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MAIN ARTICLE

In vitro induction and differentiation of newborn guinea pig hippocampus neural stem cells into cells resembling inner hair cells, using artificial perilymph

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Abstract

Objective: To investigate whether artificial perilymph can induce neural stem cells, derived from the hippocampus of newborn guinea pigs, to differentiate into inner ear hair cells, *in vitro*.

Methods: Primary neural stem cells derived from the hippocampus of newborn guinea pigs were incubated in medium containing either 10 per cent fetal bovine serum or 5, 10 or 15 per cent artificial perilymph, for three weeks. Differentiated cells were identified using immunofluorescence, Western blot and scanning electron microscopy.

Results: Both fetal bovine serum and artificial perilymph induced the neural stem cells to differentiate into cells with hair-cell-specific antibodies.

Conclusion: Neural stem cells can survive in both fetal bovine serum and artificial perilymph, and within these media can differentiate into cells with hair-cell-specific antibodies. This provides an experimental basis for transplantation of neural stem cells into the inner ear.

Key words: Ear, Inner; Stem Cells; Hair Cells, Auditory; Cell Differentiation; Perilymph; Guinea Pig

Introduction

It had previously been believed that stem cells from adult tissues could differentiate only into the mature cells of that tissue.

However, recent studies have shown that, although adult stem cells are specialised, they still possess the potential for multi-directional differentiation, and may even trans-differentiate into mature cells of other tissues.[1,2]

The differentiation of neural stem cells is known to be controlled by both internal genetic information and external factors.[3] Choosing the optimum external factors is very important for the differentiation of neural stem cells into inner ear hair cells. Reports indicate that neural stem cells may differentiate into cells resembling inner ear hair cells, within fetal bovine serum.[4] However, it has not previously been confirmed whether neural stem cells can survive and differentiate into cells resembling inner ear hair cells when cultivated in artificial perilymph, which simulates the micro-environment of the inner ear.

Therefore, this study used artificial perilymph of different concentrations to attempt to induce differentiation of neural stem cells.

Our results demonstrate, for the first time, that neural stem cells can differentiate into cells resembling inner ear hair cells, within artificial perilymph *in vitro*. This provides an experimental basis for the transplantation of neural stem cells into the inner ear.

Materials and methods

Animals

Newborn guinea pigs (i.e. younger than 24 hours) were provided by the Laboratory Animal Center, School of Medicine, Zhengzhou University, China.

Main reagents

Dulbecco's Modified Eagle Media, Nutrient Mixture F-12 (DMEM/F12) medium was provided by Hyclone (Utah, Logan, USA). Basic fibroblast growth factor (bFGF) was obtained from Beijing Shuanglu (Beijing, China). Epidermal growth factor was purchased from Peprotech (Princeton, UK), and B27NeuroMix (B27) and Alexa Fluor 594 were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was provided by Hangzhou Sijiqing (Hangzhou, China). Dispase II was obtained from

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Roche (Basel, Switzerland). Rat tail collagen and 4,6-diamino-2-phenyl indole (DAPI) fluorescent dye were provided by Sigma (Colorado, Loveland, USA). Myosin VIIa was purchased from ABR (California, los angeles, USA). Horseradish Peroxidase (HRP) labelled anti-rabbit secondary antibody was provided by Pufei Biological (Shanghai, China).

Artificial perilymph contained 137 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L NaH_2PO_4, 11 mmol/L glucose, 12 mmol $NaHCO_3$ and 1 mmol/L $MgCl_2$. The artificial perilymph was adjusted to a pH of 7.4 ± 0.2 and an osmotic pressure of 300–320 mmol/L.[5]

Neural stem cell induction and differentiation

Neural stem cells were divided into four groups and cultured in either 10 per cent fetal bovine serum, 5 per cent artificial perilymph, 10 per cent artificial perilymph or 15 per cent artificial perilymph. In addition, the basic growth medium for each group was DMEM/F12.

Neural stem cells derived from the hippocampus of newborn guinea pigs were separated and incubated according to the method described by Xi and Dong.[6] Dispase II was added to 7-day-old, primarily cultured neurospheres and incubated at 37°C for one hour, followed by mechanical dissociation into single cells, then re-suspension using DMEM/F12 medium. Finally, DAPI was added to make the final concentration up to 2 μg/ml, followed by incubation for 24 hours.

A small amount of cells were used to calculate the labelling rate under fluorescence microscopy. The labelled cells were centrifuged for 5 minutes and then washed three times using fresh culture medium, then underwent mechanical dissociation into single cells. The cell concentration was adjusted to 1×10^4/ml, distributed across a 24-well plate coated with rat tail collagen, and incubated at 37°C in an atmosphere of 5 per cent CO_2. Half of the culture medium was changed twice a week.

Immunofluorescent cytochemistry

Following three weeks' culture, cells were fixed using 4 per cent paraformaldehyde. Myosin VIIa (1:500) was applied, as the primary antibody for immunohistochemical analysis, followed by immunocytochemical detection using Alexa Fluor 594 (1:500) as the secondary antibody.[4] Ten non-overlapping microscopy fields, at a magnification power of ×400, were randomly selected. Myosin VIIa positive cells were counted twice under fluorescence microscope, using both a bright field and a dark field.

The number of myosin VIIa positive cells was expressed as $\bar{x} \pm s$. The area of a high power field was calculated according to the area (1/400 mm^2) of each small square in the blood counting grid. As the area of each cover slip (side length = 6 mm) and the number of cells (1×10^4/ml) did not vary, the concentration of myosin VIIa positive cells could be calculated.

TABLE I
MYOSIN VII A POSITIVE CELLS IN EACH CULTURE GROUP

Culture medium	MVIIa +ve cells *n* (mean ± SD)*	%
10% FBS	1.86 ± 0.86	12.8
5% AP	0.57 ± 0.26	3.9
10% AP	1.66 ± 0.77	11.4
15% AP	1.98 ± 0.92	13.6

*$x \pm s$. MVIIa +ve = myosin VIIa positive; SD = standard deviation; FBS = fetal bovine serum; AP = artificial perilymph

Western blot

Following three weeks' culture, cells were washed once using phosphate-buffered saline, dissolved using two applications of sodium dodecyl sulphate gel buffer and boiled for 5 minutes. Cells then underwent electrophoresis on 4 per cent polyacrylamide gel, were transferred onto Polyvinylidene Fluoride (PVDF) membrane and soaked in a mixture of 5 per cent milk and Tris buffer saline Tween (TBST) at room temperature for 1 hour,

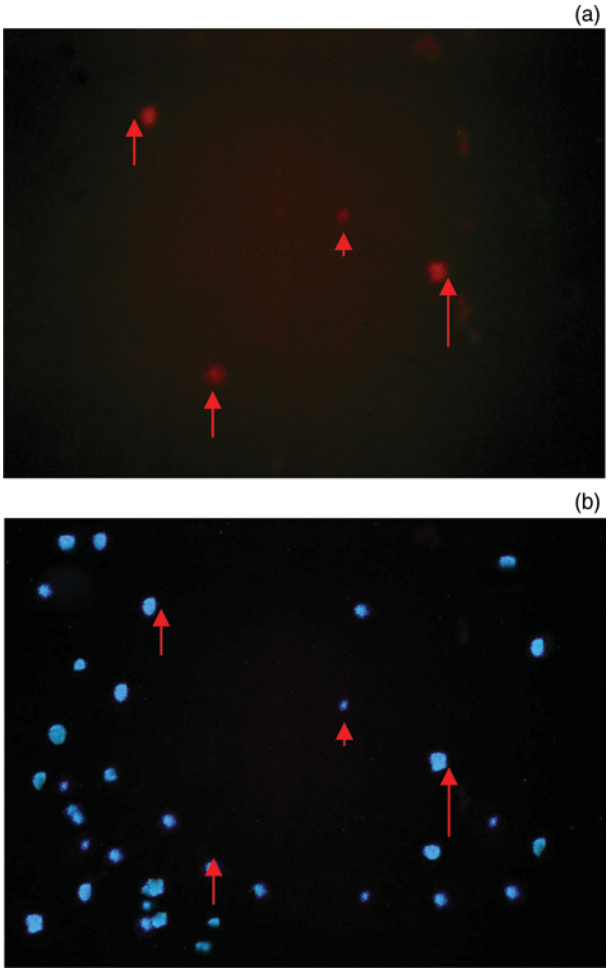

FIG. 1
Fluorescence photomicrographs for 10 per cent fetal bovine serum group, with arrows indicating (a) myosin VIIa positive cells and (b) DAPI labelled cells. (×400)

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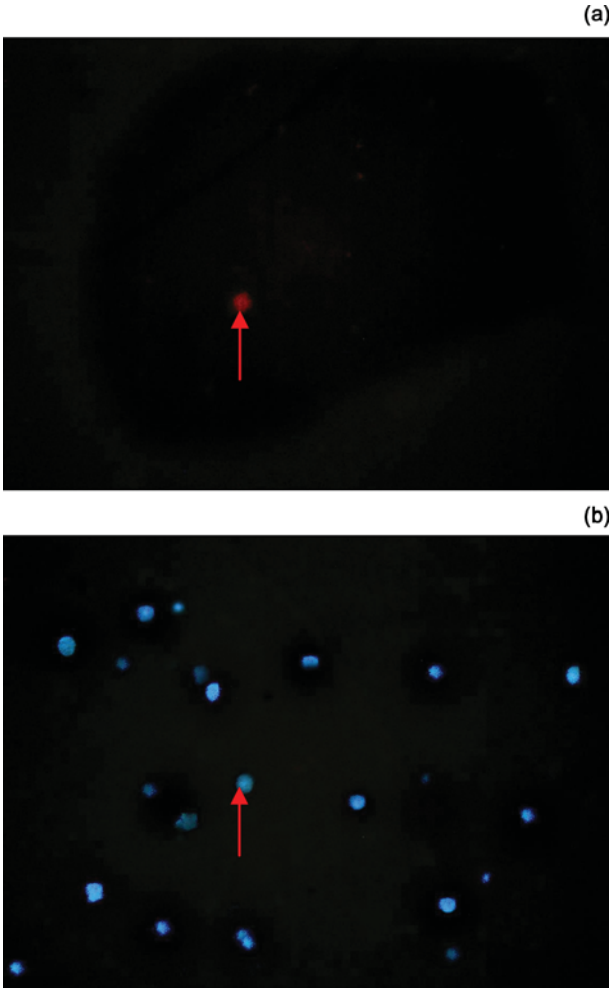

FIG. 2
Fluorescence photomicrographs for 5 per cent artificial perilymph group, with arrows indicating (a) myosin VIIa positive cell and (b) DAPI labelled cells. (×400)

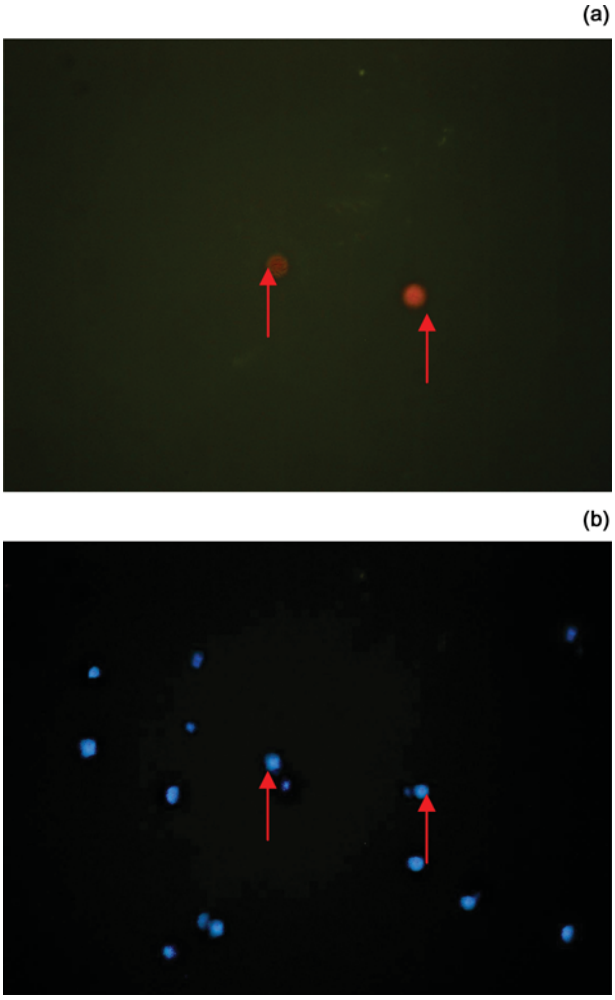

FIG. 3
Fluorescence photomicrographs for 10 per cent artificial perilymph group, with arrows indicating (a) myosin VIIa positive cells and (b) DAPI labelled cells. (×400)

to remove any nonspecific binding. The primary antibody, myosin VIIa (1:200), was then added at 4°C and left overnight. HRP labelled anti-rabbit secondary antibody (1:400) was then added at 37°C for 2 hours. Finally, Enhanced Chemiluminescence (ECL) was added for colouration.

Scanning electron microscopy

Slides containing three-week-cultured cells were fixed with 1 per cent glutaral, dehydrated in gradient ethanol and dried with gradient tertiary butyl alcohol.

Following vacuum pumping and gold spraying, slides were observed under an S-3500N scanning electron microscope (Hitachi, Japanese, Japan).

Results

Cytology

In the four groups, most neural stem cells showed adherent growth when incubated on sterile, rat tail collagen coated cover slips for three days.

A small number of stem cells showed differentiation into cells with small, short processes extending out from the bulk of the cell. These differentiated cells showed an obvious increase in size, as well as various different shapes. After one week's incubation, they also showed cellular processes which extended out to contact adjacent cells. After three weeks' incubation, differentiated cells exhibited various shapes, including: round or oval, neuron-like cells with one to two long processes; astrocyte-like cells with several long, thick processes; and other cells which were small and round, oval or pear-shaped, with no obvious processes.

Immunocytochemistry

Myosin VIIa detection was carried out in three-week-old cell cultures, and myosin VIIa positive cells were seen. Most such cells were round or pear-shaped, with red cytoplasm (on Alexa Fluor 594 staining), but had no obvious cellular processes when viewed under fluorescence microscopy.

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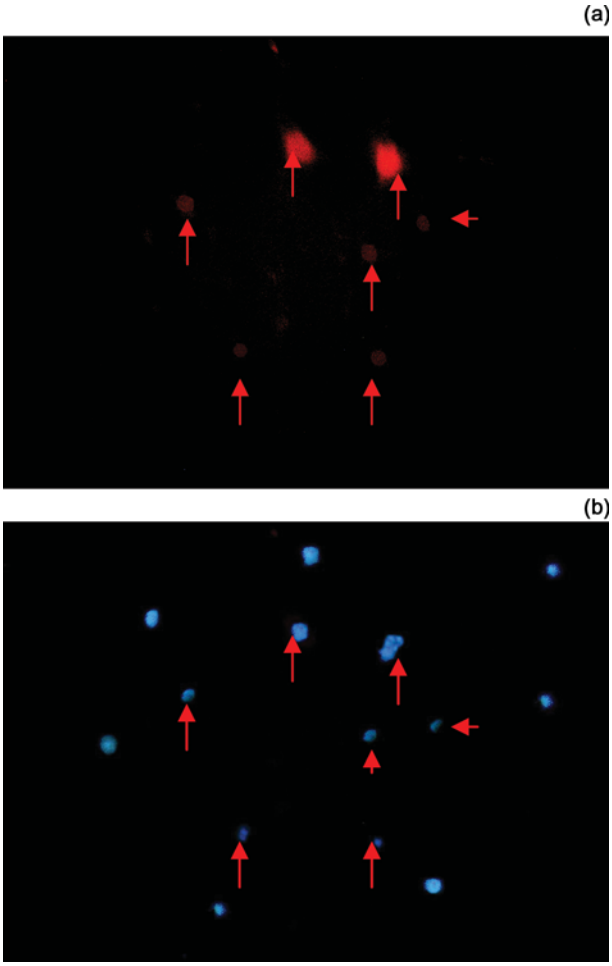

FIG. 4
Fluorescence photomicrographs for 15 per cent artificial perilymph group, with arrows indicating (a) myosin VIIa positive cells and (b) DAPI labelled cells. (×400)

Myosin VIIa positive cells accounted for 11.4–13.6 per cent of cells cultured in either 10 per cent fetal bovine serum or 10 or 15 per cent artificial perilymph, but only 3.9 per cent of cells cultured in 5 per cent artificial perilymph (Table I and Figures 1 to 4). DAPI labelled nuclei appeared blue. The labelling rate reached 93.4 per cent, and the fluorescence intensity was not obviously decreased after the eighth passage.

Western blot

Western blot results were positive for myosin VIIa expression within the cells (Figure 5).

Scanning electron microscopy

Scanning electron microscopy showed round, oval and pear-shaped cells with no obvious cellular processes (Figure 6).

Discussion

Our study findings indicate that neural stem cells can survive and differentiate into cells with inner ear hair-cell-specific antibodies, when cultivated both in fetal bovine serum and in artificial perilymph.

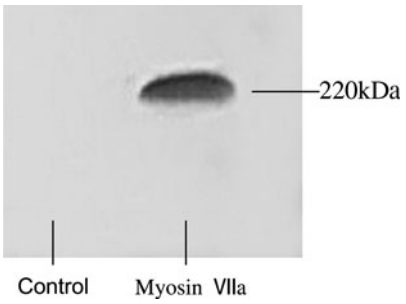

FIG. 5
Western blot.

Neural stem cells are characterised by hypoimmunity, self-renewal and multipotential differentiation. Research indicates that neural stem cells can differentiate into various kinds of nervous system cells; however, under conditions of trauma or pathology, local factors can induce neural stem cell differentiation into cells of specific types, in order to compensate for destroyed cells and to restore neural function.[7,8]

Ito was the first to report that some neural stem cells differentiated and exhibited morphological

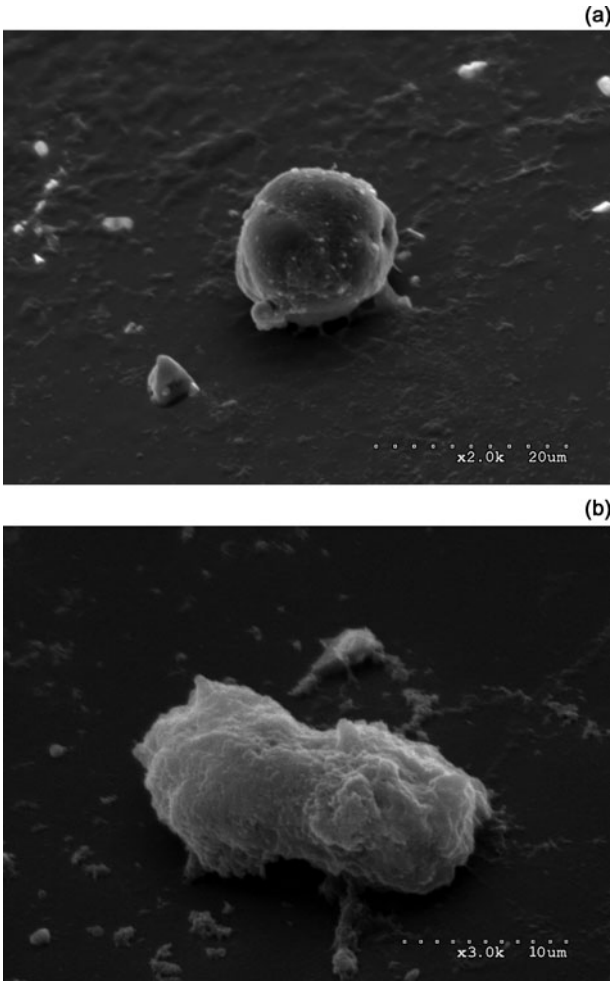

FIG. 6
Scanning electron photomicrographs showing differentiated cells: (a) ×2000; (b) ×3000.

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characteristics of inner and outer hair cells, four weeks after transplantation of cells into the cochleae of newborn rats.[9] Subsequently, Fujino *et al.* reported that neural stem cells can integrate into the vestibular epithelia.[10] Neural stem cells are known to be able to survive, migrate to sensory epithelia and express specific hair cell markers, following transplantation into ototoxic drug damaged inner ears; this demonstrates that neural stem cells can differentiate into hair cells *in vivo*.[11] The differentiation of neural stem cells is known to be controlled by internal genetic information and also by many external factors.[3] The choice of the optimum external factors is very important to encourage differentiation of neural stem cells into inner ear hair cells. To this end, Kojima *et al.* incubated neural stem cells (derived from the nervous system of fetal rats) in 10 per cent fetal bovine serum, and obtained cells which displayed the immunophenotype of inner ear hair cells and contained specific marker protein for inner ear hair cells.[4]

- **Differentiation of neural stem cells is controlled by both internal genetic information and external factors**
- **In this study in guinea pigs, artificial perilymph of different concentrations (simulating the inner ear micro-environment) was used to induce neural stem cell differentiation**
- **Neural stem cells can differentiate into cells resembling inner ear hair cells, in artificial perilymph *in vitro***
- **This finding could provide an experimental basis for transplantation of neural stem cells into the inner ear**

The current study used artificial perilymph to induce differentiation of neural stem cells, in an attempt to simulate the micro-environment of the inner ear. We found that artificial perilymph could induce neural stem cells to differentiate into cells positive for the hair-cell-specific antibody myosin VIIa, in the same manner as fetal bovine serum. Fluorescence microscopy showed round or pear-shaped cells with red cytoplasm (on Alexa Fluor 594 staining) but no obvious cellular processes. Scanning electron microscopy showed round or oval cells. Immunocytochemical analysis found that myosin VIIa positive cells accounted for 11.4–13.6 per cent of cells cultured in either 10 per cent fetal bovine serum or 10 or 15 per cent artificial perilymph, but only 3.9 per cent of cells cultured in 5 per cent artificial perilymph. Western blot identified myosin VIIa positivity in the cells.

Conclusion

Our study findings suggest that neural stem cells can survive and differentiate in artificial perilymph. This indicates, for the first time, that neural stem cells may be able to survive and differentiate within the micro-environment of the inner ear. Furthermore, this study provides an effective experimental model with which to investigate the best conditions for transplantation of neural stem cells into the inner ear.

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References

1 Clarke DL, Johansson CB, Wilbertz J, Veress B, Nilsson E, Karlstrom H *et al*. Generalized potential of adult neural stem cells. *Science* 2000;**288**:1660–3
2 Wang Yun-fang, Pei Xue-tao. Adult stem cells and their application in regenerative medicine. *Chin Sci Bull* 2003;**48**:750–4
3 Bian Lin, Hui Guo-zhen, Lu Hua, Jiang Yun-zhao, Miao Zong-ning, Wang Ling *et al*. The experiment on the normal adult cerebrospinal fluid induction of fetal brain derived neural stem cells. *Journal of Jiangsu Clinical Medicine* 2003;**7**:202–4
4 Kojima K, Tamura S, Nishida TA, Ito J. Generation of inner ear hair cell immunophenotypes from neurospheres obtained from fetal rat central nervous system in vitro. *Acta Otolaryngol Suppl* 2004;**551**:26–30
5 Su Zhen-lun, Jiang Si-chang, Gu Rui, Yang Wei-ping. Calcium channels in bullfrog saccular hair cells [in Chinese]. *Zhonghua Er Bi Yan Hou Ke Za Zhi* 1992;**27**:133–35
6 Xi Kai, Dong Ming-min. Identification and culturing of neural stem cells from guinea pig hippocampi tissue. *Journal of Zhengzhou University (Science Medical)* 2005;**40**:471–3
7 Shen Xue-li, Jia Li-hong, Shi Yu-xiu. Study on nerve stem cell orientation differentiation to cholinergic neuron. *China Journal of Modern Medicine* 2005;**15**:520–3
8 Westelund U, Morten C, Varghese M, Berg-Johnsen J, Ohisson M, Langmoen IA *et al*. Stem cells from the adult human brain develop into functional neurons in culture. *Exp Cell Res* 2003; **289**:378–83
9 Ito J. Regeneration of the auditory pathway [in Japanese]. *Nippon Rinsho* 2003;**61**:469–74
10 Fujino K, Kim TS, Nishida AT, Nakagawa T, Omori K, Naito Y *et al*. Transplantation of neural stem cells into explants of rat inner ear. *Acta Otolaryngol Suppl* 2004;**551**:31–3
11 Punnett A, Bliss B, Dupuis LL, Abdolell M, Doyle J, Sung L. Ototoxicity following pediatric hematopoietic stem cell transplantation: a prospective cohort study. *Pediatr Blood Cancer* 2004;**42**:598–603

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