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利用細胞和動物模式探討GJB3基因突變造成聽障之機制

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中 文 摘 要 ： 遺傳性聽障一種常見的感音神經性疾病，在開發中國家大約60%由於基因突變所造成的。目前已知有 59 個基因的突變會導致聽障且每個基因的致病機制皆不盡相同。gap junction 在聽力功能上扮演的角色已被許多研究所確認，其在鉀離子再循環、耳蝸內離子平衡及聽力形成過程皆扮演重要的角色。本研究計畫目的主要是著重於探討 GJB3 (CX31)基因突變對其功能的影響及建立斑馬魚為模式動物以探討聽障的成因，藉此瞭解 GJB3 基因於聽覺形成過程中所扮演的角色並瞭解其致病機制。

本研究計畫我們發現正常的 CX31 蛋白可以正常的表現和被運送到細胞膜，並在兩個鄰近細胞間形成間隙連接（gap junction）。另外，我們發現含 p.V84I 或 p.A194T 的 CX31 突變蛋白，其表現位置和正常的 CX31 蛋白一樣可被運送到細胞膜上。相反的，含 p.P18S、p.V174M 或 p.E183K 的 CX31 突變蛋白卻會堆積在細胞質中，無法正常送至細胞膜上表現。同時在共同轉殖實驗中我們發現含 p.V84I 或 p.A194T 的 CX31 突變蛋白其表現位置會與正常的 CX31 蛋白疊合在一起，且共同表現在細胞膜上，這結果代表這些突變點並不會影響正常 CX31 蛋白的表現和運輸。另外在上述的研究中我們發現轉殖入 CX31V174M 或 CX31E183K 突變的 HeLa 細胞會陸續的死亡，所以我們利用細胞存活檢測方法（MTT assay）分析細胞存活率。我們發現細胞如果含 p.V174M 或 p.E183K 的 CX31 突變蛋白，其還原 MTT 的能力有下降的趨勢，這代表細胞存活率會漸漸降低，亦即這兩個突變可能會造成細胞的死亡。綜合以上的結果，在本研究中我們已經瞭解 p.L10R、p.P18S、p.V84I、p.V174M、p.E183K 及 p.A194T 的 CX31 突變蛋白在 HeLa 細胞中的表現位置。另外，對於突變點 p.V174M 及 p.E183K 可能造成聽障的原因有一個初步的了解。這些結果讓我們初步的了解到 CX31 突變所造成的影響。未來我們需要更深入的針對這幾個突變點功能上的影響加以探討，以更清楚瞭解各突變點造成聽障的分子機制。在斑馬魚模式動物的建立方面，我們利用 Tol2 斑馬魚基因轉殖系統，已經建立好耳囊聽斑及側線會發螢光的轉殖基因魚，這些基因轉殖魚的建立將可方便我們後續對於聽障的成因或致病機轉的研究。

中文關鍵詞： 基因、突變、聽障、功能研究、斑馬魚

英 文 摘 要 ： Recently, we identified novel heterozygous missense mutation, p.L10R, p.P18S, p.V84I, p.V174M, p.E183k and p.A194T, in the GJB3 gene encoding CX31 from 513

unrelated Taiwanese patients with non-syndromic hearing loss. In the proposed project, we will focus on the study of the effect on function of GJB3 genes with mutation. Simultaneously, we will create a zebrafish model to investigate the mechanisms of hearing loss.
Here we focused on the functional properties of six CX31 mutants derived from point mutation. Immunostaining pattern of transfected cells revealed that p.P18S, p.V174M and p.E183K mutants impaired the trafficking of CX proteins to the plasma membrane leading to accumulation of the mutant proteins in the cytoplasm, whereas p.L10R, p.V84I and p.A194T mutants showed the typical punctuate pattern of gap junction channel between neighboring expression cells as CX31 wild-type. According to our previous result, we found that cells with p.V174M or p.E183K mutants were dead after transfected for 3 to 6 days. Therefore, we use MTT assay to determining viable cell number. Our results indicated that HeLa cells transfected with p.V174M or p.E183K may cause cell death. Based on above results, we suggest that CX31 p.P18S, p.V174M and p.E183K mutations have effect on the formation and function of the gap junction. Moreover, p.L10R, p.V84I and p.A194T mutations do not affect the trafficking of mutant CX31 proteins, but its functional significance remains unknown. Therefore, the functional significance of p.L10R, p.V84I and p.A194T mutations requires further investigation. In addition, we have established the transgenic zebrafish with sensory cells-specific overexpression of GFP. We believe that the transgenic zebrafish model will provide a good approach to study the etiology and mechanism of pathogenesis in hereditary hearing loss.

英文關鍵詞： GJB3, mutation, hearing loss, gap junction, zebrafish

中文摘要

遺傳性聽障一種常見的感音神經性疾病，在開發中國家大約60%由於基因突變所造成的。目前已知有59個基因的突變會導致聽障且每個基因的致病機制皆不盡相同。gap junction在聽力功能上扮演的角色已被許多研究所確認，其在鉀離子再循環、耳蝸內離子平衡及聽力形成過程皆扮演重要的角色。本研究計畫目的主要是著重於探討*GJB3* (CX31)基因突變對其功能的影響及建立斑馬魚為模式動物以探討聽障的成因，藉此瞭解*GJB3*基因於聽覺形成過程中所扮演的角色並瞭解其致病機制。

本研究計畫我們發現正常的CX31蛋白可以正常的表現和被運送到細胞膜，並在兩個鄰近細胞間形成間隙連接 (gap junction) 。另外，我們發現含p.V84I或p.A194T的CX31突變蛋白，其表現位置和正常的CX31蛋白一樣可被運送到細胞膜上。相反的，含p.P18S、p.V174M或p.E183K的CX31突變蛋白卻會堆積在細胞質中，無法正常送至細胞膜上表現。同時在共同轉殖實驗中我們發現含p.V84I或p.A194T的CX31突變蛋白其表現位置會與正常的CX31蛋白疊合在一起，且共同表現在細胞膜上，這結果代表這些突變點並不會影響正常CX31蛋白的表現和運輸。另外在上述的研究中我們發現轉殖入CX31V174M或CX31E183K突變的HeLa細胞會陸續的死亡，所以我們利用細胞存活檢測方法 (MTT assay) 分析細胞存活率。我們發現細胞如果含p.V174M或p.E183K的CX31突變蛋白，其還原MTT的能力有下降的趨勢，這代表細胞存活率會漸漸降低，亦即這兩個突變可能會造成細胞的死亡。綜合以上的結果，在本研究中我們已經瞭解p.L10R、p.P18S、p.V84I、p.V174M、p.E183K及p.A194T的CX31突變蛋白在HeLa細胞中的表現位置。另外，對於突變點p.V174M及p.E183K可能造成聽障的原因有一個初步的了解。這些結果讓我們初步的了解到CX31突變所造成的影響。未來我們需要更深入的針對這幾個突變點功能上的影響加以探討，以更清楚瞭解各突變點造成聽障的分子機制。在斑馬魚模式動物的建立方面，我們利用Tol2斑馬魚基因轉殖系統，已經建立好耳囊聽斑及側線會發螢光的轉殖基因魚，這些基因轉殖魚的建立將可方便我們後續對於聽障的成因或致病機轉的研究。

關鍵詞：基因、突變、聽障、功能研究、斑馬魚

Abstract

Recently, we identified novel heterozygous missense mutation, p.L10R, p.P18S, p.V84I, p.V174M, p.E183k and p.A194T, in the *GJB3* gene encoding CX31 from 513 unrelated Taiwanese patients with non-syndromic hearing loss. In the proposed project, we will focus on the study of the effect on function of *GJB3* genes with mutation. Simultaneously, we will create a zebrafish model to investigate the mechanisms of hearing loss.

Here we focused on the functional properties of six CX31 mutants derived from point mutation. Immunostaining pattern of transfected cells revealed that p.P18S, p.V174M and p.E183K mutants impaired the trafficking of CX proteins to the plasma membrane leading to accumulation of the mutant proteins in the cytoplasm, whereas p.L10R, p.V84I and p.A194T mutants showed the typical punctuate pattern of gap junction channel between neighboring expression cells as CX31 wild-type. According to our previous result, we found that cells with p.V174M or p.E183K mutants were dead after transfected for 3 to 6 days. Therefore, we use MTT assay to determining viable cell number. Our results indicated that HeLa cells transfected with p.V174M or p.E183K may cause cell death. Based on above results, we suggest that CX31 p.P18S, p.V174M and p.E183K mutations have effect on the formation and function of the gap junction. Moreover, p.L10R, p.V84I and p.A194T mutations do not affect the trafficking of mutant CX31 proteins, but its functional significance remains unknown. Therefore, the functional significance of p.L10R, p.V84I and p.A194T mutations requires further investigation. In addition, we have established the transgenic zebrafish with sensory cells-specific overexpression of GFP. We believe that the transgenic zebrafish model will provide a good approach to study the etiology and mechanism of pathogenesis in hereditary hearing loss.

Keywords: *GJB3*, mutation, hearing loss, gap junction, zebrafish

前言

目前約有1/1000比例的嬰兒於出生或幼年時期罹患中重度聽障,在已開發國家中發現60%的個案為遺傳因素所造成。先前針對台灣地區513位非症候群聽障患者的*GJB3*基因篩檢中，發現了6個的錯意(missense)突變(**Table 1**和 **Figure 1**) (Yang et al., 2007, 2010)，然而目前對於這些錯意突變所造成的功能影響及其致病機轉並不清楚，，且同一基因在不同位置的突變常會產生蛋白功能影響上的差異。因此本計畫我們想藉由原本已建立好的細胞模式來加以探討*GJB3*這些錯意突變對其蛋白功能的影響是如何? 進一步的瞭解這些錯意突變所造成的聽障的機制為何? 另外利用動物模式來探討人類及動物疾病，此亦為生物醫學的一個重要方向。因此本計畫的另一個研究動機則是建立和利用斑馬魚為模式動物探討造成聽障的機制，期望藉由上述兩個模式的建立和探討以釐清非症候群聽障的致病機制。

研究目的

本研究的目的為: (1)進一步探討 *GJB3* 基因突變後對其功能的影響，藉由探討這些問題以瞭解 *GJB3* 基因在聽覺形成過程中所扮演的角色，並瞭解其致病機制。(2)利用 Tol2 斑馬魚基因轉殖系統建立耳囊聽斑或側線會發螢光的轉殖基因魚。

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)。

Connexin 31(CX31)蛋白是 *CX* gene 家族成員之一，CX31 蛋白是由 *GJB3* 基因所轉譯出來的。*GJB3* 基因位於染色體 1p35.1 的位置，其基因結構包含兩個外顯子(exon)，產生一個由 270 個氨基酸構成，分子量約 31KDa 的穿膜蛋白。其蛋白結構和其他 CX 蛋白一樣具有四個穿膜區域(transmembrane domain)，一個細胞內環(cytoplasmic 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(review in Zoidl and Dermietzel, 2010)。CX31 的突變已經被指出牽涉在遺傳性聽障、皮膚疾病和神經方面疾病 (review in Dror and Avraham, 2010; review in Zoidl and Dermietzel, 2010)。Richard 等作者發現一種與皮膚過角質化有關的疾病 Erythrokeratodermia variabilis (可變性紅斑角皮病，簡稱 EKV)與 CX31 基因的突變有關，在他的研究中發現 CX31G12D 和 C86S 兩個突變會造成 EKV 的疾病 (Richard et al.1998)。最早在 1998 年發現 CX31 Q183K 及 R180X 兩種顯性突變造成遺傳性非症候群聽障(Xia et al., 1998)。同時在 2000 年首先發現 *GJB3*(CX31)基因具有 compound heterozygosity (423-425delATT / I141V)的隱性遺傳突變，在此研究中發現不管缺失或是取代的單套基因型，都會損害 CX31 的 M3 domain (Liu et al., 2000)。在 2001 年也有研究證實 CX31D66Del 突變會導致神經細胞的傳導性降低，同時也造成顯性的遺傳性聽障(Lopez-Bigas et al., 2001)。

現今，利用動物模式來探討人類的疾病，是生物醫學或轉譯醫學的一個重要方向。發現、確認及開發一種人類疾病的動物模式可以對疾病之發生、防治有更深入的了解。而目前為止嚙齒類動物(小鼠或大鼠) 在全世界的動物實驗中佔了百分之八十以上，是最常用來研究遺傳疾病的材料，藉由基因的剃除(knockout)或基因轉殖(transgene)來瞭解基因在疾病上所扮演的角色。目前針對牽涉到維持耳蝸內淋巴液高 K^+低 Na^+環境的基因(即參與 K^+循環的相關基因)有突變鼠動物模式的包括(1) gap junction 基因：*Cx26/GJB2* 基因(Cohen-Salmon et al., 2002; Kudo et al., 2003)和 *Cx30/GJB6* 基因(Teubner et al., 2003)；(2) tight junction 基因：*Claudin 14* (*Cldn 14*)基因 (Ben-Yosef et al., 2003)；(3) K^+通道：*kcne1* 基因(Vetter et al., 1996; Nicolas et al.,2001), *kcnq1* 基因(Lee et al., 2000; Casimiro et al., 2001; Rivas and Francis, 2005)和 *kcnq4* 基因(Kharkovets et al., 2006; Winter et al., 2006)。另外 *Cx29* 基因也有兩個團隊發展出突變鼠的動物模式(Eiberger et al., 2006; Tang et al., 2006)。雖然這麼多團隊都利用老鼠當動物模式，但在 Krogh's principal 中有提到一個概念"針對一個生物醫學的問題，必須去觀察、研究與實驗多

種的模式生物，然後將不同模式生物所提供的資訊，互相比較對照，以求得單一種模式生物無法提供的資訊(Editoria, 2003) 。”加上老鼠是屬於體內受精和體內發育，這特性會令許多胚胎早期即需發揮功能的蛋白質在 gain of function 及 loss of function 的情況下無法順利的著床發育。因此我們認為尋找另一種模式動物來探討聽障的機制是有其必要性。

近年來由於斑馬魚(zebrafish, *Danio rerio*)有許多優點包括：體型小、飼養容易且不需太多空間、具有光週期誘發產卵、每次產卵數量眾多(約可產 100~200 顆)、其透明的胚胎使得器官在發育時期即可利用解剖顯微鏡從外部觀察和、胚胎發育期短（約 2-3 天）(Kimmel et al., 1995)。斑馬魚的世代週期比老鼠短，約 3 個月即可發育性成熟。在早期的的胚胎發育過程中，在 24 小時(24 hpf)即可發展出一心房一心室的心臟並且具有血液循環的功能，約在 48~120 小時(48~120 hpf)左右則其餘的器官就會開始發育、形成並具有功能(Wells et al., 1999)。因此斑馬魚是目前常用於研究脊椎動物發育的動物模式。斑馬魚為一種體型約 3~5 公分的熱帶淡水魚，其在胚胎發育上的機制與哺乳類動物是非常相似。最近在一些斑馬魚突變的研究已發現在一些器官組織如耳朵、眼睛、心臟、肌肉、血液、脊柱、胰臟及腎等所產生的缺陷，與人類這些器官所產生的一些疾病之病理特徵相似，且為相同基因或同源基因突變所造成，因此，斑馬魚目前在國內外已經越來越多人利用來做為人類疾病研究的動物模式之一。

斑馬魚具有兩種 mechanosensory 器官，一種是內耳—主要負責聽覺和平衡，另一種側線(lateral line)器官—主要牽涉在決定水的動態上。科學家研究發現，若按照魚類聽覺能力的好壞來區分，可分為聽覺特化型與普通型兩大類。聽覺特化型的代表種類為鯉魚及鯰魚（都是屬於骨鰾魚類），牠們的聽覺音頻範圍可以從 200 赫茲（Hz, 每秒的週波數）到 8000 赫茲，而最靈敏的聽覺閾值約在 60 分貝左右。聽覺普通型的代表如吳郭魚的鯛類，牠們的音頻範圍局限於 200 ~ 1000 赫茲，而最靈敏的聽覺閾值則高達 90 分貝以上。聽覺特化型的魚類之所以有較好的聽覺，主要是透過一串由脊椎骨特化的小骨連接內耳與氣鰾，當氣鰾裡的氣體受到穿透身體的音波而壓縮及膨脹後，會產生共振波，經由連接的小骨傳送到內耳；沒有這種助聽構造的魚類，聽力就較差 (嚴宏洋, 2007)。斑馬魚像其他魚類一樣並無外耳構造，但具有複雜的內耳構造。但是斑馬魚的內耳也不像人類或老鼠具有明顯特化性的內耳如耳蝸(cochlea)來產生聽覺。斑馬魚像一般的骨鰾魚類(如鯉魚及鯰魚)一樣具有 3~4 塊小骨組成的韋伯氏器(Weberian ossicles)，這個組織連接於充滿氣體的魚鰾(swim bladder) 和內耳之間，負責將顫動(水下聲波) 從魚鰾(swim bladder) 傳遞至內耳(Moorman, 2001; Bang et al., 2001)。

斑馬魚的聽覺主要是藉有內耳(inner)和側線(lateral line)來偵測外來顫動(水下聲波)的刺激而產生(Moorman, 2001;)。斑馬魚的內耳可分為兩部分 ---1. 上半部(pars superion)包括兩個垂直和一個水平共三個半規管(semicircular cannals)連接到卵形囊(utricle)的區域，三個半規管

稱為膜迷路(labyrinthus membranaceus)，膜迷路內充滿內淋巴液（endolympha）；2. 下半部(pars superion)包括球形囊(saccule)和小囊(lagena)，而球形囊(saccule) 是一個主要的聽覺器官(primary audiotory organs) (Bever and Fekete, 2002) 。卵形囊(utricle)、球形囊(saccule)和小囊(lagena)內各有一塊耳石(otolith)，當聲音穿透過內耳時，因重量與惰性的差異耳石不會有移動。但位於聽斑(macula)上毛細胞(hair cell)頂端的動毛(kinocilum)，則會因受震動而彎曲與耳石產生磨擦從而產生聽覺(Popper and Platt, 2003)。此機制類似於人類聽覺的產生機制。另外，在斑馬魚的內耳構造，經顯微分析後發現在內耳聽斑區(macula)和側線(lateral line)上的毛細胞構造類似於人類耳蝸內感覺受器的毛細胞構造是相類似的(Popper and Platt, 2003)，同樣的在斑馬魚內耳中上皮細胞內襯(epithelial lining)也和在人類耳蝸內上皮細胞內襯(epithelial lining)一樣有各種細胞所組成的，目前為止至少有 granular 和 ionocytes 細胞牽涉其中(Mayer-Gostan et al., 1997; Pisam et al., 1998)。但是哺乳類動物和斑馬魚在內耳毛細胞的發育是不一樣的，在斑馬魚毛細胞的數目會隨著魚體的成長而增加，一般數目可增加到大約 10 個月大，然而這毛細胞數目的增加並沒有影響到聽覺的敏感度和頻率寬度，而哺乳類動物則在出生時，毛細胞數目已決定，當毛細胞受損後是不能再生的(Higgs et al., 2001)。

關於以斑馬魚為模式動物來研究聽覺的形成機制，在國外事實上早在 1996 年就有研究學者利用大量篩檢內耳發育缺陷的斑馬魚突變種來瞭解基因突變造成內耳缺陷的關係，在這研究中找的了 58 種內耳缺陷的突變斑馬魚(Whitfied et al., 1996)。甚至於在這之前也有部分探討基因與魚類內耳發育的相關報告被提出(Whitfield, 2002, review)。在 1998 年 Nicolson 等人，已經在斑馬魚發現幾個基因與 mechanosensory 毛細胞的功能有關，如 *orbiter*、*mercury* 和 *gemini* 基因的突變其具有正常的毛細胞形態但對於聽覺震動(acoustic-vibrational)的刺激並沒反應和 *sputnik* 及 *mariner* 基因的突變會影響到毛細胞 bundle 的正常發育。在 2000 年 Ernest 等人在研究中發現 *mariner* 的突變是由於基因表現出缺陷的 *Myosin VIIA*，因而造成毛細胞 bundle 的發育異常，此情形和人類的 *MYO VIIA* 基因異常所產生的情形類似，因此他們建立了斑馬魚來探討人類遺傳性聽障的動物模式。最近一個人類聽障基因 *TMIE* 的同源基因(*tmie*)在斑馬魚被選殖出來，這基因主要表現在受精後 24 到 51 小時之間，且在斑馬魚內耳前庭和側線毛細胞的發育扮演著重要角色，此結果和以老鼠為動物模式所看到的情形是一樣的(Shen et al., 2008)。近一年(2010 年)來越來越多的國際期刊已經慢慢針對斑馬魚內耳的基因加以探討，如找到 *parvalbumin 3a* (*pvalb3a*) and *parvalbumin 3b* (*pvalb3b*)基因表現的控制區域，來控制轉殖基因在發育中毛細胞的表現(McDermott Jr et al., 2010)。*Sox2* 基因的表現並不是在內耳發育剛開始就表現，其功能主要在斑馬魚內耳毛細胞的維持和再生(Millimaki et al., 2010)。同時有研究指出 hmx2 和 hmx3(轉錄因子)在斑馬魚內耳和側線的發育上扮演重要的角色(Feng and Xu,

2010)。在最近的研究(2010)也發現斑馬魚 Cx30.3 基因類似於人類的 *CX26* 或 *CX30* 基因，可大量表現在斑馬魚的皮膚和內耳，然此篇文章較著重在皮膚相關機制的探討(Tao et al., 2010)，不過這篇研究的出現更加讓我們覺得利用斑馬魚為模式動物來探討聽障的機制或治療是重要且可行的。另外，斑馬魚也已經被用來作為藥物篩檢的模式動物，哪些藥物會造成聽障，哪些藥物可以預防聽障（Ou et al., 2010）。在國內對於利用斑馬魚來探討聽覺機制形成的研究並不多，以中研院細胞與個體生物學研究所黃鵬鵬特聘研究員的研究團隊和嚴宏洋研究員為前鋒，在他們的研究裡，結合了聽覺腦幹反應的測試方法與控制耳石增長或縮小的方法，來探討魚類的聽覺能力是從什麼時候開始產生的、耳石在聽力形成過程中所扮演的角色和耳石形成過程的研究。

另外，轉殖基因技術是一個重要的探討基因功能的工具。過去在斑馬魚的轉殖基因技術是有其缺點，包括使用傳統的選殖技術來選殖複雜的表現質體是艱苦的、低的轉殖基因效率、在短暫表現的轉殖基因動物中會有 mosaicism 的情形和轉殖基因很少併入 germline 中等。最近的研究在斑馬魚的基因轉殖技術方面最近有一些重大的突破來克服先前在斑馬魚系統中所遇到的問題(Kwan et al., 2007)。此種方法是 multisite Gateway technology (Tol2Kit system)，此項技術 2007 年已經商品化了(Invitrogen, catalog no. 12537-023)。此技術主要是利用在斑馬魚內 Tol2 酵素和我們將[promoter]-[目標基因]-[3'端 tag]建構在 Tol2 transposon 的骨架上來達成。斑馬魚 Tol2 基因可轉譯出 649 胺基酸，此片段類似人類 TA 家族(一 transposases 家族)，根據研究顯示 Tol2 酵素是一種 transposases 可以催化切斷 trans 的 DNA(Kawakami et al., 1999)。最近此項技術在國內外已被廣泛運用在基因轉殖斑馬魚的建立上。根據上述的相關資訊我們認為以斑馬魚為模式生物來探討 *gap junction protein* 基因族突變造成聽障的機制是一個值得、重要且可行的方案。

研究方法

一、HeLa細胞模式

1. 建構正常及突變的*GJB3* (CX31)基因於螢光表現載體
2. 基因轉殖(transfection)至HeLa細胞株表現
3. 利用細胞免疫螢光染色技術觀察正常或突變CX蛋白於細胞內的表現位置
4. 建立持續穩定表現正常或突變CX蛋白的HeLa細胞株
5. MTT assay

二、斑馬魚的飼養與胚胎操作

1. 斑馬魚(*Danio rerio*)之飼育

2. 斑馬魚胚胎的受精與培養
3. 胚胎卵膜脫除及胚胎脫水固定保存
4. 草履蟲與豐年蝦的飼養

三、轉殖基因斑馬魚的建立

1. 建立Tol2基因轉殖系統
2. 建構Tol2表現質體
3. 合成*transposase* cRNA
4. 顯微注射(microinjection)
5. 世代有性交配

結果與討論

一、 *GJB3*基因(*CX31*)突變之研究 **(Figure 1~Figure 6**)

我們在此研究計畫中已將先前針對台灣 513 位非症候群聽障患者所篩檢到的六個 *GJB3* 基因錯意突變點 p.L10R、p.P18S、p.V84I、p.V174M、p.E183K 和 p.A194T 以及正常的 *GJB3* 基因完成選殖，並分別將其建構於 pLEGFP 或 pTagRFP 螢光表現載體上。自 CX31 的結構示意圖中可發現 p.L10R 和 p.P18S 突變點位於蛋白質 N 端，p.V174M 和 p.E183K 突變點位於細胞質外環(cytoplasmic loop)，而 p.V84I 和 p.A194T 突變點則是位於穿膜區域(transmembrane region)。因此本研究計畫即針對六個 *GJB3* 基因錯意突變點進行研究，利用免疫螢光法探討此錯意突變所造成的影響。研究成果證實 p.L10R、p.V84I 和 p.A194T 三個錯意突變並不會影響突變蛋白被運輸到細胞膜上而形成班塊，但在細胞膜上是否也具有和正常蛋白相同的功能仍需進一步分析。另外 p.P18S、p.V174M 和 p.E183K 三個突變蛋白皆被發現堆積於內質網、高基氏體或溶酶體中，並無法被正常運送到細胞膜以形成 gap junction，此部分結果將於 **102 年度「微免及檢驗醫學」暨「生化及藥理醫學」跨學門學術交流研討會**以壁報的方式發表。另外，在先前的實驗我們嘗試將建構在含綠螢光或紅螢光蛋白表現載體上的 CX31 突變點 p.V174M 及 p.E183K 分別轉殖到 HeLa 細胞，並以 G418 藥物做篩選，期望能達到穩定表現含 *CX31* 各突變點的細胞株以利後續實驗進行，然而始終無法達成，我們觀察細胞型態發現細胞在轉殖入這些質體後的 3 至 6 天會陸續死亡，因此我們以細胞存活檢測的方式分析。結果發現當 HeLa 細胞分別表現含有突變點 p.V174M 或者 p.E183K 後其細胞數目相較表現正常 CX31 的細胞數比有略為下降的趨勢，因此我們認為突變點 p.V174M 或者 p.E183K 會造成細胞死亡，使我們無法獲得穩定表現的細胞株。然而，這是初步的結果，後續將可進一步探討其造成細胞死亡的機轉。

二、 轉殖螢光斑馬魚的建立 (**Figure 7~Figure 12**)

在先前我們已經完成Tol2基因轉殖系統的建立，且已將可專一性調控基因表現於內耳的*pvalb3b*啟動子活性區域完成選殖以及完成建構Tol2相關質體。同時我們進一步利用顯微注射技術將Tol2表現質體注射至斑馬魚受精胚胎中，並進行世代有性交配以獲得F2世代的*pvalb3b*:TagGFP基因轉殖螢光斑馬魚。在這一年的研究計畫我們進一步獲得F3世代的*pvalb3b*:TagGFP基因轉殖魚。GFP綠螢光蛋白被標定於斑馬魚耳囊聽斑和側線系統的毛細胞。在本研究計畫我進一步的利用抗體anti-acetylated tubulin antibody或螢光染劑FM4-64分別進行聽斑及側線系統之毛細胞的標定，研究成果證實抗體anti-acetylated tubulin antibody確實可標定到聽斑之毛細胞前端的kinocillium，並和我們GFP基因轉殖的螢光蛋白表現在同一個位置，此結果讓我們確定建立了耳囊聽斑發螢光的斑馬魚的line。另外螢光染劑FM4-64亦確實可標定到側線系統之毛細胞，此結果證實側線系統的毛細胞帶有螢光的基因轉殖魚所標定到的毛細胞為具有功能性的毛細胞，可機械性通透螢光染劑。此部分結果將於**102年度「微免及檢驗醫學」暨「生化及藥理醫學」跨學門學術交流研討會**以壁報的方式發表。這些轉殖螢光斑馬魚的建立，將可幫助我們後續在耳毒性或聽力損失治療藥物的篩檢上扮演重要角色，另外，藉由這些技術的建立，將可讓我們繼續建立帶有不同聽障基因的轉殖基因斑馬魚，幫助我們瞭解這些聽障基因與聽覺形成間的關係，以及基因突變造成聽障的機制。

計劃成果結論和建議:

我們已將本計畫的研究成果以壁報的方式將發表在**102年度「微免及檢驗醫學」暨「生化及藥理醫學」跨學門學術交流研討會 (如附件)**，且另有兩篇相關的文章正在投稿中。在本研究計畫中我們已證實正常及突變*GJB3 (*CX31)基因在細胞內的表現情形及功能。另外在斑馬魚系統的建立上，我們也完成建立Tol2基因轉殖魚系統，目前我們已經篩選到*pvalb3b*:TagGFP品系的基因轉殖斑馬魚。在這一年所獲得的經驗和研究成果，提供了相關的資訊，可作為後續研究*GJB3*基因造成非症候群聽障致病機轉的參考。

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Table 1. 台灣非症候群聽障患者中所發現 *GJB3* 基因突變

Nucleotide change	Amino acid change	Domain	Predicted effect
c.29 T>G	p.Leu10Arg (L10R)	N	Missense mutation
c.53 C>T	p.Pro18Ser (P18S)	N	Missense mutation
c.250G>A	p.Val 84 Ile (V84I)	M2	Missense mutation
c.520G>A	p.Val174Met (V174M)	E2	Missense mutation
c.547 G>A	p.Glu183Lys (E183K)	E2	Missense mutation
c.580 G>A	p.Ala194Thr (A194T)	M4	Missense mutation

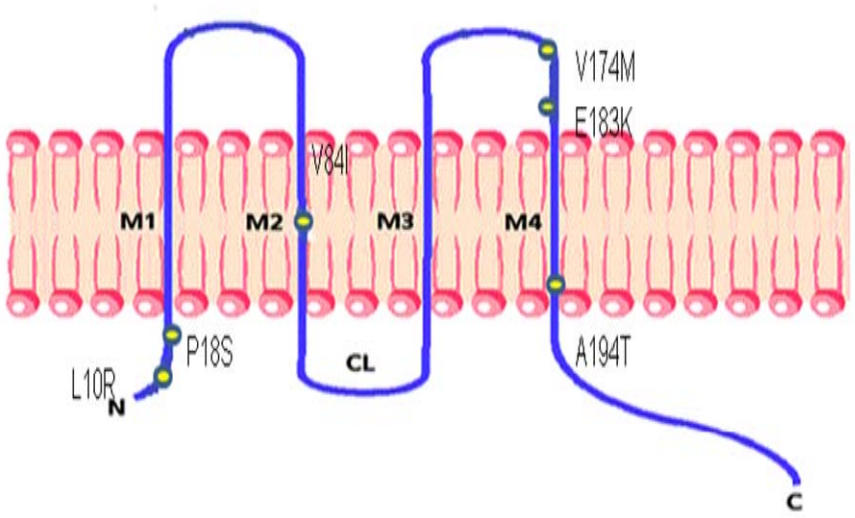

Figure 1 Schematic representation of the domain structure of the CX31 protein with indication of known missense mutations. *M1-4*: transmembrane domains; *E1-2*: extracellular domains; *CL*: cytoplasmic linking domain; *N*: N-terminal domain; *C*: C-terminal domain

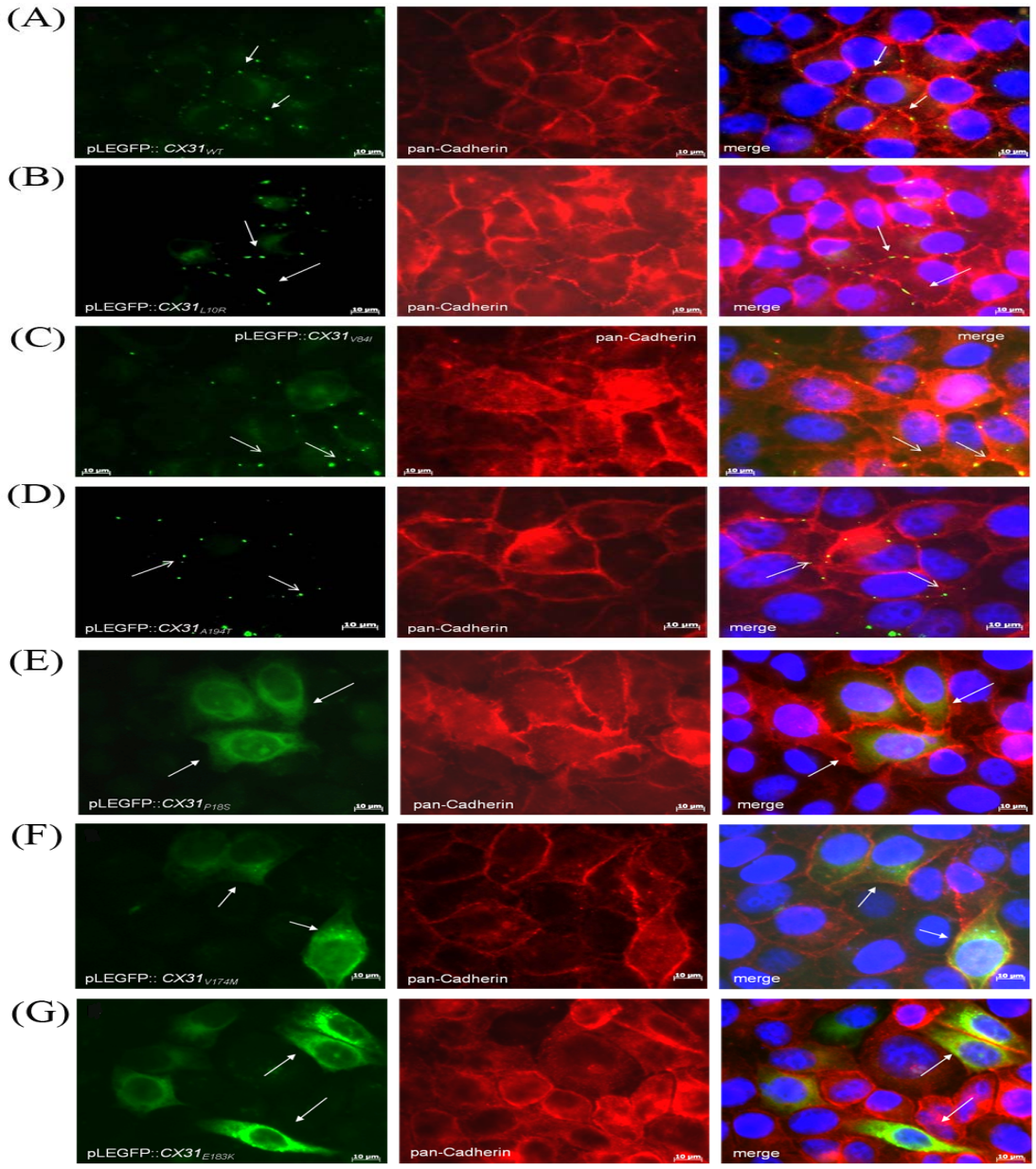

Figure 2. Intracellular localization of the wild-type and mutant CX31 proteins in transiently transfected HeLa cells. The immunocytochemistry analysis of wild-type (wt) and mutant CX31 proteins in transiently transfected HeLa cells were performed by anti-pan-cadherin antibody. All images were showed using fluorescence microscopy. Within cells transfected with wt CX31 (A), the p.L10R mutant (B), p.V84I mutant (C) or p.A194T mutant (D), each EGFP-tagged CX31 fusion protein was presented the typical plaques between adjacent cells (white arrows). However, the p.P18S (E), p.V174M (F) and p.E183K (G) mutant proteins were showed the impaired trafficking and were retained within the cytoplasm, concentrated in a region close to the nucleus (white arrows). The cells were counterstained with 4'-6'-diamidino-2'-phenylindole (DAPI) to4'-6'-diamidino-2'-phenylindole (DAPI) highlight the nuclei. Scale bar: 10 μm.

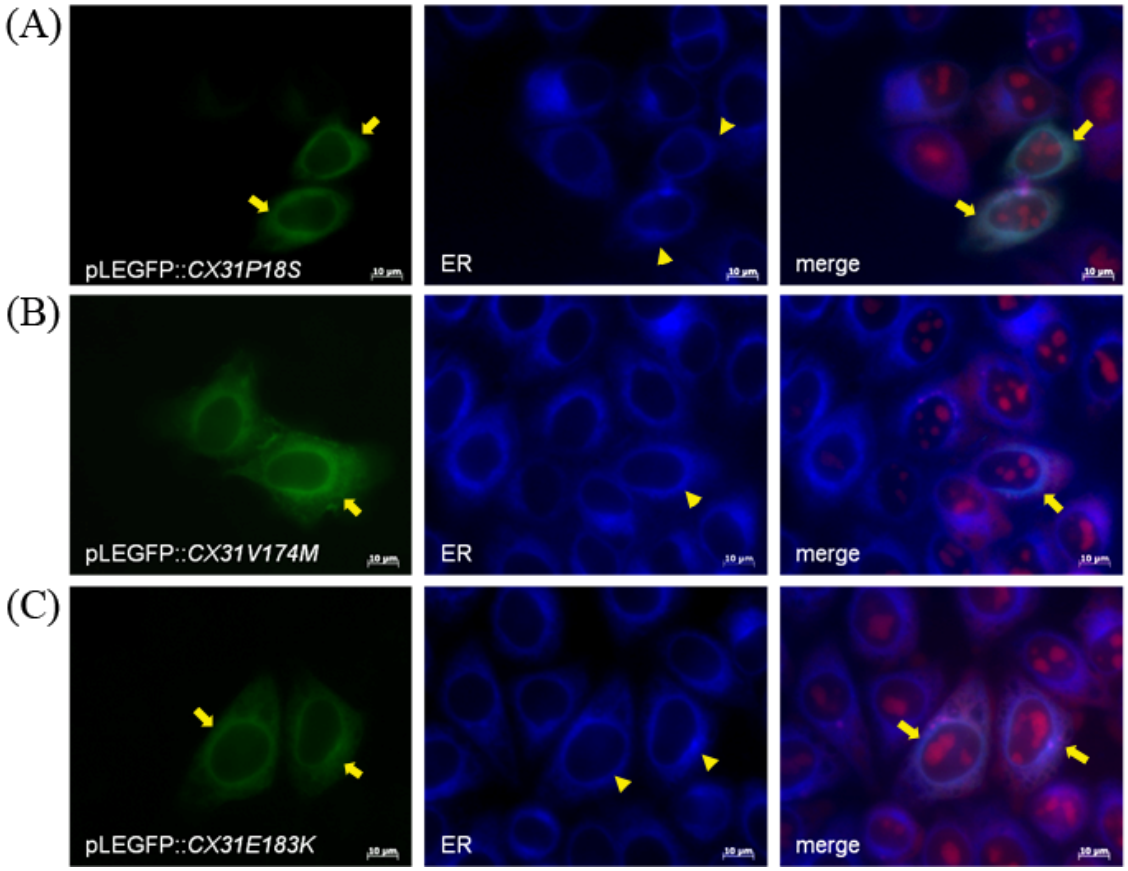

Figure 3. The mutant CX31 proteins in transiently transfected HeLa cells could intracellular co-localized with endoplasmic reticulum. Three mutant CX31proteins, p.P18S (A), p.V174M (B) and p.E183K (C) respectively, were retained within the cytosol of HeLa cells and concentrated in a region close to the nucleus. Photo-micrographs of HeLa cells transfected with each mutant *GJB3* cDNA, followed by immunostaining for markers of endoplasmic reticulum (ER). All mutant CX31 proteins were found to moderately co-localized with the ER marker. The cells were counterstained with propidium iodide (PI) to highlight the nuclei. Yellow arrows indicate the localization of CX31 mutant protein. Yellow arrowheads indicate the localization of ER. Scale bar: 10 μm.

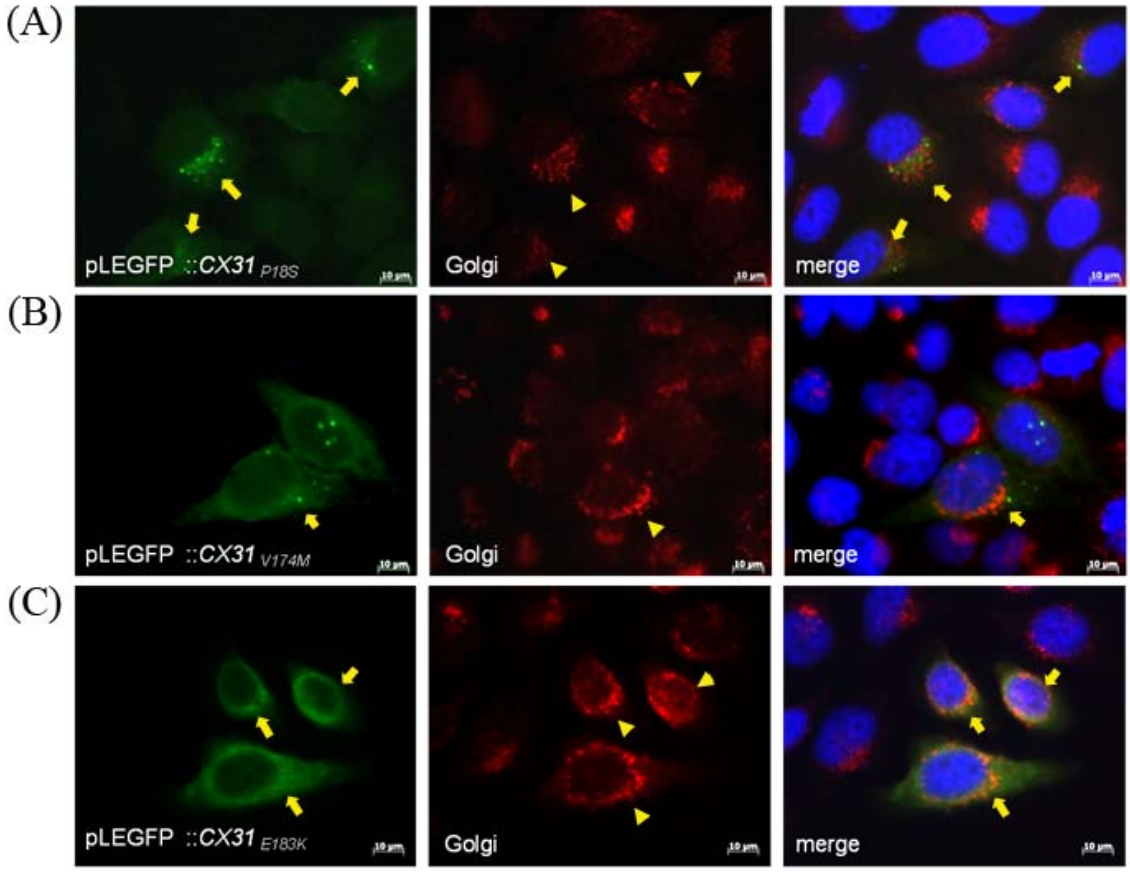

Figure 4. The mutant CX31 proteins in transiently transfected HeLa cells could intracellular co-localized with Golgi apparatus. Three mutant CX31proteins, p.P18S (A), p.V174M (B) and p.E183K (C) respectively, led to the substantial reduction in trafficking to cell membrane between adjacent cells. Photomicrographs of HeLa cells transfected with each mutant *GJB3* cDNA, followed by immuno-staining for markers of Golgi apparatus (Golgi). The immunostaining results for both mutants p.P18S and p.E183K showed the substantial co-localization in the Golgi apparatus, but the staining for mutant p.V174M proteins did not. The cells were counterstained with 4'-6'-diamidino-2'-phenylindole (DAPI) to highlight the nuclei. Yellow arrows indicate the localization of CX31 mutant protein. Yellow arrowheads indicate the localization of Golgi apparatus. Scale bar: 10 μm.

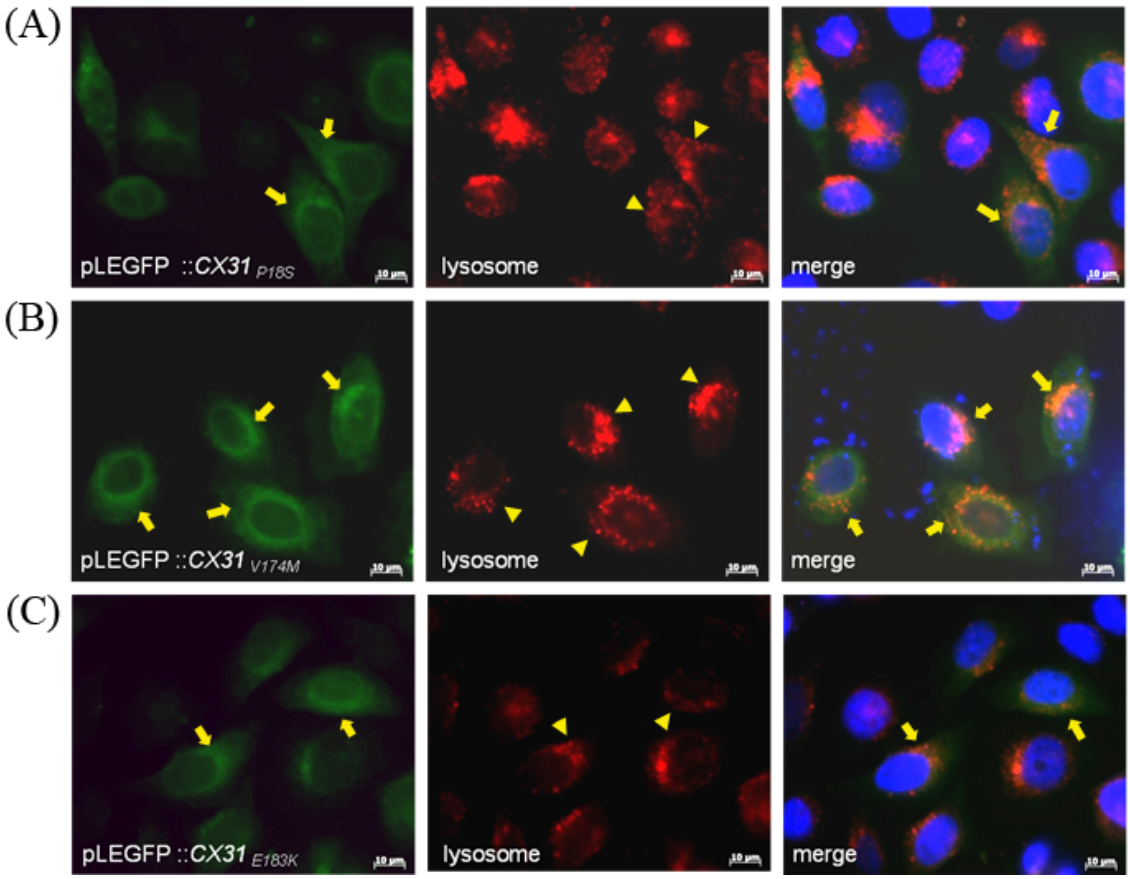

Figure 5. The mutant CX31 proteins in transiently transfected HeLa cells could intracellular co-localized with lysosome. Three mutant CX31proteins, p.P18S (A), p.V174M (B) and p.E183K (C) respectively, were diffusrly distributed within the cytosol of HeLa cells. Photomicrographs of HeLa cells transfected with each mutant *GJB3* cDNA, followed by immunostaining for markers of lysosome. The immunostaining patterns for all mutants proteins showed the substantial co-localization in the lysosome of HeLa cells. The cells were counterstained with 4'-6'-diamidino-2'-phenylindole (DAPI) to highl-ight the nuclei. Yellow arrows indicate the localization of CX31 mutant protein. Yellow arrowheads indicate the localization of lysosome. Scale bar: 10 μm.

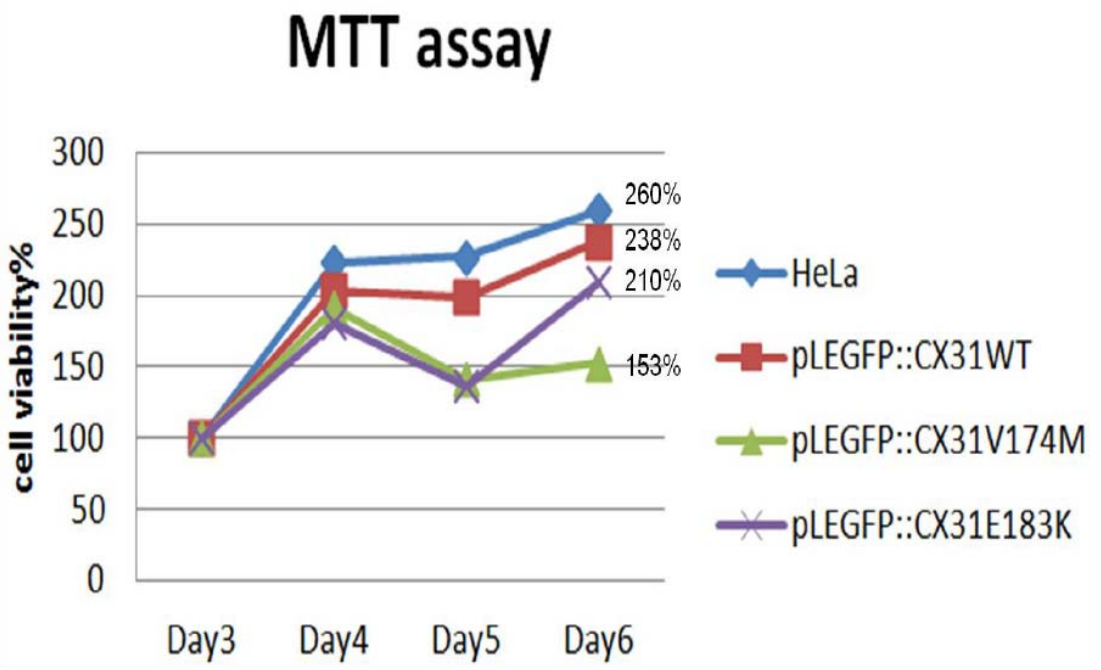

Figure 6. 含CX31突變點p.V174M或p.E183K HeLa細胞存活率之分析

我們利用 MTT assay 的方式分析含突變 CX31 表現的 HeLa 細胞之存活率。含 CX31 突變點 p.V174M 的 HeLa 細胞在轉殖入質體後的第五天細胞存活率明顯的有下降的趨勢，到轉殖後的第六天細胞的存活率 (153%) 相較未轉殖入質體的 HeLa 細胞 (260%) 或含正常 CX31 質體的 HeLa 細胞 (238%) 存活率明顯的低。含 CX31 突變點 p.E183K 的 HeLa 細胞在轉殖後的第五天細胞存活率也有明顯下降的趨勢，到轉殖後的第六天細胞的存活率 (210%) 相較 HeLa 細胞 (260%) 及含正常 CX31 表現的細胞 (238%) 存活率低。圖縱軸為細胞存活率的百分比，橫軸為細胞轉殖入質體後的天數。

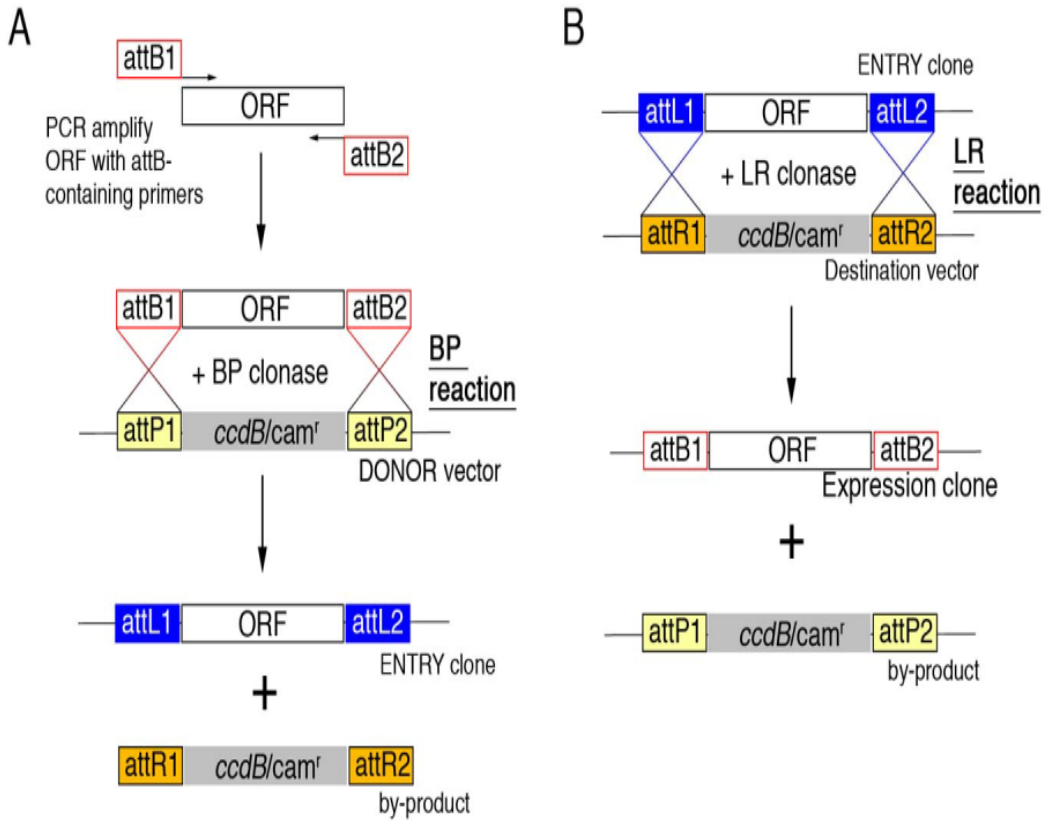

Figure 7. MultiSite Gateway cloning system (Invitrogen). (A) Description of the BP reaction for a polymerase chain reaction amplified open reading frame to get an ENTRY clone and by-product. (B) LR reaction between an ENTRY clone and a Destination vector to get an Expression clone. Note that att sites are not to scale.

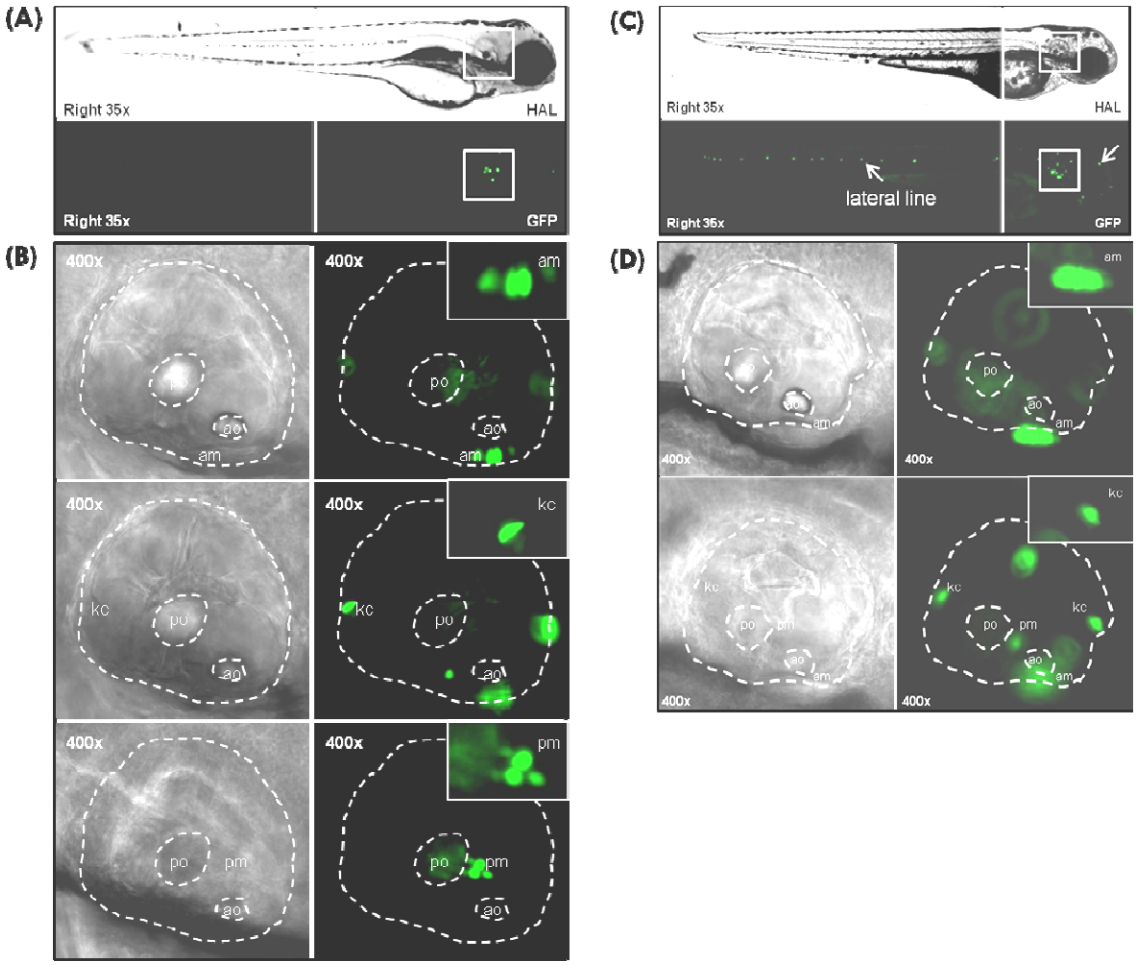

Figure 8. Cellular distribution of TagGFP in the pvalb3b transgenic line. All DIC images of 96 hpf embryos are lateral views, anterior is to the right and dorsal is up. Fluorescent images of 96 hpf embryos demonstrate TagGFP labeling in the otic vesicle (A) or/and lateral line (C). (B, D) In ventral view of embryo in 96 hpf, sensory epithelia containing TagGFP-positive cells are located beneath the two otoliths or crista. ao: anterior otolith, po: posterior otolith, am: anterior macula, pm: posterior macula, kc: Kinocilia of the crista hair cells.

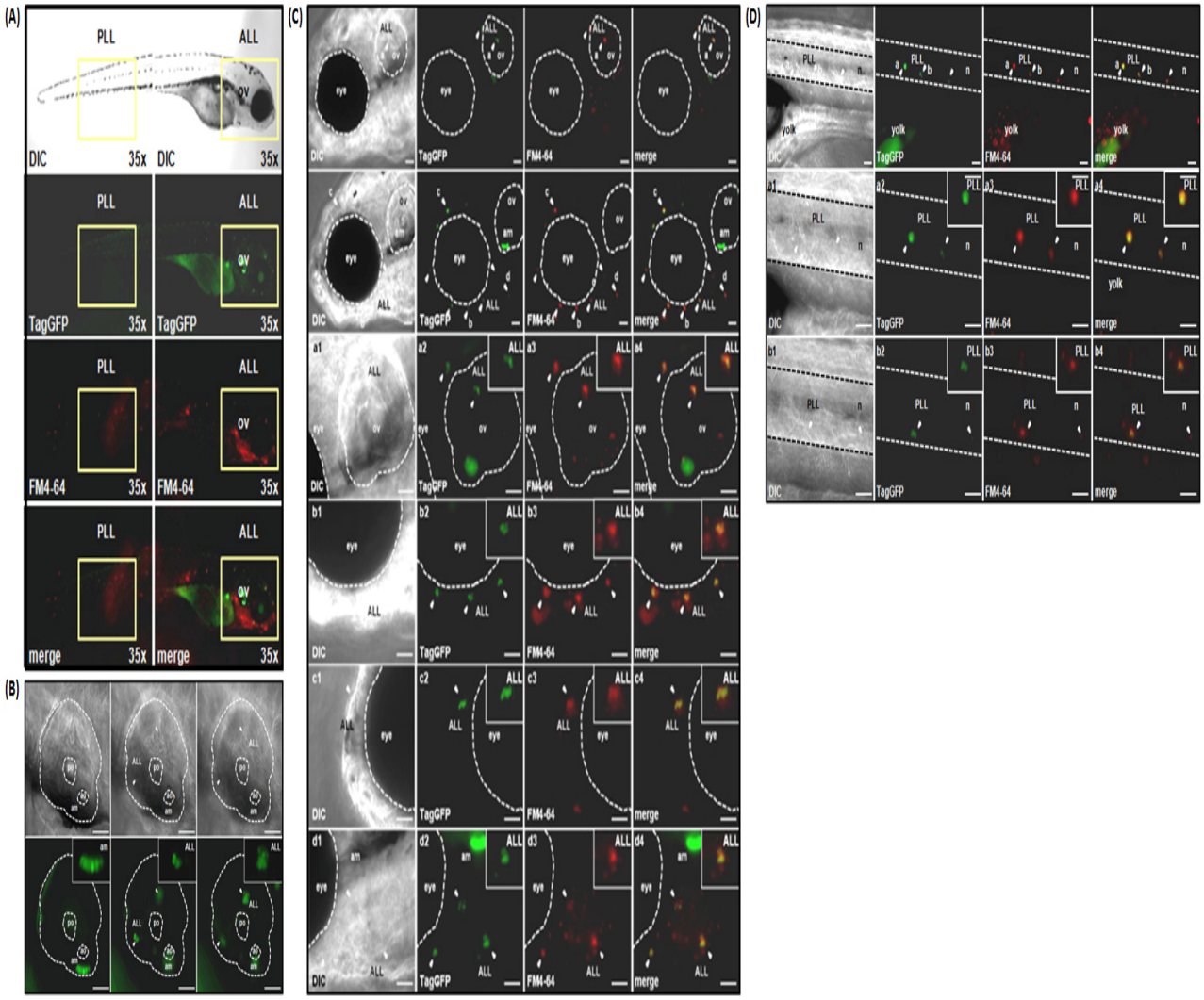

Figure 9. pvalb3b-TagGFP *gfp/wt ln1*殖基因魚的螢光表現型態。(A)於受精後5天的胚胎，TagGFP綠色螢光蛋白專一性表現於斑馬魚的內耳(ov)及側線系統(ALL和PLL)。(B)自腹面觀察受精後4天的胚胎內耳(白色虛線)，發現TagGFP綠色螢光蛋白會分別表現於相鄰耳石(otolith)的前聽斑(am)及前側線毛細胞。隨後於受精後5天的胚胎，以紅色螢光染劑FM4-64藉由具有功能性的機械傳導通道(mechanotransduction channel)進行前側線(C)及後側線(D)系統的毛細胞標定，可發現TagGFP綠色螢光蛋白確實會表現於側線系統的毛細胞(白色箭頭所示) 。ov: otic vesicle、ao: anterior otolith、po: posterior otolith、am: anterior macula、pm: posterior macula、ALL: anterior lateral line、PLL: posterior lateral line。

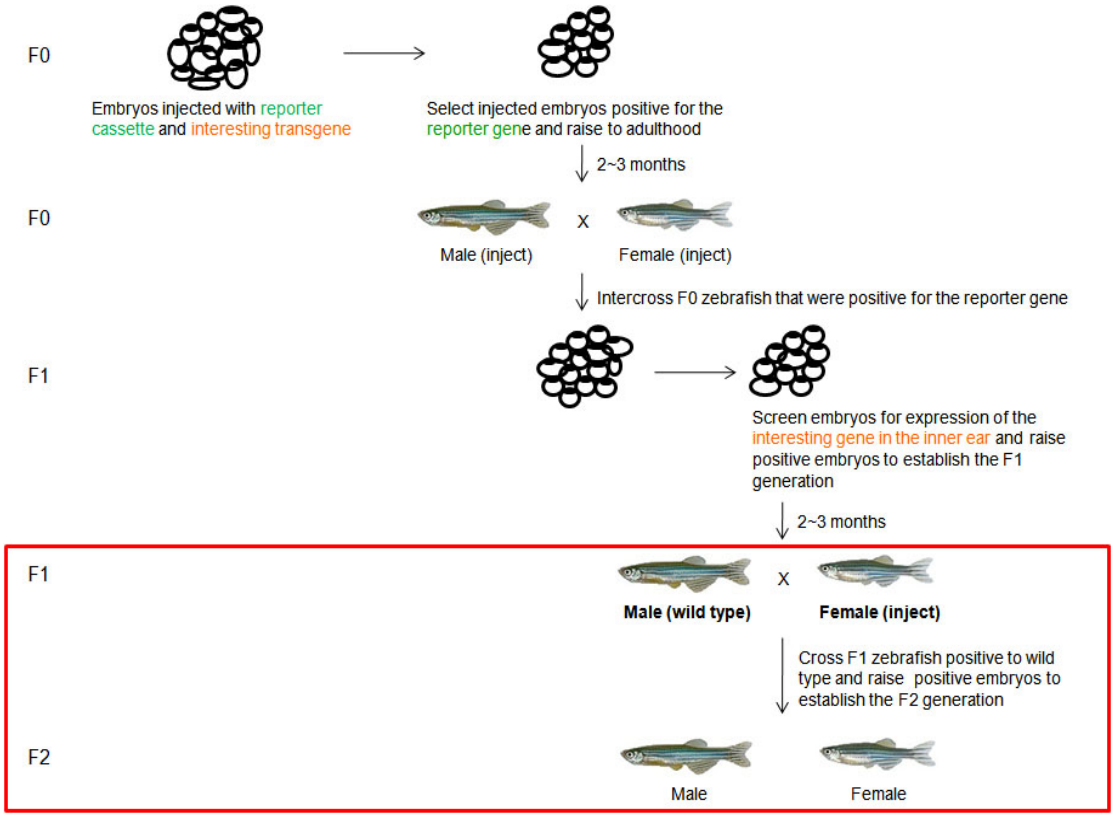

Figure 10. Transgenesis in zebrafish --Tg (*pvalb3b*: *TagGFP*)。以pvalb3b啟動子驅動TagGFP螢光蛋白於斑馬魚中表現。紅色框框為F1子代(含螢光)和wild-type交配產生F2子代。

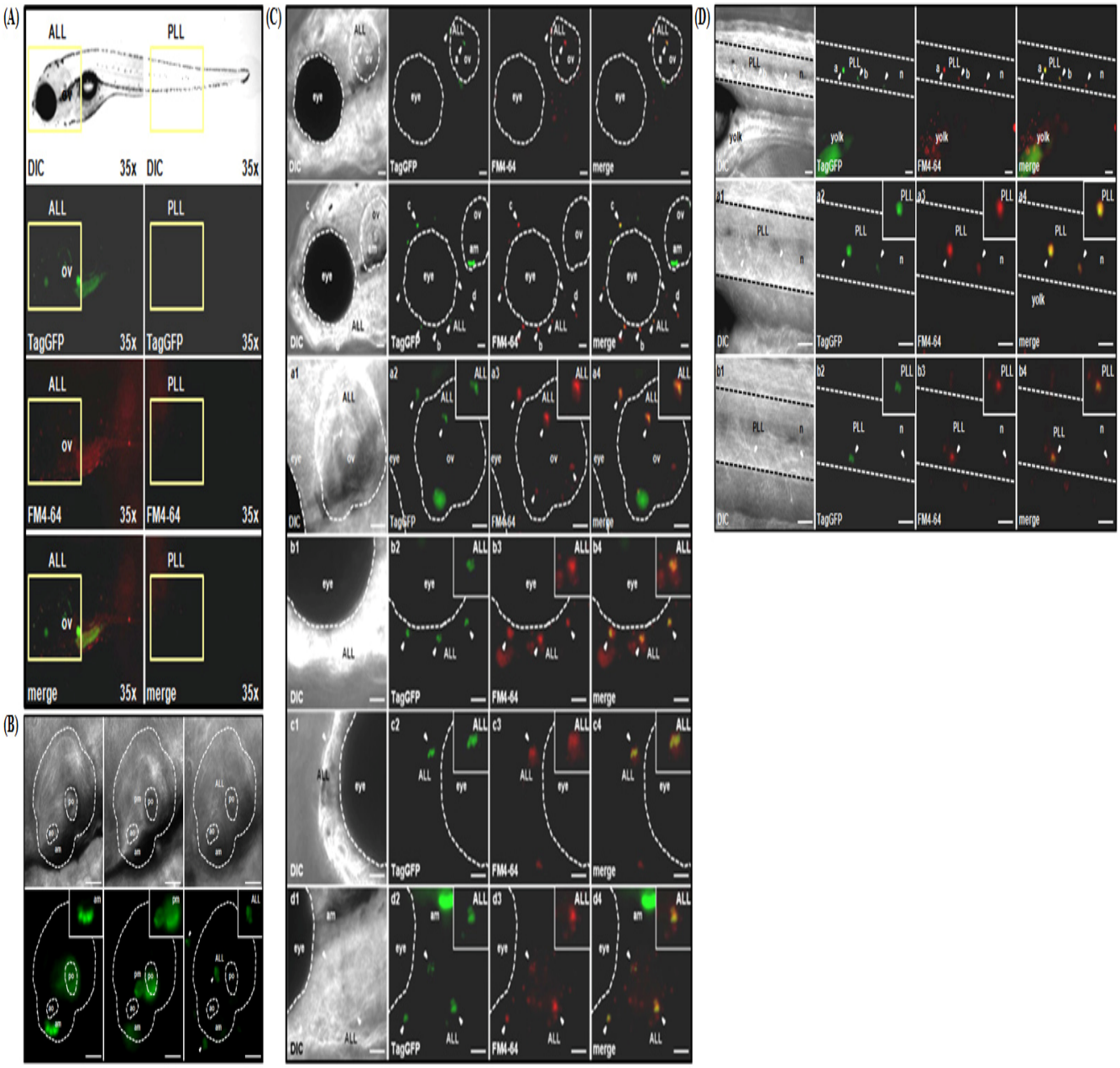

Figure 11 pvalb3b-TagGFP $^{gfp/wt\ ln2}$**轉殖基因魚的螢光表現型態。**(A)於受精後5天的胚胎，TagGFP綠色螢光蛋白專一性表現於斑馬魚的內耳(ov)及側線系統(ALL和PLL)。(B)自腹面觀察受精後4天的胚胎內耳(白色虛線)，發現TagGFP綠色螢光蛋白會分別表現於相鄰兩耳石(otolith)的前聽斑(am)和後聽斑(pm)，以及前側線的毛細胞。隨後於受精後5天的胚胎，以紅色螢光染劑FM4-64藉由具有功能性的機械傳導通道(mechanotransduction channel)進行前側線(C)及後側線(D)系統的毛細胞標定，可發現TagGFP綠色螢光蛋白確實會表現於側線系統的毛細胞(白色箭頭所示)。ov: otic vesicle、ao: anterior otolith、po: posterior otolith、am: anterior macula、pm: posterior macula、 ALL: anterior lateral line、PLL: posterior lateral line。

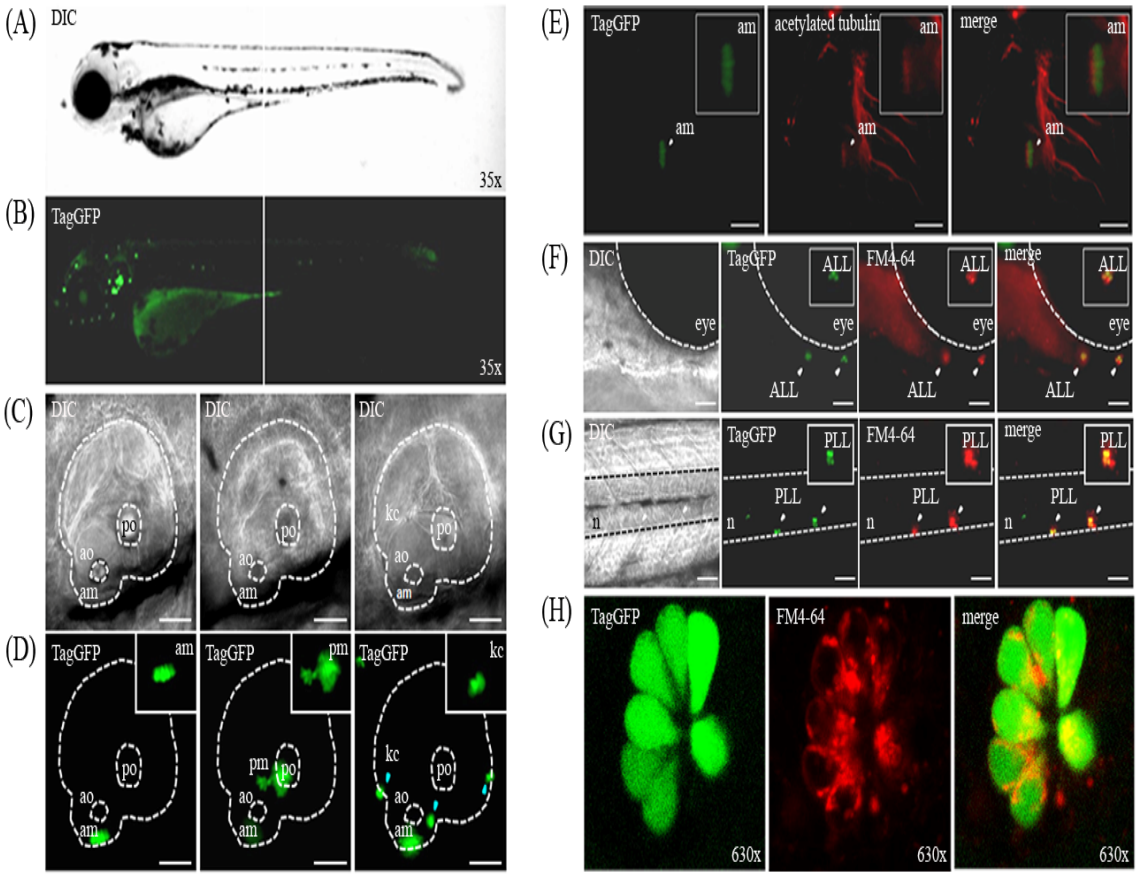

Figure 12. Cellular distribution of TagGFP in the pvalb3b transgenic zebrafish (pvalb3b-TagGFP *gfp/wt ln3*). The fluorescent images of 96 hpf embryos showed by using fluorescence microscopy (A-G) or confocol microscopy (H) were lateral views, anterior waer to left and dorsal were up. (A-B) The fluorescent images of 96-hpf embryo showed that the TagGFP-labeling sensory cells were distributed in the otic vesicle and lateral line system. (C-D) In lateral view of otic vesicle in a 96-hpf embryo, sensory epithelia containing TagGFP-positive hair cells were located beneath the two otoliths (termed anterior macula and posterior macula respectively) or crista, followed by immunostaining with anti-acetylated tubulin antibody. (E) The immunostaining patterns showed the single, long kinocillium (red) in each TagGFP-positive cell (green), indicating the TagGFP-positive cells were indeed the macular hair cells. (F-H) In addition, transgenically expressed GFP marked hair cells in 96-hpf embryo neuromasts of lateral line system including anterior lateral line (F) and posterior lateral line (G) respectively, followed by labeling with FM4-64 fluorescent dye that passed through functional mechanotransduction channels. The merge images of TagGFP (green) and FM4-64 signals (red) indicated the transgenically expressed TagGFP marked the functional hair cells. am: anterior macula, pm: posterior macula, kc: Kinocilia of the crista hair cells, ALL: anterior lateral line, PLL: posterior lateral line. Scale bar: 50 μm.

Investigation of the mechanisms of the *GJB3* gene mutations cause hearing loss with cellular and animal models

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ABSTRACT

Congenital hearing loss is a common sensory disorder worldwide, and approximately 60 % cases are attributed to genetic factors in the developed countries. The crucial role of intracellular gap junction channels (GJs), which are composed of connexin (CX) proteins, in auditory functions has been confirmed to recycle and maintain the potassium ions in the inner ear by numerous studies. Mutations in a family of *CX* gene, encoding connexin proteins have been linked to hearing loss, and zebrafish also has been considered an attractive animal model for the investigation of vertebrate inner ear development. Recently, we identified novel heterozygous missense mutation, p.L10R, p.P18S, p.V84I, p.V174M, p.E183k and p.A194T, in the *GJB3* gene encoding CX31 from 513 unrelated Taiwanese patients with non-syndromic hearing loss. Here we focused on the functional properties of six CX31 mutants derived from point mutation. Immunostaining pattern of transfected cells revealed that p.P18S, p.V174M and p.E183K mutants impaired the trafficking of CX proteins to the plasma membrane leading to accumulation of the mutant proteins in the cytoplasm, whereas p.L10R, p.V84I and p.A194T mutants showed the typical punctuate pattern of gap junction channel between neighboring expression cells as CX31 wild-type. In addition, we have established the transgenic zebrafish with sensory cells-specific overexpression of GFP. Based on above results, we suggest that CX31 p.P18S, p.V174M and p.E183K mutations have effect on the formation and function of the gap junction. Moreover, p.L10R, p.V84I and p.A194T mutations do not affect the trafficking of mutant CX31 proteins, but its functional significance remains unknown. Therefore, the functional significance of p.L10R, p.V84I and p.A194T mutations requires further investigation. Simultaneously, we believe that the transgenic zebrafish model will provide a good approach to study the etiology and mechanism of pathogenesis in hereditary hearing loss.

RESULTS

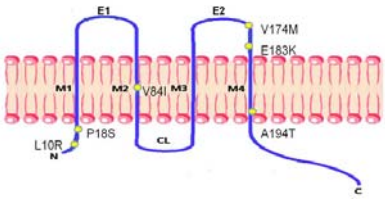

Figure 1. Mutation analysis of *GJB3* gene in 513 Taiwanese individuals manifesting nonsyndromic hearing loss. Schematic representation of domain structure of CX31 protein with indication of six missense mutations. Those mutations were respectively located in the N-terminus (p.L10R and p.P18S), cytoplasmic loop (p.V174M and p.E183K) and transmembrane region (p.V84I and p.A194T) of CX31 protein.

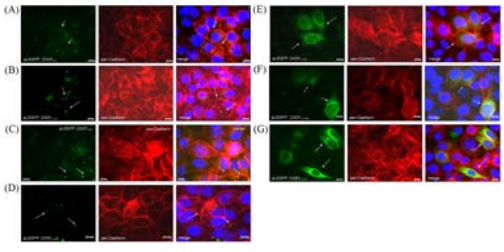

Figure 2. Intracellular localization of the wild-type and mutant CX31 proteins in transiently transfected HeLa cells. The immunocytochemistry analysis of wild-type (wt) and mutant CX31 proteins in transiently transfected HeLa cells were performed by anti-pan-cadherin antibody. All images were showed using fluorescence microscopy. Within cells transfected with wt CX31 (A), the p.L10R mutant (B), p.V84I mutant (C) or p.A194T mutant (D), each EGFP-tagged CX31 fusion protein was presented the typical plaques between adjacent cells (white arrows). However, the p.P18S (E), p.V174M (F) and p.E183K (G) mutant proteins were showed the impaired trafficking and were retained within the cytoplasm, concentrated in a region close to the nucleus (white arrows). The cells were counterstained with 4'-6'-diamidino-2'-phenylindole (DAPI) to4'-6'-diamidino-2'-phenylindole (DAPI) highlight the nuclei. Scale bar: 10 μm.

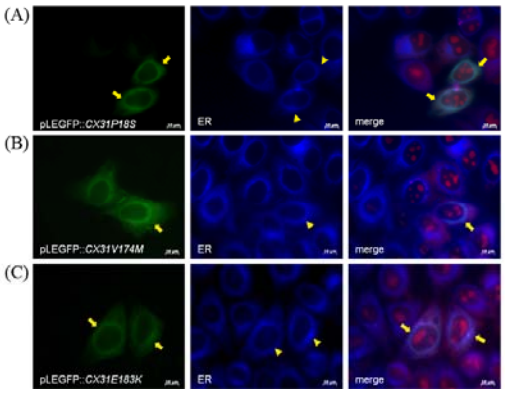

Figure 3. The mutant CX31 proteins in transiently transfected HeLa cells could intracellular co-localized with endoplasmic reticulum. Three mutant CX31proteins, p.P18S (A), p.V174M (B) and p.E183K (C) respectively, were retained within the cytosol of HeLa cells and concentrated in a region close to the nucleus. Photo-micrographs of HeLa cells transfected with each mutant *GJB3* cDNA, followed by immunostaining for markers of endoplasmic reticulum (ER). All mutant CX31 proteins were found to moderately co-localized with the ER marker. The cells were counterstained with propidium iodide (PI) to highlight the nuclei. Yellow arrows indicate the localization of CX31 mutant protein. Yellow arrowheads indicate the localization of ER. Scale bar: 10 μm.

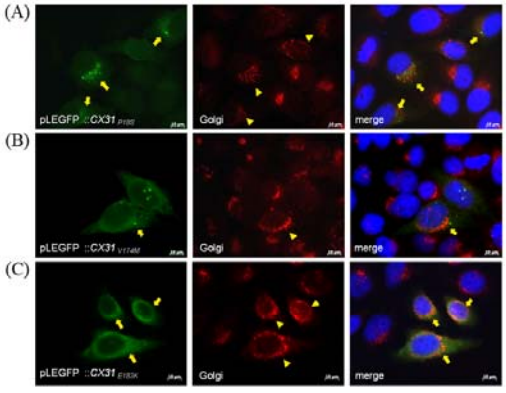

Figure 4. The mutant CX31 proteins in transiently transfected HeLa cells could intracellular co-localized with Golgi apparatus. Three mutant CX31proteins, p.P18S (A), p.V174M (B) and p.E183K (C) respectively, led to the substantial reduction in trafficking to cell membrane between adjacent cells. Photomicrographs of HeLa cells transfected with each mutant *GJB3* cDNA, followed by immuno-staining for markers of Golgi apparatus (Golgi). The immunostaining results for both mutants p.P18S and p.E183K showed the substantial co-localization in the Golgi apparatus, but the staining for mutant p.V174M proteins did not. The cells were counterstained with 4'-6'-diamidino-2'-phenylindole (DAPI) to highlight the nuclei. Yellow arrows indicate the localization of CX31 mutant protein. Yellow arrowheads indicate the localization of Golgi apparatus. Scale bar: 10 μm.

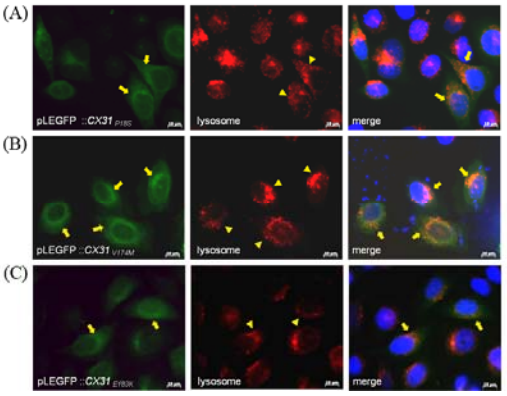

Figure 5. The mutant CX31 proteins in transiently transfected HeLa cells could intracellular co-localized with lysosome. Three mutant CX31proteins, p.P18S (A), p.V174M (B) and p.E183K (C) respectively, were diffusrly distributed within the cytosol of HeLa cells. Photomicrographs of HeLa cells transfected with each mutant *GJB3* cDNA, followed by immunostaining for markers of lysosome. The immunostaining patterns for all mutants proteins showed the substantial co-localization in the lysosome of HeLa cells. The cells were counterstained with 4'-6'-diamidino-2'-phenylindole (DAPI) to highl-ight the nuclei. Yellow arrows indicate the localization of CX31 mutant protein. Yellow arrowheads indicate the localization of lysosome. Scale bar: 10 μm.

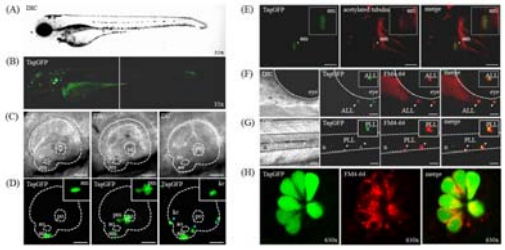

Figure 6. Cellular distribution of TagGFP in the pvalb3b transgenic zebrafish. The fluorescent images of 96 hpf embryos showed by using fluorescence microscopy (A-G) or confocol microscopy (H) were lateral views, anterior waer to left and dorsal were up. (A-B) The fluorescent images of 96-hpf embryo showed that the TagGFP-labeling sensory cells were distributed in the otic vesicle and lateral line system. (C-D) In lateral view of otic vesicle in a 96-hpf embryo, sensory epithelia containing TagGFP-positive hair cells were located beneath the two otoliths (termed anterior macula and posterior macula respectively) or crista, followed by immunostaining with anti-acetylated tubulin antibody. (E) The immunostaining patterns showed the single, long kinocillium (red) in each TagGFP-positive cell (green), indicating the TagGFP-positive cells were indeed the macular hair cells. (F-H) In addition, transgenically expressed GFP marked hair cells in 96-hpf embryo neuromasts of lateral line system including anterior lateral line (F) and posterior lateral line (G) respectively, followed by labeling with FM4-64 fluorescent dye that passed through functional mechanotransduction channels. The merge images of TagGFP (green) and FM4-64 signals (red) indicated the transgenically expressed TagGFP marked the functional hair cells. am: anterior macula, pm: posterior macula, kc: Kinocilia of the crista hair cells, ALL: anterior lateral line, PLL: posterior lateral line. Scale bar: 50 μm.

CONCLUSIONS

1. Five novel heterozygous missense mutation, p.L10R, p.P18S, p.V84I, p.V174M, p.E183k and p.A194T, in the *GJB3* gene encoding CX31 were identified from 513 unrelated Taiwanese patients with nonsyndromic hearing loss.
2. Three mutants p.P18S, p.V174M and p.E183K impaired the trafficking of CX proteins to the plasma membrane leading to accumulation of the mutant proteins in the cytoplasm.
3. However, p.L10R, p.V84I and p.A194T mutants showed the typical punctuate pattern of gap junction channel between neighboring expression cells as CX31 wild-type.
4. We have established the transgenic zebrafish with sensory cells-specific overexpression of GFP.

ACKNOWLEDGEMENT

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國科會補助計畫衍生研發成果推廣資料表

日期:2013/10/31

國科會補助計畫	計畫名稱: 利用細胞和動物模式探討GJB3基因突變造成聽障之機制
	計畫主持人: 楊建洲
	計畫編號: 101-2320-B-040-014- 學門領域: 醫學之生化及分子生物

無研發成果推廣資料

101 年度專題研究計畫研究成果彙整表

計畫主持人：楊建洲	計畫編號：101-2320-B-040-014-
計畫名稱：利用細胞和動物模式探討 GJB3 基因突變造成聽障之機制	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	2	0	100%	人次	
		博士生	2	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 ■未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

1. 學術成就：

在本研究中我們已經瞭解 p.L10R、p.P18S、p.V84I、p.V174M、p.E183K 及 p.A194T 的 CX31 突變蛋白在 HeLa 細胞中的表現位置。另外，對於突變點 p.V174M 及 p.E183K 可能造成聽障的原因有一個初步的了解。確認或證實這些突變點所造成的功能影響，可讓我們初步的了解到 CX31 突變所造成聽障的分子機制。對後續針對 GJB3 的研究有一定的幫助。

在斑馬魚模式動物的建立方面，我們利用 Tol2 斑馬魚基因轉殖系統，已經建立好兩株耳囊聽斑及側線會發螢光的轉殖基因魚，這兩株基因轉殖魚的建立將可方便我們後續對於聽障的成因或致病機轉的研究。

2. 技術創新：

建立耳囊聽斑及側線會發螢光的轉殖基因魚，後續將可建立一個藥物篩檢平台，來評估哪些藥式而毒性藥物? 哪些藥可加強聽力的功能? 這些螢光轉殖基因魚，改變現在利用免疫螢光染色法標定耳囊聽斑及側線的毛細胞，在進行後續研究所造成的不變和時間的浪費。這轉殖基因魚有其應用價值。