行政院國家科學委員會專題研究計畫　成果報告

造成聽障之 Connexin 基因族與 CLAUDIN14 基因突變的功能研究

計畫類別：個別型計畫
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執行期間：94 年 08 月 01 日至 95 年 07 月 31 日
執行單位：中山醫學大學生物醫學科學學系

計畫主持人：李宣佑

計畫參與人員：楊建洲、洪惠媚、余如珊、楊舒婷、張嘉真

報告類型：精簡報告

處理方式：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中　華　民　國 95 年 10 月 26 日

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

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處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
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一、中文摘要

目前已知有59個基因的突變會導致聽障，各基因的致病機制不盡相同，非常複雜。*Connexin*(*Cx*)基因族和 CLAUDIN14 基因在耳蝸內影響離子濃度恆定性和造成遺傳性聽障上扮演重要角色。故在聽障研究方面，*Cx* 和 *CLAUDIN14* 在耳蝸的表現及功能的探討為相當重要的一環。因此本年度計畫的研究主要是探討 *Cx29* 在耳蝸內的表現和 *CLAUDIN14* 基因的突變後對其功能所造成的影響，以瞭解 *Cx29* 在耳蝸內的表現功能和 *CLAUDIN14* 基因在聽障中的致病機制。

我們想了解 *Cx29* 基因在老鼠耳蝸中的表現位置，故以免疫組織染色法(Immunohistochmistry)和雷射顯微擷取法(Laser Capture Microdissection)來觀察 *Cx29* 的蛋白 gje1(gap junction membrane channel protein epsilon 1)在老鼠耳蝸存在的組織部位，實驗結果發現 *Cx29* 表現於 stria vascularis、spiral limbus、spiral ligament 以及 organ of Corti 部位，這些組織對耳蝸內淋巴液中高鉀低鈉的離子特性維持扮演著相當重要的角色。

在 *CLAUDIN14* 基因研究方面我們發現 52A>G/wt (M18V) 和 167-168delGG 突變後的蛋白無法形成 tight junction，其突變蛋白是均勻散布或堆積在細胞質，而 424G →A/w t (D142N)的突變蛋白可以和正常的 *CLAUDIN14* 蛋白一樣運送到細胞膜形成 tight junction。

這些資料庫的建立和功能分析系統的建立將有助我們對聽障的致病成因和機轉有更進一步的瞭解。

關鍵詞：語言學習前聽障、*Cx29*、免疫組織染色法、雷射顯微擷取法、*CLAUDIN14*、功能研究

Abstract

To date, 59 auditory genes have been identified, among which are those involved in K^+ recycling and maintenance. The importance of K^+ recycling and maintenance is underscored by the fact that mutations in each of *Connexin* gene family, and *CLAUDIN14* leads to deafness in human. In the proposed project, we will focus on study of *Cx29* expression on the cochlea and the effect on function in *CLAUDIN14* genes when mutations occur.

We have initiated a study to investigate the expression of gje1 (gap junction membrane channel protein epsilon 1), gene product of *Cx29*, in the mouse cochlea by immunohistochmistry and laser capture microdissection. We found that gje1 is present in the stria vascularis, spiral limbus, spiral ligament and in the organ of Corti, all of which presumably play important roles to maintain the ionic composition (high K^+, low Na^+).

In addition, we have found that both

CLAUDIN14 52A>G/wt (M18V) and 167-168 delGG proteins were localized or accumulated in the cytoplasm. In contrast, 424G→A/w t (D142N) resulted in a protein that was localized to the membrane.

Our data are clearly useful in our understanding of the weight of genetic factors in prelingual non-syndromic sensorineural deafness in Taiwan.

Keywords: Laser Capture Microdissection, hearing loss, *Cx26, CLAUDIN14* immunohistochmistry

二、緣由與目的

在涉及聽覺的基因中，有許多基因所編碼的蛋白質（encoded protein）會在耳蝸中表現。因此在聽覺中耳蝸的功能扮演十分重要的角色，而在耳蝸內的許多聽障基因主要會影響離子的恒定性（ionic homeostasis），由於聽覺的產生與內淋巴需具有高K^+和EP的特性有極大的關係，因此K^+循環機制的進行和維持在整個聽障中扮演重要的角色。我們已知K^+泵進入（pumped into）內淋巴液並不是來自stria vascularis 之血液供應（Konish, et al., 1978, Wada et al., 1979）。有學者認為K^+離子可能是在耳蝸管（Cochlear duct）之再循環（再循環(recycling)）（Kikuch et al., 1995, Spicer & Schulte 1998）。K^+離開毛細胞會被柯蒂氏器之支持細胞攝取，運回stria vascularis，再泵回（pump back）內淋巴液。K^+再循環（再循環(recycling)）有幾條路徑。 (1) 側面途徑（lateral route）：經由支持細胞間的網狀gap junction 流到spiral ligament的fibrocyte，再回到stria vascularis（Kikuch et al, 1995），(2) 在內淋巴液部位的上下經由perilymph 到達spiral ligament，然後再到stria vascularis（Schulte & Steel 1994），(3) 利用中間支持細胞（medial supporting cell），spiral limbus fibrocyte 及interdental cell間的gap junction 而在interdental cell 膜上Na^+-K^+-ATPase pumps泵出，進入endolymph（Kikuch et al., 1995, Spicer & Schulte 1998, Schulte & Steel 1994）。在整個K^+ 再循環(recycling)牽涉的基因很多，包括(1)形成 gap junction 的基因---connexin(Cx) 基因族--- *connexin 26 (Cx26)*、*connexin 29 (Cx29)*、*connexin 30 (Cx30)*、*connexin 30.3 (Cx30.3)*、*connexin 31 (Cx31)*及*connexin 43(Cx43)*；(2).形成tight junction 的基因 --- *CLAUDIN 11* 和 *CLAUDIN 14*；(3). *KCNQ4* 基因。在內耳gap junction主要是細胞間離子通過的管道，而內耳內的外淋巴和內淋巴需要特殊的保護機制而將他們完全的區隔以維持高K+和EP，為維持內淋巴高靜止電位，有很多研究已經發現在內淋巴的周圍細胞間須用各種 tight junction 將其緊密連接起來(Anderson and van Itallie, 1995; Schneeberger and Lynch, 1992; Tsukita et al., 2001)。

Cx29 基因在近年來被選殖(clone)

到，表現的蛋白為 Gap junction membrane channel protein epsilon 1 (GJE1)，屬於ε次群體(Sohl et al., 2001; Altevogt et al., 2002)。*Cx29* 的基因坐落於 7q22.1，含有兩個 exon 中，可轉譯出(encode) 279 個胺基酸，和其他的 connexin 蛋白一樣為一穿膜蛋白，含有四個穿膜區(transmembrane domain)及在細胞質內有單一細胞內環(single intracellular loop)和二個細胞外環(extracellular loops)，其氨基端區(amino terminal region)和羧基端區(carboxyl terminal region)皆位在細胞質內。最早 *Cx29* mRNA 在成鼠的大腦中被發現，然而越來越多的研究顯示，其在大腦和中樞神經以及周邊神經都有表現，在周邊神經的 mRNA 表現量最為豐富(Li et al., 2002)。有研究發現 Cx29 表現於寡突神經膠質細胞(oligodendrocyte)和髓鞘(myelinating)中的膠質細胞(glial cell) (Altevogt et al., 2002)。最近在耳蝸中也被發現有 *Cx29* 的 mRNA 的表現 (Ahmad et al., 2003)。 然而對於在耳蝸內的表現位置並不清楚，因此我們期望藉由免疫組織染色和雷射顯微擷取技術可以觀察到 Cx29 蛋白在老鼠耳蝸中的表現位置，以期可以對 *Cx29* 基因在耳蝸內的功能有更多的了解。

因此本年度計畫的研究主要是探討 *Cx29* 在耳蝸內的表現和 *CLAUDIN14* 基因的突變後對其功能所造成的影響，以瞭解 *Cx29* 在耳蝸內的表現功能和 *CLAUDIN14* 基因在聽障中的致病機制。

三、結果與討論

(1) *Cx29* 基因在老鼠耳蝸內的表現

先前的研究以 macroarray 的方式發現在老鼠耳蝸中有 *Cx29* mRNA 的表現(Ahmad et al., 2003)，然而對於該基因會表現在什麼位置、在聽力的形成方面會有什麼功能都尚未了解，因此我們利用老鼠(mouse 和 rat)的耳蝸為實驗材料來觀察 *Cx29* 蛋白的表現位置，以期了解 *Cx29* 在聽力方面的影響。結果發現 Cx29 在老鼠(mouse 和 rat)耳蝸中的 stria vascularis、 spiral limbus、spiral ligament 以及 organ of Corti 都有表現（Figure 1 和 Figure 2）

為了更進一步的了解 *Cx29* 在老鼠耳蝸內的表現位置，我們利用雷射顯微擷取技術(Laser Capture Microdissection ；LCM)來檢測耳蝸內各部位 *Cx29* 的 mRNA 表現，結果顯示 stria vascularis、spiral limbus、spiral ligament 以及 organ of Corti 都有表現(Figure 3A; Table 1)，另外我們將各部位 Cx29 mRNA 量化後，發現表現量最高的是在 spiral ligament，最少的則是在 stria vascularis（Figure 3B 和 3C）。我們以 spiral ligament 後方沒有被偵測到螢光表現的部份 later wall 作為負對照，*Cx29* 則有微量被偵測到，不過相較於其他偵測得到螢光的組織表現，可以很明顯的看到量的差異。此結果和免疫組織染色法所發現的結

果是一致的，因此我們確定 *Cx29* 會表現於耳蝸中特有的高鉀低鈉的循環組織的部份(stria vascularis、spiral limbus、spiral ligament 以及 organ of Corti）。

(2) *CLAUDIN 14* 基因的功能研究

在我們針對的 *CLAUDIN 14* 基因之前的研究中，我們發現有 2 個錯意突變(missense mutation)，分別是：52A>G/wt (M18V)和 424G→A/wt (D142N)。另外我們也發現一個 deletion 的突變 167-168delGG，此突變會造成第 56 個胺基酸由 Trytpphan 變成 Serine 並造成胺基酸 frameshift 而形成一個 premature 的蛋白停止在第 158 個胺基酸。因此我們為了進一步了解 *CLAUDIN 14* 這些突變點的影響，我們建構正常和突變的 *CLAUDIN 14* 的螢光蛋白表現質體，表現於 MDCK 細胞。

實驗結果證明，正常的 CLAUDN14 蛋白可以在 MDCK 細胞間形成 tight junction (Figure 4)。另外當 *CLAUDN14* 發生 167-168delGG 的突變造成縮短的蛋白時，此突變蛋白並無法運送到 MDCK 細胞膜上形成 tight junction，而是均勻的分布在細胞質中(Figure 5)。同樣的 *CLAUDN14* 52A>G /wt (M18V)錯意突變(missense mutation)的突變蛋白也無法形成 tight junction，但又不像 167-168delGG 的突變蛋白一樣的分布，此突變蛋白是在靠近細胞膜的周圍形成堆積的蛋白(Figure 6)。相反的 424G→A/wt (D142N) 錯意突變(missense mutation)的突變蛋白卻可以和正常 *CLAUDN14* 蛋白一樣被運送到細胞膜形成 tight junction (Figure 7)。雖然結果如此，但並不一定代表此 tight junction 蛋白就有功能，所以在未來我們必須進一步作功能的分析來探討此突變的 424G→A/wt (D142N) 錯意突變(missense mutation)所形成的 tight junction 是否仍有正常間隙連結的功能。

另外在這研究中我們所看到的都是 homozygous 突變的結果，而我們所發現的突變除了 167-168delGG 為 homozygous 外其餘兩個錯意突變皆為 heterozygous，因此接下來我們也將進一步的分析來探討 heterozygous 時的影響為何。

四.計劃成果自評:

在本年度的計畫執行中，在偵測*Cx29* 基因在rat和mouse耳蝸內的哪一個部位有表現方面，我們已經利用免疫組織化學染色法(Immunohistochemistry；IHC)、雷射細胞擷取技術和RT-PCR等方法證明了 *Cx29* 在老鼠(Mouse and Rat)耳蝸內的實際分佈情形。此結果已經發表在Biochemical and Biophysical Research Communications 338 (2005) 723–728 (如附件)。另外在 *CLAUDN14* 基因突變後所造成功能的影響也獲得不錯的初步結果，此部份結果我們正在收集資料撰寫論文中。因此我們認為再這一年裡所獲得的資料將可幫助我們進行接下來的實驗，讓我們對於遺傳性聽障之致病機轉更加清楚。

五、參考文獻

Altevogt BM, Kleopa KA, Postma FR, Scherer SS and Paul DL (2002) Connexin29 is uniquely distributed within myelinating glial cells of the central and peripheral nervous systems. J Neurosci 22, 6458-6470.

Ahmad S, Chen S, Sun J and Lin X (2003) Connexins 26 and 30 are co-assembled to form gap junctions in the cochlea of mice. Biochem Biophys Res Commun 307, 362-368.

Anderson JM, and van Itallie CM. (1995). Tight junctions and the molecular basis for regulation of paracellular permeability. Am. J. Physiol. 269, G467-G475.

Kikuchi T, Kimura RS, Paul DL and Adams JC (1995) Gap junctions in the rat cochlea: immunohistochemical and ultrastructural analysis. Anat Embryol 191, 101-118

Konishi T, Harick DE and Walsh PJ (1978) Ion transport in guinea pig cochlea. I. Potassium and sodium transport. Acta Otolaryngol 86, 22-34

Li X, Lynn BD, Olson C, Meier C, Davidson KG, Yasumura T, Rash JE and Nagy JI (2002) Connexin29 expression, immuno-cytochemistry and freeze-fracture replica immunogold labelling (FRIL) in sciatic nerve. Eur J Neurosci 16, 795-806.

Schneeberger EE, and Lynch RD. (1992). Structure, function, and regulation of cellular tight junctions. Am. J. Physiol. 262, L647-L661.

Schulte BA and Steel KP (1994) Expression of α and β subunit isoforms of Na, K-AT pase in the mouse inner ear and changes with mutations at the W^v or Si^d loci. Hear. Res. 78, 65-76

Sohl G, Eiberger J, Jung YT, Kozak CA and Willecke K (2001) The mouse gap junction gene connexin29 is highly expressed in sciatic nerve and regulated during brain development. Biol. Chem. 382, 973-978.

Spicer SS and Schulte BA (1998) Evidence for a medial K^+ recycling pathway from inner hair cell. Hear Res. 118, 1-12

Tsukita SH, Furuse M. and Itoh M. (2001). Multifunctional strands in tight junctions. Nat. Rev. Mol. Cell Biol. 2, 285-293.

Wada J, Kambayashi J, Marais DC and Thaimann R, (1979) Vascular perfusion of the cochlea: effect of potassium-free and rubidium-substituted media. Arch Otorhinolaryngol 225, 79-81

Yang JJ, Liao PJ, Su CC and Li SY (2005) Expression patterns of connexin 29 (GJE1) in mouse and rat cochlea. Biochem Biophys Res Commun 338: 723–728.

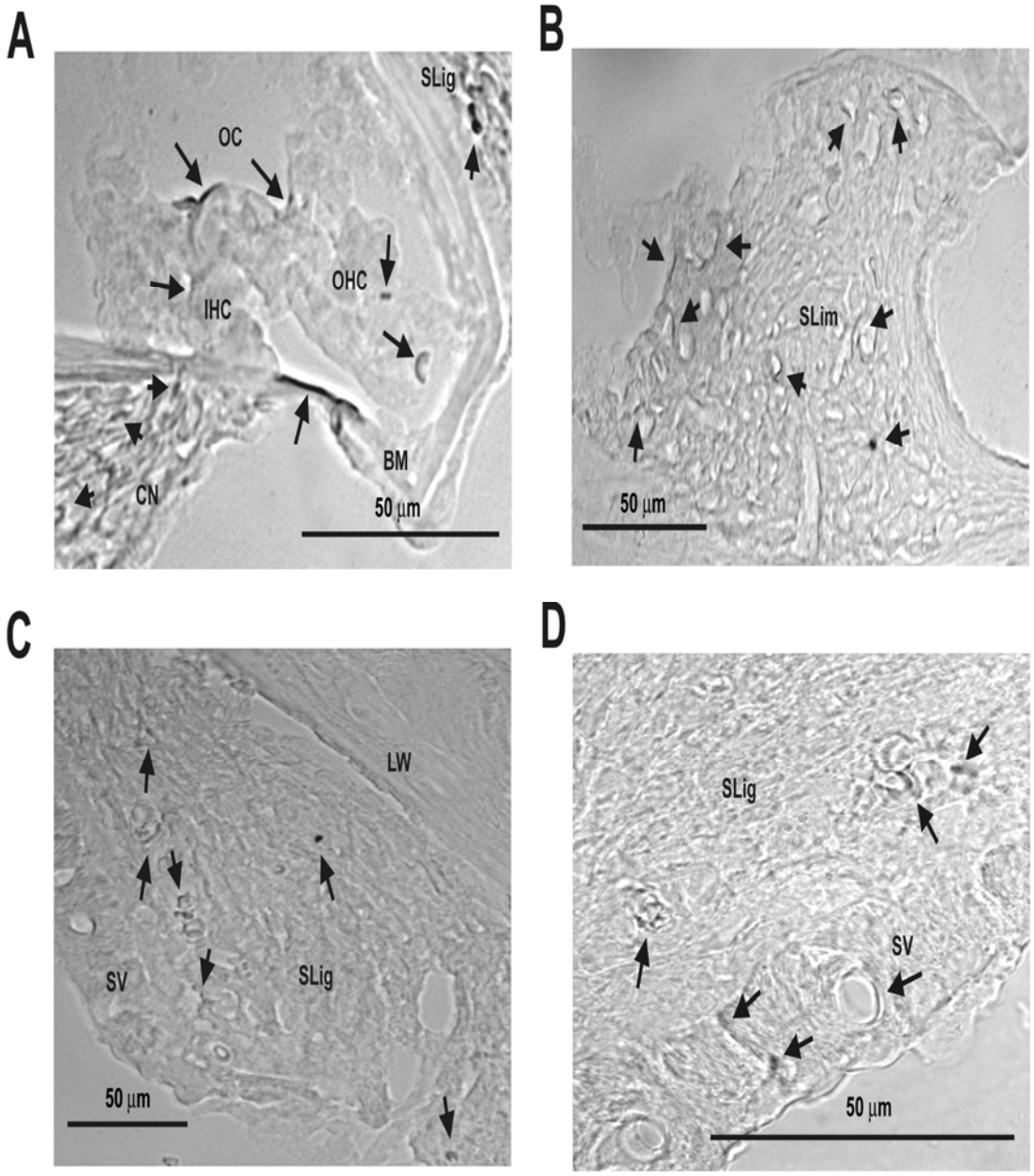

Figure 1 Immunohistochemistry of mouse cochlea for Cx29 using diaminobenzidene (DAB)/ H_2O_2 stain. Tissue parts from (A) cochlear neurons and organ of Corti., (B) spiral limbus, (C) spiral ligament and (D) stria vascularis were stained as described in Material and Methods. Arrows indicate the localization of Cx29 protein. OC: organ of Corti.; CN: cochlear neurons; SLim: spiral limbus; IHC: inner hair cell; OHC: out hair cell; SLig: spiral ligament; BM: Basilar membrane; SV: stria vascularis; LW: lateral wall. Scale bars= 50 μm.

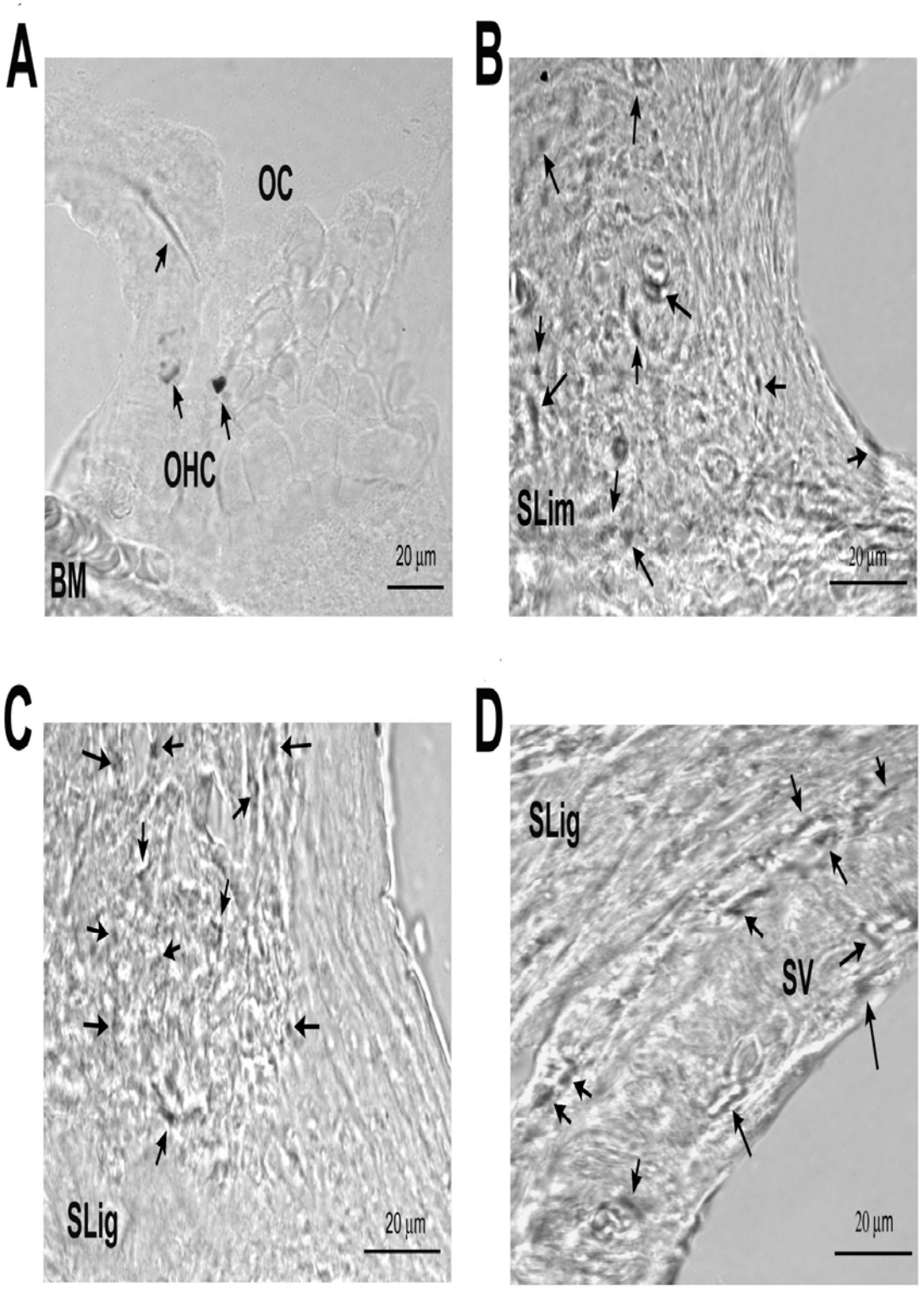

Figure 2 Immunohistochemistry of rat cochlea for Cx29 using diaminobenzidene (DAB)/ H_2O_2 stain. Expression of Cx29, indicated by arrows, in (A) organ of Corti., (B) spiral limbus, (C) spiral ligament and (D) stria vascularis. OC: organ of Corti.; SLim: spiral limbus; OHC: out hair cell; SLig: spiral ligament; BM: Basilar membrane; SV: stria vascularis. Scale bars= 20 µm.

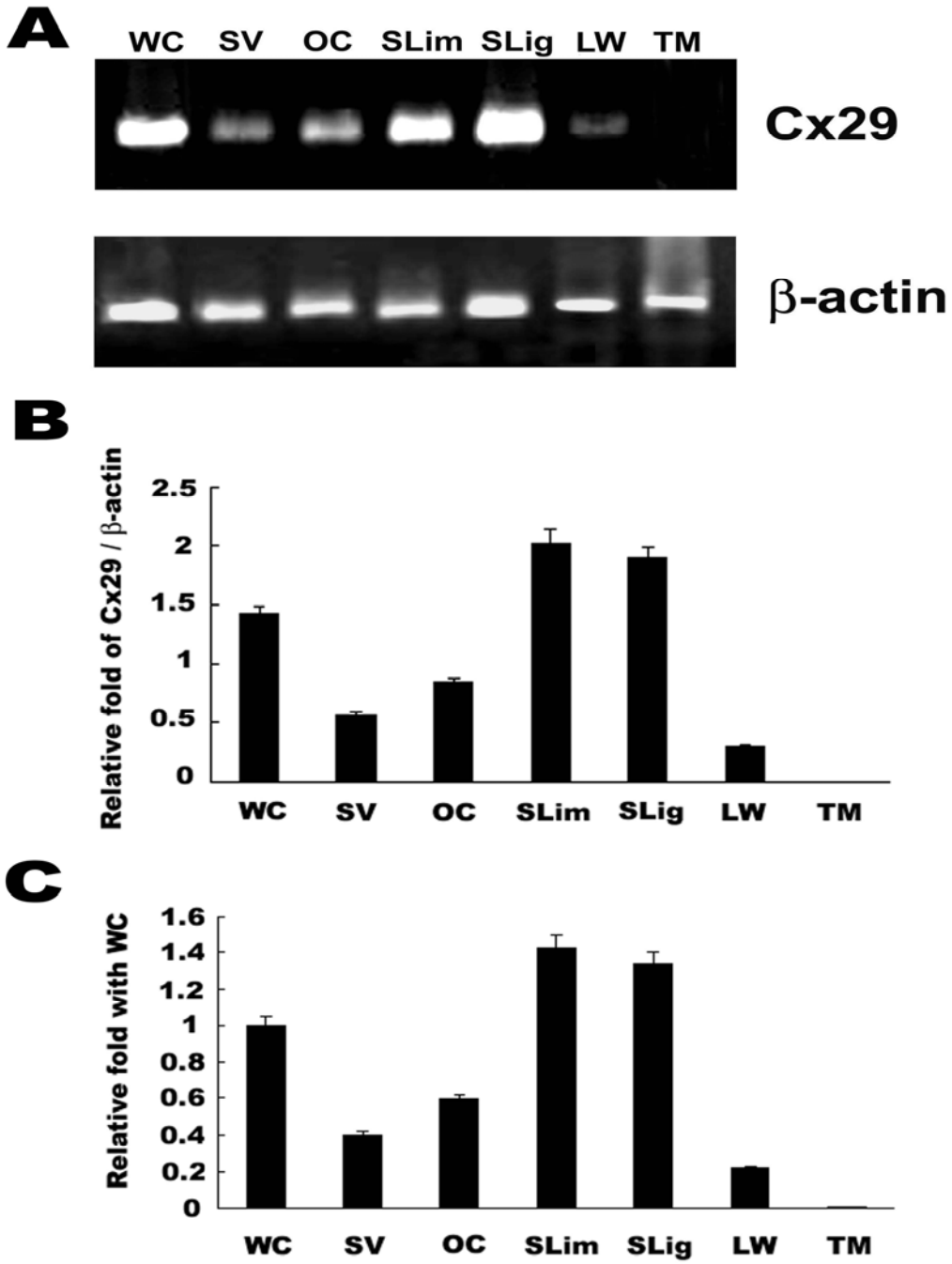

Figure 3 Expression of *Cx29* mRNA in the mouse cochlear tissues analyzed by RT-PCR and densitometry. (A) Representative an agarose gel electrophoresis of Cx29 products of RT-PCR from various parts of mouse cochlear tissues. β-actin served as reference of the loading amount of total RNA for each sample. (B) It was showed the ratios of the Cx29 band intensity over corresponding β-actin intensity. (C) Comparative level of Cx29 expression of various parts of cochlea to whole cochlea. The data are calibrated that of β-actin relative. Each bar represents the mean value of triplicates experiments. SLim: spiral limbus; SLig: spiral ligament; SV: stria vascularis; OC: organ of Corti.; LW: lateral wall; TM: tectorial membrane.

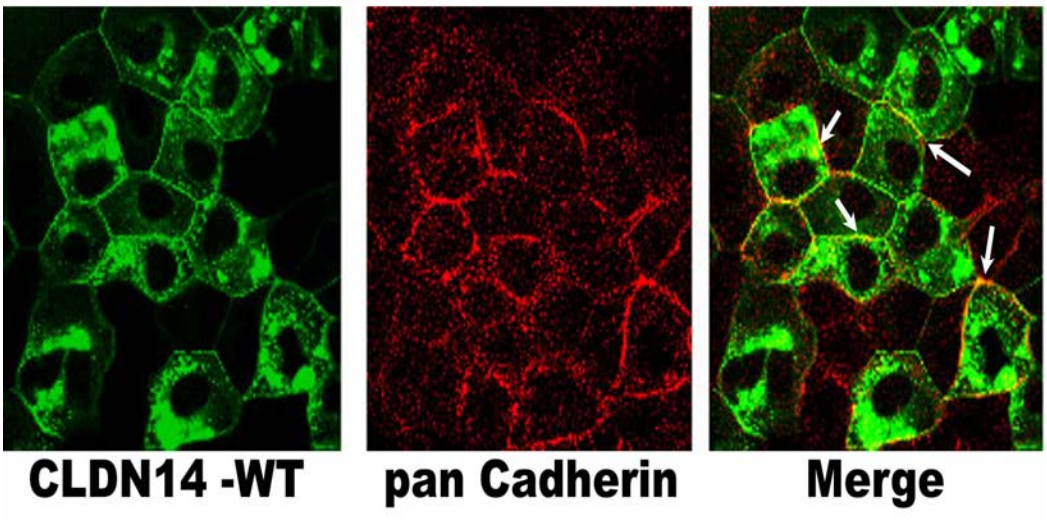

Figure 4 Localization of GFP fusion *CLAUDIN 14* (*CLDN14*) wild type (Wt) expressed in MDCK cells. The construct plasmid, pGFP-*CLDN14*$_{Wt,}$ was transiently transfected into MDCK cell lines (Green color). MDCK cell membrane was stained using anti-pan Cadherin antibody (Red color). The results showed Wt transfected MDCK cells positive reactions in the cell membranes. Arrows indicate expression protein of CLDN14 formation tight junction in the membrane.

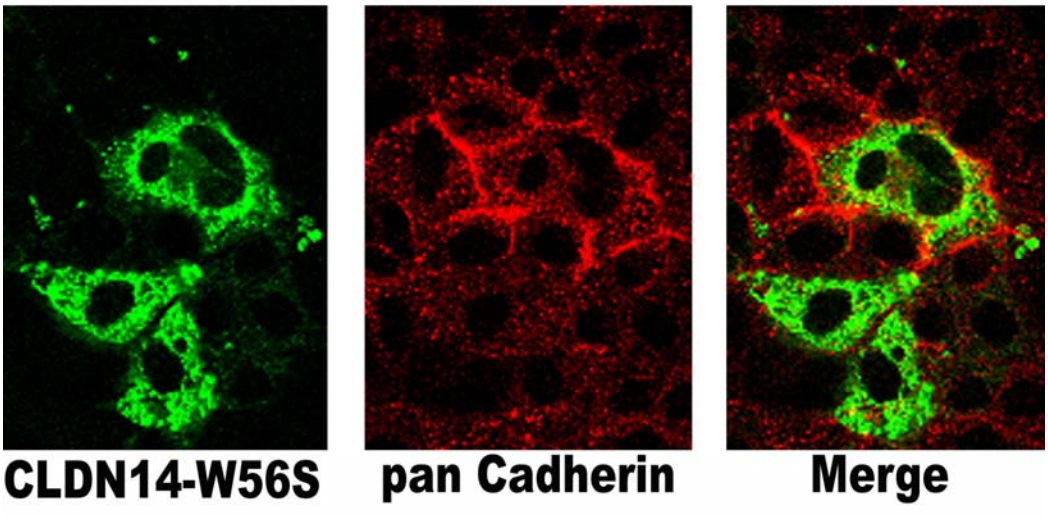

Figure 5 Localization of GFP fusion *CLAUDIN 14* (*CLDN14*) W56S mutation expressed in MDCK cells. The construct plasmid, pGFP-*CLDN14*$_{w56S,}$ was transiently transfected into MDCK cell lines (Green color). MDCK cell membrane was stained using anti-pan Cadherin antibody (Red color). The results showed W56S mutation transfected MDCK cells negative reactions in the cell membranes. The protein of W56S mutant was localized in the cytoplasma.

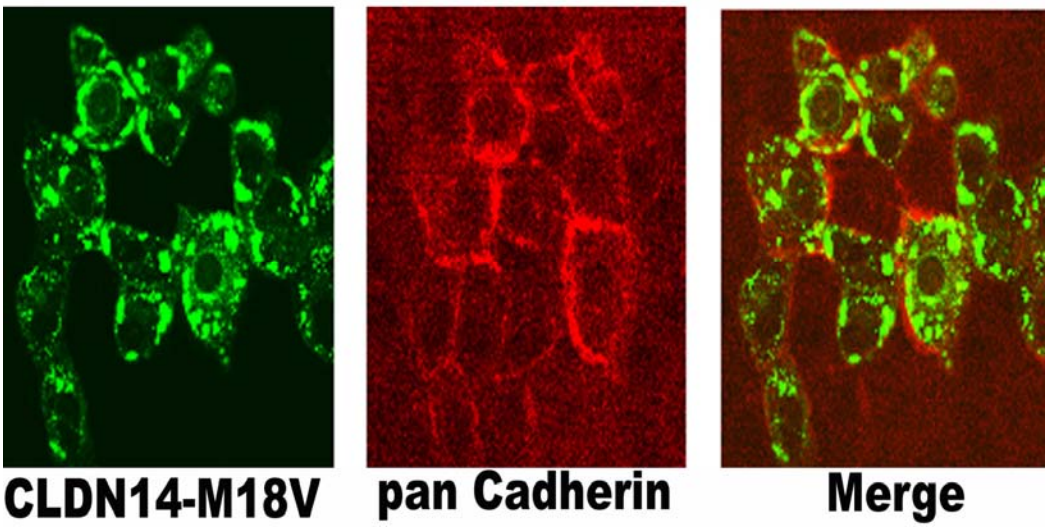

Figure 6 Localization of GFP fusion *CLAUDIN 14* (*CLDN14*) M18V mutation expressed in MDCK cells. The construct plasmid, pGFP-*CLDN14*$_{M18V,}$ was transiently transfected into MDCK cell lines (Green color). MDCK cell membrane was stained using anti-pan Cadherin antibody (Red color). The results showed M18V mutation transfected MDCK cells negative reactions in the cell membranes. The protein of M18V mutant was localized in the cytoplasma and around under in the cell membrane regions.

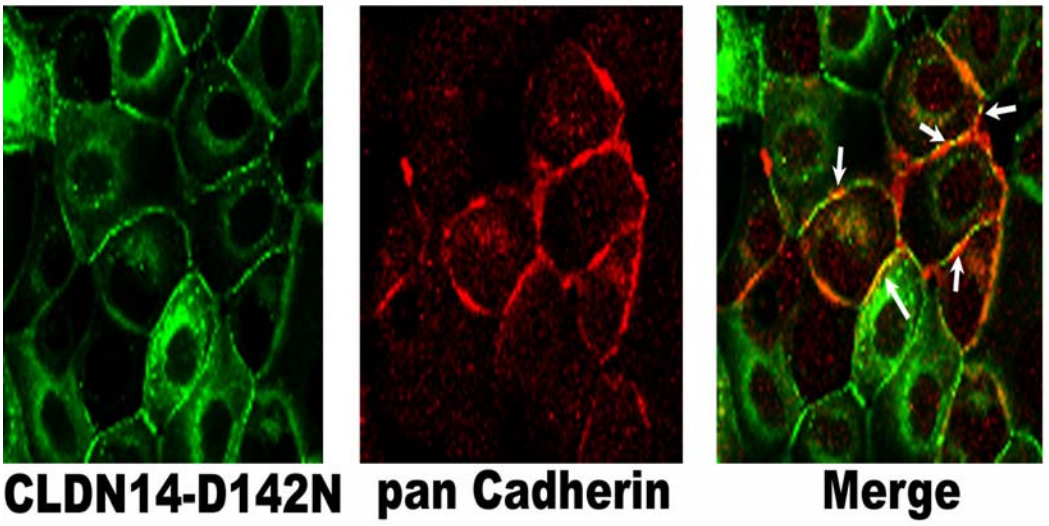

Figure 7 Localization of GFP fusion *CLAUDIN 14* (*CLDN14*) D142N mutation expressed in MDCK cells. The construct plasmid, pGFP-*CLDN14*$_{D142N,}$ was transiently transfected into MDCK cell lines (Green color). MDCK cell membrane was stained using anti-pan Cadherin antibody (Red color). The results showed D142N transfected MDCK cells positive reactions in the cell membranes. Arrows indicate expression protein of D142N formation tight junction in the membrane.

Table 1. Distribution of *Cx29* expression using immunohistochemistry (IHC) and RT-PCR among various cochlea tissues of adult mouse and rat

	Mouse						Rat					
	Spiral ligament	Spiral limbus	Organ of Corti	Stria vascularis	*Lateral wall	Tectorial membrane	Spiral ligament	Spiral limbus	Organ of Corti	Stria vascularis	*Lateral wall	Tectorial membrane
IHC	+	+	+	+	-	-	+	+	+	+	-	-
RT-PCR	+	+	+	+	+	-	+	+	+	+	+	-

+ : with expression; - : without expression ;

*** : Stars indicated difference between IHC and RT-PCR**

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Expression patterns of connexin 29 (GJE1) in mouse and rat cochlea

Jiann-Jou Yang [a,b], Pei-Ju Liao [a,b], Ching-Chyuan Su [b,c], Shuan-Yow Li [a,*]

[a] *Genetics Laboratory and Department of BioMedical Sciences, Chung Shan Medical University, Taichung, Taiwan, ROC*
[b] *Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan, ROC*
[c] *Tian-Sheng Memorial Hospital, Tong Kang, Ping-Tong, Taiwan, ROC*

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Abstract

Multiple types of connexin (*Cxs*) products, including Cx26, Cx30, Cx31, and Cx43, are found by immunolabeling in the mature cochlea. The transcript of *Cx*29, a newly discovered member of *Cx* gene family, was also discovered in the cochlea by cDNA macroarray hybridization. However, the functional roles of Cx29 in the cochlea remain unclear. To elucidate whether the Cx29 gap junction protein epsilon 1, GJE1, is localized in the adult mouse and rat cochlea, we performed an immunohistochemistry (IHC) and reverse transcription-polymerase chain reaction (RT-PCR) analysis. GJE1 was detected in the cochlea neurons, spiral limbus, spiral ligament, organ of Corti, and stria vascularis using IHC analysis. We also show that Cx29 mRNA is present in spiral limbus, spiral ligament, organ of Corti, stria vascularis, and lateral wall by the method of RT-PCR. Higher levels of *Cx29* mRNA were found in spiral ligament and spiral limbus, whereas lower level in lateral wall. Our data first provide a comprehensive and detailed pattern of *Cx29* gene expression in the mouse and rat cochlea. Knowledge of spatial distribution of Cx29 also allows the identification of candidate genes for deafness and provides important insight into mechanisms that lead to deafness due to mutations in *Cx29* gene.

Keywords: *Cx29*; *GJE1*; Immunohistochemistry; Laser capture microdissection; Mouse; Rat; Cochlea; Reverse transcription-polymerase chain reaction

The cochlea is an intricate organ which is composed of dozens of cell types and specialized regions involved in the normal process of hearing. A number of genes have been associated with deafness and some of them appear to affect ionic homeostasis in the cochlea duct. In addition, many encoded proteins have been found in the cochlea; these proteins can be grouped into functional categories and used to provide insight into the biology of hearing [1]. In the mouse model, the endolymph has high potassium and low sodium concentrations, and is maintained at a high positive resting potential of around +100 Mv. This high resting potential is essential for normal hair cell function, because when it is reduced to zero, deafness occurs [2].

Gap junctional intercellular communication (GJIC) serves many different functions, each tailored to meet the specific needs of organs, tissues or groups of cells [3]. Gap junctions are clusters of gated intercellular channels that directly connect the cytoplasm of neighboring cells and thereby allow the passage of small ions, metabolites, secondary messengers, and other small molecules from cell to cell [3,4]. The crucial role of gap junctions in auditory functions has been confirmed by numerous reports [5–9]. Cxs are gap-junction proteins which constitute a major system of intercellular communication [10]. Cxs belong to a protein family of more than 20 members, each encoded by a different gene and the numbers assigned to the various Cxs refer to their approximate molecular weights. Based on the similarities at nucleotide and amino acid levels, they can be further classified into subgroups, α, β, and ϵ [11]. Cxs share a common structure of four transmembrane segments, which extend into two extracellular and three cytoplasmic domains [12]. In the inner ear, intercellular channels formed by Cx26, Cx30, Cx31, and Cx43 proteins are thought to be key factors for maintaining a high extracellular electrical potential in the cochlea by facilitating the

* Corresponding author. Fax: +886 4 24757412.
E-mail address: syl@csmu.edu.tw (S.-Y. Li).

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local circulation of potassium ions [5–9]. In addition, many reports indicated that autosomal recessive and autosomal dominant mutations in these genes encode various mutation proteins associated with hearing loss [13–19]. Therefore, gap junction channels play an important role in the recycling of potassium ions and make up auditory process.

Ahmad et al. [20] have recently reported the four most prominently expressed Cxs in mouse cochlea in the order of *Cx26* > *Cx29* > *Cx30* ≈ *Cx43*. Their study is the first and the only one to report the presence of *Cx29* mRNA in cochlea using cDNA macroarray hybridization [20]. Human *Cx29* gene (h*Cx29*) (NM 181538), synonym *Cx31.3*, is localized on chromosome 7q22.1 and contains 2 exons with an open reading frame of 840 bp. The product of h*Cx29* gene, gap junction protein epsilon 1 (GJE 1), consists of 279 amino acid residues with a molecular weight of 31.29 kDa [11,21]. However, whether GJE1 is expressed in the cochlea and in which tissue regions it is expressed remains unclear. In this article, we used the adult mice and rats as models to answer these questions by examining the expression of *Cx29* gene in the cochlea of these animals. We found that Cx29 protein was present in the cochlea neurons, spiral limbus, spiral ligament, organ of Corti, and stria vascularis by immunohistochemistry (IHC). *Cx29* RNA transcript was also detected in the same regions of cochlea in mice and rats by RT-PCR. This is the first study which provides comprehensive and detailed patterns of the expression of the *Cx29* gene product in the mouse and rat inner ear.

Materials and methods

Immunohistochemistry (IHC) analysis. Adult female FVB mice and adult male Wistar rats free of middle ear infection were used. Cochlear tissues of adult mice and adult rats were excised out carefully using microdissecting tools under a stereomicroscope and then fixed in 4% paraformaldehyde of phosphate-buffered saline (PBS; pH 7.4) solution for 1 day at 4 °C. Ossified cochleae were perfused with 10% trichloroacetic acid (TCA), immersed in TCA for 1 h, followed by three times of wash with PBS, and decalcificated with 5% EDTA in PBS for at least 3 days. The tissue samples were then immersed in 30% sucrose of PBS for 1 day and embedded carefully in Optimal Cutting Temperature (OCT; Sakura Finetek, USA). Finally, the samples were either molded as frozen sections or stored in −80 °C until sectioning.

The serially frozen sections of tissue (10 μm) were mounted on 0.3% gelatin coated slides. Selected slides were fixed in cold acetone for 10 min, followed by blockage of nonspecific antibody binding in 10% normal horse serum for 40 min. These slides were incubated with polyclonal antibodies specific to Cx29 generated from rabbit (Zymed, San Francisco) at 1/50 dilution in 1% BSA of PBS at 4 °C overnight, followed by three washes with PBS. The slides were incubated with a goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Chemicon) at 1/500 dilution in 1% BSA of PBS for 1 h, followed by three washes with PBS. The slides were then added with diaminobenzidine/H_2O_2 for 5 min and washed three times with PBS. In the final step, the slides were mounted in an antifade medium (Molecular Probes) and examined using Axioplan 2 microscope imaging system (Zeiss, Germany).

Laser capture microdissection (LCM). Frozen sections of 10 μm were mounted on 0.3% gelatin-coated slides. The slides were fixed with 70% ethanol for 5 min followed by treatment with DEPC d_2-H_2O for 5 min to eliminate OCT. Then, the slides were dehydrated in a reaction sequence of: 70% ethanol for 30 s, 95% ethanol for 30 s, 100% ethanol for 30 s, and xylene for 1 min. They were then air-dried for 5 min in a hood. All individual tissue parts from 10 μm frozen sections of cochlea of adult mice and rat were obtained by laser capture microdissection using a PixCell lle LCM system (Arcturus, Mountain View, CA).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using the Total RNA Extraction Miniprep System according to the manufacturer's directions (VIOGENE, Sunnyvale). cDNA was synthesized according to the manufacturer's directions in a reaction of 20 μl, containing 2–5 μg RNA, random hexamer primer, and 200 U Improm-II Reverse Transcriptase (Promega, San Luis Obispo). With primers specific for the coding region of *Cx29* gene (forward 5′-ATGTGCGGCAGGTTCCTGAG-3′ and reverse 5′-CATGTTT GGGATCAGCGG-3′), PCR was performed (94 °C 30 s, 58 °C 35 s, and 72 °C 1 min) for 30 cycles in a volume of 25μl, containing 1 mM Tris–HCl (pH 9.0), 5 mM KCl, 150 μM $MgCl_2$, 200 μM dNTP, 1 U proTaq DNA polymerase (Promega, San Luis Obispo), 100 ng cDNA, and 200 μM forward and reverse primers. A fragment of approximate 700 bp was amplified from cDNA of *Cx29* gene. The PCR products were subjected to electrophoresis in an agarose gel (2 w/v%) stained with ethidium bromide. The signals were detected by Alpha Image 2200 system and the intensities of bands on the gel were quantified by densitometry (Alpha Image 2200 analysis software).

Results

In order to understand the functional relationship between Cx29 and hearing, we tried to determine the presence and expression patterns of *Cx29* products in the cochlea of mice and rats. First, sequences of *Cx29* gene from human, rat, and mouse were compared with Biology WorkBench CLUSTAL W (1.81) Multiple Sequence Alignments (http://workbench.sdsc.edu/, San Diego Supercomputer Center). At the nucleic acid level, 74% and 71% of h*Cx29* genes were identical to the sequences of rat and mouse, respectively. At the protein level, human GJE1 has 66% and 63% sequence identity to the Cx29 protein of rat and mouse, respectively. The Cx29 protein of mouse also showed a high similarity (87% identity) to that of rat.

To determine the localization of Cx29 product in cochlea, cryosections of inner ears from adult mice and rats were probed with anti-Cx29 antibody in the IHC analysis using diaminobenzidene (DAB)/H_2O_2 stain. In the mouse model, it was apparent from (DAB)/H_2O_2 stain that the expression of Cx29 was clearly observed in the cochlear neuron (Fig. 1A). Moreover, the Cx29 product was found in apical surfaces of Deiter cells and supporting cells of the organ of Corti (Fig. 1A). Cx29 was also expressed at the spiral limbus (Fig. 1B), the spiral ligament (Fig. 1C), and the stria vascularis (Fig. 1D). No Cx29 product, however, was detected at the lateral wall and tectorial membrane (data not show). A similar Cx29 expression pattern was found in the cochlea of adult rat (Fig. 2).

To further confirm the results from IHC, LCM system was first applied to separate individual tissue region from the rat and mouse cochlea and then RT-PCR analysis was performed with total RNA extracted from each region (Fig. 3). *Cx29* mRNA was found in the organ of Corti (OC), stria vascularis (SV), spiral ligament (SLig), spiral limbus (SLim), and lateral wall (LW), but not detected in the sample obtained from tectorial membrane (TM)

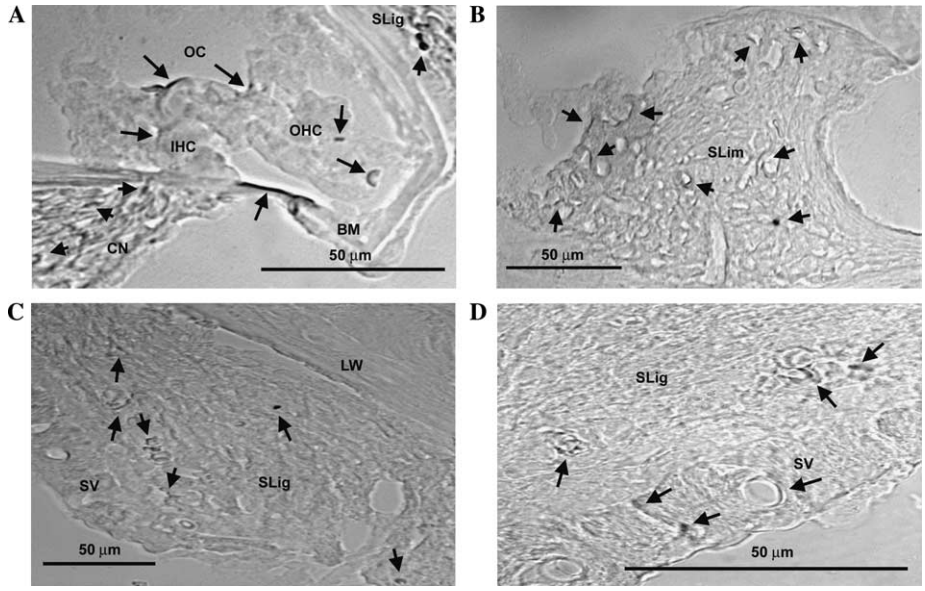

Fig. 1. Immunohistochemistry of mouse cochlea for Cx29 using diaminobenzidene (DAB)/H_2O_2 stain. Tissue parts from (A) cochlear neurons and organ of Corti, (B) spiral limbus, (C) spiral ligament, and (D) stria vascularis were stained as described under Materials and methods. Arrows indicate the localization of Cx29 protein. OC, organ of Corti; CN, cochlear neurons; SLim, spiral limbus; IHC, inner hair cell; OHC, out hair cell; SLig, spiral ligament; BM, basilar membrane; SV, stria vascularis; LW, lateral wall. Scale bars = 50 μm.

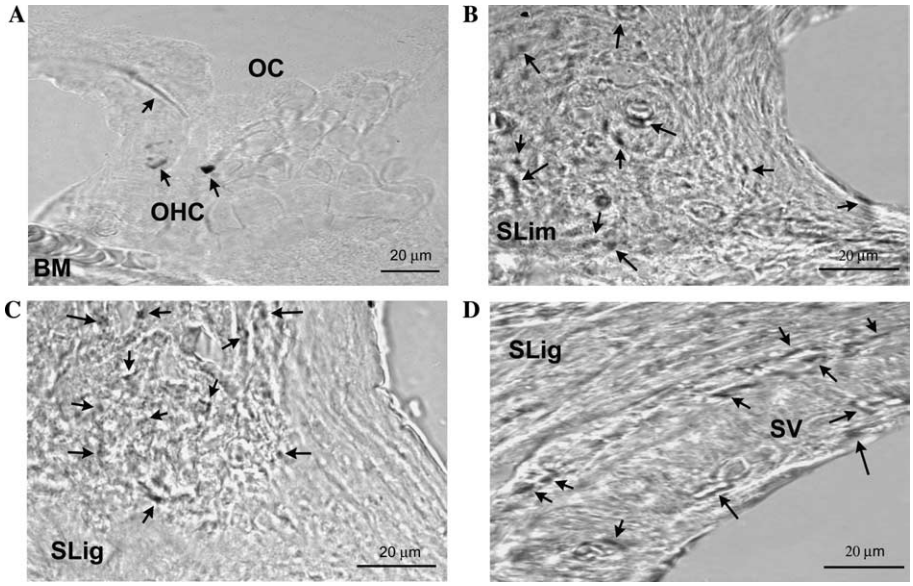

Fig. 2. Immunohistochemistry of rat cochlea for Cx29 using diaminobenzidene (DAB)/H_2O_2 stain. Expression of Cx29, indicated by arrows, is shown in (A) organ of Corti, (B) spiral limbus, (C) spiral ligament, and (D) stria vascularis. OC, organ of Corti; SLim, spiral limbus; OHC, out hair cell; SLig, spiral ligament; BM, basilar membrane; SV, stria vascularis. Scale bars = 20 μm.

(Fig. 3A). This experiment was repeated three times with similar results. These results are consistent with those of IHC and summarized in Table 1. The expression level of *Cx29* mRNA in each region was further quantified using densitometry. As shown in Fig. 3B, the ratios of *Cx29* mRNA level to β-actin mRNA ranged from 0.3- to 2-fold in various tissue regions (OC, 0.84; SV, 0.56; SLig, 1.89; SLim, 2.03; and LW, 0.30). The levels of *Cx29* mRNA in SLig and SLim were 1.3- and 1.4-fold higher than that in Whole cochlea (WC), respectively, while *Cx29* mRNA levels of the OC, SV, and LW were only 0.2- to 0.5-fold compared to that of WC (Fig. 3C). Taken together, our findings demonstrated the expression pattern of *Cx29* in the cochlea of mice and rats.

Discussion

The *Cx29* gene, a novel member of *Cx* gene family, has been cloned [11,21], and Cx29 products have been found to exist in the adult mouse central nervous system (CNS) and

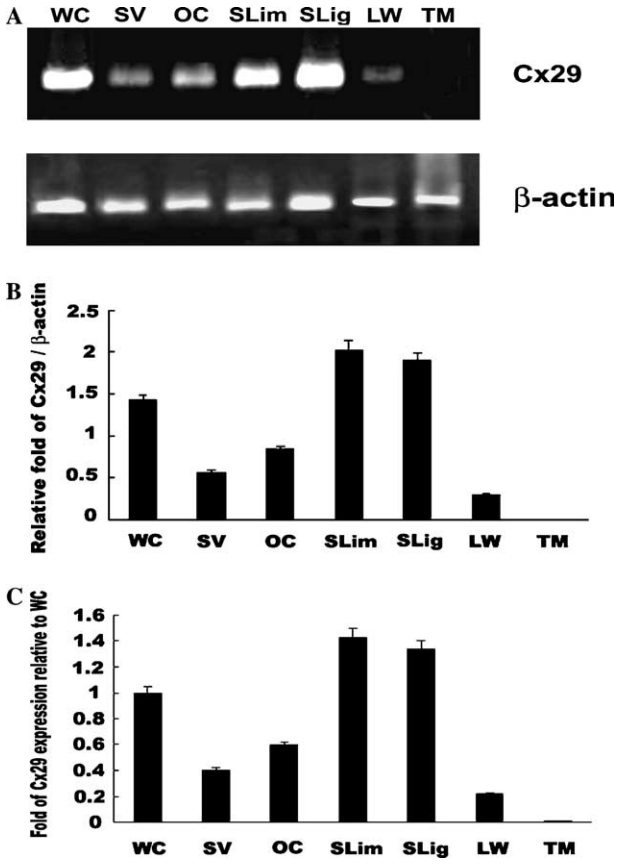

Fig. 3. Expression of *Cx29* mRNA in the mouse cochlear tissues analyzed by RT-PCR and densitometry. (A) Representative an agarose gel electrophoresis of Cx29 products of RT-PCR from various parts of mouse cochlear tissues. β-Actin served as reference of the loading amount of total RNA for each sample. (B) It shows the ratios of the Cx29 band intensity over corresponding β-actin intensity. (C) Comparative level of Cx29 expression of various parts of cochlea to whole cochlea. The data are calibrated relative to those of β-actin. Each bar represents the mean value of triplicates experiments. SLim, spiral limbus; SLig, spiral ligament; SV, stria vascularis; OC, organ of Corti; LW, lateral wall; TM, tectorial membrane.

in the sciatic nerve where it is probably expressed in Schwann cells [11,22]. Until recently *Cx29* transcript was also reported to be present in the cochlea of mouse when the techniques of cDNA macroarray hybridization were used [20]. However, the presence of Cx29 protein in cochlea has not been confirmed by other methods and the localization of Cx29 products in cochlea is unclear. With the application of immunohistochemistry (IHC) and RT-PCR, our study is the first to show the pattern of *Cx29* expression in mouse and rat cochlea. The results from both IHC and RT-PCR showed that *Cx29* products were located in the organ of Corti, spiral ligament, spiral limbus, and stria vascularis. On the other hand, only very low levels of *Cx29* mRNA were detected in the lateral wall in RT-PCR assays, a finding that is not completely consistent with the results obtained from IHC (Table 1). It is highly possible that the level of *Cx29* product is too limited to be detected by the less sensitive IHC methods.

Previous studies demonstrated that the potassium ions pumped into the endolymph are not immediately derived from the rich blood supply of the stria vascularis [23,24]. Instead, the ions may be recycled within the cochlear duct [25,26]. After leaving the hair cells, the potassium is taken up by the supporting cells of the organ of Corti and returned to the stria vascularis for pumping back into the endolymph. The gap junction system is the most likely pathway for the recirculation of cochlear K^+ [25–27]. In this study, the locations of *Cx29* expression within cochlea are very similar to gap junction systems. Therefore, we predict Cx29 protein like all other known Cxs that play an important functional role in the recirculation of potassium ions from the organ of Corti to the stria vascularis.

Cxs are classified into at least three subgroups based on their sequence similarities. *Cxs26*, *31*, and *30* belong to the β group, *Cxs43* and *50* belong to the α group, and *Cx29* belongs to the putative ε group. In general, Cxs proteins from different subgroups are not able to form functional heterotypic gap junctions [12], but it has been hypothesized that heterozygous assemblies of cochlea Cxs proteins may form gap junctions with gating properties. Gating properties give rise to the directional rectification that helps the flow of potassium ions in the appropriate direction [28]. Some other Cxs products, including Cx26, Cx30, Cx31, and Cx43, are also found in the mature mouse and rat cochlea [5,7,8,25,28]. Both Cx26 and Cx30 are widely and strongly expressed in the supporting cells of the organ of Corti, fibrocytes in the spiral ligament and spiral limbus region, and in the stria vascularis from early embryonic stages to adult mouse [5,28,29]. Cx43 are expressed in non-sensory epithelial cells, fibrocytes in the spiral ligament and spiral limbus [8]. Cx31 was detected in fibrocytes of spiral ligament and spiral limbus at 12 days after birth and found to gradually increase with age and reach an adult pattern on 60 days after birth [7]. The localization of various *Cxs* products (26, 30, 31, 43, and 29) is summarized in Fig. 4. Therefore, we guessed that gating properties may require

Table 1
Distribution of *Cx29* expression using immunohistochemistry (IHC) and RT-PCR among various cochlea tissues of adult mouse and rat

	Mouse						Rat					
	Spiral ligament	Spiral limbus	Organ of Corti	Stria vascularis	Lateral wall[a]	Tectorial membrane	Spiral ligament	Spiral limbus	Organ of Corti	Stria vascularis	Lateral wall[a]	Tectorial membrane
IHC	+	+	+	+	–	–	+	+	+	+	–	–
RT-PCR	+	+	+	+	+	–	+	+	+	+	+	–

+, with expression; –, without expression.
[a] Difference between IHC and RT-PCR.

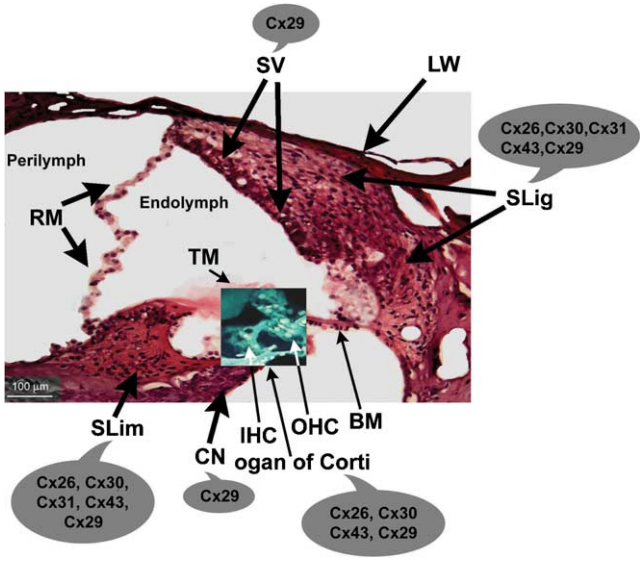

Fig. 4. Summary of the expression patterns of *Cx26*, *Cx30*, *Cx31*, *Cx43*, and *Cx29* in mouse cochlea [5,7,8,25,28]. RM, Reissner's membrane; SV, stria vascularis; LW, lateral wall; SLig, spiral ligament; BM, basilar membrane; OHC, outer hair cell; IHC, inner hair cell; CN, cochlear neurons; SLim, spiral limbus; TM, tectorial membrane. The mouse cochlea section stained with hematoxylin and eosin. Scale bars = 100 μm.

Cx29 and other *Cxs* (*Cx26*, *Cx30*, and *Cx43*) to be co-expressed in the same region of cochlea. It would be interesting to find out how these Cxs exert their functions in the potassium ions recycling in the cochlea and maintain high concentrations of potassium ions in the endolymph.

In summary, our results point out the presence and localization of *Cx29* products in the cochlea of mouse and rat, and also imply that Cx29 may play a significant role in the physiology of hearing, most probably by participating in the recycling of potassium to the cochlea endolymph.

Acknowledgments

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References

[1] B.L. Resendes, R.E. Williamson, C.C. Morton, At the speed of sound: gene discovery in the auditory system, Am. J. Hum. Genet. 69 (2001) 923–935.
[2] P. Steel, C. Barkway, G.R. Bock, Strial dysfunction in mouse with cochleo-saccular abnormalities, Hear. Res. 27 (1987) 11–26.
[3] S.S. Spice, B.A. Schutte, The fine structure of spiral ligament cells relates to ion return to the stria and varies with place-frequency, Hear. Res. 100 (1996) 80–100.
[4] S.S. Spice, B.A. Schutte, Evidence for a medial K^+ recycling pathway from inner hair cells, Hear. Res. 118 (1998) 1–12.
[5] J. Lautermann, W.J. Ten Cate, P. Altenhoff, R. Grufmmer, O. Traub, K. Jahnke, E. Winterhager, Expression of the gap-junction connexins 26 and 30 in the rat cochlea, Cell Tissue Res. 294 (1998) 415–420.
[6] P.M. Kelley, S. Abe, J.W. Askew, S.D. Smith, S. Usami, W.J. Kimberling, Human connexin 30 (GJB6), a candidate gene for nonsyndromic hearing loss: molecular cloning, tissue-specific expression, and assignment to chromosome 13q12, Genomics 62 (1999) 172–176.
[7] A.P. Xia, K. Ikeda, Y. Katori, T. Oshima, T. Kikuchi, T. Takasaka, Expression of connexin31 in the developing mouse cochlea, NeuroReport 11 (2000) 2449–2453.
[8] Z. Liu, X.J. Xia, J. Adams, Z.Y. Chen, K.O. Welch, M. Tekin, X.M. Ouyang, A. Kristiansen, A. Pandya, T. Balkany, K.S. Arnos, W.E. Nance, Mutations in *GJA1*(connexin43) are associated with non-syndromic autosomal recessive deafness, Hum. Mol. Genet. 10 (2001) 2945–2951.
[9] A. Forge, D. Becker, S. Casalotti, J. Edwards, W.H. Evans, N. Lench, M. Souter, Gap junctions and connexin expression in the inner ear, Novartis Found. Symp. 219 (1999) 134–150.
[10] T.W. White, R. Bruzzone, Multiple connexin proteins in single intercellular channels: connexin compatibility and function consequences, J. Bioenerg. Biomembr. 28 (1996) 339–350.
[11] G. Sohl, J. Eiberger, Y.T. Jung, C.A. Kozak, K. Willecke, The mouse gap junction gene connexin29 is highly expressed in sciatic nerve and regulated during brain development, J. Biol. Chem. 382 (2001) 973–978.
[12] R. Bruzzone, T.W. White, D.L. Paul, Connections with connexins the molecular-basis of direct intercellular signalling, Eur. J. Biochem. 238 (1996) 1–27.
[13] F. Denoyelle, G. Lina-Granade, H. Plauchu, R. Bruzzone, H. Chaib, F. Levi-Acobas, D. Weil, C. Petit, Connexin 26 gene linked to a dominant deafness, Nature 393 (1998) 319–320.
[14] X. Estivill, P. Fortina, S. Surrey, R. Rabionet, S. Melchionda, L. D'Agruma, E. Mansfield, E. Rappaport, N. Govea, M. Mila, L. Zelante, P. Gasparini, Connexin-26 mutations in sporadic and inherited sensorineural deafness, Lancet 351 (1998) 394–398.
[15] A. Grifa, C.A. Wagner, L. D'Ambrosio, S. Melchionda, F. Bernardi, N. Lopez-Bigas, R. Rabionet, M. Arbones, M.D. Monica, X. Estivill, L. Zelante, F. Lang, P. Gasparini, Mutations in *GJB6* cause nonsyndromic autosomal dominant deafness at DFNA3 locus, Nat. Genet. 23 (1999) 16–18.
[16] P.M. Kelley, S. Abe, J.W. Askew, S.D. Smith, S. Usami, W.J. Kimberling, Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss, Am. J. Hum. Genet. 62 (1998) 792–799.
[17] X.Z. Liu, X.J. Xia, L.R. Xu, A. Pandya, C.Y. Liang, S.H. Blanton, S.D. Brown, K.P. Steel, W.E. Nance, Mutations in connexin31 underlie recessive as well as dominant non- syndromic hearing loss, Hum. Mol. Genet. 9 (2000) 63–67.
[18] J.H. Xia, C.Y. Liu, B.S. Tang, Q. Pan, L. Huang, H.P. Dai, B.R. Zhang, W. Xie, D.X. Hu, D. Zheng, X.L. Shi, D.A. Wang, K. Xia, K.P. Yu, X.D. Liao, Y. Feng, Y.F. Yang, J.Y. Xiao, D.H. Xie, J.Z. Huang, Mutations in the gene encoding gap junction protein beta-3 associated with autosomal dominant hearing impairment, Nat. Genet. 20 (1998) 370–373.
[19] L. Morle, M. Bozon, N. Alloisio, P. Latour, A. Vandenberghe, H. Plauchu, L. Collet, P. Edery, J. Godet, G. Lina-Granade, A novel C202F mutation in the connexin26 gene (GJB2) associated with autosomal dominant isolated hearing loss, J. Med. Genet. 37 (2000) 368–370.
[20] S. Ahmad, S. Chen, J. Sun, X. Lin, Connexins 26 and 30 are co-assembled to form gap junctions in the cochlea of mouse, Biochem. Biophys. Res. Commun. 307 (2003) 362–368.
[21] B.M. Altevogt, D.L. Paul, D.A. Goodenough, Cloning and characterization of a novel central nervous system specific connexin, mouse connexin 29, Mol. Biol. Cell 11 (Suppl.) (2000) 330a.

[22] X. Li, B.D. Lynn, C. Olson, C. Meier, K.G. Davidson, T. Yasumura, J.E. Rash, J.I. Nagy, connexin29 expression, immunocytochemistry and freeze-fracture replica immunogold labeling (FRIL) in sciatic nerve, Eur. J. Neurosci. 16 (2002) 795–806.
[23] T. Konishi, D.E. Harick, P.J. Walsh, Ion transport in guinea pig cochlea. I. Potassium and sodium transport, Acta Otolaryngol. 86 (1978) 22–34.
[24] J. Wada, J. Kambayashi, D.C. Marais, R. Thaimann, Vascular perfusion of the cochlea: effect of potassium-free and rubidium-substituted media, Arch. Otorhinolaryngol. 225 (1979) 79–81.
[25] T. Kikuchi, R.S. Kimura, D.L. Paul, J.C. Adams, Gap junctions in the rat cochlea: immunohistochemical and ultrastructural analysis, Anat. Embryol. 191 (1995) 101–118.
[26] S.S. Spicer, B.A. Schulte, Evidence for a medial K^+ recycling pathway from inner hair cell, Hear. Res. 118 (1998) 1–12.
[27] B.A. Schulte, K.P. Steel, Expression of α and β subunit isoforms of Na, K-AT pase in the mouse inner ear and changes with mutations at the W^v or Si^d loci, Hear. Res. 78 (1994) 65–76.
[28] A. Forge, D. Becker, S. Casalotti, J. Edwards, N. Marziano, G. Nevill, Gap junctions in the inner ear: comparison of distribution patterns in different vertebrates and assessement of connexin composition in mammals, J. Comp. Neurol. 467 (2003) 207–231.
[29] J. Sun, S. Ahmad, S. Chen, W. Tang, Y. Zhang, P. Chen, X. Lin, Cochlear gap junctions coassembled from *Cx26* and 30 show faster intercellular Ca^{2+} signaling than homomeric counterparts, Am. J. Physiol. Cell Physiol. 288 (2005) 613–623.