2003; Goldberg et al., 2004) have been previously published. A few recent reviews on GJs in the cochlea are available (Martinez et al., 2009; Nickel and Forge, 2008; Zhao et al., 2006). This review focuses on the molecular structural basis of hemichannels and GJ-mediated intercellular communication network in the cochlea, as well as on the diverse mechanisms for deafness caused by various human Cx mutations (Table 1 and Fig. 2). A classification of Cx26 mutations based on *in vitro* functional studies of reconstituted GJs is presented (Table 1). Advances in understanding deafness mechanisms by studying multiple Cx-mutant mouse models (Table 2) and current theories about the mechanisms of deafness caused by Cx mutations are also critically reviewed.

2. Molecular structural basis of GJ networks in the cochlea

Cx26 and Cx30 are the two major Cx subtypes in the cochlea that co-assemble to form GJs

The molecular composition of GJs determines their unitary conductance, gating and rectification properties, and influence permeability and intracellular trafficking of hemichannels (Elfgang et al., 1995; Niessen et al., 2000; Rackauskas et al., 2007a; Rackauskas et al., 2007b; White and Bruzzone, 1996). Furthermore, defining the molecular assembly of cochlear GJs is essential for understanding functional consequences of Cx mutations. For example, Cx26 and Cx30 are colocalized in most cochlear GJ plaques and coimmunoprecipitation of the two Cxs suggest they coassemble in cochlear GJs (Ahmad et al., 2003; Forge et al., 2003a). Fig. 1 illustrates some of the possible molecular configurations of GJs when two Cxs are coassembled. If most GJs are heteromeric in the cochlea, a direct functional implication is that cochlear GJs are not necessarily eliminated by null expression of either Cx26 or Cx30 individually (Fig. 1). The functional properties and the number of remaining homomeric GJs, however, may differ significantly from that of the native Cx26/Cx30 hybrid GJ channels (Sun et. al., 2005; Jagger and Forge, 2006; Yum et. al., 2007).

At the mRNA level, gene transcription profiles of Cxs in the cochlea have been investigated by low-density gene array (Ahmad et al., 2003) and *in situ* hybridization (Buniello et al., 2004) approaches. Dot-blot analyses (Ahmad et al., 2003) revealed the presence of Cxs 26, 29, 30, 31 and 43. In addition, mRNA of Cx30.2, Cx37 and Cx46 was detected in the cochlea by *in situ* hybridization (Buniello et al., 2004). However, mRNA transcript levels do not necessarily faithfully reflect protein expressions (Nelson and Keller, 2007). Therefore, we will mainly examine expressions of Cxs in the cochlea based on immunolabeling and western blot results in the following sections.

Cx26 and Cx30

Immunolabeling of Cx26 (Kikuchi et al., 1995) and Cx30 (Lautermann et al., 1998) in the cochlea of adult rats localized the Cxs in the spiral limbus, the spiral ligaments, the supporting cells of the organ of Corti and the stria vascularis. Although the low-resolution images of co-immunolabeling did not show colocalization at the level of single GJ plaque (Lautermann et al., 1998), the results suggested a similar cellular expression pattern for Cx26 and Cx30 in the cochlea. Higher resolution images, at the level of a single GJ plaque observed either at optical (Ahmad et al., 2003; Forge et al., 2003a; Sun et al., 2005) or electron (Forge et al., 2003a) microscope levels, were later obtained. Results demonstrate extensive co-localization of Cx26 and Cx30 immunoreactivities in most (>85%) cochlear GJ plaques (Sun et al., 2005). In the adult mouse cochlea, the only region that showed minimal Cx26 and Cx30 colocalization (5%) was in the Deiters' cells, where expression of Cx30 dominated. Direct interactions of the two Cxs have been demonstrated by co-immunoprecipitation (Ahmad et al., 2003; Forge et al., 2003a), further supporting that Cx26 and Cx30 are co-assembled in the same GJs. General co-

localization of Cx26 and Cx30 in cochlear GJs seems to be a universal phenomenon found in many animal species, including rats (Lautermann et al., 1999), mice (Sun et al., 2005), guinea pigs (Zhao and Yu, 2006), as well as in humans (Liu et al., 2009). These two predominant cochlear Cxs also co-localize in the saccule, utricle, and cristae of the vestibular organs, where Cx26 and Cx30 are found in most GJ plaques in supporting cells and connective tissue cells (Qu et al., 2007).

During the development, embryologic expressions of Cx26 and Cx30 in the human cochlea were detected as early as 11 weeks of gestation (Kammen-Jolly et al., 2001) and adult level was reached at week 20 (Kammen-Jolly et al., 2001; Lautermann et al., 1999). In the prenatal mouse cochlea, these Cxs are sparsely expressed and the early pattern differed significantly from that of the adult (Lautermann et al., 1999; Sun et al., 2005). Both Cxs are detected as early as E14.5 (14.5 days of gestation) (Sun et al., 2005) and the immunoreactivities of the two Cxs are generally found in the same cochlear regions at all developmental stages. From E14.5 until early postnatal days, both Cx26 and Cx30 expressions are absent from the sensory epithelia of the developing organ of Corti. They are found in cells of the nascent spiral limbus and in part of the lateral wall. Before the onset of hearing, strong expressions of the two Cxs are found in the spiral limbus and in a band of fibrocytes adjacent to the basal cells of the stria vascularis (Sun et al., 2005). Expression in supporting cells gradually intensifies, giving a dynamic pattern during postnatal development of the organ of Corti. At the onset of hearing, Cx26 and Cx30 expressions in the lateral wall quickly changes from primarily a band of cells bordering the stria vascularis to almost all the cells above the spiral ligament. Studies agree that Cx26 and Cx30 are not expressed in both inner and outer hair cells, nor in marginal cells of the stria vascularis (Ahmad et al., 2003; Forge et al., 2003a; Frenz and Van De Water, 2000; Kikuchi et al., 1995; Lautermann et al., 1998; Liu and Zhao, 2008; Zhao and Yu, 2006). Whether intermediate cells of the stria vascularis express functional level of Cx26 and Cx30 is still controversial. Compared to their expression levels in basal cells of the stria vascularis, a majority of studies (Ahmad et al., 2003; Forge et al., 2003a; Wangemann et al., 2004; Xia et al., 1999) found a significantly lower, if any, expression of Cx26 and Cx30 in the intermediate cells, although a positive labeling result was also reported (Liu and Zhao, 2008).

Cx29

Cx29 is mostly expressed by myelinated glial cells (e.g., oligodendrocytes, Bergmann astroglia cells and Schwann cells), but not by astrocytes (Altevogt et al., 2002; Eiberger et al., 2006; Kleopa et al., 2004; Nagy et al., 2003). In the cochlea, Cx29 mRNA expression was first detected by cDNA dot-blot hybridization (Ahmad et al., 2003). Immunolabeling of Cx29 in wild-type (WT) mice and localization of the *LacZ* reporter gene in Cx29 null mice indicate that Cx29 is highly expressed in the cochlear Schwann cells ensheathing the afferent fibers of the eighth nerve up to the glial junction (Eiberger et al., 2006; Tang et al., 2006). After the afferent auditory fibers enter the brainstem, they are surrounded by astrocytes which are not labelled by Cx29 antibody. Low levels of Cx29 in the stria vascularis were also detected by immunolabeling (Eiberger et al., 2006; Tang et al., 2006).

Functional examinations of the Cx29 null mice (Tang et al., 2006) indicate that the absence of the Cx29 gene, with a penetrance of about 50%, causes a delay in the maturation of hearing. Auditory thresholds of about half of the Cx29 null mice tested at 3 weeks postnatally were at least two standard deviations above the averaged results of Cx29 WT littermate controls. By 6 weeks of age, however, most of the Cx29 null mice tested (13 out of 16) showed hearing thresholds not statistically different from WT controls. Hearing thresholds measured by another group from Cx29 null mice at 4–10 weeks postnatally also show no difference comparing to WT animals (Eiberger et al., 2006). However, as the Cx29 null mice mature to 6 months, they display early loss of high-frequency sensitivities. An elevated susceptibility to noise at high

frequencies (12, 18 & 24 kHz) is also observed. Cochlear morphology examined at the electron microscopic level show specific demyelination of the soma, but not the fibers, of the spiral ganglion neurons. Tang *et al*. (Tang et al., 2006) suggests that Cx29 is a candidate gene to study auditory neuropathies. Currently, few studies have screened human mutations in the Cx29 gene. Interestingly, one study in Taiwan reported Cx29 mutations in some non-syndromic deaf patients (Yang et al., 2007).

Cx31

Cx31 is one of the earliest Cx genes expressed in the embryo (Dahl et al., 1996). At adult stages, its expressions are found in the skin (Hoh et al., 1991), cochlea (Xia et al., 2000), peripheral auditory nerve (Lopez-Bigas et al., 2001), seminiferous epithelium of rat testes (Mok et al., 1999) and placenta (Plum et al., 2001).

Although genetic data linking mutations in Cx31 to deafness are strong, its cellular expression in the rodent cochlea is still controversial. Two studies, one observing the expression of the *LacZ* reporter gene that replaces Cx31 gene in Cx31 null mice (Plum et al., 2001) and the other using immunolabeling (Lautermann et al., 1998), failed to detect Cx31 expression in the cochlea. On the other hand, Cx31 mRNA transcripts have been detected in the cochlea by cDNA macroarray hybridization (Ahmad et al., 2003), *in situ* hybridization (Lopez-Bigas et al., 2002) and RT-PCR amplifications (Forge et al., 2003a; Xia et al., 2000). However, cellular patterns of Cx31 in the cochlea, as detected by immunolabeling, show poor consensus among published results. Cx31 was localized among type II fibrocytes below the spiral prominence where both Cx26 and Cx30 appear to be weakly expressed (Forge et al., 2003a). Other studies also found Cx31 in fibrocytes of the spiral ligament and spiral limbus (Xia et al., 2000), and in supporting cells of the organ of Corti (Liu et al., 2008).

Human mutations in Cx31 have been linked to the skin disorder erythrokeratodermia variabilis (Richard et al., 1998a) as well as to autosomal dominant (Xia et al., 1998) and recessive (Liu et al., 2000; Uyguner et al., 2003) non-syndromic deafness. However, these phenotypes did not correlate well with the corresponding mouse model. Cx31 null mice displayed a transiently abnormal placental development and a reduced viability (60%) of homozygote embryos, but neither epidermis nor auditory malfunctions were observed in the surviving mice (Plum et al., 2001). Human genetic studies also revealed an interaction between Cx26 and Cx31. *GJB3* mutations occurring in compound heterozygosity with the *GJB2* mutations have been identified in three unrelated Chinese families. Direct interaction of Cx26 with Cx31 has been shown by coimmunoprecipitation, supporting the idea of an interaction between these two Cxs that results in hearing loss in human digenic heterozygotes (Liu et al., 2008).

Cx32

Cx32 is generally expressed in oligodendrocytes and Schwann cells. It is believed to contribute to the myelination process and to participate in the K^+ buffering during neuronal activities. Genetic mutations in *GJB1* were the first to be associated with a human disease, the X-linked Charcot-Marie-Tooth disease, which is a demyelinating neuropathy (Bergoffen et al., 1993).

Cx32 expression has been studied during cochlear development by *in situ* hybridization (Lopez-Bigas et al., 2002) and by immunocytochemistry from the adult cochlea (Tang et al., 2006). Cx32 mRNA transcripts have been detected as early as E12 in the otocyst. At neonatal stages until P7 (one week after birth), expression was widespread in the cochlea. Labeling obtained from cochleae older than P13 restricted Cx32 to type II and IV fibrocytes of the spiral ligament. In the adult cochlea, however, no Cx32 transcripts were detected (Lopez-Bigas et al., 2002). These findings were corroborated by another study (Forge et al., 2003a) in which RT-PCR amplifications from mouse mature cochleae (6–8 week-old) and immunoblots failed

to detect Cx32 expression. In the adult cochlea Tang et al. (Tang et al., 2006) reported Cx32 expression in astrocytes located outside the glial juncture, which are the cells surrounding the central portion of the auditory nerve fibers in the brainstem. In support of a minor role played by Cx32 in auditory functions, no severe hearing loss in *Gjb1*$^{-/-}$ mice was reported (Scherer et al., 1998).

Cx43

Cx43 is widely expressed in the human body (Laird, 2006). In the mouse cochlea, its expression has been investigated by cDNA dot-blot hybridization (Ahmad et al., 2003), RT-PCR amplification and western blotting (Forge et al., 2003a). Its cellular localization has been studied by immunolabeling (Lautermann et al., 1998) and by the *LacZ* reporter gene expression in Cx43 null mice (Cohen-Salmon et al., 2004b). By localizing the *LacZ* reporter expression pattern, Cohen-Salmon *et al.* showed that Cx43 is expressed in the cochlea as early as E15.5. During early development, Cx43 expression is more widespread than in mature stage, with staining observed in fibrocytes in the later wall, mesenchymal cells below the basilar membrane, and capillaries in the stria vascularis. In the adult cochlea, Cx43 is localized to the cochlear bony shell only (Cohen-Salmon et al., 2004b). Inconsistent results are reported by other groups about the Cx43 expression in the cochlea (Lautermann et al., 1998; Liu et al., 2001; Suzuki et al., 2003). Liu *et al*. (Liu et al., 2001) reported that mutations in *GJA1* are linked to deafness in the African American population. However, later studies showed that the reported mutations (L11F, V24A) may be located in *GJA1* pseudogene on chromosome 5 (Paznekas et al., 2003).

Cx45

Cx45 expression has not been detected by RT-PCR amplifications in mature mouse cochleae or by western blotting (Forge et al., 2003a). However, Cx45 expression has been reported by studying the expression of the *LacZ* reporter gene in Cx45 null mice (Cohen-Salmon et al., 2004a). During the development, expression of *LacZ* reporter was detected as early as E17.5. One day later, all cochlear cells, apart from the hair cells, were labelled (Cohen-Salmon et al., 2004a). Starting at P4, *LacZ* reporter expression increased in capillaries. By P8, expression remained only in capillaries and mesenchymal cells lining the basilar membrane.

Pannexins—Three subtypes of Panxs (Panx1, Panx2 and Panx3) have been reported (Panchin et al., 2000). Functional expression of Panxs in *Xenopus* oocytes indicated that at least some Panxs can form functional intercellular GJ channels and hemichannels (e.g., homotypic Panx1 and heterotypic Panx1/Panx2) (Bruzzone et al., 2003a; Bruzzone et al., 2005). In the cochlea, Panxs 1 and 2 have been detected by immunoblots and RT-PCR amplifications. Immunolabeling localized Panx1 to the inner and outer sulcus cells, as well as to the Claudius cells. Additionally, both Panxs are expressed in the spiral ganglion and Scarpa's ganglion neurons (Tang et al., 2008). A more widespread cochlear expression of Panx1 and Panx2 has been reported and the expression of Panx3 has also been detected in the cochlear bone by a recent study (Wang et al., 2009).

3. Functional classifications of various types of deafness-linked Cx mutations

So far, more than 100 mutations associated with human deafness have been identified in the coding region of the Cx26 gene (Ballana et al., 2005; Chang et al., 2003; White et al., 1998). Deafness linked mutations in the regulatory region of Cx26 have also been reported (Wilch et al., 2006). The locations of some of the reported human Cx26 mutations are summarized in Fig. 2. Classifications of Cx mutations may be based on structural alterations (e.g., truncation and frame shift vs. single amino acid substitution). Truncation and frame-shift mutations (e.g.,

35delG, E147X) which only produce partial Cx26 protein, represents about 28% of Cx26 mutants illustrated in Fig. 2. Most of the Cx26 mutations (~79% shown in Fig. 2) belong to the category of point mutations (e.g., R75Q, L214P, delE42) that are produced by a single base substitution or in-frame deletion in the Cx26 coding sequence. It is interesting to note that all autosomal dominantly inherited Cx26 mutations found so far are linked to Cx26 point mutations. Many of them also cause skin disorders (Lee et al., 2008). In addition, at least four human Cx30 point mutations, including T5M (Grifa et al., 1999), 63delG, G11R, A88V (Common et al., 2002; Lamartine et al., 2000; Xia et al., 1998) and two large deletion mutations in *Gjb6* (del Castillo et al., 2002; Lerer et al., 2001; Pallares-Ruiz et al., 2002) have been linked to deafness. Since most functional studies focused on Cx26 and Cx30 mutations, this section will present a summary of their functional effects based mainly on results obtained from *in vitro* approaches.

The first step of *in vitro* functional studies is to reconstitute WT or mutant GJs in a heterologous system by either injecting mRNA into oocytes, or by transfecting cell lines lacking endogenous GJs (e.g., HEK293 or Hela cells). By transfecting cells with plasmid constructs containing the Cx coding sequence fused in frame to that of the enhanced green fluorescent protein (eGFP) or any other fluorescent proteins, homomeric and hybrid GJ plaques can be directly identified *in vitro* (Sun et al., 2005). GJ functions can then be assessed by hemichannel dye loading and single cell dye injection assays, and by double-electrode patch-clamp recording and optical recording methods (Guo et al., 2008; Hernandez et al., 2007; Sun et al., 2005; Yum et al., 2007; Zhang et al., 2005; Zhao et al., 2005). Large numbers of *in vitro* studies (Table 1) suggest that the effects of various Cx26 mutations can be classified into at least four distinct mechanisms according to their effects on GJ functions.

I. Mutations preventing the formation of GJs in the cell membrane

The life cycle of Cxs includes protein synthesis, trafficking/targeting to plasma membrane, membrane insertion and assembly into connexins, and degradation (Laird, 2006). Cx mutations belonging to this category may cause dysfunction in any of the steps, or premature degradation of Cxs before they reach the cell membrane. Potentially, this type of Cx26 mutations could also affect binding of Cx26 with other intracellular partners that normally interact with the Cx protein subunit.

II. Mutations resulting in GJ formation with null functions

Both intercellular ionic and biochemical coupling are lost for Cx26 mutants belonging to this category, although they still form GJs in the cell membrane. Most mutants in this group also lose hemichannel activities. However, mutations at the two extracellular loops of Cxs may specifically affect the docking/alignment of two connexons. Thus, hemichannel permeability may be intact.

III. Mutations resulting in a specific loss of intercellular biochemical coupling

A subgroup of structurally-mild Cx26 mutations, most of them located in the second transmembrane domain, selectively affect the permeation of molecules larger than simple ions. Although *in vitro* studies identified a specific loss of GJ-mediated permeability to inositol 1,4,5-trisphosphate (IP_3) (Beltramello et al., 2005; Zhang et al., 2005), whether it is a major molecule required for the *in vivo* function of cochlear GJs is unclear. Using Cx30 null mice, Chang *et al*. showed a dramatic reduction of GJ-mediated glucose transportation and elevated free radical concentrations in cochlear supporting cells (Chang et al., 2008). Similar deficiency in GJ-mediated biochemical coupling could happen in the cochlea of Cx26 mutant mice, although this hypothesis has not been directly tested yet. Since glucose is the major energy source for cellular metabolic activities, a chronic shortage of glucose in the organ of Corti

where microcirculation is generally poor may have extensive damaging effects on cell survival and functions.

IV. Mutations causing a gain-of-function effect: abnormal hemichannel opening at resting state

One deafness-linked Cx26 mutation, G45E, has been linked to a fatal form of keratitis–ichthyosis–deafness syndrome (Griffith et al., 2006; Janecke et al., 2005). Morphological examinations revealed that this mutation disrupts cochlear differentiation and causes dysplasia of the cochlear and saccular neuroepithelium (Griffith et al., 2006). G45 (Fig. 2) is located in the first extracellular loop, next to an aspartic acid, a previously reported Ca^{++} binding site for the hemichannels (Gomez-Hernandez et al., 2003). The G45E mutation changes the charge of the amino acid side chain from neutral to negative, therefore it is likely to affect Ca^{++} binding to the hemichannels. Stong *et al.* (Stong et al., 2006) reported that G45E mutation resulted in apoptosis and cell death within 24 hours of transfection. Increasing the extracellular Ca^{++} concentration ($[Ca^{++}]_o$) rescued the transfected cells in a dose-dependent manner. Dye loading assay suggest that the Cx26 G45E mutation causes leaky GJ hemichannels when cells are bathed in normal $[Ca^{++}]_o$, which overloads the cellular homeostatic mechanisms and ultimately leads to cell death. Other Cx26 mutations in this category are also reported by other groups (Gerido et al., 2007; Lee et al., 2008; Matos et al., 2008). The Cx26 mutants belonging to this category usually show dramatic phenotypes including death (Griffith et al., 2006; Janecke et al., 2005). Interestingly, a mutation in Cx32 that results in the formation of leaky hemichannels has been found to be responsible for a severe type of neuropathy due to imbalanced ions and metabolites (Liang et al., 2005). Evidence of the presence of functional hemichannels within the cochlea is suggested by membrane-impermeable fluorescent dye uptake assays carried on dissociated cochlear cells and in acute or cultured preparation of the cochlear epithelium (Zhao et al., 2005; Anselmi et al., 2008). These studies suggest that one of the functions of cochlear hemichannels is to release ATP into the extracellular space, which could modulate the electromotility of outer hair cells and therefore exert a control on hearing sensitivity.

The fifth category of Cx26 mutants (Table 1) can form functional GJs, but no apparent impairment in intercellular coupling is detected by *in vitro* assays. They are likely to represent polymorphism in the Cx26 coding region. The Cx26 mutations in the sixth category are reported human mutations apparently linked to deafness, but they are not tested by thorough *in vitro* studies yet. Finally, mutations in the non-coding region of Cx26 are also linked to deafness in patients (Wilch et al., 2006).

4. Mouse models of Cx mutations display diverse pathogenesis processes in the cochlea

In vitro studies suggest that deafness-linked Cx mutations can be classified into two general categories, loss-of-function (categories I, II &III in Table 1) and gain-of-function (category IV in Table 1) mutations. Four Cx mutant mouse models are generated either by targeted deletion of Cx genes that results in null expression (Cohen-Salmon et al., 2002;Teubner et al., 2003) or by expression of a dominant-negative Cx26 mutant protein (R75W) (Kudo et al., 2003;Maeda et al., 2007). These animal models are appropriate for studying *in vivo* effects of Cx mutations belonging to categories I & II (Table 1). Currently, mouse models for investigating Cx26 mutants belonging to categories III & IV are not yet available. Genetic deletion of *Gjb6* is achieved by replacing the Cx30 gene with a reporter gene *LacZ* and a neo resistance cassette (Teubner et al., 2003). Germline deletion of *Gjb2* is embryonically lethal due to ~60% reduction in GJ-mediated glucose transfer across the placenta (Gabriel et al., 1998), The problem is circumvented by utilizing a Cre-loxP system (Cohen-Salmon et al., 2002;Kudo et al., 2003). In another study, transient expression of the dominant-negative Cx26

R75W mutant protein in the cochlea of adult mice was achieved by lipofection through the round window route (Maeda et al., 2007). Table 2 summarizes major findings obtained from these animal models. All mouse models show significant hearing loss. The most severe threshold elevations are displayed by Cx30 null and Cx26 R75W mice, with hearing thresholds measured at over 100 dB SPL in adult mice (Kudo et al., 2003;Teubner et al., 2003). Only a 15–20 dB threshold increase is detected transiently by click ABR in the model reported by Maeda et al. (Maeda et al., 2007). In general, none of the mouse models display obvious endolymphatic hydrops and degeneration of stria vascularis when observed at the level of light microscope. Hair cell and supporting cell loss after the time of hearing onset are observed in all mouse models (Cohen-Salmon et al., 2002;Kudo et al., 2003;Teubner et al., 2003).

No gross developmental defects in cochlear morphology are detected in both Cx26 and Cx30 mutant mice, suggesting that the two cochlear GJs do not play essential roles in cochlear development. One interesting exception is that the opening of the tunnel of Corti and the Nuel's space, which normally happens around P9 just before the onset of hearing, is absent in the Cx26R75W mutant mice (Inoshita et al., 2008; Kudo et al., 2003). Another surprising difference is the effect on the EP generation between the Cx26 and Cx30 mutant mice. Normally, the EP starts to develop around P5 in mice and reaches adult-like value at P11 to P20 depending on the location in the cochlea (Sadanaga and Morimitsu, 1995). The EP develops normally either initially in one model (Cohen-Salmon et al., 2002) or even reaches normal level in the adult stage in the Cx26 R75W mice (Kudo et al., 2003). In contrast, EP is never developed in the Cx30 null mice (Teubner et al., 2003). Normal EP suggests that GJ-mediated ionic coupling is not affected by the Cx26 mutations, which is not consistent with the K^+ recycling hypothesis for the Cx26 mutations (Kikuchi et al., 1995).

In the study by Cohen-Salmon *et al*. (Cohen-Salmon et al., 2002), null expression of Cx26 in the cochlea is targeted specifically to the epithelial GJ system. In contrast, the expression of R75W dominant-negative Cx26 mutant is driven by a ubiquitous CAG promoter (Kudo et al., 2003), which theoretically results in the mutant expression in the whole cochlea including cells that normally do not express Cxs. The precise cellular pattern of the mutant expression, however, is unknown. In general, the phenotypes (in terms of both hearing loss and morphological deteriorations) displayed by Cx26 R75W mice are more severe than those shown by the conditional Cx26 null mice, in which a floxed *Gjb2* is specifically deleted by *Otog*-driven Cre. The deafness phenotypes displayed in human patients are extremely heterogeneous. Although in most cases deafness caused by Cx26 mutations is congenital, some patients may not show hearing impairments until a few months after birth (Orzan and Murgia, 2007; Pagarkar et al., 2006). Some of the phenotypic differences in various mouse models may be due to technical approaches used in different studies to generate mutant mice, or caused by heterogeneous deafness mechanisms. The surprisingly different effects on the EP generation observed between the Cx26 and Cx30 null mice also hint that the underlying deafness mechanisms may not necessarily be the same, despite the observations that Cx26 and Cx30 are coassembled in most cochlear GJs (Sun et al., 2005). Until more Cx mutant mouse models are generated and validated by independent methods, clear answers to these questions will not be possible. In addition, mouse models for Cx mutants in categories III and IV are not currently available. More Cx26 mutant mouse models are certainly needed for further understanding molecular mechanisms of the most common form of inherited deafness in humans.

5. Current theories on mechanisms of deafness caused by Cx26 and Cx30 mutations

The extracellular fluid in the endolymphatic space of the cochlea has a high K^+ concentration ($[K^+]_o$) that is similar to normal intracellular $[K^+]_o$ found in most cells. The ~160 mM of extracellular $[K^+]_o$ and ~+80 mV positive potential in the endolymph (endolymphatic potential,

or EP) give an unusual electrochemical environment on the apical side of hair cells that is essential for the sensitive mechanical transduction of hair cells (Wangemann, 2002). Clinical phenotypes of deafness caused by most Cx mutations are non-syndromic (Chang et al., 2003). Expressions of both Cx26 and Cx30, however, are widespread in the body (Sohl and Willecke, 2004). In order to explain the distinctive phenotype of deafness, investigators generally link GJ dysfunctions to disturbance of the unique endocochlear environment. Many theories focus on possible scenarios about how the maintenance of high concentration of K^+ in the scala media and/or endolymphatic potential (EP) could be disrupted. A more recent hypothesis (Chang et al., 2008) considers the contribution of cochlear GJ network to homeostasis of the avascular sensory epithelium of the organ of Corti. Chang et al. found that GJs in the cochlear supporting cells play a vital role in maintaining biochemical coupling and delivering glucose to these cells.

a) Disruption of cochlear K^+ recycling theory

During the auditory transduction, endolymphatic K^+ enters hair cells through mechanotransducton channels. The intracellular K^+ concentration is balanced by the exit through hair cells' basolateral K^+ channels (e.g. Kcnq4) (Wangemann, 2002). Since high extracellular K^+ is generally considered toxic and data support that K^+ is recycled back to the endolymph (Konishi et al., 1978; Sterkers et al., 1982), the K^+ ions around the base of hair cells are believed to be quickly absorbed by cochlear supporting cells and recycled back to the endolymph (Kikuchi et al., 1995; Spicer and Schulte, 1996). Because no local mechanisms of returning K^+ ions back to the endolymph seem to exist in the cochlear supporting cells and the apparent source of generating high $[K^+]_o$ of the endolymph is in the stria vascularis, K^+ ions are thought to be transported through a relatively long route, first along the epithelial cell GJ network to the spiral ligament, then through the connective tissue GJ system in the lateral wall and finally are moved by the stria vascularis back to the endolymphatic space (Kikuchi et al., 1995; Zhao et al., 2006). However, direct measurements of current flux show an alternative route for K^+ recycling. A standing current in the perilymph of the scala tympani can be measured exiting the basilar membrane and flowing towards the spiral ligament in the scala tympani (Zidanic and Brownell, 1990). The current suggest that K^+ ions in the scala tympani are absorbed back to the connective tissue GJ system in the lateral wall, which bypasses the epithelial GJ system located in the sensory epithelium. In support of the K^+ route through the epithelial GJ network, however, targeted deletion of Cx26 specifically in the sensory epithelia of the cochlea clearly shows that GJ coupling in the cochlear supporting cells is required for normal hearing (Cohen-Salmon et al., 2002). In addition, the driving force and the active membrane mechanisms that move K^+ laterally through the GJs of the sensory epithelium and then upward through the lateral wall towards the stria vascularis are unclear. Immunolabeling studies provide indirect support that some necessary active mechanisms (e.g., ion pumps and transporters) exist along the proposed recycling route (Crouch et al., 1997; Schulte and Adams, 1989; Spicer and Schulte, 1996).

Contrary to the notion that mutation of cochlear GJs disrupts K^+ recycling, recent data indicate that the intercellular conduit provided by GJs is not significantly disrupted by the absence of either Cx26 or Cx30 individually. Immunolabeling data obtained from the cochlea of conditional Cx26 (Cohen-Salmon et al., 2002) and Cx30 (Chang et al., 2008; Teubner et al., 2003) null mice support the existence of homomeric GJs in the cochlea of mutant mice. Double-electrode patch clamp recordings made from Cx30 null mice demonstrated that the ionic coupling among the cochlear supporting cells is indistinguishable from that of WT animals (Chang et al., 2008), suggesting that the GJ-mediated intercellular K^+ movement is not significantly affected in the epithelial GJ system of the Cx30 null mice. Additionally, Cx26 mutations specifically affecting biochemical coupling (e.g., V84L, V95M, and A88S) are sufficient to cause deafness in humans (Beltramello et al., 2005; Zhang et al., 2005), indicating

that loss of GJ-mediated transfer of molecules larger than K^+ ions may underlie the deafness mechanism. The most direct evidence against a disruption of K^+ recycling as the basis for Cx-mutation-linked deafness is the finding that the EP is normal in the deaf Cx26R75W mutant mice (Kudo et al., 2003).

b) Endothelial barrier breakage theory

In Cx30 null mice, the time course of cell death in the organ of Corti substantially lags that of hearing loss, indicating that hair cell loss is not directly responsible for deafness. One major finding is that the EP is never formed in these mutant mice (Teubner et al., 2003). It is well known that loss of EP directly results in deafness (Flagella et al., 1999; Gow et al., 2004; Kitajiri et al., 2004; Marcus et al., 2002). To investigate mechanism for the failure in EP generation, Cohen-Salmon *et al*. (Cohen-Salmon et al., 2007) reported that ion channels and transporters required for EP generation (e.g., KCNQ1, KCNE1, KCNJ10, and H^+/K^+-ATPase) and tight junctions that enclose the intrastrial fluid space appeared to be normal in the Cx30 null mice. Further examination at the electron microscope level showed that the endothelial barrier of the capillaries in the stria vascularis was disrupted before the EP is developed. Conceptually, damaged endothelial cells lining the microvessels in the stria vascularis could provide a short-circuit leak conductance to overload the EP generation machinery in the stria vascularis. The electric shunt is believed to be sufficient to account for the total loss of EP in Cx30 null mice. However, neither Cx26 nor Cx30 are expressed in the endothelial cells in the stria vascularis (Cohen-Salmon et al., 2007). It is therefore unclear how GJs dysfunctions can result in damages to the endothelial barrier, although a marker for endothelial dysfunction (an increase in homocysteine) has been found. Moreover, the report of a normal EP in deaf Cx26R75W mutant mice (Kudo et al., 2003) indicates that this electric shunt theory may not be generalized to explain deafness caused by all Cx mutations. Apparently, more experiments are needed to test the relatively new theory of deafness caused by a breakdown of endothelial barrier in the stria vascularis.

c) Deficiency in GJ-facilitated metabolite transportation theory

The sensory epithelium of the organ of Corti is an avascular organ where direct microcirculation to hair cells and supporting cells is lacking. In contrast, GJs co-assembled from Cx26 and Cx30 are highly expressed in the non-sensory cells of this region (Ahmad et al., 2003; Forge et al., 2003a; Kikuchi et al., 1995; Sun et al., 2005). Recent studies directly demonstrated that glucose from the cardiovascular circulation could reach the cochlear supporting cells (Chang et al., 2008), fibrocytes in the lateral wall (Suzuki et al., 2008) and spiral limbus (Matsunami et al., 2006) in a GJ-dependent manner. Chang et al. reported (Chang et al., 2008) that GJ-mediated intercellular diffusion of many fluorescent tracers among cochlear supporting cells of Cx30 null mice, including a fluorescent analogue of glucose (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose, or 2-NBDG), is dramatically reduced. In contrast, ionic coupling among the same group of cells measured directly by two-electrode patch-clamp recordings show no change of the intercellular conductance by the deletion of the *Gjb6* gene. These results suggest that a chronic shortage of glucose, but not an eradication of the pathway for K^+ recycling, exists in the cochlea of Cx30 null mice.

In general, O_2 consumed by mitochondria is reduced fully to water and only about 2% of electrons leak out of the oxidative chain to generate superoxide anions (O_2^-) and H_2O_2. Deficiency in glucose supply exacerbates ATP exhaustion and increases the generation of reactive oxygen species (ROS) (Moley and Mueckler, 2000). Co-assembly of Cxs in cochlear GJs indicates that a total elimination of GJ-mediated intercellular biochemical and ionic coupling is an unlikely consequence (Fig. 1). Decreased glucose transportation through GJs and increased ROS production are directly detected in cochlear supporting cells of Cx30 null mice. Based on these results, Chang et al. (Chang et al., 2008) proposed that deafness linked

to loss-of-function Cx mutations is caused by a reduction in the efficiency in delivering energetic metabolites (e.g., glucose) through the GJ intercellular network, especially in cochlear regions where microcirculation is poor (e.g., the organ of Corti). The further speculate that the accumulated damaging effects to the cellular homeostasis become destructive when large amount of ROS is generated that ultimately lead to cell death and cochlear dysfunction.

6. Conclusions and perspectives

It is clear that we are only at the beginning stage of revealing the molecular mechanisms of deafness caused by Cx mutations. Evidences support that Cx26, Cx30 and perhaps Cx31 are the major Cx subtypes present in both epithelial and connective tissue GJ networks in the cochlea. Other Cxs and Panxs (Cx29, Cx43, Panx1 and Panx2) are either expressed in cochlear Schwann cells, neuronal cells, or capillary cells that do not play a direct role in the auditory transduction or EP generation. Co-immunolabeling and co-immunoprecipitation data support that the combinations of Cx26 and Cx30 (Ahmad et al., 2003; Forge et al., 2003b; Yum et al., 2007), Cx26 and Cx31 (Liu et al., 2008) are co-assembled in cochlear GJs. As pointed out earlier, a direct functional implication draw from these structural studies is that targeted deletion of one Cx gene is unlikely to totally eliminate GJ-mediated ionic coupling in the cochlea. In addition, co-assembly of cochlear GJs complicates interpretations of the inheritance patterns of Cx mutational effects. Studies have reported that at least some Cx26 recessive mutations have transdominant effects on Cx30 (Marziano et al., 2003). This transdominant effect on Cx30 is, however, unlikely to be universally true for all Cx26 mutations. More investigations are certainly needed to understand the meanings of genetic dominant and recessive inheritance patterns under the context of heteromeric GJs. Although most studies show that Cx26 and Cx30 generally co-localize in the cochlea, the relative proportion of GJs that exist in each molecular configurations (Fig. 1), their precise locations in the cochlea and whether the proportion is dynamically regulated during development or after injury are unclear. If Cx26 and Cx30 are not expressed in a synchronized manner during certain development stages or after stress/injuries, it is possible that a local elimination of GJ-mediated intercellular communication may occur temporally or spatially. Thus, further investigations into the temporal and spatial expression patterns of various subtypes of Cxs and their protein interactions are indispensible information for advancing the studies on the function of cochlear GJs. Other urgently-needed investigations concern the regulatory mechanisms of *Gjb2* and *Gjb6* gene expression and their interactions. Some recent *in vitro* studies have begun to address these issues (Ortolano et al., 2008). Cx26 over-expression in Cx30 null mice completely rescues hearing in these deaf mice (Ahmad et al., 2007), suggesting a novel therapeutic strategy for Cx30 null expression patients. However, the translation of the finding into clinical applications is not possible until the genetic regulatory mechanisms of Cx26 and Cx30 are fully understood and a safe and effective pharmacological intervention method is found.

Development of better mouse models will greatly help further testing the new theories about deafness mechanisms caused by Cx mutations (Cohen-Salmon et al., 2007; Martinez et al., 2009) and various aspects of the glucose deficiency hypothesis (Chang et al., 2008). The efforts should help to answer whether Cx26 play any significant roles in cochlear development, which is uniquely demonstrated by Cx26 R75W mutant mice (Inoshita et al., 2008). According to the hypothesis proposed by Chang et al. (Chang et al., 2008), reduction in glycolysis due to glucose shortage should decrease concentration of ATP and mitochondrial ROS production should increase in the cochlea of Cx mutant mice. Cell death should first occur in the most vulnerable regions of the sensory epithelium, particularly at the onset of hearing when cellular energy demand rapidly increases. All these aspects need to be tested experimentally. It is interesting to note that a recent paper proposed a similar general scheme in GJ-connected astroglia metabolic network in the brain to efficiently delivers energetic metabolites from blood vessels to distal neurons in an activity-dependent manner (Rouach et al., 2008). The observations that

Cx-mutation-linked defects tend to happen in areas where either microcirculation is poor (e.g., lens in the eyes (White and Paul, 1999)) or transportation of metabolites via GJ intercellular coupling is demanding (e.g., placenta (Gabriel et al., 1998)) suggest a unified theory that dysfunctions of GJ-mediated metabolite transfer underlie many pathogenesis processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2-NBDG
: 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose

Cx
: connexin

eGFP
: enhanced green fluorescent protein

EP
: endolymphatic potential

GJ
: gap junction

IP_3
: inositol 1,4,5-trisphosphate

Panxs
: pannexins

ROS
: reactive oxygen species

WT
: wild type

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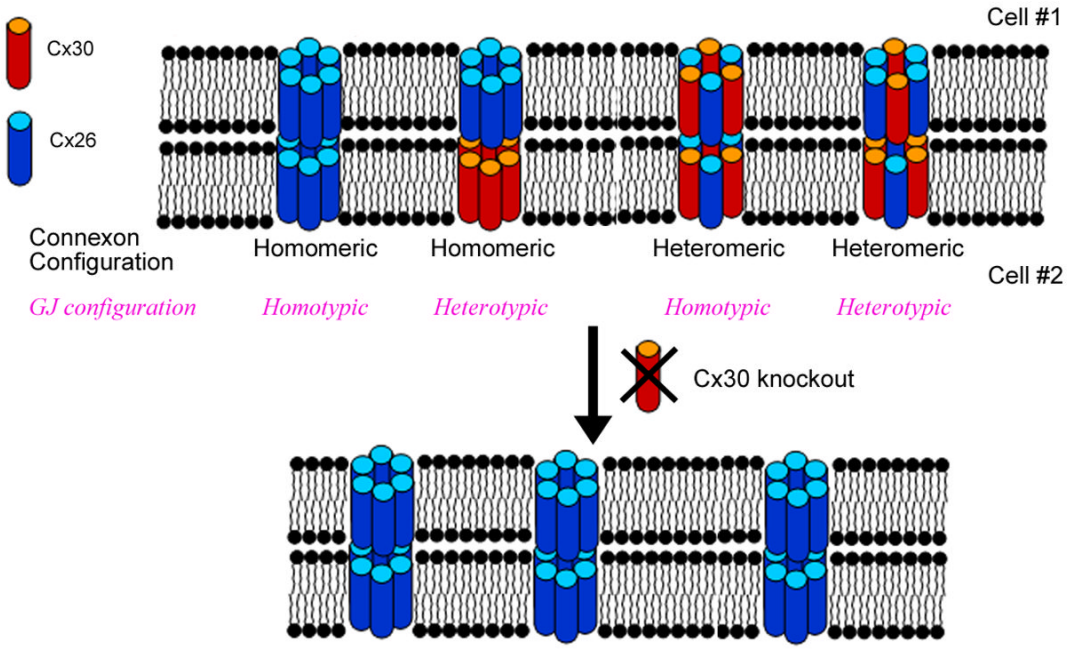

Figure 1.

A diagram showing molecular configurations of co-assembled cochlear GJs from Cx26 and Cx30 in the cell membrane and the predicted effect of genetic knockout of *GJB6*. Cxs26 and 30 normally co-assemble into GJs in the cochlea. Connexons are called homomeric or heteromeric, respectively, depending on whether a single or more than one Cx subtype are used as building blocks. A heterotypic GJ channel is formed by the docking of two different connexons whereas a homotypic channel is constituted from the same connexons. The ablation of a single Cx subtype may eliminate GJs in regions where it is the only Cx expressed. However, significantly amounts of GJs may remain in areas where co-asssembly of Cxs dominates, although disruption of the expression of one Cx subtype is likely to affect biophysical properties of GJs that are highly dependant on the composition Cxs. The number of GJs may also be reduced as a gene dosage effect.

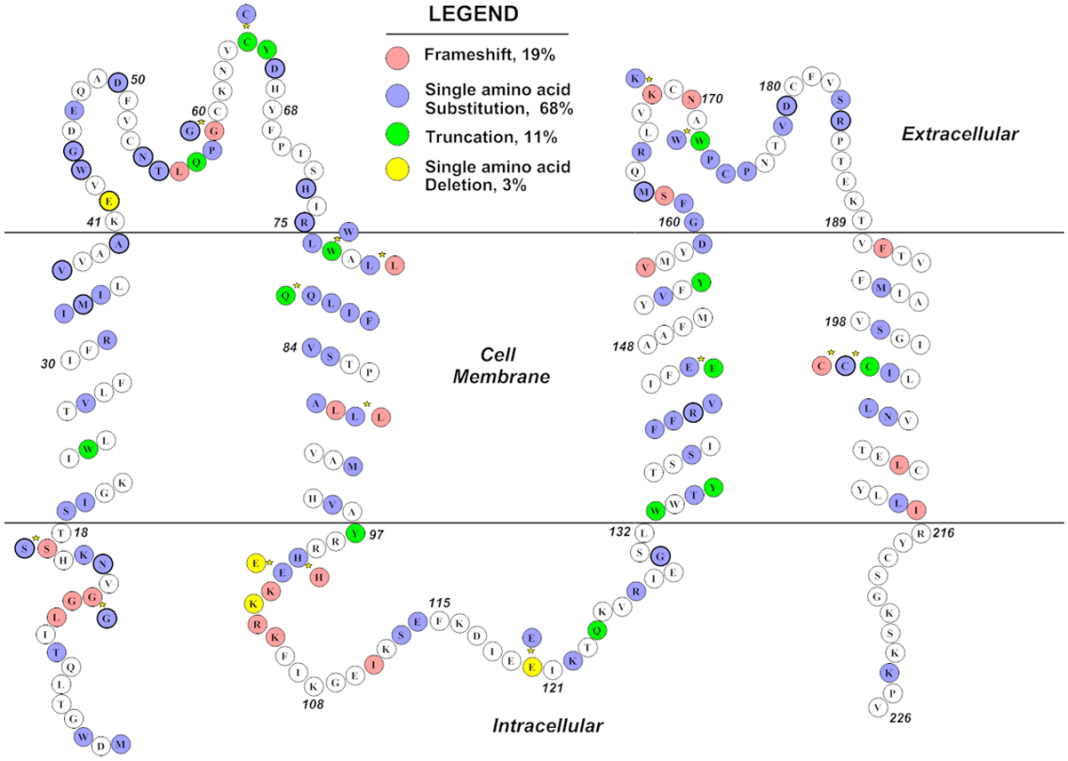

Figure 2.

A diagram showing the location of reported human Cx26 mutations in the Cx26 protein that are linked to hearing loss. Four types of mutations (frame shift, truncation, single amino acid substitution or in-frame deletion) are color coded. Stars denote the location where more than one types of mutations are reported. References for these deafness-linked Cx26 mutations are given in the Table 1 and supplemental Table 1.

Table 1

Classifications of Cx26 mutations linked to deafness in human patients according to data obtained from *in vitro* studies.

Classification	Cx 26 mutations	References
I	M1V, N14D, 30delG, 35delG, I20T, I35S, DeltaE42,, D50Y, D50N, T55N, G59A, G59V, C64S, D66H, H73R, I82M, L90P, Y136X, V153I, M163V, 167delT, P173R, D179N, R184P, L214P, 235delC, E147K, F142L	(Brown et al., 2003; Bruzzone et al., 2003; Choung et al., 2002; de Zwart-Storm et al., 2008a; de Zwart-Storm et al., 2008b; Di et al., 2005; Frei et al., 2004; Haack et al., 2006; Kudo et al., 2000; Kupka et al., 2000; Mani et al., 2008; Marziano et al., 2003; Melchionda et al., 2005; Mese et al., 2004; Oshima et al., 2003; Palmada et al., 2006; Primignani et al., 2003; Rouan et al., 2001; Thomas et al., 2004; Thonnissen et al., 2002; Yotsumoto et al., 2003; Zelante et al., 1997; Zhang et al., 2005)
II	G12R, N14K, W24X, I33T, M34 T, del 42, V37I, A40V, W44C, W44S, E47 K, R75W, R75D, R75Q, W77R, C119T, E120del, T135A, F161S, W172R, R184P	(Beahm et al., 2006; Bicego et al., 2006; Bruzzone et al., 2001; Bruzzone et al., 2003; Chen et al., 2005; D'Andrea et al., 2002; Evans et al., 1999; Gerido et al., 2007; Griffith et al., 2000; Lee et al., 2008; Mani et al., 2008; Martin et al., 1999; Marziano et al., 2003; Montgomery et al., 2004; Oshima et al., 2003; Piazza et al., 2005; Purnick et al., 2000; Richard et al., 1998; Richard et al., 2002; Rouan et al., 2001; Skerrett et al., 2004; Stong et al., 2006; Thonnissen et al., 2002; White et al., 1998)
III	T8M, G12V, N14Y, S19T, M34A, F83L, V84L, A88S, V95M, R127H, N206S,	(Arita et al., 2006; Beltramello et al., 2005; Bruzzone et al., 2003; D'Andrea et al., 2002; Lee et al., 2008; Mese et al., 2004; Mese et al., 2008; Oshima et al., 2003; Thonnissen et al., 2002; Wang et al., 2003; Zhang et al., 2005)
IV	G45E, M163L, G12R, D50N, S17F	(Gerido et al., 2007; Lee et al., 2008; Matos et al., 2008; Stong et al., 2006)
V	E114G, R143W, I128I, S183F, Q80R, V27I, V37I, I203T, c.- 15C>T, p.Met34Thr, p.Ala40Ala, p.Gly160Ser	(Batissoco et al., 2009; Choung et al., 2002; de Zwart-Storm et al., 2008a; de Zwart-Storm et al., 2008b; Kudo et al., 2000; Mese et al., 2004; Prasad et al., 2000; Wang et al., 2003)
VI	R32C, P58A, K168R, N54K, L205P, 465T-->A, 31del14, S113P, S199F, C202F, K224Q, F142L, 313del14, 176–191 del (16), p.Lys168Arg, c. 684C>A, p.Leu81Val (c.G241C), p.Met195Val (c.A583G), Q80K and P173S, S199F, T55G, D159V, 605ins46, 313del14, 355del9, 573delCA	(Antoniadi et al., 2000; Batissoco et al., 2009; Brown et al., 2003; Christiani et al., 2007; Gualandi et al., 2004; Gualandi et al., 2002; Kalay et al., 2005; Kelley et al., 1998; Kudo et al., 2000; Kupka et al., 2002; Leshinsky-Silver et al., 2005; Marlin et al., 2005; Murgia et al., 1999; Prasad et al., 2000; Primignani et al., 2007; Tamayo et al., 2009; Tekin et al., 2003; Uyguner et al., 2003; Xiao and Xie, 2004; Yuge et al., 2002)

Class I mutations are mutations preventing the formation of GJs. Class II comprises mutations that do not affect formation of GJs, but the mutated GJs display null functions. Class III refers to mutations specifically impaire the GJ-mediated biochemical coupling. Class IV consists of mutations causing a gain-of-function due to abnormal hemichannel openings. Mutations without reported functional effects on GJ functions (likely to represent polymorphism) are grouped in category V. Finally, class VI consists of mutations that have not been thoroughly studied in vitro. Detailed criteria for the classification are given in the text.

Table 2

Comparison of results obtained from various Cx26 and Cx30 mutant mouse models

Animal models	Cx30 null	cCx26 null	Cx26R75W-Kudo	Cx26R75W-Maeda
Reference of the first report	(Teubner et al., 2003)	(Cohen-Salmon et al., 2002)	(Kudo et al., 2003)	(Maeda et al., 2007)
Approach used	Targeted replacement of *Gjb6* by LacZ/neo	*Gjb2* is flanked by loxP. Excision of *Gjb2* by otogelin-driven Cre expression from a BAC	hCx26 R75W is expressed under universal CAG promoter	p*GJB2*$_{R75W}$-eGFP plasmid (CMV promoter) delivered by lipofection applied to the round window membrane
Time and location of Cx deletion	Germline deletion of *Gjb6*	*Gjb2* presumably is deleted at E10 in sensory epithelium of the cochlea	The dominant-negative *Gjb2* mutant is presumably expressed before the first meiotic division. Detailed cellular pattern of expression is unknown	The dominant-negative *Gjb2* mutant is expressed in adult cochlea. Many cochlear cells expressed the mutant Cx as detected by the GFP immunolabeling
Inner and outer hair cell loss	Hair cell losses begin at the third week postnatally. and increase gradually with age. Outer hair cells are affected first and more severely	Gross morphology of inner hair cells appears to be normal in most animals. Outer hair cell loss starts at P15. The two most internal rows are affected first	Inner hair cells are present but show changed shape. Outer hair cells are present at P14 but show shape changes and they degenerated at the seventh week	No hair cell loss and hearing loss is transient. Auditory sensitivities recover in 5 days after introducing mutant
Vestibular morphology	Vestibular hair cell loss specifically in the saccule is observed (Qu et al., 2007)	Normal up at least to P60	Normal by functional assessment	No data reported
Supporting cell loss	Not degenerated	Initial damage observed at P15	Initial damage observed at P14	Not affected
Is there SG neuron loss?	No description provided.	No SG neuron degeneration observed	Degeneration of SG neurons in basal turn noted at seventh week postnatally	
Is the opening of the tunnel of Corti affected?	No	No	Yes (Inoshita et al., 2008)	
Hearing threshold elevation	At P17–18, click ABR threshold elevation is about is 50 dB. Adult mice show no ABRs at >100 dB	About 30 dB elevation at the most sensitive frequencies on average.	Greater than 100 dB threshold elevation	About 15–20 dB threshold elevation as assessed by click ABR
EP value	At P13/P14: 0±4 vs. 74 ±9mV in control mice. In adults: 3±3 vs. 148±15 mV in controls	At P12–13: 56 ±12 vs. 58 ±12mV in control mice. In adults: 38±14 *vs*. 110±12mV in controls	In adults: 87±2.5 vs. 97.4±7.1 mV in control mice	
Endolymphatic K^+ concentration	At P13/P14: 100±39 vs. 102±24 mM in control mice. In adults: 44±19 *vs*. 148±15mM	In adult: 85±21 vs. 153±7mM in the control mice		
Morphology changes in the organ of Corti, spiral limbus, stria vascularis, firbrocytes in the lateral wall	No gross morphological changes in stria vascularis, lateral wall is observed. No displacement of	Disruption of the reticular lamina, missing of some interdental cells. Gross cochlea	No gross changes in gross cochlear morphology observed. No opening of the	

Animal models	Cx30 null	cCx26 null	Cx26R75W-Kudo	Cx26R75W-Maeda
	Reissner's membrane observed	structure appear to be normal	tunnel of Corti. The Nuel's space is absent. Microtubule abnormality in Inner pillar cells.	