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非症候群聽障病因之探討:CONNEXIN29(GJE1)和 CLAUDIN 14 基因突變之功能研究(第 2 年)
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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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中文摘要 關鍵詞：基因突變、功能研究、非症候群聽障

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。Gap junction和 tight junction在聽力功能上的重要角色已被許多研究確認，其在鉀離子循環、耳蝸內離子平衡及聽力產生的過程中扮演重要的角色。本研究的目的主要是想要更進一步的探討*Cx29*基因和*CLDN14*基因突變後對其功能的影響，藉由探討這些問題以瞭解*Cx29*基因和*CLDN14*基因在聽覺形成過程中所扮演的角色並瞭解其致病機制。本次研究結果在Cx29基因方面我們發現Cx29E269D突變蛋白會在細胞質內堆積，並且這突變蛋白會被堆積在ER。另外在co-transfection和teo-on系統的實驗我們發現E269D的突變會影響正常的Cx29蛋白到細胞膜上形成gap junction。在CLDN14基因方面，我們發現D142N的突變雖然會被送到細胞膜，然而他並沒有tight junction barrier的功能，另外M18V的突變蛋白是堆積在lysosome。另外我們也發現薑黃素(curcumin)似乎可以挽回CLDN14變後所造成的功能喪失。 綜合以上結果我們建議Cx29基因E269D突變有dominant negative的影響(此部分已經發表)。而CLDN14 D142N這個突變點確實會影響到tight junction的功能。

Abstract Keywords: Cx29, CLDN14, mutation, functional study, nonsyndromic hearing loss

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 gap junction and tight junction gene family lead to deafness in human. In the proposed project, we will focused on study of the effect on function of *Cx29* and *CLDN14* genes with mutation. Our results indicated that E269D missense mutation of *Cx29* resulted in accumulation of Cx29 mutant protein in lysosome instead of targeting to cytoplasmic membrane. Co-expression Cx29 and Cx29E269D proteins by either co-transfection or bi-directional tet-on expression system demonstrate that the heteromeric connexon accumulated at cytoplasma. Our previous study showed that CLDN14 D142N mutant protein was expressed as wild-type CLDN14, with tight junctional plaques at zones of cell-to-cell apposition. However, we found that CLDN14 D142N retained its ability in trafficking, but lost significance in its function as a barrier of tight junction. In addition, we found that CLDN14 M18V mutant protein accumulated in the lysosome. The mutant protein can be rescued by curcumin. In summary, *Cx29* E269D has dominant negative effect on normal *Cx29* resulting in accumulation of the Cx29 mutant protein in cytoplasma that impaires formation of the gap junction. CLDN14 D142N affects the normal function of CLDN14 protein.

前言

先前我們實驗室針對非症候群聽障患者和部分家屬的分析中發現病人帶有*Cx29*基因突變，此結果已發表在*Audiology & Neurotology 2007;12:198-208*。然而對於*Cx29*基因的真正功能或突變後所造成功能的影響機制為何?或和其他耳蝸內的基因(Cx基因族或tight junction protein)之間是否有交互影響? 到目前為止都還不清楚。另外在我們實驗室在*CLDN14*基因分析發現有5位帶有*CLDN14*基因的突變，且在初步的功能研究方面我們也發現這些突變點在細胞的表現位置是不一樣的，然而確切突變後所造成功能的影響我們還不是很清楚。

研究目的

本研究的目的主要是想要更進一步的探討 *Cx29* 基因和 *CLDN14* 基因突變後對其功能的影響，藉由探討這些問題以瞭解 *Cx29* 基因和 *CLDN14* 基因在聽覺形成過程中所扮演的角色並瞭解其致病機制。

文獻探討

耳蝸(Cochlea)內充滿液體，由基底膜(Basilar membrane)和覆膜（Reissner's membrane）畫分成三個充滿液體的腔室，當聲波傳入耳蝸，會產生像水波的振動，因基底膜向覆膜的垂直移動，活化位於基底膜上的柯蒂氏器內的內毛細胞，產生電化學物質傳送到聽神經，使聽神經傳遞訊息至大腦而產生聽覺。此過程是將聲波轉換成電神經衝動(electrical nerve impulses)，其中涉及許多離子的進出(Coulogigner et al., 2006)。耳蝸(Cochlea)是一個很複雜的器官，由數十種細胞及特化的區域組成，有許多與聽覺相關的基因，部份顯示其影響了耳蝸管內的離子恆定(ion homeostasis)，如果發生突變，鉀離子和鈉離子濃度不正常，造成聽障。在老鼠，內淋巴有高鉀離子及低鈉離子濃度，並保留在高正靜止電位約+100mV，這個高靜止電位是正常毛細胞功能所必須，當電位還原成零時，耳聾即發生(Steel et al., 1987)。Gap junction和 tight junction在聽力功能上的重要角色已被許多研究確認，其在鉀離子循環、耳蝸內離子平衡及聽力產生的過程中扮演重要的角色。

在人類已發現約20種connexin gene 家族成員(Willecke et al., 2002)，每個都由不同的基因編譯，並根據其分子量(molecular weight in kDa)給予命名，再基於核酸及胺基酸層級上的相似性分成α、β、ε子群體。(Sohl and Willecke,2003)相同(homomeric)或不同的(heteromeric) connexins可以組成多種不同的connexon isoforms，由於所組成的蛋白大小及電荷不同，因此也會改變通道(channel)對分子的選擇性及調節的敏感度，如:正常*Cx26*蛋白所形成的gap

junction可使Leucifer yellow(457 Da)通過，但在*Cx26*與*Cx30*形成heteromeric connexon時，細胞只能使neuroobiotin(287 Da)通過(Marziano, et al., 2003)。

Connexin29(*Cx29*)基因近幾年才在老鼠中被選殖(clone)出來(Altevogt et al., 2000; Sohl et al.,2001)，是相當新的 Cx 蛋白家族成員，人類的 *Cx29* 基因(*hCx29*)同義於 *Cx31.3*，座落於染色體 7q22.1，包含兩個 exons，840bp 開放式讀碼框(open reading frame)，其表現的蛋白為 GJE1(gap junction protein epsilon 1)，含 279 個胺基酸，分子重為 29kDa(Yang et al., 2005)。2007 年 Cx29 已被更名為 GJC3。*Cx29* mRNA 在中樞及周邊神經系統(central and peripheral nervous systems) 都有表現，而在周邊神經系統表現特別豐富(Sohl et al.,2001)。以免疫螢光標定發現多分布在坐骨神經(sciatic nerve)的雪旺細胞(Schwann cells)(Sohl et al.,2001)、寡突神經膠質細胞(oligodendrocyte)和髓鞘(myelinating)的膠質細胞(glial cell)(Altevogt et al., 2002)，另外 *Cx29* 和其他的 connexin 基因族成員如 *Cx32*、*Cx47* 在寡突神經膠質細胞(oligodendrocyte)中會共同表現(Kleopa et al.,2004)。在 Ahmad et al.,2003 研究報告中，首度指出 *Cx29* mRNA 亦存在於老鼠耳蝸，且其表現量僅次於 *Cx26*。而我們實驗室先前的研究，以免疫組織染色法(Immunohistochmistry) 和雷射顯微擷取法(Laser Capture Microdissection)觀察 *Cx29* 的蛋白 GJE1(gap junction protein epsilon 1)在老鼠耳蝸存在的組織部位，發現 GJE1 表現在耳蝸神經(cochlear neurons)、螺旋韌帶(spiral ligament)、螺旋紋(spiral limbus)、柯蒂氏器(organ of Corti)及血管紋(stria vascularis)，而這些組織對耳蝸內淋巴高鉀離子及低鈉離子濃度有重大的影響(Yang et al., 2005)。*Cx29* 也高度表現在耳蝸的雪旺細胞(Schwann cells)，且為耳蝸正常功能所必須(Tang et al., 2006)。另外在先前文獻指出正常 *Cx26* 蛋白在細胞膜所形成的 gap junction channel 可使較大的分子 Cascade blue(548 Da)和 Leucifer yellow(457 Da)通過，但在 *Cx26* 與 *Cx30* 形成 heteromeric connexon 時，改變了通道的特性，使 channel 無法讓 Leucifer yellow 通過，只能讓分子較小的 neuroobiotin(287 Da)通過 gap junction 轉移到鄰近的細胞(Marziano, et al., 2003)。然而對於 Cx29 的功能目前並不清楚，值得我們進一步探討。

另外一個在維持耳蝸內淋巴離子濃度相當重要的系統是 tight junction 系統。在內皮細胞和上皮細胞 tight junction 是主要頂部細胞內 junctional complexes 的組成成分。可區分為頂部(apical)和基底膜(basolateral)細胞表面 domains(a fence 功能)，主要的功能是抑制液體和水經細胞間的空隙流過是一種屏障(barrier)的功能。形成 tight junction 的蛋白質很多約有 12 種包括 occludin、claudins、cingulin、ZO-1、ZO-2 和 ZO-3 等，由最近的研究發現在老鼠的耳蝸內不同區域有至少 10 種的 claudin 表現包括 claudin 1、claudin 2、claudin 3、claudin 8、claudin 9、claudin 10、claudin 11、claudin 12、claudin 14 和 claudin 18 等(Kitajiri, et al., 2004a)。到目前

為止已知 claudin 有 20 種家族成員，claudin 也有 4 個穿膜蛋白區域(transmembrane domains)，在一些研究發現 claudin 直接參與 tight junction 的形成且也會在單層上皮細胞形成一個屏障(barrier)的功能(Sonoda, et al., 1999)。

CLDN14 所編碼之 claudin14 是存在於柯蒂氏器的 tight junction (Wilcox et al., 2001)，對維持內淋巴與其周圍組織間的電化學梯度差(electrochemical gradient)是非常重要的。同時 Wilcox 等人在遺傳性聽障的病人中發現 *CLDN14* 基因發生了突變會造成 Corti 氏器內 tight junction 屏障(barrier)功能降低使的耳蝸內 compartmentalization 改變而影響到聽覺功能(Wilcox, et al., 2001) ；在最近的研究也在 Greek 和 Spanish 的聽障家族中發現新的突變點，並且也證明了不同的突變點所造成影響形成 tight junction 的機制是不同的(Wattenhofer et al., 2005)。另一方面在型態學和生理學的研究發現 claudin 形成的 tight junction 在不同的細胞型態和生理需要他們也可形成一個具有選擇性的通道(Tsukita et al.,2001)。同時也有研究利用 knock out 老鼠將 *claudin 11* 剔除發現確實會造成 EP 的降低而造成聽障(Kitajiri, et al., 2004)。因此 *CLDN14* 和 *CLDN11* 在聽障的成因上扮演著一定的角色。

最近有一些研究報告指出在不同組織都有發現 tight junction 和 gap junction 蛋白彼此間會互相影響，如 *Cx26* 在 Caco-2 細胞內會調控 gap junction 促進 *Claudin 4* 的表現而強化 tight junction 的屏障(barrier)的功能(Morita, et al., 2004); *Cx40* 和 *Cx43* 在肺的內皮細胞內可能被需要去維持內皮細胞屏障的功能(Nagasawa et al., 2006)；在肝臟細胞株也發現 Cx32 形成的 gap junction 能夠誘導 tight junction 的表現和功能(Kojima et al., 2002)；在呼吸道上皮細胞株 Calu-3 的實驗發現 claudin-14 可以和 Cx26 共同表現在相同位置，且 Cx26 可能調節 tight junction 的屏障和 fence 功能(Go et al., 2006)。然而到目前為止在耳蝸組織內並沒有相關的報導，加上在我們的基因篩檢中我們有發現 1 位聽障患者同時帶有 *Cx29* 和 *CLDN 14* 的突變，因此在本計劃中我們也想了解 *CLDN14* 基因和耳蝸內有表現的 Cx 基因族之間的關係或 *CLDN 14* 基因突變後對這些基因的影響。

最近對於兩個基因之間的交互作用有一個新的研究方法---Tet-On 蛋白表現系統(Tet-On inducible expression system)(Koreen et al., 2004)，此方法是利用在一個表現質體上(pBI vector, clontech) 同時接上兩個不同的基因，而利用 Doxycycline 來調控這兩個基因的表現量，因此我們將可利用這方法來瞭解不同基因或異型結合子(heterozygous)的突變所造成的交互影響。

綜合以上所述，我們將針對 *Cx29* 和 *CLDN14* 基因突變後在非症候群聽障的功能影響，

利用分子生物學和細胞生物學、電子顯微鏡等研究法來加以探討。

研究方法

一、 Tet-on蛋白表現系統(Tet-On inducible expression system)

Tet-On蛋白表現系統(Tet-On inducible expression system)(Koreen, et al., 2004)，是利用在一個表現質體上(pBI vector, Clontech)同時接上兩個不同的基因。在pBI質體上有tetracycline reponse element(TRE)，因此可接受reverse tetracycline-controlled transactivator (rtTA)來調控其基因表現，而rtTA的表現可被tetracycline或doxycycline來調控Tet operator DNA sequence (*tetO*)產生，因此此方法可由tetracycline或doxycycline來調控穩定等量的表現兩個不同基因並可大量的表現這兩個基因產物進行研究。首先我們要建立一個可經由tetracycline或doxycycline來調控穩定表現rtTA的HeLa細胞株，是將帶有Tet operator DNA sequence (*tetO*)的質體(clontech)轉殖進入HeLa細胞或MDCK細胞中用含800μg/ml G418的培養液加以篩選(T-HeLa或T-MDCK)。另外利用基因重組的技術將不同的兩個基因或兩個不同突變點subclone至pBI vector (clontech)使其兩端接了不同基因或形成異型結合子(heterozygous)，將這質體和pTK-Hyg質體(篩選穩定表現細胞株用)以10:1的比例同時送入T-HeLa或T-MDCK中經24-48小時後，改用含有1μg/ml的doxycycline取代舊的培養，在37℃的培養箱培養24小時後，可進行細胞免疫螢光染色法來觀察基因在細胞內的表現情形。同時加入Hygomycin 400μg/ml（此濃度再先前的預備實驗中所獲得）來進行穩定表現HeLa株的篩選。

1. 建構正常和突變*Cx29*或*CLDN14*基因在 pBI 載體上

以先前實驗室選殖好在含有正常或突變的*Cx29*或*CLDN14*基因pGFP(含綠螢光蛋白)或pDsRed(含紅螢光蛋白)的質體為模板，利用基因重組的技術利用兩組不同的引子(primers)--PBI-I sense–ATGGCTAGCACGACTCACTATAGGGAGAC、PBI-I antisense-ATGGATATCCTAGAGGCACAGTCGAGGCTGAT和PBI-II sense-ATGCTGCAAACGACTCACTATAGGGAGAC、PBI-II antisense-ATGGATGACCTAGAAGGCACAGTCGAG GCTGAT將不同的兩個基因或兩個不同突變點subclone至pBI vector (clontech)使其兩端接了不同基因或形成異型結合子(heterozygous)。

2. 基因轉殖(transfection)到Tet-On HeLa(T-HeLa)或Tet-On MDCK(T-MDCK)細胞株技術

首先，將 0.9-4.0×10^5 的 T-HeLa 細胞或 T-MDCK 細胞株培養在 3.5 cm^2 培養皿中(NUNC)，細胞培養液含 89 % MEM with non-essential amino acids and Earle's BSS，10% Fetal bovine serum，1% Penicillin and streptomycin (GIBCO BRL)，當細胞濃度達到 80 %

時即可使用微脂粒法基因轉殖感染(Lipofectamine 2000; Invitrogen)，將建構好的質體送入T-HeLa細胞或T-MDCK細胞株，需同時將pTK-Hyg質體(篩選穩定表現細胞株用) 以10:1的比例同時送入T-HeLa細胞或T-MDCK細胞株，此培養皿置於5 % CO_2，37℃恆溫培養箱內培養24-48小時後將transfection medium置換掉即可進行下一步的實驗。

二、細胞免疫螢光染色技術: 特異性抗體(anti-Golgi antibody 或anti-pan cadherin)細胞免疫螢光染色來觀察正常或突變的Cx29或CLDN14基因的表現位置

細胞在微脂粒法基因轉殖感染(Lipofectamine 2000; Invitrogen)後的24小時，將細胞拆至22mm^2 coversplit的3.5 cm^2 培養皿中(NUNC)中，待24-48小時後移除舊培養液，改用含有1μg/ml的doxycycline取代舊的培養來誘導基因的表現，在37℃的培養箱培養24小時後。以1倍PBS沖洗5分鐘重覆3次，予2 ml 4% paraformaldehyde將細胞固定於室溫下作用20 分鐘後，以1倍PBS沖洗5分鐘重覆3次，繼加入2 ml 含1% BSA- 0.1% Triton X-100之PBS溶液對細胞進行通透，於室溫下作用25分鐘，以1倍PBS沖洗5分鐘重覆3次，取含5 ng/ml之anti-Golgi antibody 或anti-pan cadherin 100 μl 滴於細胞上置於4℃作用16小時後，以1倍PBS沖洗5分鐘重覆3次，再取 20 ng/ml 之Alex Fluor 488 (綠色螢光：excitation =488; emission=507) or Alex Fluor 594 (紅色螢光：excitation =593; emission=608) conjugated secondary antibody (Molecular Probes) 100 μl滴於細胞上置於37℃作用60分鐘後，以1倍PBS沖洗5分鐘重覆3次，最後以d_2H_2O沖洗，以Mounting medium (biomedia)封片，使用螢光顯微鏡 (Zeiss, Axioplam)或共軛焦(Confocal)顯微鏡 (Zeiss, LSM510) 觀察基因在細胞內之分佈螢光位置及細胞型態。

三、建立基因穩定表現T-HeLa或T-MDCK細胞株

pBI vector上並沒有攜帶篩選的基因，所以在基因轉殖(transfection)時，我們同時將pTK-Hyg質體(篩選穩定表現細胞株用) 以10:1的比例同時送入T-HeLa細胞或T-MDCK細胞株，如此就可以在建立穩定表現T-HeLa或T-MDCK細胞株時可利用hygomycin B (Sigama)來篩選，在預備實驗中我們已經得到400μg/ml的hygomycin B含量可以在10~14天殺死無transfectiont成功的細胞，因此我們將使用此濃度來進行帶有transfection基因的篩選。另外我們將利用RT-PCR和western blot來確定是否篩選到基因穩定的T-HeLa或T-MDCK表現細胞株。

四、利用染料轉移(dye transfer) 的方法來研究gap junction channel 通透性(Gap junction intercellular communication; GJIC)的功能差異

各種Cx蛋白所形成的gap junction 的功能不近相同，因此我將利用不同的染料(dye)---

Lucifer yellow (charge: -2; MW: 443Da)、Rhodamine dextra (charge: 0; MW: 1000Da)和Neurobiotin (charge: +1; MW: 287Da)，經由Scrape-loading分析方法來探討GJIC的功能和基因突變後對GJIC功能的影響。Scrape-loading dye transfer assay: 我們主要參考 El-Fouly et al., 1987和Nagasawa et al., 2006的研究方法來進行。首先將細胞養在組織培養盤(tissue culture plate)，細胞培養成monolyer細胞後，用刮刀在組織培養盤刮一條溝，細胞用PBS洗2次，然後將染料(dye)加到這括掉細胞的位置，5分鐘後用PBS洗3次，然後使用倒立螢光顯微鏡觀察(Zeiss, Axioplam)染料通透情形，來判斷gap junction的通透性功能是否正常。

五、利用穿透性電子顯微鏡(transmission electron microscope)來觀察基因在細胞內的表現位置

1. 樣本的準備和觀察

單層細胞用0.5% Triton X-100/PHEM buffer (60mM PIPES、25mM HEPES、10mMEGTA、2mM $MgCl_2$ pH6.9)在4℃作用10分鐘後，再以PHEM buffer洗2次。細胞被固定在3% (v/v) glutaraldehyde/PHEM (pH 7.4) 在室溫作用10分鐘。細胞被固定後用1% (w/v) osmium tetroxide / 0.1 M phosphate buffer進行後固定作用。接下來用酒精進行一連串的脫水作用和經過兩次propylene oxide的作用後將細胞包埋在Poly/Bed 812 resin (Polysciences Inc.)。包埋樣本利用REICHERT-JUNG切片機進行薄切片(thin sections)，最後薄切片使用uranyl acetate和lead citrate染色，再用d_2H_2O 洗3次，然後將其mounted在copper grids後用JEOL 1200EX transmission electron microscope(TEM; JEOL, Tokyo, Japan)觀察。

2. Immunogold labeling

樣本使用特異性的抗體(如rabbit anti-Cx29 antibodies)加以lable在24℃ 1~1.5小時，樣本用labeling-blocking buffer(0.15 M SPB, 10% heat-inactivated goat serum and 0.5% teleost gelatin)洗四次，每次10分鐘，再用10-nm, 20-nm, and/or 30-nm gold conjugated secondary antibody (Chemicon)作用2小時後，用labeling-blocking buffer洗二次和用d_2H_2O洗三次，每次10分鐘，最後將樣本風乾(air-dried)。然後將樣本mounted在copper grids後用JEOL 1200EX transmission electron microscope(TEM; JEOL, Tokyo, Japan)觀察。

六、transepithelial electrical resistance (TER)的測量方法

將1-2×10^5穩定表現正常或突變*CLDN14*的HeLa細胞培養在兩個Transwell chambers的內層chamber (6.5mm, 孔洞大小5.0μm)中(Corning Life Sciences, Corning, NY)，並加入

100μl細胞培養液(89 % MEM with non-essential amino acids and Earle's BSS，10% Fetal bovine serum，1% Penicillin and streptomycin (GIBCO BRL))，在外層chamber則加入600μl細胞培養液。當細胞形成單層細胞時，使用Millipore electrical resistance system來測量單層細胞transepithelial electrical resistance (TER)的值，可清楚的瞭解穩定表現正常或突變*CLDN14*的單層HeLa細胞的barrier的功能。

結果與討論

一、*Cx29*基因突變的研究：

我們將wild type及mutant type分別建構在螢光蛋白載體peGFP-N1及pDsRed1-N1上，再將construct送入Hela cells中，觀察Cx29螢光融合蛋白在細胞內的表現情形。目前我們已成功篩選出Cx29wt-eGFP、Cx29wt-DsRed、CX29E269D-DsRed及Cx29E269D-eGFP四株stable cell line，並且進一步的利用RT-PCR來確認轉殖基因在細胞內RNA levels (Figure 1)。使用細胞免疫螢光染色法及利用正立顯微鏡或共軛焦顯微鏡觀察的結果發現：Cx29wt-eGFP表現在細胞膜上，並且呈現連續性表現，不會形成gap junction plaque(Figure 2a)。然而Cx29E269D-eGFP會在細胞質堆積(Figure 2b)。同樣的結果也發現在使用DsRed融合蛋白的研究(Figure 3a和3b)。為了更進一步瞭解蛋白堆積位置，我們分別以抗體anti-PDI及anti-Golgi以及染劑lysosome tracker標定內質網、高爾基氏體及溶酶體(Figure 4)，結果發現E269D突變蛋白與ER colocalization。此外，由於我們所發現在病人中的突變是heterozygote，因此我們cotransfect wild type及mutant type constructs到HeLa cell，來觀察突變的Cx29蛋白對wild type是否會造成影響。在cotransfect peGFP-Cx29wt及pDsRed-Cx29E269D的transient結果發現，Cx29E269D-DsRed會阻礙Cx29wt-eGFP，使無法送到細胞膜，共同堆積在細胞質內(Figure 5)，Cx29E269D-DsRed會對Cx29wt-eGFP造成dominant negative effect。另外為了確定我們使用的螢光蛋白(eGFP和DsRed)不會影響我們觀察的結果，我們也轉殖eGFP和DsRed的質體當一個控制組，結果我們發現兩個單獨螢光蛋白的表現是均勻的散布在細胞質中(Figure 6)。以往研究heterozygote都是利用cotransfection的方式，此方法有人任為我們無法控制兩質體等量進入細胞中，而不等量的質體可能會影響實驗結果，因此我們實驗室建立了tet-on inducible gene expression system。將兩不同質體建構在pBI雙向表現載體上，再送入Tet-on HeLa cells，在觀察蛋白表現前30分鐘加入doxycyclin誘導基因表現，此系統除了能摒除傳統cotransfection兩質體不等量進入的疑慮，並且能快速並提高蛋白表達量。目前我們已將Cx29 WT-GFP和Cx29 E269D-DsRed 的pBI construct建構完成。將這construct送入Tet-on HeLa cells，利用免疫螢光染色及正立顯微鏡觀察

兩融合蛋白在細胞內的的表現情形及交互作用。在transient的data顯示Cx29wt-eGFP表現在細胞膜(Figure 7)，Cx29E269D-DsRed堆積在細胞質(Figure 8)，雙向表現Cx29wt-eGFP與Cx29E269D-DsRed的細胞，兩融合蛋白共同堆積在細胞質內(Figure 9)。這些結果與我們在螢光蛋白載體系統所觀察到的結果相同。另外蛋白堆積在內質網(ER)會引起Unfolded Protein Response (UPR)造成細胞的apoposis，因此我們進一步的利用DNA fragmentation 和 flow cytometry來分析Cx29E269D突變蛋白堆積在ER是否會造成細胞的apoposis(Figure 10)，結果我們發現Cx29E269D突變蛋白堆積在內質網並未引起細胞的apoposis。**以上的部分結果已被接受將發表在Human Genetic國際期刊 (附件一)。**

另外Cx29是在近幾年才被發現，因此目前對於他的正常功能仍不清楚，未來我們需要進一步的建立Cx29正常channel的特性及功能的特性，如利用dye transfer或ATPase的釋放功能….等等，藉由以上實驗結果來進一步的釐清Cx29在聽覺產生中所扮演的角色和釐清造成聽障的致病機轉。

二、*CLDN 14* 基因突變的研究

先前研究我們已經發現正常的 CLDN14 蛋白可以在 MDCK 細胞間形成 tight junction。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。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，因此需要更進一步的去確認他實際的表現位置。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。雖然結果如此，但並不一定代表此 tight junction 蛋白就有功能，所以我們必須進一步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。

在本研究中我們分別以抗體 anti-PDI 及 anti-Golgi 以及染劑 lysosome tracker 標定內質網、高爾基氏體及溶酶體，結果我們發現 *CLDN14* M18V(E269D)與 lysosome colocalization，推測此突變蛋白會被運送至 lysosome 降解(Figure 11)。由於 CLDN14 蛋白質有著 PDZ Domain，會與細胞中的 ZO-1、ZO-2、ZO-3 結合(Itoh et al., 1999)，所以我們進一步的用 ZO-1 抗體標定細胞膜上的 ZO-1，來觀察 ZO-1 與 CLDN14 正常和突變蛋白之間的交互作用。同時我們以 MDCK 細胞當一個對照組(Figure 12a)。結果我們發現正常的 CLDN14WT 蛋白可以和 ZO-1 共同表現在一起(Figure 12b)。相對的 CLDN14W56S 和 CLDN14M18V 是無法和 ZO-1 表現在共同的位

置(Figure12 c 和 d)。然而另一個突變點 CLDN14D142N 表現的和 CLDN14WT 一樣，可以和 ZO-1 共同表現在一起 (Figure 12e)

tight junction 具有閘道屏障的功能，調控離子與分子在細胞間的通過，因此藉由 transepithelial electrical resistance (TER)的測定可以知道 tight junction 的功能是否正常。我們也利用此方法來分析 CLDN14 突變後是否造成屏障功能的喪失，結果發現 CLDN14D142N 的突變蛋白雖然可以和正常 CLDN14 蛋白一樣被運送到細胞膜形成 tight junction。但是此突變的 tight junction 蛋白屏障功能，並無法像正常的 CLDN14 蛋白一樣具有形成的 tight junction 正常間隙連結的功能(Figure 13)。另外兩個突變(CLDN14W56S 和 CLDN14M18V)的蛋白同樣的也喪失了 tight junction 屏障功能(Figure 13)。

最近以 CFTR(血纖維囊腫)為例的研究指出薑黃素(curcumin)可以降低內質網中鈣離子與不當摺疊蛋白間的結合力，進而減少 Proteasomal degradation 的比例(Zeitlin P, 2004)。由於我們研究中發現 CLDN14M18V 在細胞中的表現大多都堆積在細胞質，因此，我們想瞭解薑黃素(curcumin)是否亦具有改善 CLDNM18V 突變蛋白的功能。首先我們將帶有 CLDNM18V 細胞養在 18cm^2 的玻片上培養 24 小時後加入 40μM (14.7 μg/ml) 薑黃素(curcumin)到培養液中，我們分別作用 1、3、5 小時後作免疫螢光染色並用螢光顯微鏡觀察，可以發現 M18V 突變蛋白在經過 1 小時作用後，此突變蛋白會有往細胞膜上轉移的現象(Figure 14)。 得知 curcumin 能夠使 CLDN14M18V 的蛋白質往細胞膜上轉移，我們進一步的利用 TER 的測定來分析其功能，希望藉由電阻值的測量能夠觀察出 tight junction 的功能是否有改善。分別測定 20μM 薑黃素(curcumin)作用下與無薑黃素(curcumin)作用下 CLDN14M18V 細胞株的電阻值，可以發現在 curcumin 作用下的 CLDN14M18V 細胞株測出來的電阻數值有逐漸升高的趨勢，然而在無 curcumin 作用下的 CLDN14M18V 細胞株電阻值並無顯著變化，但是若移除薑黃素(curcumin) 後 24 小時電阻值又有降低的現象(Figure 15)。另外我們也利用相同的方法分析 CLDN14D142N 的突變，結果與 CLDN14M18V 的細胞株一樣電阻都有明顯上升的趨勢(Figure 15)。上述的結果讓我們瞭解到薑黃素(curcumin)似乎可以改善 CLDN14 突變蛋白的功能，但其機制如何我們並不清楚? 這需要進一步的去分析，如能了解其機制或許將來可以可以利用薑黃素(curcumin)來改善因 CLDN14 突變所造成的聽力損失。

最近幾年有些報告已經使用冷凍蝕刻(Freeze-fracture)或 Freeze-fracture replica immunogold labeling (FRIL) 等技術來研究 gap junction 和 tight junction 在組織細胞內或培養細胞(cell culture) 內的表現位置(subcellular localization)(Rash et al., 2001; Go et al., 2006)。不過這技術在我們實驗室或學校是無法執行的，我們原本想利用薄切片(thin sections)的方式利用穿

透性電子顯微鏡(transmission electron microscope)來取代上述的方法來觀察基因在細胞內的表現位置，然而我的實驗結果是失敗並沒有發現到 CLDN14 tigh junction 的表現，因此這一部分的實驗可能需要進一步的克服才可觀察到我們預期的結果。

同時因在我們所發現的 *CLDN14* 的突變除了 167-168delGG 外都是 heterozygous，因此我們也將利用 tet-on inducible gene expression system 來研究這些突變點，目前我們已經將這些突變點完全建構好這些表現質體。然而我們將他送入 tet-on HeLa 細胞株中，並無法看到這些基因的表現。因此我們為了解決這個問題，我們將建立 tet-on MDCK 細胞株，目前我們以精挑到幾個 clone，接下來我們將要進行測試來瞭解哪些 clone 是可以用來進行蛋白表現的。

計劃成果自評:

本篇研究結果對於我們研究*Cx29*和*CLDN14*基因族在聽障的致病機轉有進一步的了解，尤其對於我們在聽障病人中所發現的*Cx29* E269D、*CLDN14* M18V和*CLDN14* D142N基因的突變點所造成功能的影響，有更直接的證據來證實這些突變點確實會造成聽覺障礙。此研究成果也符合我們當初對於計畫內容所要追求的目標。在Cx29E269D基因突變的研究部分我也已經被接受將發表在Human Genetics國際期刊。在CLDN14的研究方面，我們也發現其突變所造成的一些功能喪失。另外我們也發現薑黃素(curcumin)可以挽回對於CLDN14的突變所造成功能喪失，雖然機制尚需進一步的釐清，不過這是一個重要的發現。綜合以上所述，本計畫的完成已經提供一些重要的資訊，可以提供作為進一步釐清Cx29和CLDN14基因在聽力形成和造成聽障的成因的重要參考依據。

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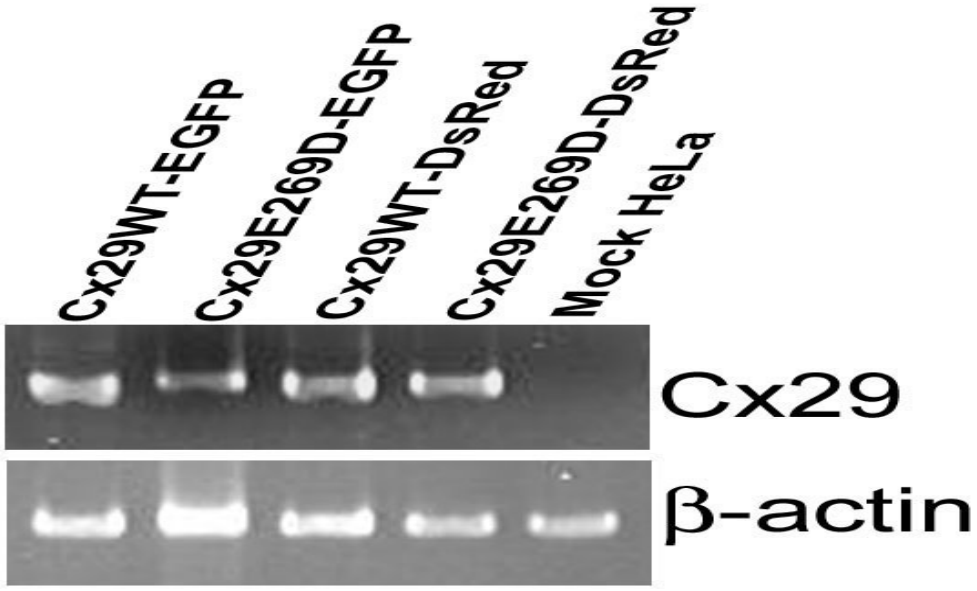

Figure 1. Expression analysis of *Cx29* mRNA in the four transfected HeLa cells by RT-PCR. RT-PCR analysis of total RNA from HeLa cells expressing Cx29WT-EGFP, Cx29WT-DsRed, Cx29E269D-EGFP, and Cx29E269D-DsRed confirms expression of the corresponding mRNAs in stably transfected HeLa cell lines (up panel). β-actin served as reference of the loading amount of total RNA for each sample (low panel). Mock HeLa is a negative control.

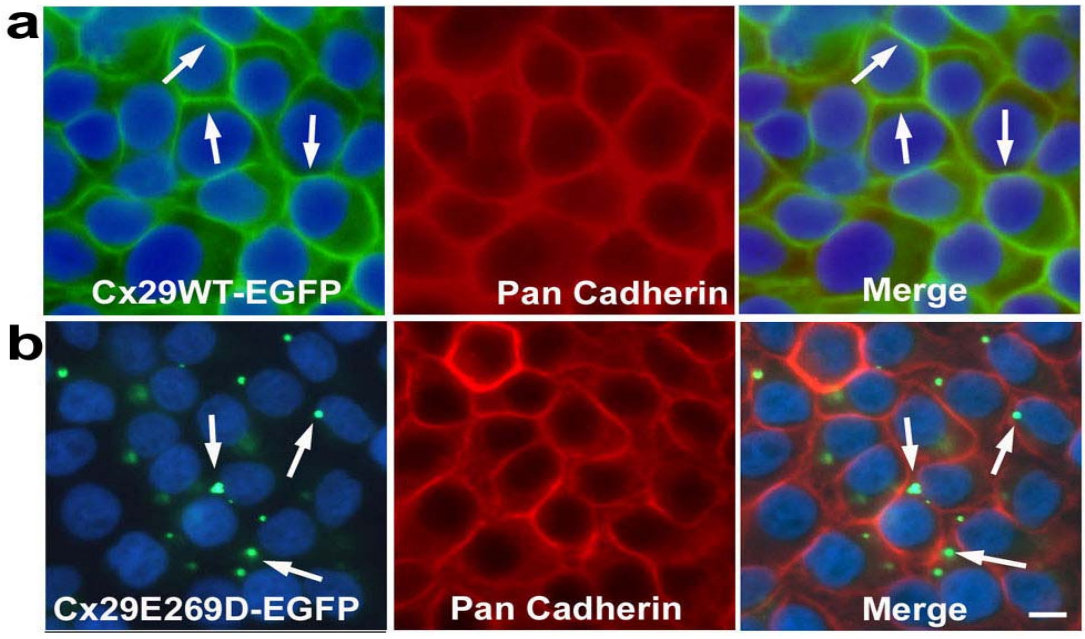

Figure 2. Expression analysis of Cx29WT and Cx29E269 in stably transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells expressing Cx29WT-EGFP (a) shows expression of the Cx29 fusion protein in the plasma membranes. However, Cx29E269D-EGFP (b) transfected HeLa cells show impaired trafficking of the Cx29 protein with localization near the nucleus. The cells were counterstained with 4'-6-Diamidino-2-phenylindole, DAPI, (blue) to highlight the nuclei. Scale bars: 10 μm.

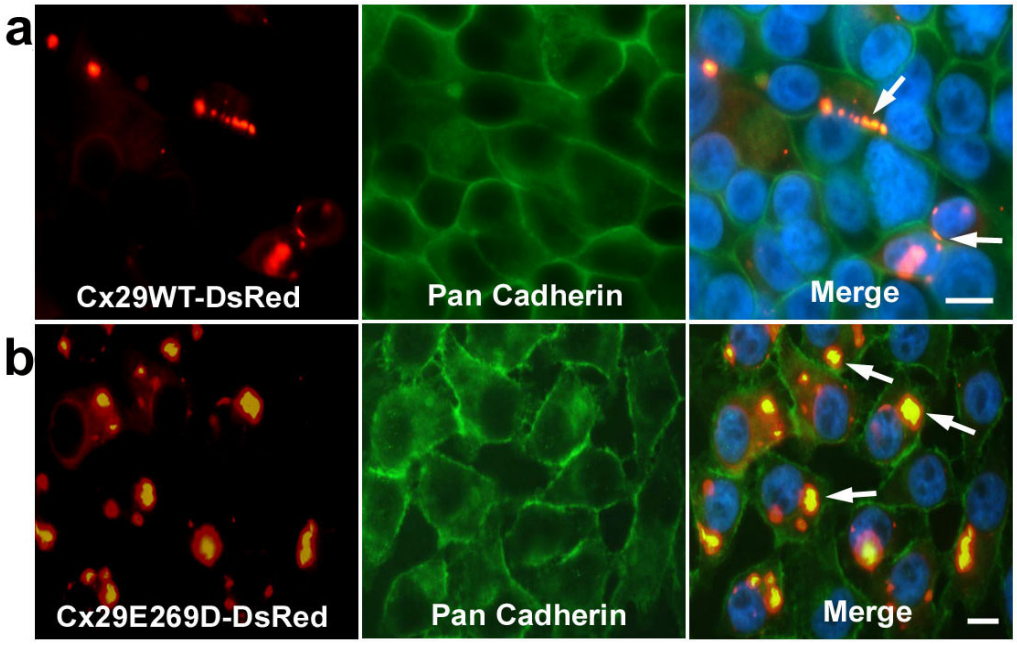

Figure 3. Expression analysis of Cx29WT and Cx29E269 in transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells expressing Cx29WT-DsRed (a) shows expression of the Cx29 fusion protein in the plasma membranes. However, Cx29E269D-DsRed (b) transfected HeLa cells show impaired trafficking of the Cx29 protein with localization near the nucleus. The cells were counterstained with 4'-6-Diamidino-2-phenylindole, DAPI, (blue) to highlight the nuclei. Scale bars: 10 μm.

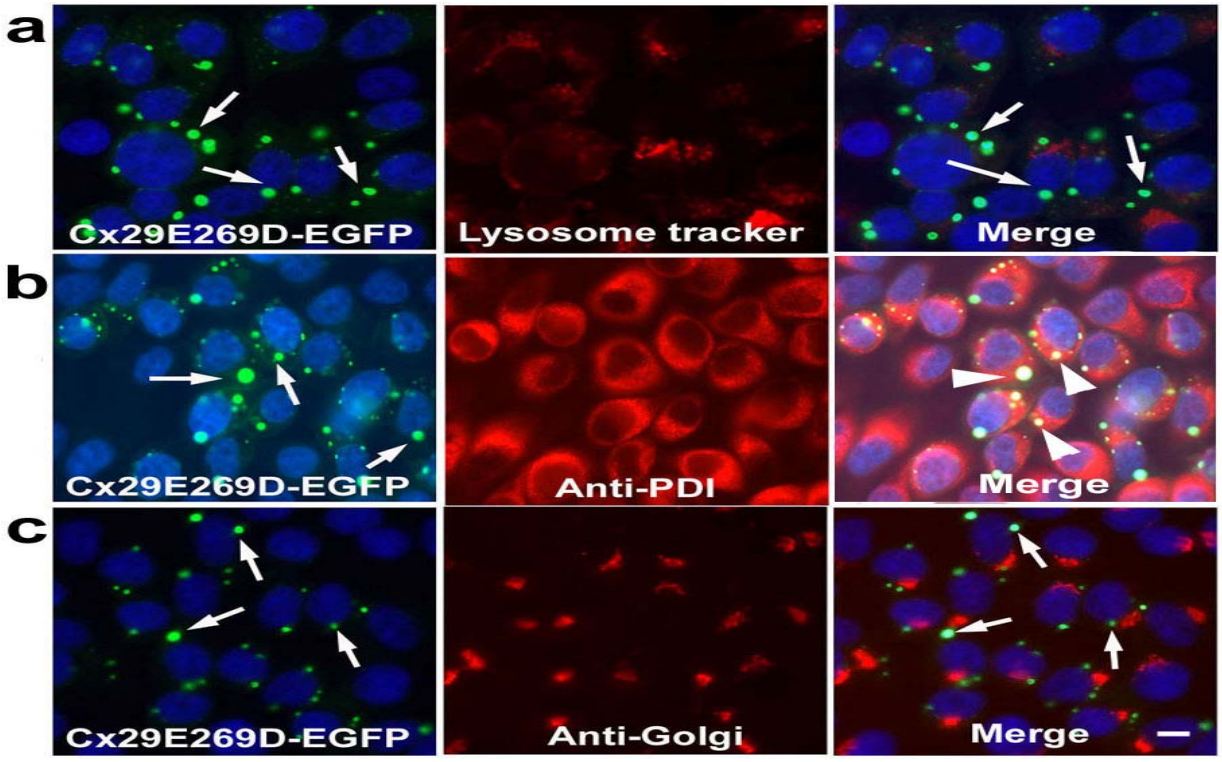

Figure 4. Intercellular localization of mutant Cx29 proteins. Photomicrographs of HeLa cells transfected with Cx29E269D-EGFP cDNA after immunostaining for markers of the lysosome, ER (anti-PDI), and Golgi apparatus (red in (a)–(c), respectively). Yellow signal in the image overlays (right column) indicates co-localization of Cx29E269D-EGFP and the organelle of interest. Mutant Cx29 shows moderate co-localization with the ER marker. The cells were counterstained with 4'-6-Diamidino-2-phenylindole, DAPI, (blue) to highlight the nuclei. Scale bars: 10 μm.

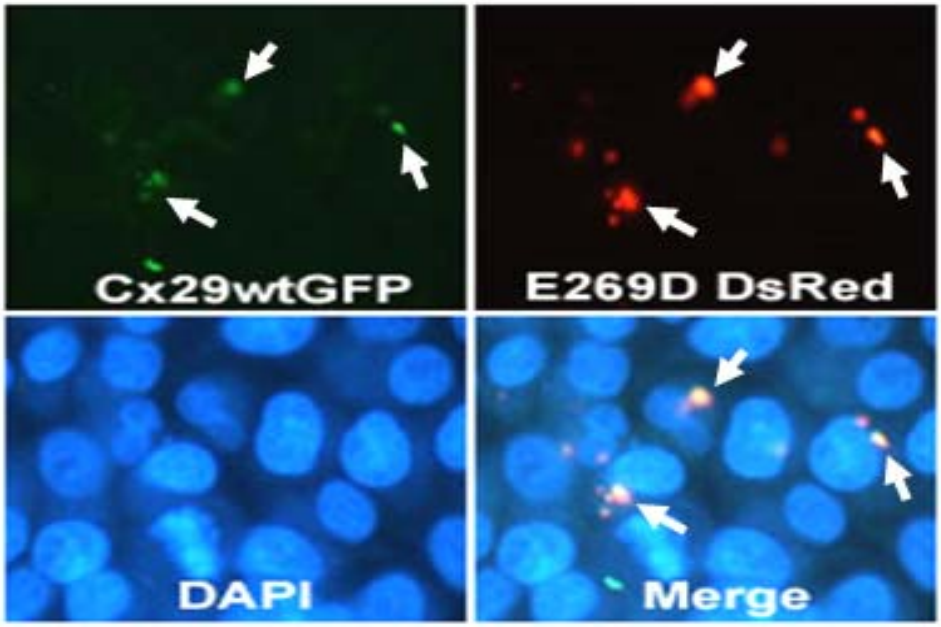

Figure 5. *Cx29*E269D 錯意突變之 Cx29 蛋白對正常 Cx29 蛋白的影響

左上圖為正常的 Cx29 蛋白表現(綠色)；右上圖為突變(E269D)的蛋白表現(紅色)；左下圖為 DAPI 染細胞核(藍色)；右下圖為 merge 在一起的結果。觀察白色箭頭所指的地方，我們可以明顯發現在細胞質內有呈現黃色的堆積物，顯示正常 *Cx29* 蛋白會被突變的 Cx29E269D 蛋白所抑制而無法運送到細胞膜上表現。

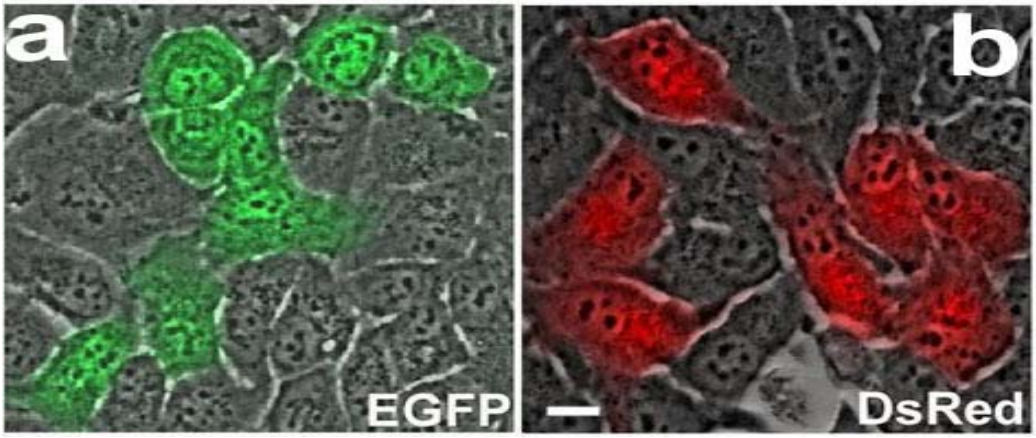

Figure 6. Expression analysis of EGFP and DsRed fusion protein in transiently transfected HeLa cells. Fluorescence microscopy of EGFP (a) and DsRed (b) HeLa cells shows uniform spread expression of these fusion proteins in the cytoplasmic of HeLa. Scale bars: 10 μm.

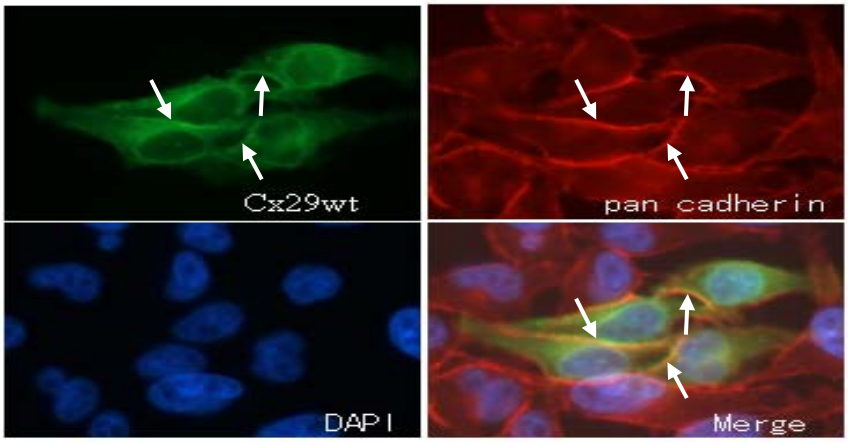

Figure 7. 利用基因重組技術將正常的 *Cx29*-eGFP 基因建構於 pBI 雙向表現載體上，轉殖感染 Tet-on HeLa cells，以細胞免疫螢光染色法染細胞膜觀察正常 Cx29-*eGFP* 蛋白在細胞內的表現情形。
左上圖為正常的 Cx29 螢光蛋白表現(綠色)；右上圖為抗體染細胞膜(紅色)；左下圖為 DAPI 染細胞核(藍色)；右下圖為 merge 在一起的結果。觀察白色箭頭所指的地方，我們可以發現在細胞膜處呈現黃色，顯示正常 Cx29 蛋白會被送到細胞膜上表現。

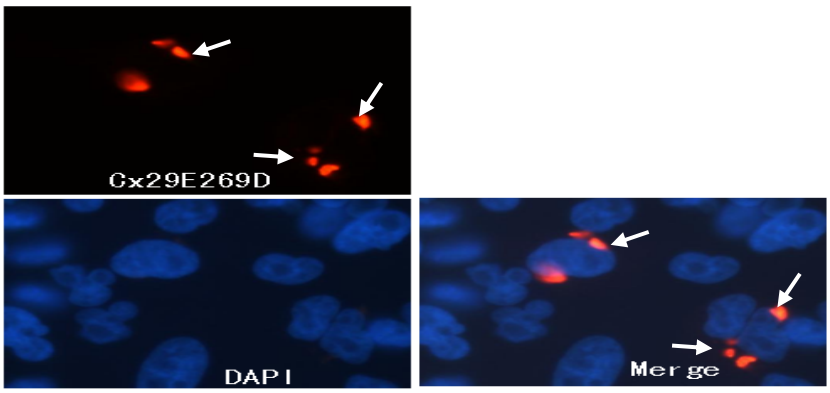

Figure 8. 利用基因重組技術將 *Cx29*E269D- DsRed 基因建構於 pBI 雙向表現載體上，轉殖感染 Tet-on HeLa cells，以細胞免疫螢光染色法染細胞膜觀察 Cx29E269D-DsRed 蛋白在細胞內的表現情形。
左上圖為正常的 Cx29 螢光蛋白表現(紅色)；左下圖為 DAPI 染細胞核(藍色)；右下圖為 merge 在一起的結果。觀察白色箭頭所指的地方，我們可以發現 Cx29E269D-DsRed 蛋白堆積在細胞質。

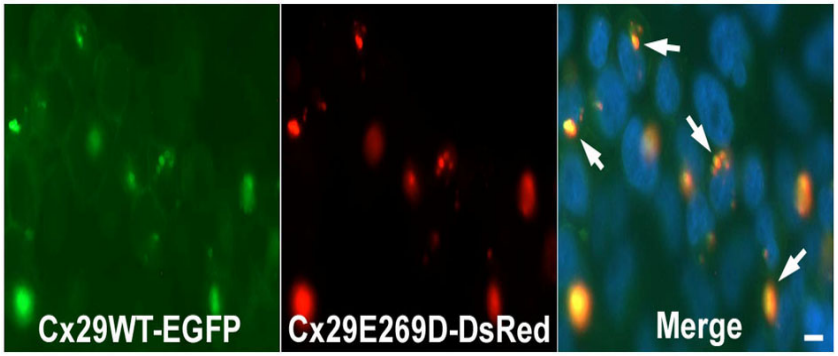

Figure 9. Co-expression of mutant proteins and Cx29WT using the tet-on protein expression system. HeLa cells co-expressing Cx29WT-EGFP and Cx29E269D-DsRed reveal co-localization of the two proteins near the nucleus. Arrows indicate co-expression of Cx29. The cells were counterstained with 4'-6-Diamidino-2-phenylindole, DAPI, (blue) to highlight the nuclei. Scale bars: 10 μm.

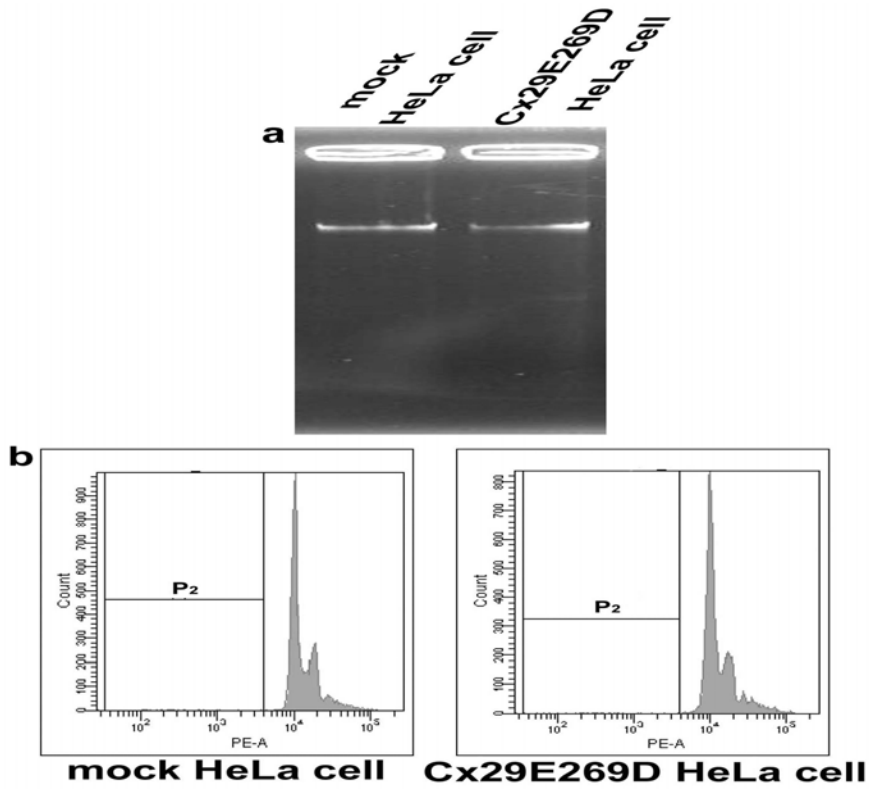

Figure 7. Cell viability analysis on mock and stably expressed Cx29E269D HeLa cells. Both cells were incubated in DMEM medium for 24 h and were then harvested for DNA fragmentation assay (a) and analysis of flow cytometry (b). (a), DNA was prepared for agarose gel electrophoresis as described in the Materials and Methods. Results are representative of three separate experiments. Lane 1: mock HeLa cell. Lane 2: stably expressed Cx29E269D HeLa cell. (b), After harvested, the cells were stained with PI and then analyzed by flow cytometry. Cells undergone apoptosis are characteristically distributed within the sub-G1 population (P_2).

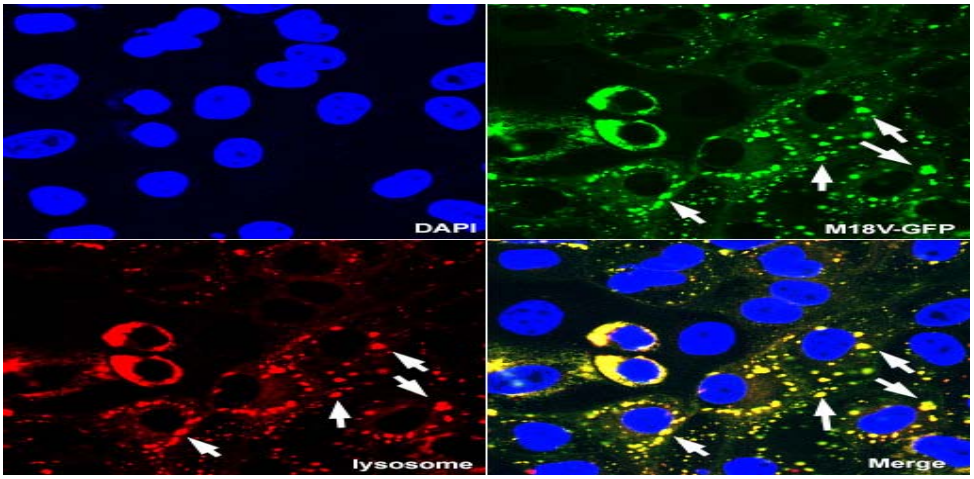

Figure 12. 確定 CLDN14 M18V 突變蛋白堆積的位置，使用免疫螢光染色法利用染劑標定溶酶體 右上圖為突變的 M18V 螢光蛋白表現；左下圖為染劑標定溶酶體；左上圖為 DAPI 染細胞核(藍色)；右下圖為 merge 在一起的結果。結果 M18V 突變蛋白大部分與 lysosome colocalization，推測突變蛋白被送到 lysosome 降解。

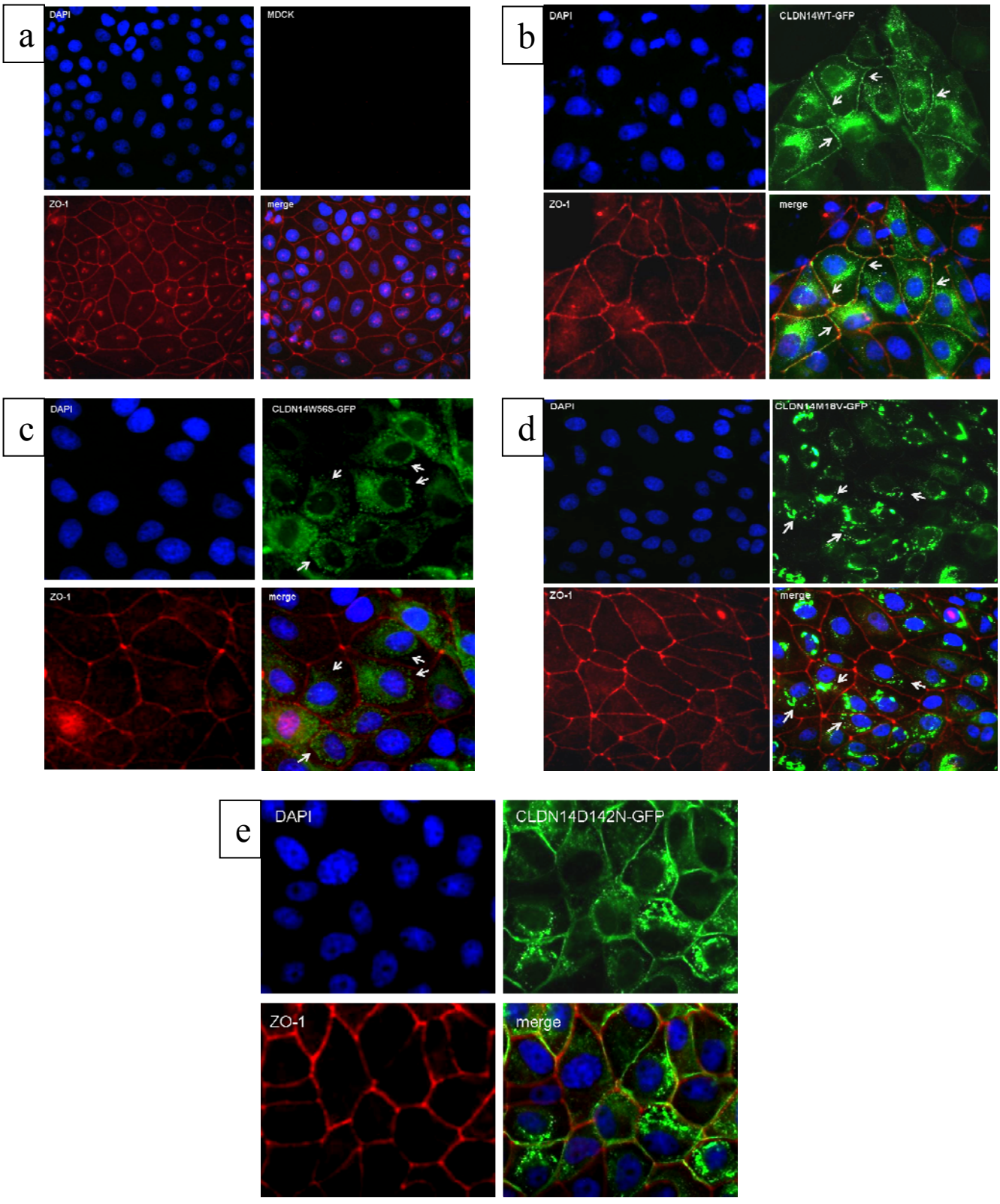

Figure 12. 觀察CLDN14正常和突變蛋白和細胞膜上ZO-1的交互作用

以ZO-1抗體分別標定(a) MDCK細胞 (b) CLDN14WT-GFP細胞 (c)CLDN14W56S-GFP細胞 (d)CLDN14M18V-GFP細胞和(e)CLDN14D142N-GFP細胞，細胞膜上ZO-1後，經過螢光顯微鏡觀察。結果我們發現CLDN14WT和CLDN14D142N會和ZO-1共同表現(黃色的部分)。然而CLDN14W56S和CLDN14M18V並不會和ZO-1有共同的表現。箭號指的是CLDN14蛋白所表現的位置。

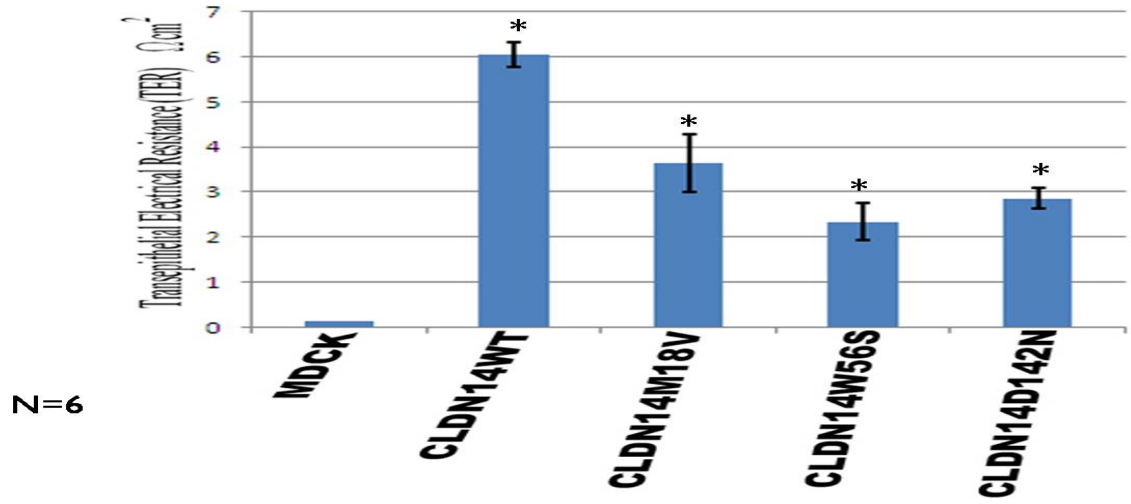

Figure 13. 利用 transepithelial electrical resistance (TER)的方法來探討 CLDN14 突變後對 barrier 功能的影響

經過測量後發現各個突變點的電阻值明顯低於正常 CLDN14WT 細胞株，沒有送入 *CLDN14* 基因的 MDCK 幾乎沒有電阻值的存在，因為 MDCK 不具有內生性的 tight junction，而 CLDN14WT 的細胞株電阻值遠高於不含 CLDN14 的 MDCK 細胞株，數值大約在 6 個電阻單位附近，而突變的 *CLDN14* 細胞株例如 CLDN14M18V 的細胞株電阻值約為 3.6， CLDN14W56S 細胞的電阻值約為 2.2，CLDN14D142N 細胞的電阻值為 2.9，由以上結果可以看出突變的 *CLDN14* 並無法像正常的 *CLDN14* 一樣具有形成的 tight junction 正常間隙的屏障功能，顯示出 tight junction 的功能性在正常與突變的 *CLDN14* 細胞株之間確實有著顯著的差異。

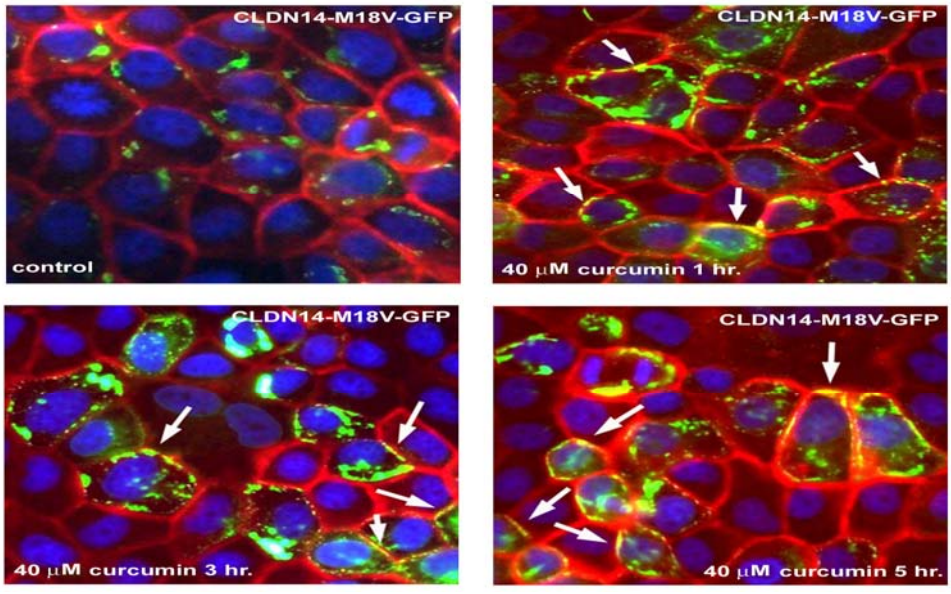

Figure 14. Curcumin rescued expression of CLDN14-M18V-GFP mutant protein from cytoplasm to membrane in MDCK cell. MDCK cells expressing the mutant protein of CLDN14-M18V-GFP (green) were treated with the indicated concentrations of curcumin for 1, 3 and 5 hours, respectively. The results indicated CLDN14-M18V-GFP mutant protein escaped degradation and appeared on the cell surface (red) in the 40 μM curcumin condition after 1 hour. Arrows indicate (yellow color) expression protein of

CLDN14-M18V-GFP in the membrane. The cells were counterstained with 4'-6-Diamidino- 2-phenylindole, DAPI, (blue) to highlight the nuclei.

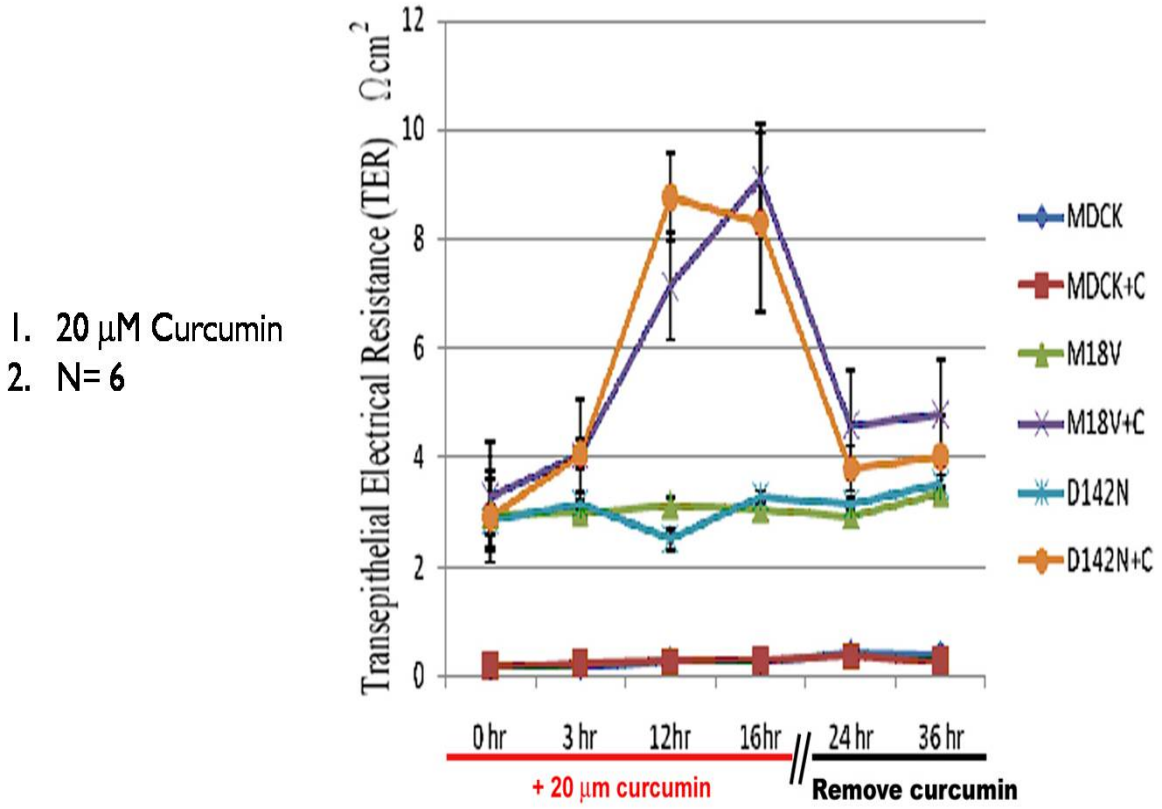

Figure 15. curcumin作用後的TER測量值

分別測定20μM curcumin作用下與無curcumin作用下CLDN14M18V細胞株的電阻值，可以發現在curcumin作用下的CLDN14M18V細胞株測出來的電阻數值有逐漸升高的趨勢，由原本的2.2個電阻單位上升到8.5個電阻單位，與無curcumin下CLDN14M18V細胞株的電阻值有明顯的差異，在無curcumin作用下的CLDN14M18V細胞株電阻值並無顯著變化，始終維持在2.2個電阻單位左右，但是若移除curcumin 24小時後電阻值又有降低的現象，若是以CLDN14D142N做相同的實驗，結果與CLDN14M18V 的細胞株一樣電阻都有明顯上升的趨勢。

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ORIGINAL INVESTIGATION

A novel mutation in the connexin 29 gene may contribute to nonsyndromic hearing loss

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Abstract Connexins (Cxs) are homologous four-transmembrane domain proteins and constitute the major components of gap junctions. Among a cohort of patients with nonsyndromic hearing loss, we recently identified a novel missense mutation, E269D, in the *GJC3* gene encoding connexin 29 (Cx29), as being causally related to hearing loss. The functional alteration of Cx29 caused by the mutant *GJC3* gene, however, remains unknown. This study compared the intracellular distribution and assembly of mutant Cx29 (Cx29E269D) with that of the wild-type Cx29 (Cx29WT) in HeLa cells and the effect the mutant protein had on those cells. Cx29TW showed continuous staining along apposed cell membranes in the fluorescent localization assay. In contrast, the p.E269D missense mutation resulted in accumulation of the Cx29 mutant protein in the endoplasmic reticulum (ER) rather than in the cytoplasmic membrane. Co-expression of Cx29WT and Cx29E269D proteins by a bi-directional tet-on expression system demonstrated that the heteromeric connexon accumulated in the cytoplasm, thereby impairing the formation of the gap junction. Based on these findings, we suggest that Cx29E269D has a dominant negative effect on the formation and function of the gap junction. These results provide a novel molecular explanation for the role Cx29 plays in the development of hearing loss.

Introduction

Gap junction (GJ) channels mediate direct cell-to-cell communication by allowing the passage of small biological molecules (<1 kDa) including electrolytes, second messengers and metabolites from one cell to the other (Gilula et al. 1972; White and Bruzzone 1996). GJ channels are thought to have diverse functions, including the propagation of electrical signals, metabolic cooperation, growth control, spatial buffering of ions and cellular differentiation (Bruzzone et al. 1996). GJ channels are double membrane protein structures that form by the head-to-head docking of two half channels to create hydrophilic pores across the membrane (Makowski et al. 1997). Each half channel, or connexon, is composed of six polytopic transmembrane protein subunits, termed connexins (Cxs). The Cxs within a connexon can be the same (homomeric) or different (heteromeric), and the two connexons docking together can be identical (homotypic junctions) or different (heterotypic junctions) (Willecke et al. 2002). Studies have also demonstrated that connexins can assemble into functional hexameric connexons in the ER membrane (Falk et al. 1997).

H.-M. Hong and J.-J. Yang contributed equally to this publication.

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Subcellular fractionation studies and immunocolocalization analyses suggest that connexins pass through the Golgi apparatus to reach the plasma membrane (Musil and Goodenough, 1991; Falk et al. 1994; Laird et al. 1995).

Connexins compose a large and highly homologous gene family encoding plasma membrane proteins. Each Cx contains four transmembrane domains linked by one cytoplasmic and two extracellular loops. The N- and C-termini are located on the cytoplasmic side. Transmembrane domains bear conserved amino acids, whereas the cytoplasmic loop and the C-terminal region are the most variable parts of connexins. More than 20 mammalian connexins have been described (Willecke et al. 2002) and, therefore, there are potentially a large number of different kinds of hemichannels in different tissue (Richard, 2000). The importance of the physiological functions of connexins is illustrated by the identification of connexin mutations as the molecular cause of various human diseases (Krutovskikh and Yamasaki 2000), such as X-linked Charcot–Marie–Tooth peripheral neuropathy, cataract, and hearing loss.

At least six *Cx* genes (*Cx26*, Cx29, *Cx30*, *Cx30.3*, *Cx31* and *Cx43*) are known to be involved in human genetic deafness (Kelsell et al. 1997; Xia et al. 1998; Grifa et al. 1999; López-Bigas et al. 2002; Yang et al. 2007; Wang et al. 2010). The proteins they encode are located in gap junction-rich regions of the cochlear duct, suggesting that all six connexin proteins are essential components of gap junctions. The loss of connexin in gap junction complexes in the cochlea would be expected to disrupt the recycling of potassium from the synapses at the base of hair cells through the supporting cells and fibroblasts back to the high potassium-containing endolymph of the cochlear duct, thereby resulting in hearing loss due to local potassium intoxication of the Corti's organ (Kikuchi, et al. 1995).

Cx29 is a relatively new member of the Cx protein family (Sohl et al. 2001; Altevogt et al. 2000). The *Cx29* gene (NM 181538), which contains two exons and an open reading frame of 840 base pairs, is localized on chromosome 7q22.1. The *Cx29* gene product contains 279 amino acid residues and has a molecular weight of 31.29 kDa (Sohl et al. 2001; Altevogt et al. 2000). Cx29 has been shown to be highly expressed in the cochlea (Ahmad et al. 2003). Animal studies indicate that the Cx29 protein is expressed in the cochlea neurons, spiral limbus, spiral ligament, organ of Corti, stria vascularis, Schwann cells myelinating the soma, and spiral ganglion (SG) neurons in mouse and in rat cochlea (Yang et al. 2005; Tang et al. 2006). At least six heterozygous mutations [c.807A>T (E269D), c.43C>G (R15G), c.230C>G (T77S), c.525T>G(L175L), c.781+10 C.>G, and c.781+15 C>T] and two heterozygous polymorphisms (781+62 G>A and c.*+2 T>G) of the *Cx29* gene have been detected in Taiwanese patients with nonsyndromic deafness (Yang et al. 2007; Wang et al. 2010). These findings demonstrate the requirement of Cx29 for normal cochlear function and suggest that *Cx29* is a new candidate gene for studying auditory neuropathy. To better understand the pathogenic role of Cx29 mutation in nonsyndromic hearing loss, it is necessary to investigate the functional properties of mutant Cx29 gap junctions. In the present study, we investigated the effect of the E269D (c.807A>T) mutation on the functional properties and the subcellular localization of the heterozygous mutant Cx29 protein in HeLa cells and in tet-on HeLa cells.

Materials and methods

Molecular cloning of wild-type and mutant Cx29 gene

The mammalian expression vector pcDNA3.1-CT used in this study was constructed as previously described (Griffin et al. 1998). We designed and synthesized a tight-binding pair of molecular components comprising a small receptor domain composed of as few as six natural amino acids (–Cys–Cys–Xaa–Xaa–Cys–Cys– tags) in the C-tail of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). The open reading frames of human Cx29 were obtained by RT–PCR (Superscript II; Invitrogen, Carlsbad, CA) from human glioma cells using oligonucleotide primers (forward primer Cx29F: 5′-ATGTGTGGCAGGTTCCTGCG-3′ and reverse primer Cx29R: 5′-TCAGGCATCTC TGGGTCC AA-3′) and Platinum Pfx DNA polymerase. The cDNA containing the full-length coding region of human Cx29 was used as the template. PCR was carried out with the following oligonucleotide primers: forward primer was Cx29F-Kpn1 5′-ATG**GGTACC**ATGTGTGGCAGGTTC CTGCG-3′ and corresponded to nucleotides 1–20 of the human Cx29 coding region; reverse primer was Cx29R-*Xho1* 5′-ATG**GAGCTC**CCGGCATCTCTGGGTCCAAC T-3′ and corresponded to nucleotides 817–836 of the human Cx29 coding region. The PCR DNA product (855 bp) of the human Cx29 coding region was cloned into the pcDNA3.1-CT vector. Mutant Cx29 gap junction proteins were obtained by performing oligonucleotide-directed mutagenesis using the Stratagene Quickchange site-directed mutagenesis kit (Stratagene,La Jolla,CA). The following oligonucleotide primers (mutated nucleotide is underlined) were used to prepare the mutant Cx29 gene: Cx29 E269D sense 5′-AGAAGCTTAGCCCAGGATAA ACAAAGACCAGTTG G-3′; Cx29 E269D antisense 5′-C CAACTGGTC TTTGTTTATCCTGGGCTAAG CTTCT-3′.

For fusion protein generation, cDNA sequences of the autofluorescent reporter proteins GFP (pGFPN1 vector; Clontech, Palo Alto, CA) and DsRed (pDsRedN1 vector; Clontech, Palo Alto, CA, USA) were fused in-frame to the C-terminus of wild-type (wt) and mutant *Cx29* genes. The

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open reading frames (ORFs) of *Cx29* were obtained from the pcDNA3.1 clone after digestion with *Kpn*I and *Sac*II, and then subcloned into the *Kpn*I and *Sac*II restriction sites in vectors pGFPN1 and pDsRedN1 (Clontech, Palo Alto, CA, USA). The dideoxy DNA sequencing method, using a DNA sequencing kit (Applied Biosystems Corporation, Foster city, CA, USA), an ABI Prism 3730 Genetic Analyzer (Applied Biosystems Corporation, Foster City, CA, USA) and restriction digest were used to confirm the DNA sequence of all constructs.

Expression of Cx29 gap junction proteins in HeLa cells

Human epitheloid cervix carcinoma cells (HeLa, ATCC CCL 2; American Type Culture Collection, Rockville, MD, USA) lacking the *GJIC* gene were used throughout this study. Cell lines were maintained in standard cell culture medium supplemented with 10% of fetal bovine serum, 2 mM of L-glutamine, and 50 units/ml of penicillin–streptomycin. Cell cultures were maintained at 37°C in a humidified 5% CO_2 incubator. The vectors, pcDNA3.1-CT, pGFPN1 and pDsRedN1, containing the DNA fragment encoding the wild-type or mutant Cx29 protein were transfected to HeLa cells using lipofectAMINE (Invitrogen Corporation, California, USA). To obtain HeLa cell colonies that stably expressed Cx29WT or Cx29 mutants, 1 mg/ml of G418 (Geneticin, Gibco–BRL, Grand Island, NY, USA) was added to the growth medium. The growth medium was renewed at 2–3-day intervals. After 2–3 weeks, single cell colonies were obtained. Under a fluorescence microscope, cells displaying either green or red fluorescence were chosen for further culture. After individual colonies had been chosen, a FACSAria™ cell sorter (BD Biosciences, USA) was used to sort positive cells. The positive stable cell line was used for the subsequent functional analyses.

Immunofluorescence staining of post-transfection HeLa cells

FlAsH-EDT_2 labeling reagent (Invitrogen, Carlsbad, CA, USA) was used at final concentrations of 1 mM in the presence of 10 mM of EDT (1,2-ethanedithiol). The labeling was performed for 1 h at 37°C in 1X Hank's balanced salt solution (HBSS, Gibco-BRL, Invitrogen Corporation, California, USA) supplemented with D+ glucose (1 g/l). Free and nonspecifically bound ligands were removed by washing with EDT (250 mM) (in HBSS with glucose). FlAsH-labeled cells were examined with a Zeiss Axioplan 200 M fluorescence microscope imaging system (Zeiss Axioplam, Oberkochen, Germany).

Wild-type or mutant Cx29 protein expression in HeLa cells was analyzed by a direct fluorescent protein fusion method involving fusion of GFP or DsRed to the C-terminal ends of the Cx29 proteins. Briefly, post-transfection HeLa cells grown on coverslips were fixed with 4% paraformaldehyde in 0.1 M of PBS for 20 min and then rinsed three times in PBS. Then, the coverslips were immersed in 10% normal goat serum and 0.1% Triton X-100 for 15 min. The primary antisera and dilutions were as follows: mouse anti-pan-cadherin antibody at 1:200 (anti-CH19; abcan) for cell membrane, mouse anti-Golgin-97 at 1:200 (Invitrogen, Carisbad, CA, USA) for Golgi apparatus, and rabbit anti-PDI at 1:200 (Invitrogen, Carisbad, CA, USA) for endoplasmic reticulum (ER). After incubation with primary antiserum at 4°C overnight, the cells were rinsed in PBS three times before adding Alexa Fluor 488 and/or Alexa Fluor 594 conjugated secondary antibodies (Invitrogen, Carisbad, CA, USA). Lysosomes were stained with LysoTracker® Probes (Invitrogen, Carisbad, CA) for 20 min at room temperature. The nuclei of cells were counterstained with DAPI (2 μg/ml) for 5 min and rinsed with PBS. Mounted slides were visualized and photographed using a fluorescence microscope (Zeiss Axioplam, Oberkochen, Germany).

Generation of Cx29 wt/Cx29 mutant Chimerae for tet-on expression system

The coding region of Cx29WT and that of mutant Cx29 were amplified from plasmids containing the Cx29 cDNA (Cx29wt-EGFP or Cx29E269D-DsRed) using two pairs of primers containing recognition sequences 5′- *Sal* I and 3′- *Not* I or 5'-*Nhe I* and 3′-*EcoR V*, respectively, and Platinum Pfx DNA polymerase (Invitrogen, Carisbad, CA, USA). Purified products were subcloned into the corresponding site of the bi-directional expression vector pBI (Clontech, Palo Alto, CA, USA). The dideoxy DNA sequencing method, using a DNA sequencing kit (Applied Biosystems Corporation, Foster city, CA, USA), an ABI Prism 3730 Genetic Analyzer (Applied Biosystems Corporation, Foster city, CA, USA) and restriction digest were used to confirm the DNA sequence of all constructs.

Transfection and expression of Cx29WT/Cx29 mutant chimerae protein in tet-on HeLa cell line

The tet-on HeLa cell line deficient in the *GJIC* gene was purchased from BD Biosciences Clontech (Palo Alto, CA, USA) and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% FBS (Gibco-BRL, Gaithersburg, USA), 100 μg/ml of G418, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a moist atmosphere containing 5% CO_2. Transfection was carried out using LipofectAMINE reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. A ratio of 1 μg DNA versus 2 μl LipofectAMINE 2000 was used

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for the tet-on HeLa cells. Cells were harvested at 24 h post-transfection and grown on a coverslip for 24 h at 37°C in a humidified 5% CO_2 incubator. Then, tet-on HeLa cells were treated with 1 μg/ml doxycyclin (Dox) (Sigma-Aldrich Corporation, St. Louis, MO, USA) in cell culture medium to induce Cx29WT or Cx29E269D mutant protein expression. Cells were exposed to Dox for 5 h prior to immunofluorescence staining. Tet-on HeLa cells were fixed with 4% paraformaldehyde in 0.1 M PBS for 20 min, rinsed three times in PBS, stained with DAPI for 5 min and then washed three times with PBS. Mounted slides were visualized and photographed using a fluorescence microscope (Zeiss Axioplam, Oberkochen, Germany).

Reverse transcription-polymerase chain reaction (RT–PCR)

Total RNA was isolated from four positive stable cell lines 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 volume of 20 μl, containing 2–5 μg RNA, random hexamer primer, and 200 units Improm-II™ Reverse Transcriptase (Promega, San Luis Obispo). With primers specific for the coding region of the *GJC3* gene (forward 5′-ATGTGCGGC AGGTTCCTGAG -3′ and reverse 5′-CATGTTT GGGAT CAGCGG-3′), PCR was performed (94°C for 30 s, 58°C for 35 s, 72°C for 1 min) for 35 cycles in a volume of 25 μl containing 1 mM of Tris–HCl (pH 9.0), 5 mM of KCl, 150 μM of $MgCl_2$, 200 μM of dNTP, 1 unit of proTaq DNA polymerase (Promega, San Luis Obispo), 100 ng of cDNA and 200 μM of forward and reverse primers. A fragment of approximately 700 bp was amplified from cDNA of the *GJC3* gene. The PCR products were subjected to electrophoresis in an agarose gel (2 w/v%) stained with ethidium bromide. The signals were detected by an Alpha Image 2200 system (Alpha Image 2200 analysis software).

Cell viability analysis

Cell viability was analyzed by flow cytometry for the presence of sub-G1 population. Both mock HeLa cells and stably expressed Cx29E269D HeLa cells were cultured in DMEM medium. After 24 h, both cells were harvested and stained with propidium iodide (PI) and then analyzed by flow cytometry (FACScan; BD Biosciences, San Juan, CA, USA).

DNA fragmentation analysis

Both mock and stable expressed Cx29E269D HeLa cells (5×10^6 cells) were cultured in DMEM medium for 24 h. After removing the nonadherent dead cells in the cultures by rinsing with PBS, the adherent cells were collected by centrifugation for 5 min (1,000 rpm) at room temperature. DNAs were purified as previously described (Liu et al. 2002). DNA was resolved in a 1.5% (w/v) agarose gel in 1× TAE buffer. The DNA bands were stained with ethidium bromide (0.5 μg/ml) and photographed (Alpha Image 2200 analysis software).

Results

Cx29 consists of four transmembrane domains (TM): TM1 (amino acid 20–42), TM2 (amino acid 78–100), TM3 (amino acid 133 ~ 155) and TM4 (amino acid 197–219), linked by one cytoplasmic and two extracellular loops with cytoplasmic C- and N-terminal ends. The p.E269D substitutions detected in this study occurred in the putative C-terminal cytoplasmic domain of the Cx29 protein (Fig. 1). To understand the effect of the p.E269D missense variant, we examined amino acid sequences of Cx29 using a basic ConSeq analysis system (http://conseq.tau.ac.il/). After the protein sequence of Cx29 had been deposited into the system, the system automatically detected homologous sequences of Cx29 and conducted multiple alignments. A total of 114 PSI-BLAST hits were detected by the system, of which 96 were unique sequences. In the next step, calculation was performed automatically by the system on 50 sequences with the lowest E-values. The result revealed that p.E269 is only moderately conserved (Conseq score = 3–4) in the C-terminal domain (Supplemental Fig. 1).

To understand the effects of p.E269D on the functional properties and subcellular localization of the Cx29 protein,

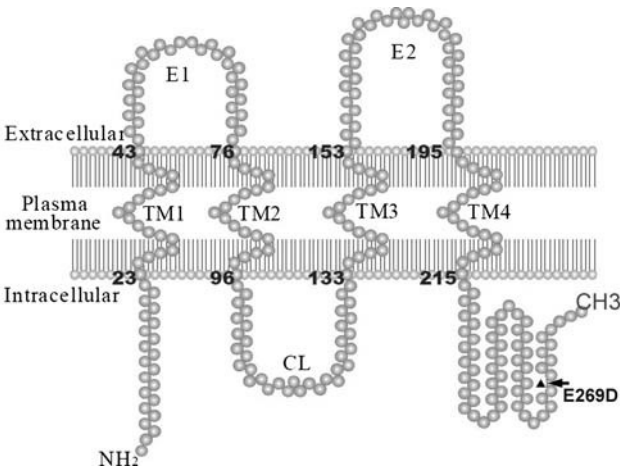

Fig. 1 Schematic representation of the domain structure of the Cx29 protein with indication of known variants. The *black triangle* and *arrow* indicate the c.807A >T (p.E269D) variant in Cx29. M1-4: transmembrane domains; *E1-2* extracellular domains; *CL* cytoplasmic linking domain, *N* N-terminal domain, *C* C-terminal domain

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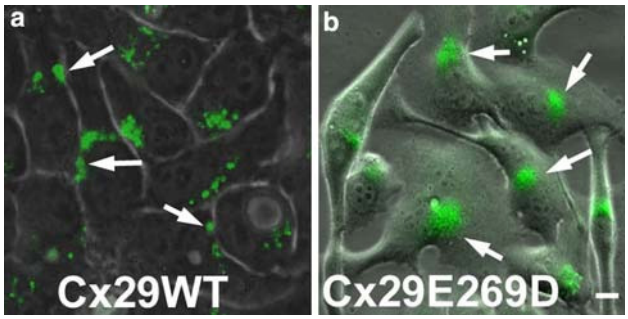

Fig. 2 Expression analysis of Cx29WT and Cx29E269D in transiently transfected HeLa cells using the FlAsH™-EDT$_2$ Labeling Kit. (**a**) Fluorescence microscopy of Cx29WT HeLa cells shows expression of the wild-type protein in the plasma membranes. (**b**) In contrast, Cx29E269D-transfected HeLa cells show expression of the mutated protein near the nucleus. *Arrows* indicate expression of Cx29. *Scale bars* 10 μm

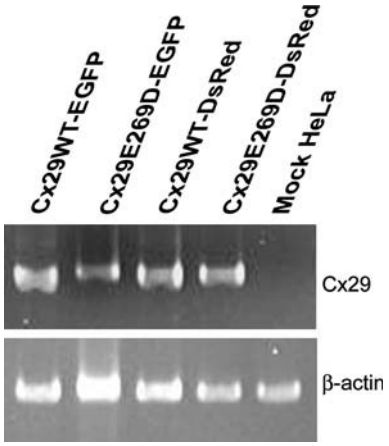

Fig. 3 Expression analysis of *Cx29* mRNA in the four transfected HeLa cells by RT–PCR. RT–PCR analysis of total RNA from HeLa cells expressing Cx29WT-EGFP, Cx29WT-DsRed, Cx29E269D-EGFP and Cx29E269D-DsRed confirms expression of the corresponding mRNAs in stably transfected HeLa cell lines (*up panel*). *β*-actin served as reference of the loading amount of total RNA for each sample (*low panel*). Mock HeLa is a negative control

we used lipofection to transiently transfect gap junction-deficient HeLa cells with cDNA constructs of wild-type (Cx29WT-pCDNA3.1-CT) or mutant Cx29 (Cx29E269D-pCDNA3.1-CT). The labeling reagent FlAsH-EDT$_2$, which becomes fluorescent upon binding to recombinant proteins containing the TC-tag, was used for site-specific detection of recombinant proteins in live mammalian cells. Immunolabeling with FlAsH-EDT$_2$-specific stain against the TC-tag in the C-terminus of Cx29 revealed that Cx29WT localized at the plasma membrane as small plaques at points of contact between adjacent cells, indicating the formation of gap junction-like structures (Fig. 2a). However, the assay revealed that Cx29E269D was distributed in the cytoplasm near the nucleus (Fig. 2b).

To confirm the localization patterns seen in the immunolabeling assay, HeLa cells were transfected with Cx29 constructs that were directly 'tagged' with GFP or DsRed at the C-terminal end of the protein. HeLa cells were then transfected with plasmids driving the expression of one or more wild-type or mutant Cx. Cells that stably integrated the WT or mutant Cx29 gene were selected. RT–PCR was performed to assess the expression of transgenes in stable cell lines (Fig. 3). Four cell lines expressed a transcript for Cx29 (Cx29WT and Cx29E269D). No Cxs were detected in non-transfected HeLa cells (Fig. 3, lane 5). In the Cx29WT-GFP stable expression cell line, fluorescence resulting from GFP expression was observed along apposed cell membranes (Fig. 4a, right pannel). This membrane localization was confirmed by colocalization with pan-cadherin (Fig. 4a, left pannel). Similarly, Cx29WT-DsRed also localized to the cell membrane (supplemental Fig. 2a). However, as seen in the immunolabeling assay, both Cx29E269D-GFP (Fig. 4b) and Cx29E269D-DsRed (supplemental Fig. 2b) were concentrated in the cytoplasm close to the nucleus. We then determined whether the p.E269D mutation affects the assembly, trafficking or degradation of the Cx29 protein. We also investigated which organelles in the cytoplasm, the mutant Cx29 localized in. HeLa cells that had been transfected with Cx29E269D-GFP cDNA were immunostained with markers for lysosome, ER (anti-PDI) and Golgi apparatus (Fig. 5). The results of the assay showed that the Cx29E269D protein was typically found in a reticular pattern co-localized with protein disulfide isomerase (PDI), a resident of the endoplasmic reticulum, indicating that the E269D mutation interferes with normal Cx29 trafficking (Fig. 5b). In addition, using HeLa cells that had been transfected with an "empty" expression plasmid (pGFP or pDsRed plasmid) as negative control, we found that only GFP- or DsRed-tagged protein were uniformly spread in the cytoplasm of HeLa cells (supplemental Fig. 3).

Previously, we found that the p.E269D mutation in Cx29 is a heterozygous mutation in patients with nonsyndromal hearing loss (Yang et al. 2007). Consequently, co-expression studies were carried out to examine the effects of the mutant protein on Cx29WT in tet-on HeLa cells using a bi-directional tet-on protein expression system with equal amounts of the two respective expression proteins. The expression pattern in cells expressing Cx29WT-EGFP and Cx29E269D-DsRed was similar to that in cells expressing only Cx29E269D (Fig. 6). Based on this finding, the p.E269D mutation appears to have a dominant negative effect on Cx29WT.

To determine the possibility of the accumulation of a great quantity of Cx29E269D mutant proteins in the ER of the HeLa cell switch on unfolded protein response (UPR) within the ER that leads to the programmed cell death (apoptosis), we further analyzed cell apoptosis using two

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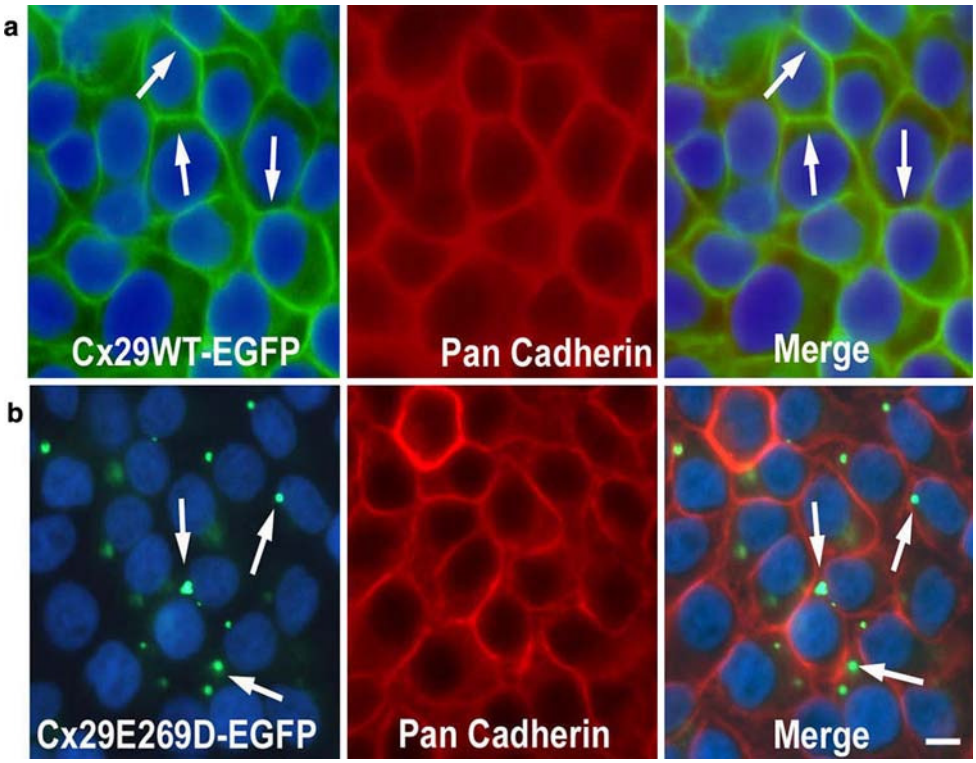

Fig. 4 Expression analysis of Cx29WT and Cx29E269 in stably transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells expressing Cx29WT-EGFP (**a**) shows expression of the Cx29 fusion protein in the plasma membranes. However, Cx29E269D-EGFP (**b**) transfected HeLa cells show impaired trafficking of the Cx29 protein with localization near the nucleus. The cells were counterstained with 4′-6-diamidino-2-phenylindole, DAPI, (*blue*) to highlight the nuclei. *Scale bars* 10 μm

methods. Both mock and stably expressed Cx29E269D HeLa cells were incubated 24 h before subjecting to cell viability assays by DNA fragmentation and flow cytometry. DNAs were purified from mock and stably expressed Cx29E269D HeLa cells and then resolved by conventional agarose gel electrophoresis to evaluate the potential apoptotic DNA fragmentation. The results clearly confirmed the absence of the characteristic DNA laddering of those cells expressing Cx29E269D (Fig. 7a). Consistent with those results, the number of sub-G1 cells that are characteristic of apoptosis was essentially the same between the mock and stably expressed Cx29E269D HeLa cells determined by flow cytometry analysis (Fig. 7b). Therefore, we suggested that the accumulation of Cx29E269D mutant protein in the ER did not trigger apoptosis.

Discussion

Functional studies of connexins have been carried out in expression systems by transfecting mammalian cells (e.g., HeLa cells) devoid of Cxs with relevant cDNAs to reconstitute gap junction communication (Beltramello et al. 2003). In this study, we studied the intracellular distribution and assembly of mutant Cx29 (Cx29E269D) in HeLa cells and in tet-on HeLa cells. The immunolabeling assay with EGFP revealed that the Cx29WT-EGFP protein was expressed in a continuous band along apposed cell membranes. This finding is consistent with that reported by Ahn et al. (2008). Although the Cx29WT-EGFP was transported to the plasma membrane, it did not evoke the typical punctuate fluorescence pattern of a gap junction channel. The detectable signals were instead equally distributed on the plasma membrane in a manner similar to the distribution of mouse Cx23 (mCx23) in HeLa cells (Sonntag et al. 2009) and pannexins in NRK cells (Penuela et al. 2007). It has been reported that mCx23 does not form functional gap junction channels, but causes enhanced ATP release from HeLa cells. In addition, mCx23 seems to share functional properties with pannexin (hemi) channels rather than gap junction channels (Sonntag et al. 2009). Further investigation into the functional roles of Cx29 in the cell is needed.

In our previous study, we found a novel c.807A>T mutation in the C-tail coding region of the *GJC3* gene in two patients with nonsyndromic deafness. This A>T transversion leads to a heterozygous glutamic acid (E) → aspartic acid (D) substitution at codon 269 (p.E269D) (Yang et al. 2007). Glutamic acid is a negatively charged, polar amino acid. It, therefore, prefers to substitute for the other negatively charged (and very similar) amino acid aspartic acid. Being charged and polar, glutamic acid (and aspartic acid) prefers to be on the surface of proteins, exposed to an aqueous environment. When buried within the protein, glutamates (and aspartates) are frequently involved in salt-bridges, where they pair with a positively charged amino acids to create stabilizing hydrogen bonds that are important for protein stability (Betts and Russell 2003).

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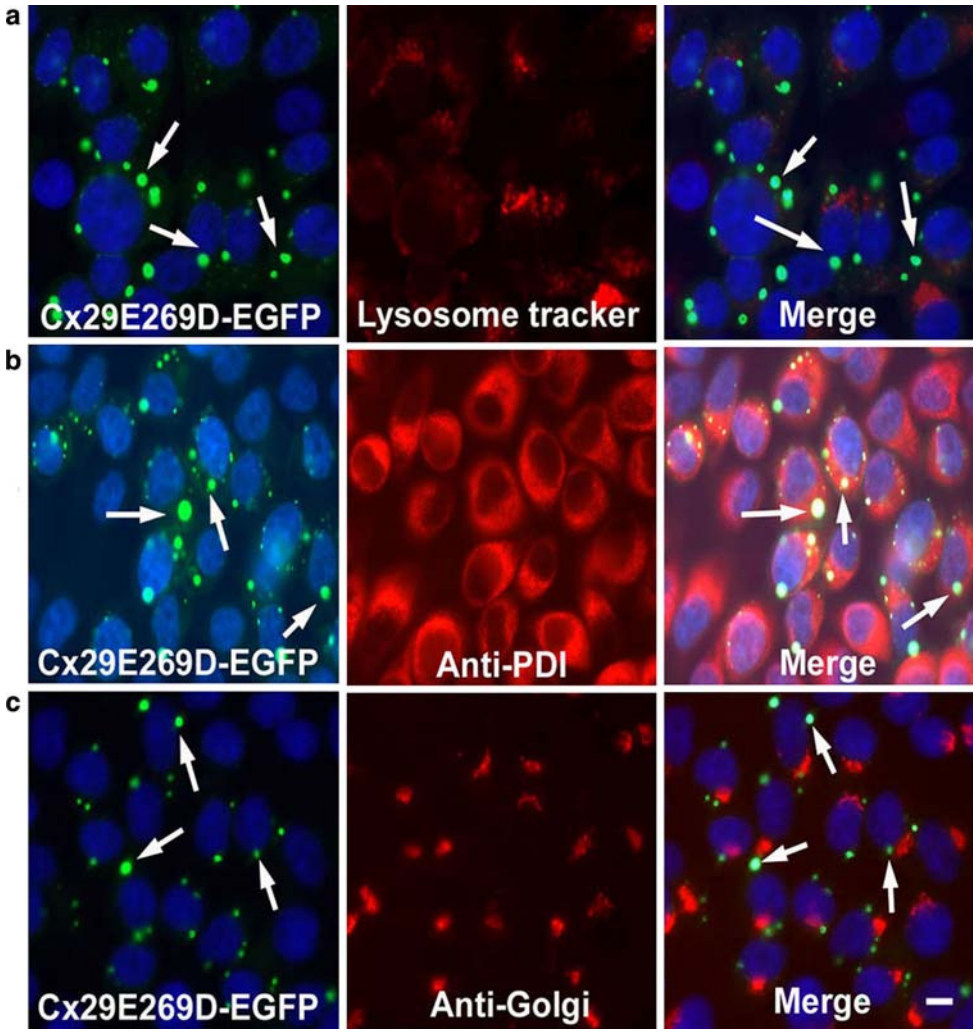

Fig. 5 Intercellular localization of mutant Cx29 proteins. Photomicrographs of HeLa cells transfected with Cx29E269D-EGFP cDNA after immunostaining for markers of the lysosome, ER (anti-PDI), and Golgi apparatus (*red* in (**a**)–(**c**), respectively). *Yellow signal* in the image overlays (*right column*) indicates co-localization of Cx29E269D-EGFP and the organelle of interest. Mutant Cx29 shows moderate co-localization with the ER marker. The cells were counterstained with 4′-6-diamidino-2-phenylindole, DAPI, (*blue*) to highlight the nuclei. *Scale bars* 10 μm

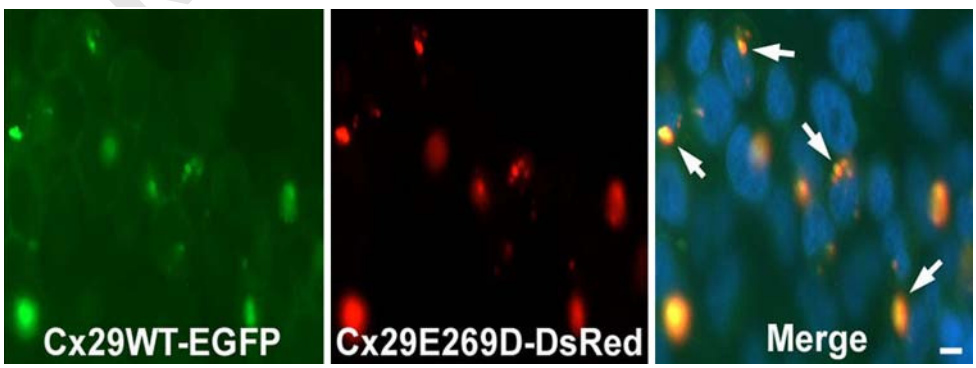

Fig. 6 Co-expression of mutant proteins and Cx29WT using the tet-on protein expression system. HeLa cells co-expressing Cx29WT-EGFP and Cx29E269D-DsRed reveal co-localization of the two proteins near the nucleus. *Arrows* indicate co-expression of Cx29. The cells were counterstained with 4′-6-diamidino-2-phenylindole, DAPI, (*blue*) to highlight the nuclei. *Scale bars* 10 μm

ConSeq is a Web site server that can identify biologically important residues in protein sequences (Berezin et al. 2004). Using the ConSeq server, we found that p.E269 is only moderately conserved (Conseq score = 3–4) in the C-terminal domain. Based on this finding, it is unlikely that the transversion of glutamic acid to sspartic acid at codon 269 of the Cx29 gene plays a critical role in the function of the Cx29 protein. However, our results showed that the p.E269D missense mutation resulted in the accumulation of the Cx29 mutant protein in the endoplasmic reticulum (ER) instead of targeting the cytoplasmic membrane.

In addition, the results from the bi-directional tet-on protein co-expression system showed that Cx29E269D has a dominant negative effect on the function of normal Cx29, indicating that the p.E269 mutation leads to loss of function of the Cx29 protein. The 3D structure of the Cx29 protein needs to be studied to further understand the influence this mutation has at the protein level.

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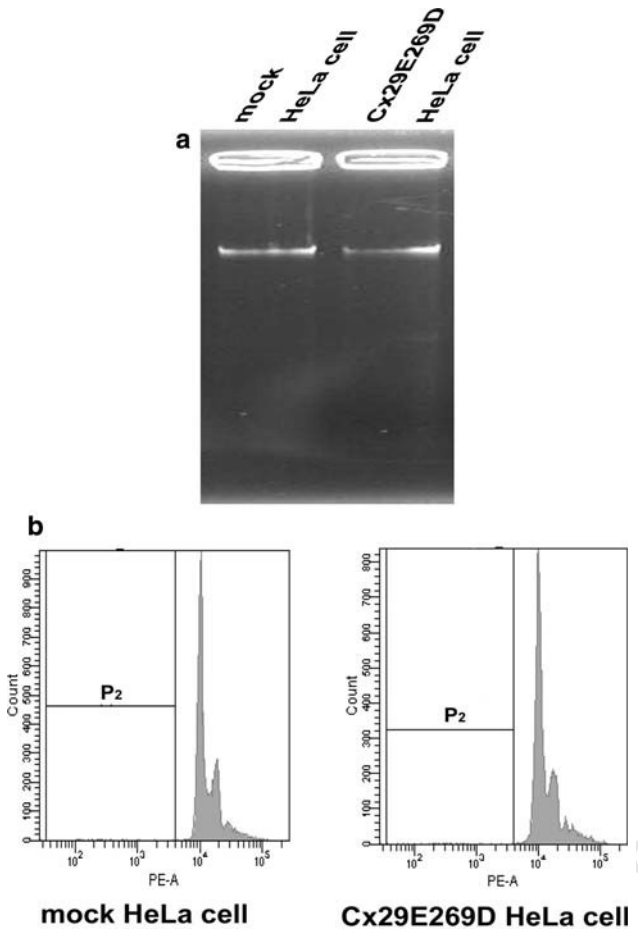

Fig. 7 Cell viability analysis on mock and stably expressed Cx29E269D HeLa cells. Both cells were incubated in DMEM medium for 24 h and were then harvested for DNA fragmentation assay (**a**) and analysis of flow cytometry (**b**). **a** DNA was prepared for agarose gel electrophoresis as described in "Materials and methods". Results are representative of three separate experiments. Lane 1: mock HeLa cell. Lane 2: stably expressed Cx29E269D HeLa cell. (**b**) After harvesting, the cells were stained with PI and then analyzed by flow cytometry. Cells that had undergone apoptosis were characteristically distributed within the sub-G1 population (P_2)

Generally, connexins are synthesized by ribosomes on the rough endoplasmic reticulum. They are then delivered to the plasma membrane in the form of membrane vesicles along the classical secretory pathway (Evans et al. 1999). Although it is generally believed that integration of connexins into membrane vesicles and the formation of hexameric connexons occur at the endoplasmic reticulum (Kumar et al. 1995; Falk and Gilula 1998), some studies point to a connexin-specific site for oligomerization, such as a trans-Golgi compartment, at least for Cx43 and Cx46 (Musil and Goodenough 1993; Koval et al. 1997; Sarma et al. 2002). In addition, it has been shown that Cx43 travels along microtubules to reach the plasma membrane (Lauf et al. 2002). The transportation of Cx43 to the plasma membrane can be inhibited by nocodazole (Paulson et al. 2000), suggesting the necessity of the microtubule network for gap junction formation. A recent study indicated that actin filaments of the cytoskeleton are important components in the processes of assembly, trafficking and stabilization of Cx30 gap junctions (Qu et al. 2009). In this study, we found that the p.E269D mutation resulted in the accumulation of the Cx29E269D protein in the endoplasmic reticulum (ER) and that it had a dominant negative effect on the function of normal Cx29. Further investigations involving sucrose gradient analysis and immunoprecipitation might provide confirmation of our findings and help to better understand the trafficking of the Cx29E269D protein.

It has been known that the accumulation of mutant proteins in the ER might cause the unfolded protein responses (UPR). The UPR is a cellular stress response that is activated in response to an accumulation of unfolded or misfolded proteins in the lumen of ER (Zhang and Kaufman, 2004). The ER is capable of recognizing malfolding proteins without causing disruption in the functioning of the ER. In such circumstances, the protein is guided through ER-associated degradation (ERAD). Here, it enters the ubiquitin–proteasome pathway, as it is tagged by multiple ubiquitin molecules, targeting it for degradation by cytosolic proteasomes (Cox et al. 1993; Ron, 2004). During conditions of prolonged stress, however, the goal of the UPR changes from being one that promotes cellular survival to one that commits the cell to a pathway of apoptosis (Fribley et al. 2008). In our study, we did not find that the accumulation of Cx29E269D mutant protein caused cell apoptosis by cell viability analysis. We suggested that the accumulation of Cx29E269D mutant proteins in the ER triggered their degradation, which was insufficient to cause cell apoptosis.

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