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Report:

In vitro culture of mammalian inner ear hair cells*

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Abstract: Auditory function in vertebrates depends on the transduction of sound vibrations into electrical signals by inner ear hair cells. In general, hearing loss resulting from hair cell damage is irreversible because the human ear has been considered to be incapable of regenerating or repairing these sensory elements following severe injury. Therefore, regeneration and protection of inner ear hair cells have become an exciting, rapidly evolving field of research during the last decade. However, mammalian auditory hair cells are few in number, experimentally inaccessible, and barely proliferate postnatally in vitro. Various in vitro primary culture systems of inner ear hair cells have been established by different groups, although many challenges remain unresolved. Here, we briefly explain the structure of the inner ear, summarize the published methods of in vitro hair cell cultures, and propose a feasible protocol for culturing these cells, which gave satisfactory results in our study. A better understanding of in vitro hair cell cultures will substantially facilitate research involving auditory functions, drug development, and the isolation of critical molecules involved in hair cell biology.

Key words: Inner ear; Hair cell; In vitro culture system
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1 Introduction

Hearing loss is one of the major health concerns in the world, and seriously affects the lives of millions of people. It may be caused by a number of factors, including genetic defects, aging, noise trauma, and excessive use of ototoxic drugs (Abdouh et al., 1993; Friedman and Griffith, 2003; Dror and Avraham, 2009; Li-Korotky, 2012; Smith et al., 2016). In general, hearing loss is irreversible in mammals (Ruben, 1967; Roberson and Rubel, 1994; Smith et al., 2016). Hair cells, as the primary sensory receptors, play an irreplaceable role in sound transmission and conversion (Hudspeth, 1997; Brandon et al., 2012). Understanding the mechanism of hair cell regeneration and repair will significantly improve prognosis in patients with hearing loss. However, there are still many unsolved technical problems in this field. For instance, the inner ear is encased in the hardest bone of the body and, hence, is experimentally inaccessible. In vitro culturing of inner ear hair cells has become an ideal alternative for investigating hair cell functions, such as transformation of supporting cells into hair cells (White et al., 2006; Shang et al., 2010) and the mechanisms involved in the rapid elimination of apoptotic hair cells (Meiteles and Raphael, 1994; Bird et al., 2010; Bucks et al., 2017), as well as to screen key factors influencing hair cell differentiation (Malgrange et al., 2002).

Sounds received by the ear are conveyed through a series of in-built systems consisting of the outer ear, ear canal, eardrum, middle ear ossicles, inner ear, cochlea, and nerves running into the brain (Fig. 1a). Briefly, sound passes through the external auditory

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canal, reaches the eardrum, and causes vibration, which is then transmitted by the ear ossicles to the cochlear, causing deflection of stereocilia on hair cells. The movement of stereocilia causes changes in the electrical potential inside the hair cells, and finally the electrical signals are passed to the cerebral cortex (Hudspeth, 1997). Inner ear hair cells are specialized cells that are tightly anchored to nonsensory supporting cells in an epithelial sheet and play an irreplaceable role in both body balance and hearing (Brandon et al., 2012). These hair cells in mammalian inner ears are very fragile and barely proliferate (Oesterle et al., 1993; Roberson and Rubel, 1994). One of the most notable features of these cells is their mechanically sensitive appendage, known as the hair bundle. These hair bundles comprise of dozens to hundreds of modified microvilli, called stereocilia, stacked in rows of increasing height (Furness et al., 1989). In addition, the number and height of stereocilia vary with hair cell location (Figs. 1b–1d), reflecting their specialized functions (Gubb and García-Bellido, 1982; Hudspeth, 1989a, 1989b; Eatock, 2000; Corns et al., 2014). The cochlea (Fig. 1b), utricle (Fig. 1c), saccule, and crista ampullaris (Fig. 1d) are sensory epithelial tissues found in the inner labyrinth in mammals, with only cochlea being capable of perceiving sound.

There is a single row of inner hair cells and three rows of outer hair cells in the spiral organ (organ of Corti) of humans. The cochlea of a newborn human infant contains approximately 16000 hair cells (Géléoc and Holt, 2014). According to the hypothesis of the travelling-wave proposed by von Békésy (1956), hair cells in different areas of basilar membranes perceive sound of different frequencies. In general, humans can perceive sounds between 20 and 20000 Hz (Meyer-Bisch, 2005). Loss of apical hair cells in the cochlea inhibits the transformation of low-frequency sound into electrical signals, eventually resulting in low-frequency hearing loss. Loss of base turn hair cells in the cochlea leads to high-frequency hearing loss (Fig. 2a).

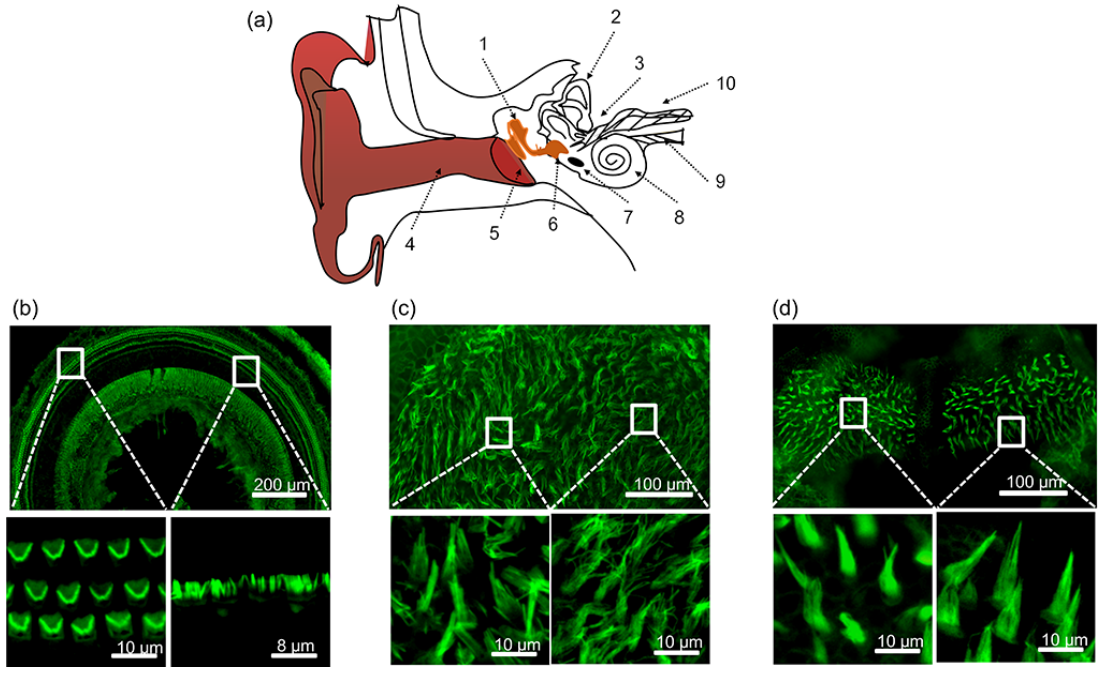

Fig. 1 A complex set of "audio devices"

(a) Schematic diagram of sound transmission: 1, ossicles; 2, semicircular canal; 3, crista ampullaris; 4, external ear; 5, tympanic membrane; 6, oval window; 7, round window; 8, cochlea; 9, auditory vestibular nerve; 10, vestibular nerve. (b–d) One of the most notable features of hair cells in the mammalian inner ear is the hair bundle. These hair bundles comprise numerous modified microvilli, called stereocilia, stacked in rows of increasing height. (b) Mouse cochlear hair cells at middle turn, one row of inner hair cells and three rows of outer hair cells exhibit typical "W" arrangement. (c, d) Hair cells in the mouse utricle (c) and crista ampullaris (d). The zoomed-in images show the stereocilia of the type I (lower left in c, d) and type II (lower right in c, d) hair cells. All stereocilia were stained with phalloidin (b–d)

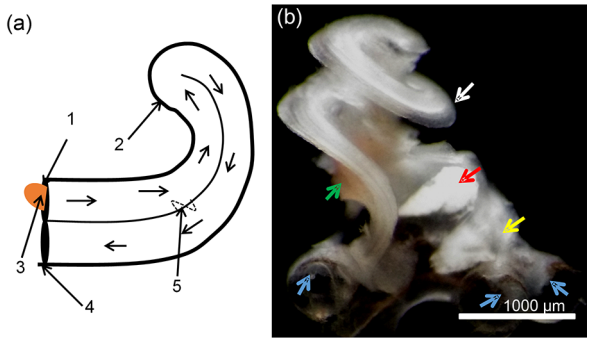

Fig. 2 Structure of cochlear duct

(a) Schematic diagram of cochlear duct: 1, oval window; 2, cochlear apex; 3, tympanic membrane; 4, round window; 5, representative position of sound resonance. (b) The inner ear labyrinth of a P7 C57BL/6 mouse. The white arrow indicates the basal membrane, the red arrow indicates the saccule, the green arrow indicates the cochlear axis, the yellow arrow indicates the utricle, and the blue arrows indicate the three cristae ampullaris

The membranous labyrinth is a continuous system of ducts filled with endolymph, and it lies within the bony labyrinth, surrounded by the perilymph. Because of the complex architecture, hair cells in the inner ear are difficult to access. Corti used surface preparations to observe the mammalian organ of ear in vitro for the first time in 1851 (Betlejewski, 2008). With the application of this technology, researchers successfully developed hair cell cultures, thus making it possible to study the physiology and pathophysiology of the inner ear in vitro (Table 1). Sobkowicz et al. (1975) established an in vitro method to culture the organ of Corti of the newborn mouse, whereas Yamashita and Vosteen (1975) adopted the Rose chamber method to culture hair cells from the guinea pig. In 1978, the crista ampullaris of postnatal mice was successfully isolated and cultured in vitro for 7 d

Table 1 Different systems of in vitro hair cell cultures

Study	Specimen	System/culture medium	Supplement	Culture/ experimental time
Yamashita and Vosteen (1975)	Guinea pig Corti	Rose chamber method	Horse serum, fetal calf serum, human ascitis fluid, Gey's balanced salt solution	20 d
Sobkowicz et al. (1975)	Mouse cochlea	Collagen slice; MEM	Horse serum, HEPES, modified Simms salt solution, L-glutamine, D-glucose	27 d
Anniko and van de Water (1978)	Mouse crista ampullaris	Neaman/Tytell medium	1% L-glutamine, 1% Na-pyruvate, 10% fetal calf serum	7 d
Oesterle and Rubel (1993)	Chicken cochlea	Floating culture; BME	Earle's balanced salt solution, D-glucose, FBS	7 d
Rastel et al. (1993)	Rat Corti	Collagen gel drop floating; MEM	40% horse serum, L-glutamine, glucose, HEPES, HBSS	12 d
Romand and Chardin (1999)	Cochlea	DMEM/F12	D-glucose, L-glutamine, sodium selenium, apotransferrin, albumin bovine	10 d
Spencer et al. (2008)	Chicken cochlea	Artificial perilymph media	N1, N2	5 d
Lelli et al. (2009)	Mouse cochlea	MEM	10 mmol/L HEPES	8 d
Parker et al. (2010)	Mouse cochlea	DMEM	5% FBS, 5% horse serum	7–10 d
Ding et al. (2011)	Mouse cochlea	Collagen gel drop; BEM	BSA, serum-free supplement, 20% glucose, penicillin G, 200 mmol/L glutamine, 1× BME	48 h
May et al. (2013)	Mouse utricle	Floating culture; DMEM/F12	5% FBS	24 h
Ou et al. (2013)	Mouse utricle	In bone culture; DMEM	1% FBS	3 d
Lin et al. (2015)	Mouse utricle	Floating culture; DMEM/F12	1% N2, 2% B27, b-FGF, IGF-1, EGF, heparin sulfate	10 d
Werner et al. (2015)	Rat utricle	DMEM	1% N1, 10% FBS, glucose	28 d
Taura et al. (2016)	Mouse utricle	Matrigel-coated glass coverslips; DMEM	10% FBS	10 d
Landegger et al. (2017)	Cochlea	DMEM	1% FBS, 1% N2, and 1% ampicillin	7 d

MEM: minimal essential medium; BME: basal minimal Eagle's medium; DMEM: Dulbecco's modified Eagle's medium; F12: Ham's F12 medium; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FBS: fetal bovine serum; HBSS: Hank's balanced salt solution; N1: N1 supplement; N2: N2 supplement; BSA: albumin from bovine serum; B27: B27 supplement; b-FGF: basic fibroblast growth factor; IGF-1: insulin-like growth factor 1; EGF: epidermal growth factor

(Anniko and van de Water, 1978). To date, microdissection and surface preparation of the cochlea have been applied as key histological techniques by investigators worldwide.

The first paper on in vitro culturing was published more than 40 years ago (Betlejewski, 2008). Since then, researchers worldwide have been trying to improve the culturing systems to achieve long-term hair cell cultures. Different culture media, such as Dulbecco's modified Eagle's medium (DMEM), DMEM/Ham's F12 medium (DMEM/F12), and basal minimal Eagle's medium (BME), were adopted for hair cell cultures in different groups (Oesterle and Rubel, 1993; Kalinec et al., 2003; Gaboyard et al., 2005; Lelli et al., 2009; Werner et al., 2012; Ding et al., 2013; Ou et al., 2013; Lin et al., 2015; Taura et al., 2016). Most of the media used to culture spiral ganglion cells found in the cochlear axis can be also applied to culture hair cells located on the basilar membrane. Unlike regular tissue culture methods, only penicillin G or ampicillin was added to these hair cell culturing systems. The use of antibiotics such as gentamicin, kanamycin, neomycin, amikacin, and streptomycin, which have ototoxic side effects, should be avoided (Hawkins et al., 1969; Quint et al., 1996). Even low doses (1–10 μg/ml) of these ototoxic antibiotics in the culture medium may cause a primary morphological damage of vestibular and cochlear hair cells. According to the current recommendations of the American Academy of Otolaryngology-Head and Neck Surgery (Matz et al., 2004), only fluoroquinolone antibiotics are considered safe to administer in the ear. Moreover, it is widely accepted that N2, B27, and growth factors, such as insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), and basic fibroblast growth factor (b-FGF), play important roles in maintaining hair cell viability in vitro (Romand and Chardin, 1999; Yamahara et al., 2015; Smith et al., 2016; Landegger et al., 2017). Most researchers prefer adding these supplements in their in vitro hair cell cultures so as to achieve prolonged culture time (Gaboyard et al., 2005; Koehler and Hashino, 2014; Chang et al., 2015; Lin et al., 2015; Werner et al., 2015). To improve the adhesion and survival of hair cells, various types of substrates have been tested, including collagen (Ding et al., 2013), Pura Matrix (Gaboyard et al., 2005), 2-hydroxyethylmethacrylate hydrogels (Spencer et al., 2008), Matrigel (Taura et al., 2016), and poly-L-lysine (Qian et al., 2007). Spencer et al. (2008) compared the survival rates of chick cochleae hair cells cultured in vitro with different coat substrates and suggested that Pura Matrix is the most suitable substrate for such cultures. Parker et al. (2010) used glass coverslips coated with a nutrient-rich mixture (consisting of laminin, poly-L-ornithine, and fetal bovine serum (FBS)) in their culture systems and maintained the primary explants for 7–10 d.

Gel embedding methods have been developed for inner ear hair cell cultures. Rastal et al. (1993) established a floating-drop method to study the effects of growth factors on the development of auditory cells. Ding et al. (2002, 2011, 2013) introduced a gel-embedding culture system using collagen and BME, which has been proved to effectively culture the utricle and cochlea in vitro compared with other cultures without coating materials. Transwell assays, which ensured consistent gas and liquid environments in the tissues and organs, also have been applied to the utricle cultures in vitro (Werner et al., 2012, 2015; May et al., 2013). With the development of biomaterials, three-dimensional (3D) culturing has emerged as a powerful technique for optimizing hair cell culture systems to mimic the in vivo conditions of the organism. Chang et al. (2015) reported that elastin-like protein hydrogel preserves the 3D architecture of neonatal cochlea in short-term cultures, which can also be applicable to in vitro studies of the physiology and pathophysiology of the inner ear. Nevertheless, until recently, "in-bone" culturing is widely accepted as a simplified, atraumatic technique for in situ cultures of the adult mouse utricle (Ou et al., 2013). In situ culturing of the utricle within the temporal bone can resolve several regular technical problems faced by researchers, such as variability in preservation of the sensory epithelium. However, it is difficult to standardize hair cell culture conditions, resulting in a higher rate of apoptosis in these cells near the opened sites of the vestibule. In addition, "in-bone" utricle culture systems could introduce more variability due to the influence of unknown additional survival factors generated by other tissues/cells.

2 Materials and methods

2.1 Mice

C57BL/6 mice were purchased from SLAC laboratory Animal Co. (Shanghai, China). The mice

were housed in temperature- and humidity-controlled rooms with 12-h light/dark cycles and with free access to food and water. All protocols were approved by the Animal Care and Use Committee of Zhejiang University, Hangzhou, China, and were in compliance with the "Guidelines for the Care and Use of Laboratory Animals" published by the National Academy Press (NIH Publication No. 85-23, Revised in 1996; https://www.nabr.org/animal-welfare-2/animal-welfare-in-practice/guide-for-the-care-and-use-of-laboratory-animals).

2.2 Reagents

DMEM (GBICO, cat: SH30243.01); fetal bovine serum (GBICO, cat: 10099141); heat-inactivated horse serum (GBICO, cat: 26050070); N2 supplement (Thermo Fisher, cat: 17502048); B27 (GBICO, cat: 12587-010); Hank's balanced salt solution (HBSS, Life Technologies, cat: 14025076); phosphate-buffered saline (PBS, Sangon, cat: E607016-0500); rat tail collagen, Type 1 (BD Biosciences, cat: 4236); BME (Sigma-Aldrich, cat: B9638); penicillin (Sigma-Aldrich, cat: A6140); phalloidin (Enzo, cat: ALX-350-268-MC01); 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, cat: D9542); 4% paraformaldehyde (PFA, Beijing Leagene Biotechnology, cat: DF0135-500ml); Triton X-100 (Sangon, cat: 9002-93-1).

2.3 Equipment

Surgical forceps (VETUS, #5 Tweezers 3C); surgical scissors (ROBOZ, cat: RS-6702); 4-well plates (BD Falcon, cat: 353654); microscope slide coverslips (Thermo, cat: D9542); dissecting microscope (Lecia, EZ4); sterilizer (VORTEX-5); biosafety cabinet (BCM-1300); cell incubator (Heal Force).

2.4 Procedure

2.4.1 In vitro culture of mouse cochlear hair cells

(1) Postnatal mice (3 to 5 d after birth) are anaesthetized using isoflurane-saturated gas (Fig. 3a). (2) Bisect the mouse head and remove the brain tissue (Fig. 3b). (3) Remove the temporal bone, which is not continuous with the skull, by pushing in the junctions, and then place it into ice-cold HBSS with 1× penicillin until further dissection (Figs. 3c and 3d). (4) Perform further dissection of the temporal bone under the hood. Carefully separate the cochlea from the extraneous bones; clean and store it in HBSS without penicillin on ice (Figs. 3e and 3f). (5) After removing the lateral wall, isolate the complete basilar membrane containing the organ of Corti and spiral ganglion neurons (Fig. 3g). (6) Subsequently, carefully remove the spiral ligament and the connected bone plate as well as the Reissner's membrane and tectorial membrane, followed by the cochlear axis and the spiral ganglion (Fig. 3h). (7) Carefully transfer the basilar membrane into a 4-well chamber slide coated with a high concentration of 15 μl collagen gel matrix (rat tail collagen, Type I, 10× BME, 0.02 g/ml sodium carbonate; 9:1:1) containing 130 μl of DMEM (supplemented with 5% horse serum and 5% FBS), which was maintained in a cell culture incubator for 4−6 h. (8) Subsequently, replace the previous culture medium with 200 μl of fresh culture medium (DMEM supplemented with 10% FBS, 1% N2, and 1% B27) and incubate it overnight (37 °C in 5% CO_2). (9) Monitor the cultures daily under a microscope, and carefully replace half of the culture medium with fresh medium daily. Ensure that the stereocilia of the hair cells face upwards during culturing.

2.4.2 In vitro culture of mouse utricle hair cells

(1) Mice were sacrificed, and the temporal bone was obtained as mentioned above (Figs. 3a−3d). (2) Remove the cochlea and open the vestibule. (3) Subsequently, carefully remove the three cristae ampullaris and other tissues (Fig. 3f). (4) The utricles (Fig. 2b, yellow arrow) were transferred into a 4-well chamber slide coated with a high concentration of collagen gel matrix and containing 130 μl DMEM (supplemented with 10% FBS, 1% N2, and 1% B27). (5) Monitor the cultures daily under a microscope, and carefully replace half of the culture medium with fresh medium daily.

2.5 Fluorescence staining

The organotypic cultures were fixed in 4% PFA (4 °C/overnight) and permeabilized with 0.01% Triton X-100 (5 min/room temperature). Then, the hair cells were stained with phalloidin (1:200, in PBS) for 20 min at room temperature. The slides were washed three times in PBS, and then the nuclei were labeled by DAPI (10 μmol/L stock solution; 1:1000 in PBS) for 10 min at room temperature. The slides were washed three more times in PBS, covered with a glass coverslip over fluorescent mounting media, and left to dry in the dark before the edges were sealed with nail hardener. All staining processes were manipulated in a light avoidance condition.

3 Results and discussion

Viable culture preparations from the mammalian inner ear are still problematic. Currently, there is no satisfactory way to culture inner ear tissues for a very long time period. Utricle, one of the sensory epitheliums in the inner ear membranous labyrinth, is easier to obtain for culturing hair cells in vitro. As shown in Fig. 4a, utricles from newborn mice were isolated and cultured in hair cell medium (DMEM supplemented with 10% FBS, 1% N2, and 1% B27). The number of hair cells can be preserved for about 3 d, and most of the cells lost their hair bundles on Day 5 of in vitro culture (Fig. 4b).

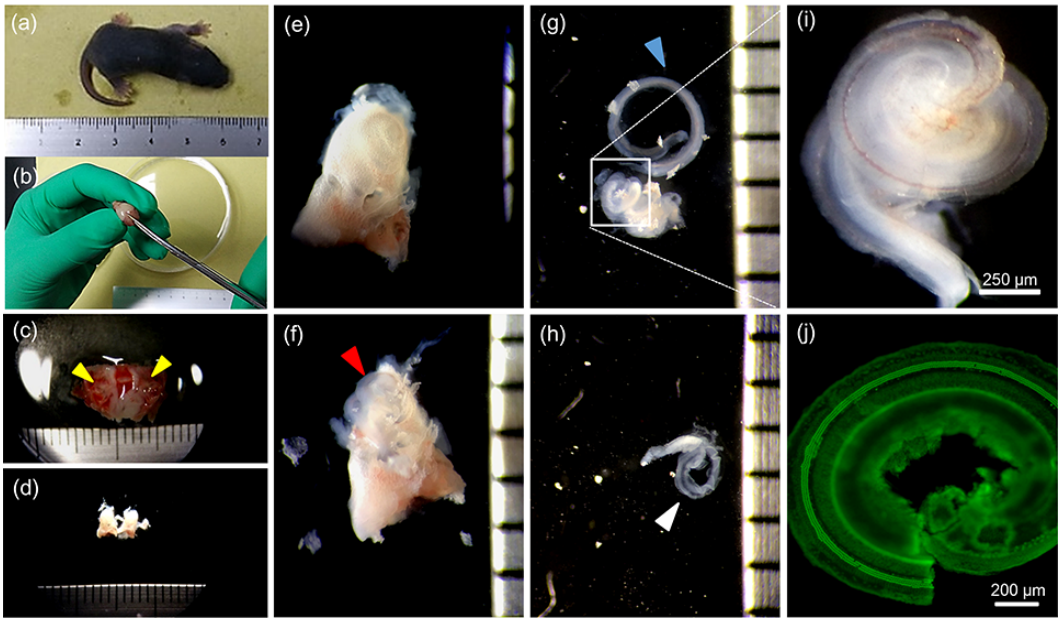

Fig. 3 Diagrams of dissecting steps

(a) Postnatal 5-d-old C57BL/6 mice were sacrificed for inner ear dissection. (b) Bisect the mouse head and remove the brain tissue. (c) The yellow triangles show the locations of inner ear. (d–f) Carefully separate the cochlea from the extraneous bones. The red triangle shows the cochlea. (g, h) Remove the stria vascularis, spiral bone plate, Reissner's membrane, and tectorial membrane. The blue triangle shows the Reissner's membrane; the white triangle shows the basilar membrane. (i) Representative structure of the basilar membrane. (j) The hair cells were labeled with phalloidin. (a–h) Bar=1 mm (each grid)

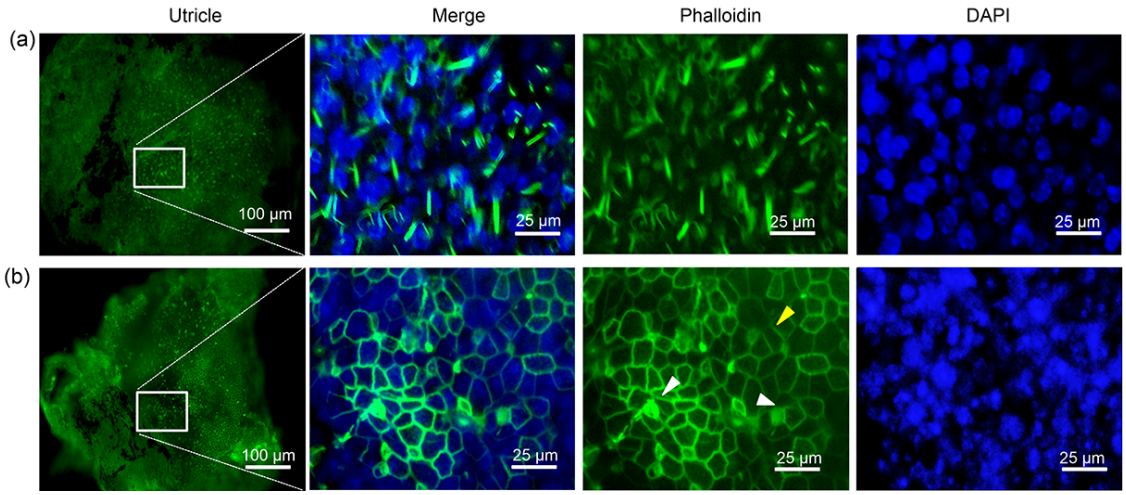

Fig. 4 In vitro culturing of mouse utricle hair cells

(a) Mouse utricles were isolated and cultured in vitro for 1 d. (b) After 5 d's culture, most of the cells lost their hair bundle (the white triangles indicate the hair cells and the yellow triangle shows the supporting cells). The stereocilia were stained with phalloidin (green). The nuclei were labelled by DAPI (blue)

For cochlear cell culture, mice of postnatal 3–5-d old were used. Compared with adult mice, the cochleae of newborn mice can be easily dissected to obtain the culture specimen since the temporal bone is not fully calcified (Fig. 5a). Our procedure is modified from the original description by Parker et al. (2010) and May-Simera (2016). After 4–6 h of seeding, part of the epithelial cells with a cobblestone morphology emerged from the basilar membrane. These cells expressed early hair cell markers such as Brn3.1 and calretinin (Zhao, 2001). After overnight incubation, the spiral ganglion cells could be also observed if they were not completely removed during the dissection procedure. With increasing culture time, hair cells on the basal membrane gradually stuck to the dishes, and the peripheral epithelial cells gradually formed a dome outside the hair cells (Fig. 6a). In our studies,

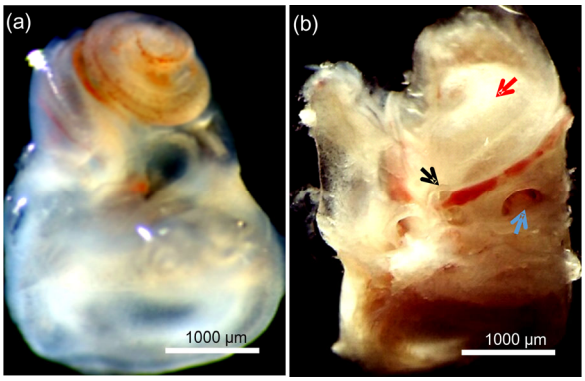

Fig. 5 Isolation of the inner ear from mouse

(a) Representative image of inner ear from P7 C57BL/6 mouse. (b) The inner ear of adult mouse was embedded within the bony labyrinth. The red arrow indicates the cochlea, the black arrow indicates the oval window, and the blue arrow indicates the round window

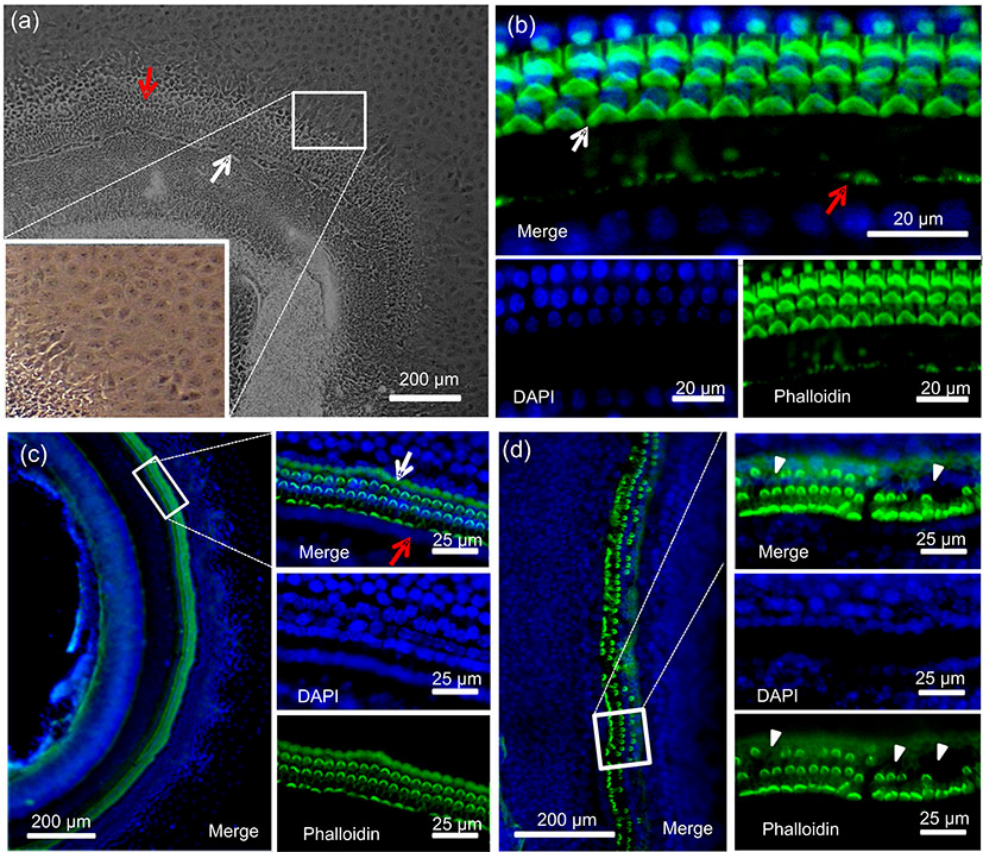

Fig. 6 In vitro culturing of mouse cochlea hair cells

The cochlea was isolated from P5 C57BL/6 mouse for both immunohistochemical staining and in vitro culturing. (a) Epithelial cells were observed emerging from the basilar membrane. (b) The fresh isolated cochlea cells were stained with phalloidin. The nuclei were stained with DAPI. (c) The cochlea maintained a good shape and cell viability after culturing for 5 d. (d) Outer hair cells gradually disappeared with the increase of culturing time. The red arrows show the inner hair cells, the white arrows show the outer hair cells, and the white triangles show the loss of hair cells

most cochlear cells maintained these typical structures for about 5 d (Figs. 6b–6d), suggesting that this method could be used as a suitable model for a short-term analysis.

In the last decade, rapid progress has been made in the area of in vitro culture of mammalian inner ear hair cells, and various culturing systems have been published to date. The development of in vitro hair cell cultures not only improves our understanding of the molecular control of progenitor cell fates within the developing cochlea, but also facilitates the study of drug delivery and gene therapy targets. However, gradually increased apoptosis of hair cells continues to be an issue. In general, hair cells cultured in vitro survive for only 1–2 weeks. Recently, 3D cell culture systems have gained increasing importance in tissue engineering and drug testing because of their advantages in providing physiologically relevant information. Using these 3D cell culture strategies, researchers have succeeded in maintaining primary cultures with greater stability and longer lifespans than those in regular 2D cultures. Further research should be conducted to enable prolonged survival of hair cells and eliminate the influence of antibiotics, thereby significantly contributing to the understanding of hair cell biology.

Compliance with ethics guidelines

Lu-wen ZHANG, Xiao-hui CANG, Ye CHEN, and Min-xin GUAN declare that they have no conflict of interest.

All applicable institutional and/or national guidelines for the care and use of animals were followed.

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中文概要

题　目：哺乳动物内耳毛细胞的体外培养

概　要：由于内耳的血脑屏障作用，药物渗入到内耳比较困难。新生小鼠内耳毛细胞的体外培养体系的建立，为体外进行支持细胞转分化机制的研究和进行体外药物损伤毛细胞实验等提供实验技术的前提。

为了避免毛细胞体外培养过程中污染杂菌，解剖内耳耳蜗的整个过程十分重要。处死小鼠后，将其浸泡在 75%酒精中 1~3 分钟，防止鼠毛污染培养基。打开内耳耳蜗之前，选用添加了青霉素的磷酸盐缓冲液（1× PBS）；培养过程中使用的是仅仅添加青霉素的培养基来减少对毛细胞的损伤。在基底膜培养的第一步，选用 DMEM（包含 5%马血清体积比和 5%胎牛血清）作为组织粘附培养的培养基，保证足够的营养，同时更好地维持整个基底膜培养状态下的形态。在之后的培养中，选用 DMEM（添加了 10%胎牛血清、1%N2 和 1%B27）作为长期的培养基。使用含有表皮生长因子的 N2 和 B27 的培养基进行基底膜以及椭圆囊之后的培养，有助于维持毛细胞的体外生长时间。选用鼠尾胶包被盖玻片后培养，可以增加基底膜和椭圆囊的粘附作用，保证毛细胞静纤毛的向上生长。

该文章展现了哺乳动物毛细胞的体外培养的具体方法，能够较好地维持耳蜗基底膜在体外培养的形态，并增加毛细胞体外培养的存活时间。

关键词：内耳；毛细胞；体外培养