Original Article
Favorable proliferation and differentiation capabilities of neural precursor cells derived from rat cochlear nucleus

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Abstract: NSCs/NPCs could be used for Sensorineural hearing loss treatment, because of the extensive capacity for self-renewal and pluripotency. In order to isolate and identify neural precursor cells (NPCs), we established a strategy to isolate and cultivate NPCs. Immunohistochemistry, immunofluorescence, Western blotting, and electron microscopy were used to characterize the cells and compare their differentiation patterns with those of olfactory bulb and olfactory epithelium NPCs. Furthermore, NPCs from the cochlear nucleus were sustained good cell viability and cloning efficiency after cryopreservation and thawing. Finally, high capacity to differentiate into astrocytes, oligodendrocytes, and neurons of NPCs was found. In conclusion, NPCs isolated from the cochlear nucleus can proliferate and differentiate into functional neurons, which offers a potential strategy for sensorineural hearing loss treatment. In addition, the storage method developed here will benefit further exploration of NPCs.

Keywords: NPCs, cochlear nucleus, isolation, identify, differentiation

Introduction

Sensorineural hearing loss can arise from damage to hair cells and neurons caused by inner ear diseases, forte stimulates, ototoxic drugs, aging, or viral infection, and it has become one of the most challenging and active areas of research in audiology and otology worldwide [1-3]. Unfortunately, the deficiency of mammalian auditory hair cells is not reversible, which impedes their regeneration [4]. Common therapeutic strategies currently rely on gene conversion, cochlear implants, and stem cell transplantation. Cochlear implants play an important role in clinical treatment of sensorineural hearing loss [5, 6], but their use is limited because of the susceptibility of spiral neuron to changes and individual patient eligibility. Gene conversion can induce functional molecules that protect and regenerate hair cells in the inner ear [7], but the ototoxicity of viral vector to target cells is difficult to predict and avoid. Stem cell transplantation technology appears promising, and it has been frequently applied in clinical medicine, including as therapy for sensorineural hearing loss [8-10].

Various types of stem cells have been discovered, including embryonic stem cells (ESCs), neural stem cells (NSCs), neural precursor cells (NPCs), and hematopoietic stem cells, among others. NPCs are known to have self-renewal capability and pluripotency which underlines the formation of NPCs as well as cells lost during normal turnover [11-13]. Adult NSCs/NPCs have been shown to have extensive capacity for self-renewal and pluripotency in vitro. Importantly, their use would avoid the potential ethical issues associated with the use of ESCs for regenerative therapies.

Recently, NSCs/NPCs have been isolated from areas of the developing brain, including the cerebral cortex, hippocampus, striatum, olfactory bulb, spiral ganglion neurons, and olfactory epithelium [14-18]. However, it is still not completely clear whether functional NSCs/NPCs exist in central auditory nerve nuclei. NSCs have been isolated from the neonatal rat cochlear nucleus [19], and NSCs/NPCs with self-renewing ability from the cochlear nucleus may be the ideal sources for autogenous NPC transplantation. Additionally, this animal model

may be ideal for the analysis of NSCs/NPCs because the hearing system of rats improves after birth [20, 21]. This study was to investigate the existence as well as the isolation of NPCs in the cochlear nucleus of newborn rats and verified their proliferation and differentiation characteristics *in vitro*.

Materials and methods

NPC isolation and culture

NPCs were derived from newborn female SD rats (P1, P3, P7, P9, P11) which were provided by the animal center of the Fourth Military Medical University. After intraperitoneal injection of anesthetic (0.8 ml/kg), rats were surface sterilized with disinfectant (75% alcohol) and killed by decapitation. The skin and subcutaneous tissue were peeled back from the skull along the center of the head, and the skull was opened along the sagittal suture and forward to expose olfactory bulb. The bone flap and the brain tissue were removed consecutively, along with the cranial nerve and the complete brainstem. The brain was placed in 4°C HBSS with 100 IU/ml penicillin and 100 μg/ml streptomycin and washed three times after the mater was peeled away. The entire process was carried out with great care to avoid damaging the brain tissue. **Figure 1B** shows the specific dissected level of cochlear nuclei. This study was approved by the Ethics Committee of the Fourth Military Medical University.

The tissue from the cochlear nuclei, olfactory bulbs [14], and olfactory epithelium [22] was cut into 0.5-mm^3 fragments on ice using iris scissors under a microscope. The fragments underwent mild trypsinization (0.125%) for 20 min at 37°C, followed by centrifugation at 1000 *g* for 5 min. The cells were gently resuspended and dispersed by using a flame-polished pasteur pipette and then filtered through a 100-mesh copper filter. The cells were subsequently placed in Dulbecco's modified Eagle's medium (DMEM) with F12 (1:1), B27 (1:50) (Invitrogen, Carlsbad, CA), 100 IU/ml penicillin, and 100 streptomycin. FGF-2 (Fibroblast Growth Factor, 20 ng/ml) and EGF (Epidermal Growth Factor, 20 ng/ml) were added daily to enhance the proliferation of precursor cells. Cells were passaged every 5-7 days at a viable cell density of 5×10^5/ml, and the culture conditions were kept at 37°C, 5% CO_2.

Cell purification and amplification

The limited dilution method was used to obtain brainstem NPCs from the cochlear nucleus amplified from a single cell clone. Cells were gently mixed by pipetting and collected by centrifugation. Prior to secondary cloning by 7–10 days, cells were pipetted into single-cell suspension and diluted in serum-free medium supplemented with EGF and FGF-2 at eight gradient concentrations of 2000, 1000, 500, 250, 125, 63, 32, and 16 living cells/ml. Next, cells were placed in a 96-well plate and cultivated at 37°C in a 5% CO_2 atmosphere for 2 h. As the monoclone formed, continuous passage was undertaken to gain abundant subclones. Inverted phase contrast microscopy was used to observe the growth, differentiation, and division status every day. After 16 days, the culture medium contained mostly separated NPCs and postmitotic neurons. Separated cells were collected by mechanical dissociation after trypsinization (0.05% + 0.53 mM EDTA), while single-cell suspensions were centrifuged at 1400 rpm for 5 min. Cells were then resuspended in precoated plates at a density of 1.2×10^6 cells per 150-mm plate. Cells were harvested upon reaching approximately 75% confluence, which occurred within 5 or 6 days. After approximately 20 passages, cells from various passages were frozen and stored in liquid nitrogen.

Immunohistochemistry and immunofluorescence

For immunohistochemistry, cells were first fixed with 150 μl of 3.7%-4% paraformaldehyde for 15 min in plates, washed three times, and then blocked with phosphate-buffered saline (PBS) containing 5% normal goat serum and 0.25% Triton X-100 for 20 min. The supernatant was then discarded, and the cells were incubated overnight at 4°C together with primary antibodies, such as mouse anti-nestin (1:250; Chemicon), rabbit anti-Musashi (1:200; Chemicon), mouse anti-NeuN (1:300; Chemicon), etc. Goat anti-species conjugates of Cy3 (Boster Biological Technology Company, Wuhan, China) was used following the list of the primary antibodies. After being rinsed three times with PBS, cells were exposed to secondary antibodies for 3 h at room temperature. A DAB kit was used for staining after the cells were washed three times with PBS. Following gradient alcohol dehydration, xylene treatment, and neutral

gum sealing, the cells were observed and photographed under an optical microscope. Immunofluorescence double-staining of nestin/musashi1 was done on cells that had been incubated for 2 h at 37°C on glass slides pretreated with polylysine. Familiar with immunohistochemistry, after PBS washing, fixation, adding confining liquid and antibodies, observing with Olympus optic microscope using appropriate fluorescence filters are all finished in order. Imagepro-plus 6.0 software was applied to analyze images achieved.

RT-PCR

Total RNA extraction was carried out by using an RNeasy Mini Kit with on-column deoxyribonuclease digestion (Qiagen, Valencia, CA), and RNA was reverse transcribed with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed on an ABI PRISM 7000 sequence detection system with TaqMan universal PCR master mix (Applied Biosystems) or SYBR green PCR master mix (Applied Biosystems) with the following primers: nestin, 5'-TCGCTTAGAGGTGCAACAGC-3' (sense) and 5'-CCTATTCCGTCTCAACGTA-3' (antisense), and Musashi-1, 5'-CGAGCTCGACTCCAA-AACAAT-3' (sense) and 5'-AGCTTTCTTGCATTCC-ACCA-3' (antisense). Each experiment was performed in triplicate. TaqMan probes/primers were used for rat RORα, C/EBPα, C/EBPβ, C/EBPδ, C/EBP homologous protein (CHOP), PPARγ, SREBP1, aP2, perilipin, fatty acid synthase (FAS), stearoyl CoA desaturase-1 (SCD-1), acetyl CoA carboxylase1 (ACC1), ACC2, adiponectin, tribbles homolog 3 (TRB3), and 18S (Applied Biosystems).

Cell viability and growth curve

Fourth generation cell clones at different days of age were suspended into single-cell solution and diluted in DMEM at an approximate concentration of 1×10^6 cells/ml. Nine drops of each solution were inoculated into a tube with one drop of 0.4% trypan blue. After thorough mixing, living and dead cells were observed by optical microscopy and counted within 3 min. Growth curves were also obtained for fourth generation cells. Cells in single-cell suspensions were seeded into 96-well plates at a concentration of 1×10^5 cells/ml. Cells were then divided into four groups: three treatment groups with either EGF+FGF-2, EGF, or FGF-2 added and a control group with no added growth factors. The cells were incubated at 37°C in a 5% CO_2 atmosphere, and their growth curves were characterized by using the CCK-8 (cell counting kit-8) method, which is commonly used for cell proliferation and cytotoxicity detection. Cells showing perfect growth were then seeded into 96-well plates at 37°C, 5% CO_2. Three days later, cells were randomly seeded in 96-well plates with 10 μl of CCK-8 in each well, and the optical density was detected at 450 nm after 1-4 h of incubation.

Cell proliferation and differentiation assay

For the cell proliferation study, bromodeoxyuridine (BrdU; Sigma Chemical, St. Louis, MO) was used to mark cells in the S phase. Fourth generation cells of the cochlear nuclei, olfactory bulbs, and olfactory epithelium were cultured with BrdU at 30 μg/L at 37°C for 40 min. The proportions of BrdU-positive cells were calculated as the number of positive cells divided by the number of total cells, multiplied by 100.

For the differentiation assay, passage 4 cells from cochlear nuclei, olfactory bulbs, and olfactory epithelium were cultured with BrdU (6 μg/ml) for 24 h, and then inoculated into DMEM/F12 (1:1) with 10% fetal bovine serum (FBS), without EGF and FGF-2. Seven days later, a single-cell suspension in the logarithmic phase was added to 24-well plates containing polylysine-coated coverslips. Within 1-2 days, the growing cells adhered to the coverslips, which were subsequently carefully removed and washed with PBS prior to immunocytochemistry. Ten regions of interest (under × 200 magnification) were randomly selected from each sample to calculate the number of nestin-negative cells and the total number of cells. Then the cell differentiation rates were obtained by dividing the nestin-negative cell number by the total number of cells.

Cell cryopreservation and recovery

Third passage cells were resuspended in different frozen stock solutions (dimethyl sulfoxide [DMSO], glycerin, 10% DMSO + FBS, or 10% glycerinum + FBS). After being held at -70°C for 8-12 h, the cells were transferred into liquid nitrogen and stored at -196°C. After 3 months, the cells were thawed at 37°C and resuspend-
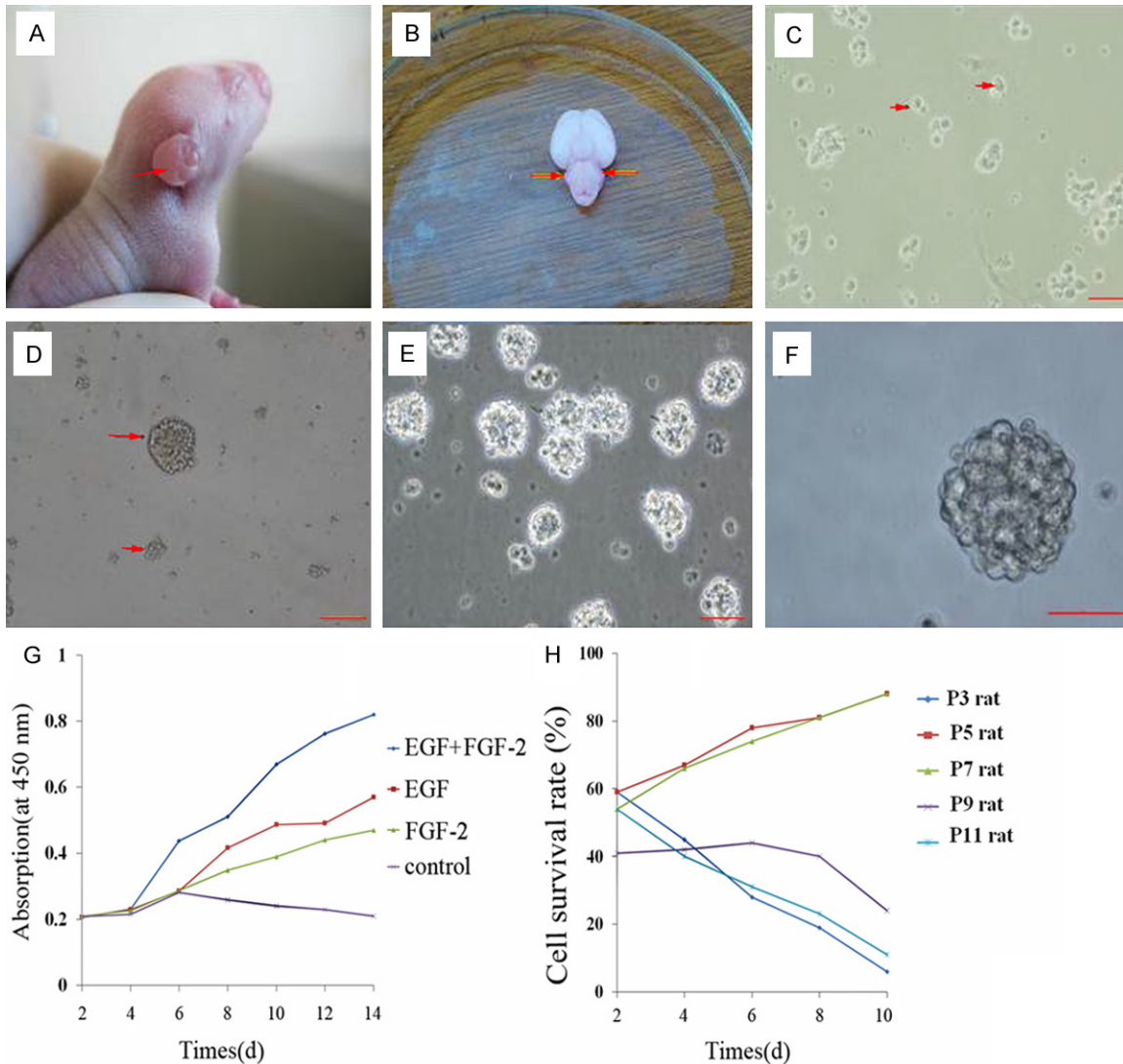
Figure 1. Isolation and cultivation of NPCs derived from the cochlear nucleus of a rat brain. A, B. The experimental rats and the isolated rat brain. C-E. Primary cultured cells with rounded morphology. F. Passage 100 cells retained high cell viability. G. Cell viability with different growth factors, EGF or FGF-2, added. H. Cell survival rates derived from rats of P 1, 3, 5, 7, 9, and 11 (scale bar = 50 µm).

ed. Following gradient dilution, trypan blue exclusion and a cell viability study were carried out by optical microscopy to count living cells and dead cells. The cell growth curves were constructed by the CCK-8 method to assess cell survival after cryopreservation and recovery. In addition, the clone formation rate together with the differentiation ability was also evaluated to assess any changes in those qualities.

Clone formation rate

After the thawing, passage 3 cells were collected after centrifugation. Cells were diluted to eight concentrations with serum-free DMEM

containing EGF and FGF-2; 25 µl of each concentration was inoculated in 96-well plates, and cells were incubated at 37°C in a 5% CO_2 atmosphere for 2 h. Wells were observed with only one cell in a dynamic state. Clone formation rates were recorded when a single colony formed.

Statistical analysis

Statistical analysis was performed using SPSS l1.0 statistical software package (SPSS Inc, Beijing, China). Numerical data were expressed as means ± standard deviation, and intergroup comparisons were made using Student's
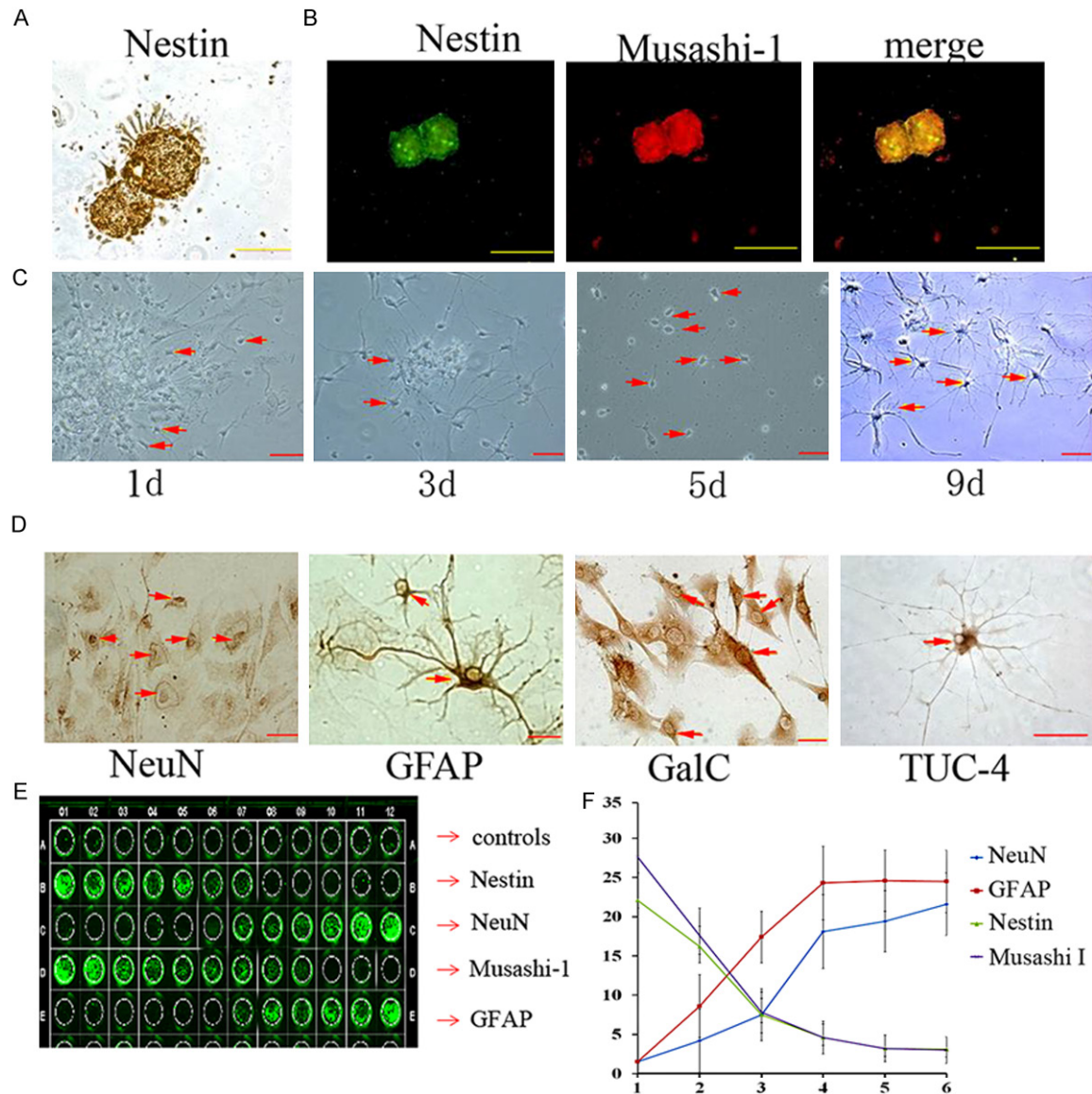
Figure 2. Identification of NPCs and assessment of differentiation. A, B. NSCs/NPCs spheres were identified by using specific markers for nestin and Musashi-1 by using immunohistochemical and immunofluorescence staining. C. The morphology of differentiating cells was observed from the first to the ninth day. D. Immunostaining analysis showed different types of cells positive for NeuN, GFAP, GalC, and Tuc-4 (scale bar = 100 μm in picture). E, F. Immunofluorescence staining analysis detected expression of nestin, NeuN, Musashi1, and GFAP (Scale bar = 50 μm).

t-tests. A *P* value of less than 0.05 was considered statistically significant.

Results

Isolation of stem cells from the cochlear nucleus

To isolate local NSCs in the cochlear nucleus, a cell suspension was prepared from cochlear nuclei of newborn rats (**Figure 1A**, **1B**) and plated under tissue culture conditions with the

presence of FGF-2 and EGF. Some cells from P5 and P7 rats retained their proliferation ability 2 days later and began to divide (**Figure 1C**, arrowheads). Cells were inspected daily, and they were found to display continuous division, which resulted in the formation of cell aggregates or spheres (**Figure 1D**, **1E**). With the inoculation of second-generation cells into culture medium containing EGF and FGF-2, subclones were formed. Cells retained high cell activity after 30 generations, and the formation of sub-
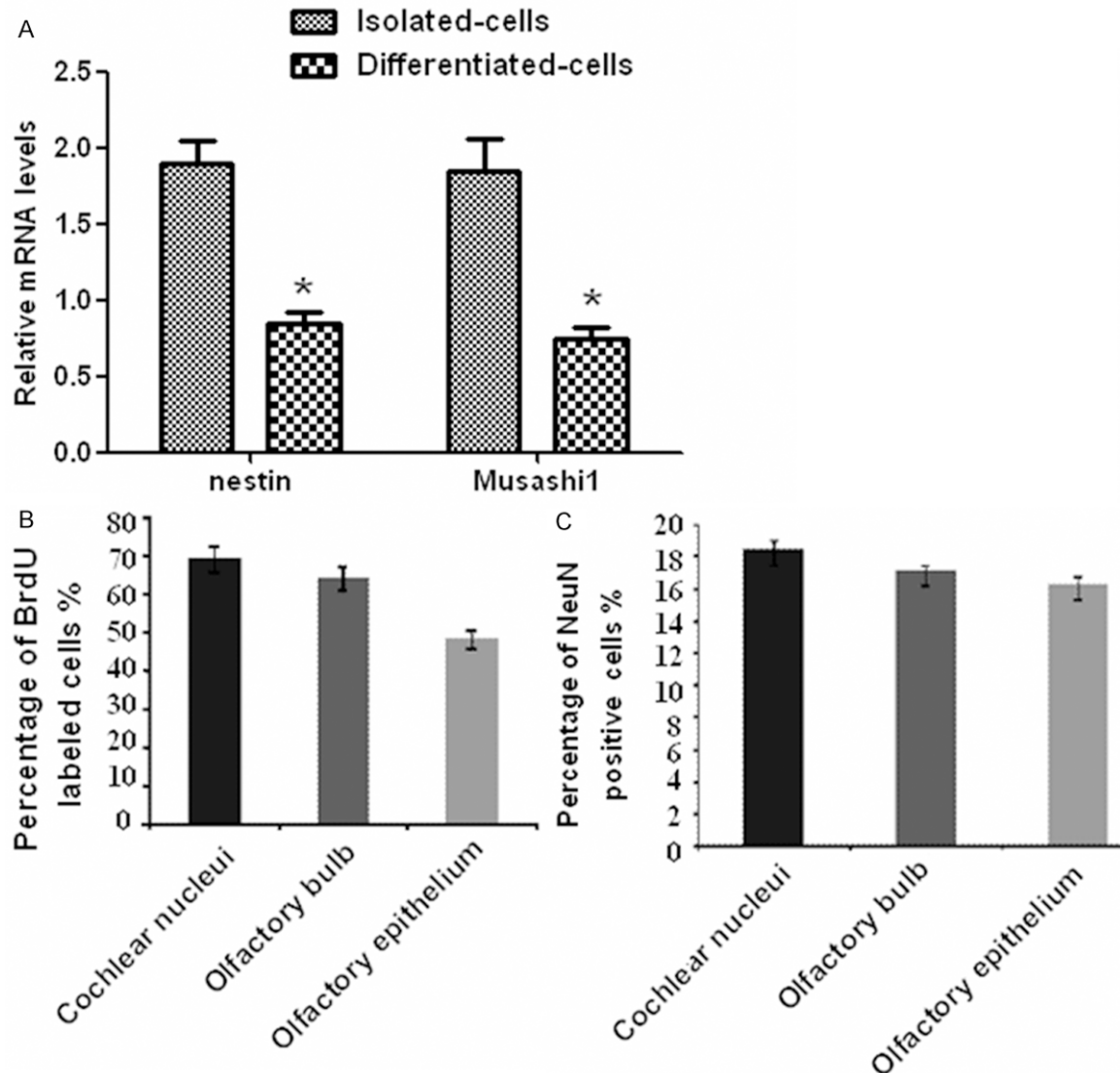
Figure 3. Comparison of proliferation and differentiation ability of NPCs. A. Relative mRNA levels of isolated cells and differentiated cells. B. Cell proliferation ability was analyzed using the BrdU-label assay. C. Cell differentiation ability was evaluated by NeuN-positive versus BrdU-labeled cells.

clones of different sizes is shown in **Figure 1E**. Under standard growth conditions, cells could be passaged every 4-7 days for at least 9 months (approximately 100 passages; **Figure 1F**), while maintaining vigorous proliferation activity.

Next, we next optimized the cultural conditions of NPCs. The requirement for EGF and FGF-2 was assessed, and NPCs were found to have a relatively higher level of proliferation with both factors; further growth did not occur 6 days later in the absence of EGF or FGF-2 (**Figure 1G**). Meanwhile, the different viabilities of NPCs

from specific distinct day-old rats were researched under the optimized cultural condition already described. As shown by the growth curves in **Figure 1H**, most of the cells from P1, P3, P9, and P11 rats were dead 2 days after plating (**Figure 1H**), while cells of from P5 and P7 rats retained their proliferation ability.

Identification of NSCs/NPCs

To preliminarily identify the isolated cells, we analyzed the expression levels of Musashi1 and nestin, specific markers of NSCs/NPCs differentiation. Immunocytochemistry analysis
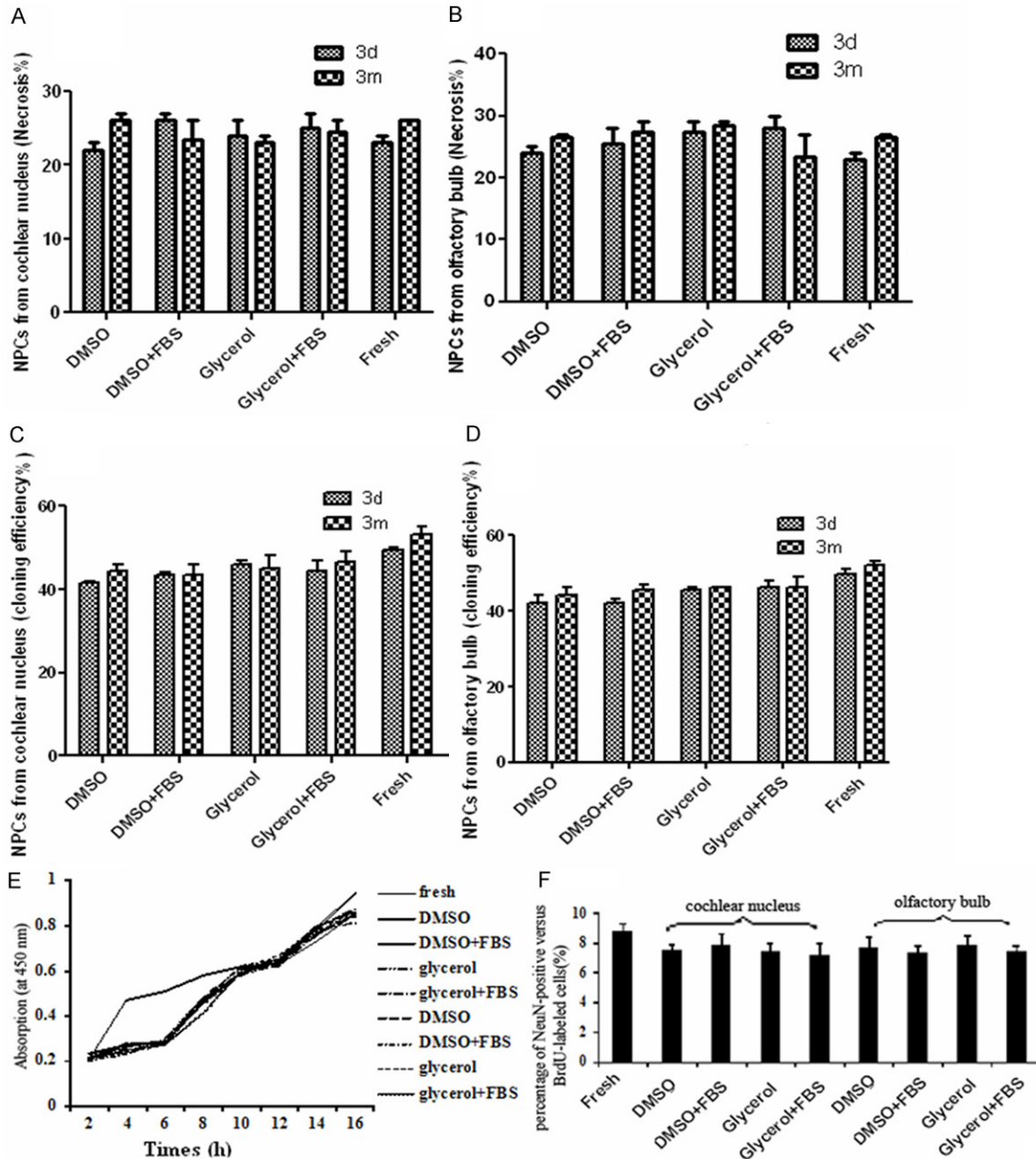
Figure 4. Cryopreserved cell viability and differentiation ability observation. A. Viability of cells derived from cochlear nuclei after thawing. B. Viability of cells derived from olfactory bulbs after thawing. C. The cloning efficiency of cells from cochlear nuclei. D. The cloning efficiency of cells from olfactory bulbs. E. The cell growth curve. F. Differentiation ability of NPCs from the cochlear nucleus and olfactory bulb.

showed that Nestin was present (**Figure 2A**), and further immunofluorescence analysis revealed Musashi-1 (red) and Nestin (brown) (**Figure 2B**). In addition, different forms of cells began to appear at cell culture days 5-6; some cells formed obvious dendrites and axons, which elongated from 1 to 9 days in culture (**Figure 2C**).

RT-PCR revealed changes in the nestin and Musashi1 levels when differentiation occurred.

Significant declines of nestin and Musashi1 were found in comparison with the undifferentiated isolated cells (**Figure 3A**). Immunohistochemistry directly confirmed that most cells were positive for NeuN (a marker for mature neurons) [23], GFAP (marker for astrocytes) [24], TUC-4 (early neuron marker), and GalC (oligodendrocyte marker) [25]. Cells were thus found to have the capacity to differentiate into mature neurons, astrocytes, and oligodendrocytes (**Figure 2D**).

The expression levels of Nestin and Musashi1 were found to gradually decrease as cells differentiated, which was accompanied by a gradual increase in GFAP and NeuN expression (others not detected) (**Figure 2E**, **2F**).

Comparison of cell proliferation and differentiation ability

The BrdU-positive rates of the three groups may be viewed as the index of cell proliferation and division. As the results show, the BrdU-positive rates were 68.17%, 66.27%, and 47.12% for the cochlear nucleus, olfactory bulb, and olfactory epithelium, respectively (**Figure 3B**), suggesting a relatively high reproductive capacity of the isolated NPCs. In addition, the cell differentiation rates (percentage of NeuN-positive cells) were 18.57 ± 1.26% for the cochlear nucleus, 17.23 ± 1.59% for the olfactory bulb and 16.36 ± 1.87% for the olfactory epithelium, respectively (**Figure 3C**). These results confirm that differentiation activity was comparable among the three groups ($p > 0.05$).

Cell viability and cloning efficiency after thawing

Cells were cryopreserved in 10% DMSO, 10% DMSO + FBS, 10% glycerol, or 10% glycerol + FBS at -196°C in liquid nitrogen after being frozen for 12 h at -70°C. After thawing, cell viability and clone formation rate were assessed. Trypan blue staining analysis showed that cell death rates were lower than 30% (**Figure 4A**). Furthermore, no significant differences were observed in cell viability of cochlear nucleus NPCs after cryopreservation compared with cells from the olfactory bulb or fresh cells (**Figure 4B**). The average cloning efficiency of thawed cells after cryopreservation was about 43% (**Figure 4C**, **4D**). There was no significant difference between cochlear nucleus NPCs and olfactory bulb NPCs based on cryopreservation time or protective agent, and both had similar growth curves ($p > 0.05$) (**Figure 4E**). For further exploration of the differentiation capacity of thawed cells, culture medium containing FBS, but no growth factor, was added. After 2 h, the cultured NPCs were spontaneously free from cell spheres and started to differentiate. Further immunofluorescence analysis confirmed the percentage of NeuN-positive cells under microscope. The results showed that there were no significant differences among different groups between cochlear nucleus NPCs and olfactory bulb NPCs (**Figure 4F**).

Discussion

Sensorineural hearing loss is a clinically common disorder [26], and increasing attention has been focused on introducing exogenous stem cells into the inner ear as a potential treatment. NSCs/NPCs possess crucial application value for this disease [27, 28]; however, transplantation of most exogenous NSCs/NPCs has generated glial cells with little differentiation ability, leading to its limited application. The isolation of endogenous stem cells appears promising because endogenous stem cells of the inner ear have been shown to successfully differentiate into hair cells. Until now, there have been only a few reports about the isolation of NPCs from cochlear nucleus. More evidence is needed regarding the isolation of functional NSCs/NPCs from the cochlear nucleus.

In this study, we successfully isolated cells from the cochlear nucleus. These cells possessed proliferative capability and were able to differentiate into astrocytes, oligodendrocytes, and neurons. Interestingly, cells from 5- to 7-day-old rats were successfully isolated, but cultures could not be established with cells from rats that were less than 5 days old or more than 9 days old. This outcome may arise from the initial hearing development stage of rats, but further research is needed to more precisely detail the underlying mechanism. EGF and bFGF have essential roles in the growth of olfactory bulb and olfactory epithelium NSCs/NPCs [29], and cochlear nucleus NPCs/NSCs were similarly found to grow well in the presence of EGF and bFGF. This finding further proves the significance of EGF and bFGF in NPC culture [30].

Recently, stem cell-based therapy, using stem cells to generate neural tissue to repair the nervous system, has been used to restore damaged neural circuitry by transplanting NSCs/NPCs. The foundation of the clinical application is the ability of NSCs/NPCs to differentiate. During the differentiation test, a striking expression of nestin and Musashi1 was observed. Nestin is a member of the intermediate filament protein family, which is especially abundant in neuroepithelial stem cells of the rat. Musashi1 is an evolutionarily conserved marker for neural stem cells [31]. Once the NSCs/NPCs differentiate into neurons and glial cells, the

expression of Nestin and Musashi1 is down-regulated [32]. Immunofluorescence results showed positive expression of Nestin and Musashi1 in isolated NPCs. More evidence of differentiation was gained from positive signals for TUC-4, NeuN, GFAP, and GalC, which are known to be the specific markers of postmitotic and mature neurons, astrocytes, and oligodendrocytes. Compared with the characteristics of olfactory bulb and olfactory epithelium NPCs, cochlear nucleus NPCs had almost the same activities; in some cases they were even better. Decreased expression of nestin/Musashi1 and the enhanced level of NeuN/GFAP further verified the differentiation of NPCs. The successful achievement of functional NPCs from the cochlear nucleus may provide a new possibility for NPCs for transplantation.

Essential goals for a cell replacement strategy in the auditory system are high survival rates and long-term survival. In the present study, NPCs from the cochlear nucleus, olfactory bulb, and olfactory epithelium were cryopreserved with four different cryoprotective agents at -196°C in liquid nitrogen after being frozen for 12 h at 70°C. After thawing, similar cell activity and cloning efficiency were confirmed in NPCs from the cochlear nucleus with olfactory bulb and the olfactory epithelium. Hence, we successfully used our strategy to cryopreserve cells that maintained their inherent biological characteristics for high reproduction and ability to differentiate after thawing. The strategy may be beneficial for using NPCs in a cell replacement strategy to treat disorders related to the auditory systems.

In conclusion, we successfully isolated NPCs from the cochlear nucleus that had desired self-renewal and differentiation functions. Furthermore, a successful cryopreservation strategy was confirmed. Therefore, our results expand NSC research in a way that may benefit the development of stem cell transplantation for treating sensorineural hearing loss.

Acknowledgements

Disclosure of conflict of interest

None.

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