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Potential of Embryonic Stem Cell-Derived Neurons for Synapse Formation With Auditory Hair Cells

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Recent studies have indicated that embryonic stem cells (ESCs) can be a source for the replacement of spiral ganglion neurons (SGNs), auditory primary neurons, and neurite projections from ESC-derived neurons to auditory sensory epithelia. However, the potential of ESC-derived neurons for synapse formation with auditory hair cells (HCs) has not been elucidated. The present study therefore aimed to examine the ability of ESC-derived neurons to form synaptic connections with HCs in vitro. Mouse ESC-derived neural progenitors expressing enhanced green fluorescence protein (EGFP) were cocultured with explants of cochlea sensory epithelia obtained from postnatal day 3 mice. After a 7-day culture, neurites of ESC-derived neurons predominantly elongated toward inner hair cells (IHCs), which play a crucial role in sound transmission to SGNs. Immunohistochemical analyses revealed the expression of synapsin 1 and synaptophysin in the nerve endings of ESC-derived neurons adjacent to IHCs, indicating the formation of synaptic connections. Transmission electron microscopy demonstrated synaptic contacts between nerve endings of ESC-derived neurons and IHCs. The present findings show that ESC-derived neurons can make synaptic connections with IHCs.

Key words: embryonic stem cell; hair cell; cochlea; transplantation; spiral ganglion neuron

Sensorineural hearing loss (SNHL) is one of the most common disabilities in our society today, but, because mammalian inner ear cells have limited regenerative activity, treatment options are limited to cochlear implants and the use of hearing aids. Spiral ganglion neurons (SGNs) located in the bony axis (modiolus) of the cochlea are bipolar auditory primary neurons that make synaptic connections with auditory hair cells (HCs) and the cochlear nucleus in the brainstem. They consist of two types of neurons: type I neurons making synaptic connections with inner hair cells (IHCs) that play a predominant role in the transduction of sound stimuli to neural stimuli, and type II neurons making synaptic connections with outer hair cells (OHCs) that assist in controlling the sensitivity of regions of the auditory epithelia to specific sound stimuli (Spoendlin, 1987; Rubel and Fritzsch, 2002). The loss of SGNs therefore compromises auditory function. SGN degeneration resulting in SNHL occurs during the aging process (Keithley et al., 1989), and can also be caused by several ototoxic agents (Lee et al., 2003) or acoustic trauma (Pujol and Puel, 1999). It compromises the clinical benefits of cochlear implants (Nadol et al., 1989), which rely on direct stimulation of SGNs to improve impaired hearing.

Recent investigations have indicated the potential for SGN regeneration using cell transplantation into the cochlea. Several candidates are available as a source of transplant; previous studies have demonstrated the survival and neurite outgrowth of neural stem cells (Tamura et al., 2004; Hu et al., 2005b) and dorsal ganglion neurons (Hu et al., 2005a) after transplantation into the cochlea. Bone marrow-stromal cells have also shown the capacity for settlement and differentiation into neuronal cells after engraftment into the cochlea (Naito et al., 2004). More recently, the survival of bone marrow stromal cell-derived neurons in the cochlear modiolus has been reported (Matsuoka et al., 2006). Embryonic stem cells (ESCs) are another possible transplant source with the ability to dif-

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ferentiate into neurons, and several neural-inducing methods have been established for this cell type. ESC-derived neural progenitors have a high potential for survival and differentiation into neurons after transplantation into the cochlear modiolus where they were previously shown to massively elongate their neurites toward the peripheral and central auditory systems, reaching host SGNs and the osseous spiral lamina, which is located between SGNs and auditory sensory epithelia (Okano et al., 2005; Corrales et al., 2006). In addition, transplantation of ESC-derived neurons into the cochlear modiolus contributes to the recovery of SGN functions, as monitored by electrically evoked auditory brainstem responses (Okano et al., 2005). In vitro analyses such as coculture studies of ESC-derived neural progenitors with cochlear or vestibular sensory epithelia have demonstrated their differentiation into bipolar neurons (Coleman et al., 2007) and neurite outgrowth that attaches to sensory HCs and expresses synaptophysin at the attachment site (Kim et al., 2005; Matsumoto et al., 2005).

Although there was no direct evidence of synapses establishment, there was functional evidence to indicate the establishment of functioning neural connection (Okano et al., 2005). The aim of the present study was therefore to determine the synapse formation ability of ESC-derived neurons with auditory HCs. In the cochlea, there are the afferent synapses between the peripheral fibers of spiral ganglion neurons and sensory HCs (Sobkowicz, 1992) and the efferent synapses, both axosomatic with the sensory HCs and axodendritic with the peripheral afferents (Sobkowicz et al., 1997, 2002). We cocultured mouse ESC-derived neural progenitors with explants of mouse auditory sensory epithelia and morphologically examined the neural connections that arose between ESC-derived neurons and HCs.

MATERIALS AND METHODS

The Animal Research Committee, Graduate School of Medicine, Kyoto University, approved all experimental protocols. Animal care was under the supervision of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. All experimental procedures were performed in accordance with the NIH *Guide for the care and use of laboratory animals*.

ESCs and Neural Induction

We used the mouse ESCs G4-2 (generously donated by Dr Hitoshi Niwa of Riken CDB, Kobe, Japan), derived from the E14tg2a ESC line (Hooper et al., 1987) and carrying the EGFP gene driven by a ubiquitous strong promoter (CAG promoter), because our previous experiments demonstrated the potential of neurons derived from this cell line for neurite outgrowth toward inner ear HCs (Kim et al., 2005; Matsumoto et al., 2005). Stromal cell-inducing activity (SDIA) was used for the neural induction of ESCs (Kawasaki et al., 2000). Briefly, ESCs were cultured to form differentiated colonies on a feeder layer of PA6 stromal cells derived from newborn mouse calvaria (RCB1127; RIKEN Cell Bank, Japan) in Glasgow's modified Eagle's medium (GMEM; Invitrogen, Carlsbad, CA) supplemented with 5% knockout serum replacement (KSR; Invitrogen), 1 mM pyruvate (Sigma, St. Louis, MO), 0.1 mM nonessential amino acids (Invitrogen), and 0.2 mM 2-mercaptoethanol (2-ME; Wako, Osaka, Japan). Colonies that formed on the PA6 monolayer after 6 days of culture were isolated by incubation with collagenase B (Roche Diagnostics, Tokyo, Japan) for 5 min at room temperature (RT).

Isolation of Auditory Epithelia and Coculture

Postnatal day (P) 3 ICR mice were deeply anesthetized with diethyl ether and decapitated. The temporal bones were dissected, and the cochleae were freed from surrounding tissues in 0.01 M phosphate-buffered saline (PBS) at pH 7.4, supplemented with 0.2% glucose. After removal of cochlear lateral walls, auditory epithelia were dissected from the cochlear modiolus. SGNs were carefully removed from the explants using fine forceps as described previously (Matsumoto et al., 2005), resulting in complete removal of neural elements from explant cultures (Fig. 1). Auditory epithelia were explanted intact on a sterile membrane (Falcon BD Biosciences, San Jose, CA) into MEM (Gibco, Grand Island, NY) supplemented with 0.3% glucose in 24-well culture plates. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 hr. The cultured explants were then provided for coculture with ESC-derived neural progenitors. Explants including SGNs were excluded from analysis. Finally, 20 explants were provided for coculture with SDIA-treated ESCs, and an additional four explants cultured alone in the same medium were used as controls.

Cultured auditory epithelia were transferred into GMEM supplemented with 5% KSR, 1 mM pyruvate, 0.1 mM nonessential amino acids, and 0.2 mM 2-ME. Cell suspensions of SDIA-treated ESCs (2 μl, 1×10^4 cells/μl GMEM) were placed inside cultured auditory epithelia, where SGNs are normally located, using a Hamilton syringe (Hamilton, Reno, NV). The medium was changed daily, and cultures were maintained for 7 days.

Tissue Preparation

Histological analysis was performed on whole mounts or cryostat sections. After the culture period, whole mounts (cocultures n = 5; controls n = 2) were fixed in 4% paraformaldehyde in PBS for 15 min at RT. Cryostat sections (cocultures n = 15; controls n = 2) were fixed in 4% paraformaldehyde with 0.1% glutaraldehyde in PBS for 30 min and then cryprotected by immersion in 30% sucrose in PBS at 4°C overnight. Specimens were mounted in OCT compound (Tissue-Tek, Sakura, Tokyo, Japan) and cut at 15 μm using a cryostat.

Immunohistochemistry

Whole-mount specimens were doubly immunostained for myosin VIIa and βIII tubulin to identify the location of HCs in the auditory epithelia and ESC-derived neurons and their neurites, respectively. The latter location was also determined by EGFP expression. We focused on neurite extension

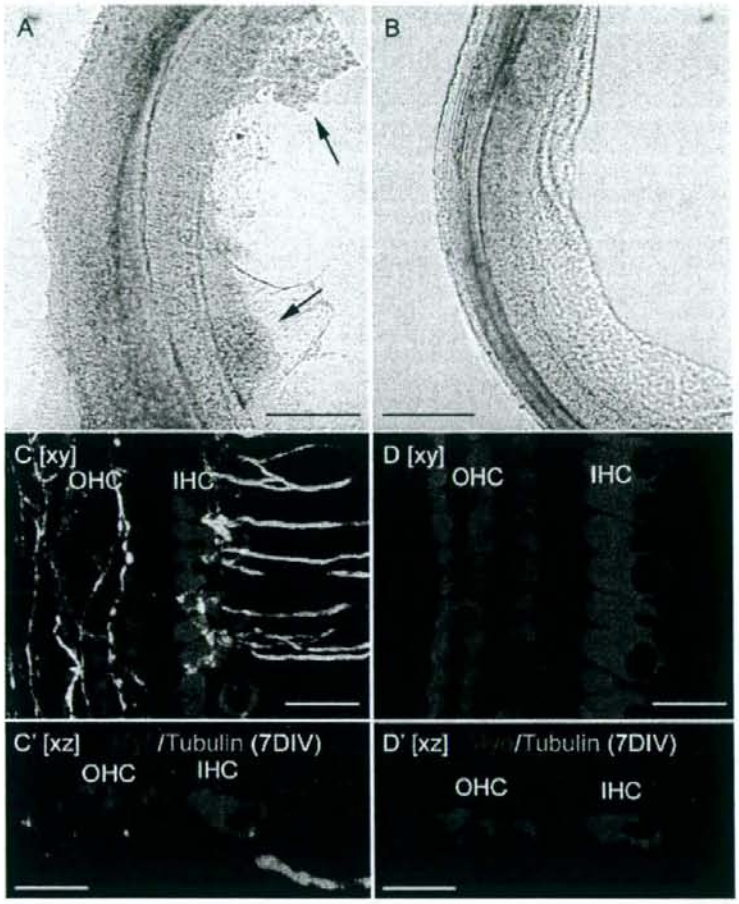

Fig. 1. Explants of auditory epithelia with or without spiral ganglion neurons. On 7 days in vitro (DIV), an explant of the auditory epithelium with spiral ganglion neurons (**A**) exhibits the presence of neurites and nerve endings expressing βIII tubulin (blue) adjacent to hair cells (red; **C**: xy projection; **C'**: xz projection). Arrows in A indicate the remaining spiral ganglion neurons. An explant of the auditory epithelia after elimination of spiral ganglion neurons (**B**) demonstrates three rows of outer hair cells and a single low of inner hair cells expressing myosin VIIa (red) and no neural elements in the auditory epithelium (**D**: xy projection; **D'**: xz projection). Myo, myosin VIIa; tubulin, βIII tubulin; IHC, inner hair cell; OHC, outer hair cell; xy, xy projection image; xz, xz projection image; 7DIV, 7 days in vitro. Scale bars = 200 μm in A,B; 20 μm in C,C',D,D'.

of ESC-derived neurons and the relationship between HCs and their extended neurites.

Immunohistochemistry for myosin VIIa, βIII tubulin, synapsin 1, synaptophysin, growth-associated protein 43 (GAP43), C-terminal binding protein 2 (ctbp2)/ribeye, and calretinin was carried out on cryostat sections. Synapsin 1 and synaptophysin were used as markers for synaptic vesicles and GAP43 to identify growing neurites. Ctbp2/ribeye was used as a specific marker for synapse ribbons and calretinin as a maker for mature SGNs and their neurites. We focused on the expression of these marker proteins in the nerve endings of ESC-derived neurons that were attached to HCs. Cochlear specimens obtained from P4 (before hearing onset), P12 (onset of hearing), and adult (10-week-old) mice were also stained for synapsin 1 and synaptophysin to correlate the expression patterns of these proteins with the development of mouse auditory function. Cochlear specimens obtained from adult mice were used as controls for ctbp2/ribeye or calretinin.

Whole mounts and cryostat sections were permeabilized and blocked with 10% normal goat serum (Chemicon, Teme-

cula, CA) in PBS-T (PBS containing 0.02% Triton X) for 30 min at R.T, followed by overnight incubation at 4°C in the presence of primary antibodies diluted in an appropriate blocking solution. On the following day, sections were washed three times in PBS-T and incubated in diluted secondary antibody for 1 hr at R.T. The primary antibodies used were rabbit antimyosin VIIa polyclonal antibody (1:250; Proteus BioSciences, Ramona, CA), mouse anti-βIII tubulin monoclonal antibody (1:250; Covance Research Products, Berkeley, CA), mouse antisynapsin 1 monoclonal antibody (1:50; BD Biosciences Pharmingen, San Diego, CA), mouse antisynaptophysin monoclonal antibody (1:100; BD Biosciences Pharmingen), mouse anti-GAP43 monoclonal antibody (1:200; Chemicon), mouse anti-ctbp2/ribeye monoclonal antibody (1:50; BD Biosciences Pharmingen), and rabbit anti-calretinin polyclonal antibody (1:250; Chemicon). The secondary antibodies used were Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 633 goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR). Fluorescent images were obtained with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems Inc., Wetzlar, Germany).

The ratio of synapsin 1, synaptophysin, or GAP43 expression in nerve endings of ESC-derived neurons adjacent to IHCs was quantitatively assessed by counting the number of nerve endings positive for each marker in three randomly selected cryostat sections from each specimen. The ratio was determined by dividing the number of positive nerve endings by the total number of nerve endings. Values are given as mean ± SD.

Transmission Electron Microscopy

Cryostat sections adjacent to the section in which synaptophysin-positive nerve endings of ESC-derived neurons attached to IHCs were stained by immunohistochemistry using an anti-GFP rabbit polyclonal primary antibody (diluted 1:500 in PBS; Molecular Probes). GFP immunoreactivity was visualized using a Vectastain ABC kit and DAB substrate kit (Vector Laboratories, Burlingame, CA). Specimens were then postfixed with 1% OsO_4 in phosphate buffer for 90 min at 4°C. After dehydration in a graded ethanol series, specimens were embedded in Epon-812 and cut into 80-nm ultrathin sections. The sections were stained with lead citrate and viewed with a transmission electron microscope (TEM; H-7000, Hitachi, Tokyo, Japan).

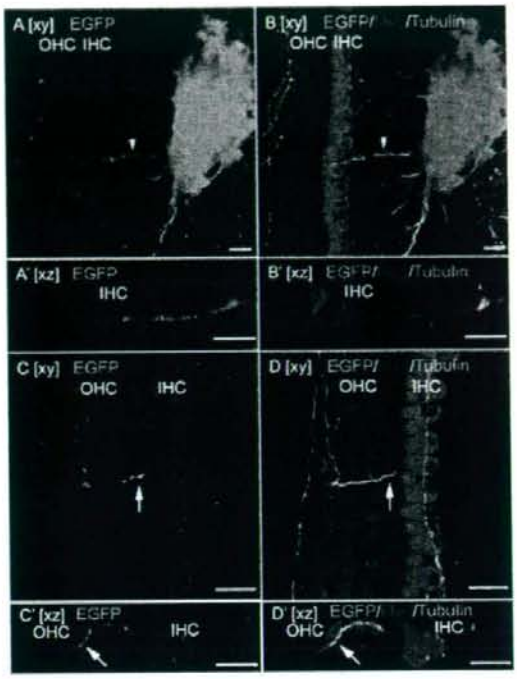

Fig. 2. Embryonic stem cell-derived neurons project neurites to auditory hair cells. A–D show xy projections and A′–D′ show xz projections of cocultures with embryonic stem cell (ESC)-derived neurons and auditory epithelia on 7 days in vitro (DIV). **A,B:** ESC-derived neurons labeled with EGFP (green) and βIII tubulin (blue) extend many processes toward IHCs (red). **A′,B′:** EGFP- and βIII tubulin-expressing fibers (arrowhead in A,B) extend beneath the auditory epithelium and attach to the basal portion of IHCs. **C,D:** ESC-derived neurons project a few processes toward OHCs like cross-tunnel fibers (arrow in C,D). **C′,D′:** ESC-derived neurites attach to the basal portion of OHCs (arrow in C′,D′). EGFP, enhanced green fluorescent protein; Myo, myosin VIIa; tubulin, βIII tubulin; IHC, inner hair cell; OHC, outer hair cell. Scale bars = 20 μm.

RESULTS

Projection of eurites From ESC-Derived Neurons to HCs

Auditory epithelia explants were cocultured with ESC-derived neural progenitors labeled with EGFP for 7 days. After this, βIII tubulin expression was detected in ESC-derived cells, as previously observed (Kim et al., 2005; Matsumoto et al., 2005), indicating that ESC-derived neural progenitors had differentiated into neurons. Seven days after plating, ESC-derived neurons formed colonies inside of a single row of IHCs and projected their neurites toward IHCs of the auditory epithelia (Fig. 2A,B). EGFP-expressing fibers were shown to extend from ESC-derived neurons beneath the auditory epithelium and attach to the basal portion of IHCs (Fig. 2A′,2B′), where nerve fibers and nerve endings from type I SGNs also attach in vivo conditions. Some EGFP-labeled neurites were observed around OHCs (Fig. 2C,D). Three of twenty cocultured specimens contained ESC-derived neurites running through the auditory epithelium like the cross-tunnel fibers (Fig. 2C′,D′). These findings demonstrate that ESC-derived neurons have the ability to project neurites to auditory HCs and that these neurites exhibit the same distribution as SGN-derived nerve fibers.

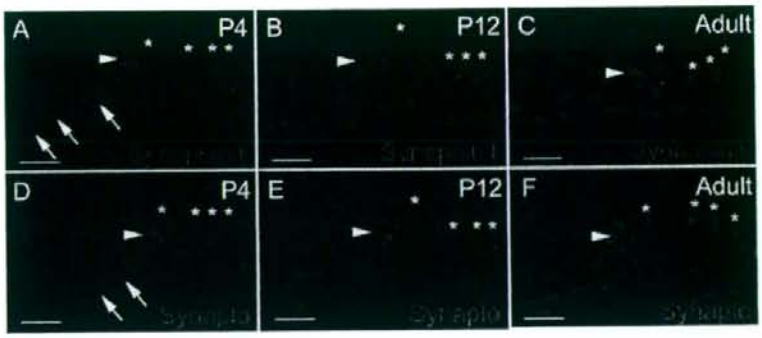

Fig. 3. Synapsin 1 and synaptophysin expression in auditory epithelia of postnatal day (P) 4, P12, and adult mice. **A:** In P4 mice, immunoreactivity for synapsin 1 is observed in the nerve fibers (arrows) and nerve endings adjacent to inner hair cells (arrowhead). **B,C:** In P12 and adult mice, the distribution of synapsin 1 immunoreactivity is limited to the nerve endings adjacent to inner hair cells (arrowhead). **D:** P4 mice exhibit synaptophysin immunoreactivity in the nerve fibers (arrows) and nerve endings adjacent to inner hair cells (arrowhead) and in the cytoplasm of hair cells. **E,F:** In P12 and adult auditory epithelia, synaptophysin expression is accumulated in the nerve endings adjacent to inner hair cells (arrowhead). Cellular nuclei are stained with DAPI (blue). Asterisks indicate the location of hair cells. Synapto, synaptophysin. Scale bars = 20 μm.

Expression of Synapsin 1, Synaptophysin, GAP43, Ctbp2/Ribeye, and Calretinin in ESC-Derived Neurons

To investigate the synapse formation of ESC-derived neurites with HCs, we examined the expression of the synaptic proteins synapsin 1, which is expressed in small synaptic vesicles containing neurotransmitters (De Camilli et al., 1983a,b; Huttner et al., 1983; Navone et al., 1984), and synaptophysin, which is an intrinsic membrane protein of small synaptic vesicles (Wiedenmann and Franke, 1985; Navone et al., 1986; Sudhof et al., 1987) under normal conditions. In auditory epithelia of P4 mice, before the onset of hearing, synapsin 1 immunoreactivity was observed in the nerve fibers and nerve endings adjacent to HCs (Fig. 3A). In P12 mice, at the onset of auditory function, and in adult mice, the distribution of synapsin 1 immunoreactivity was limited to the nerve endings adjacent to HCs (Fig. 3B,C). P4 mice exhibited synaptophysin immunoreactivity in nerve fibers and nerve endings adjacent to IHCs and in the cytoplasm of HCs (Fig. 3D), whereas, in P12 and adult auditory epithelia, synaptophysin expression was observed in the nerve endings adjacent to HCs (Fig. 3E,F).

In cocultured specimens, the expression of synapsin 1 was identified in the nerve endings of ESC-derived neurons adjacent to the base of IHCs (Fig. 4), equivalent to the case in P12 mice. Synaptophysin immunoreactivity was found in the nerve endings of ESC-derived neurons adjacent to the base of IHCs and in the cytoplasm of IHCs (Fig. 5), equivalent to P4 mice. We quantified the ratio of the expression of synapsin 1 or synaptophysin in nerve endings of ESC-derived neurons adjacent to the base of IHCs as 90.5% ± 16.2% and 95.2% ± 12.6%, respectively. These findings indicate active synapse formation between ESC-derived neurons and IHCs in cocultured specimens. We also examined GAP43 immunoreactivity, which reflects neurite elongation and synaptic sprouting (Meiri et al., 1986; Skene et al., 1986; Skene, 1989). GAP43 expression was found in 81.0% ± 17.7% of the nerve endings of ESC-derived neurons adjacent to the base of IHCs and the nerve fibers (Fig. 6).

We also performed immunohistochemistry for ctbp2/ribeye and calretinin to examine the types or maturation status of synaptic contacts between IHCs and ESC-derived neurons. We identified expression of calretinin in SGNs, their nerve fibers, and the HCs in adult mouse cochleae (Fig. 7). In cocultured specimens, the expression of calretinin was not observed in ESC-derived nerve fibers and endings, although HCs were positive (Fig. 7). The expression of ctbp2/ribeye was found in both the nucleus and the base of cytoplasm of IHCs in adult mouse cochleae (Fig. 7). In cocultured specimens, ctbp2/ribeye expression was also observed in the nuclei and the base of cytoplasm of IHCs, although few ctbp2/ribeye-positive dots were identified in IHC cytoplasm (Fig. 7).

Transmission Electron Microscopy

To investigate synaptic contacts between ESC-derived neurons and IHCs, we carried out TEM analysis of cryostat sections next to those containing the nerve endings of ESC-derived neurons expressing both EGFP and synaptophysin (Fig. 8A). EGFP expression was visualized by DAB staining, which was identified as electronically dense regions (Fig. 8B). High magnification revealed vesiculated nerve endings of ESC-derived neurons and their contacts with IHCs (Fig. 8C) as well as a parallel apposition of the two membranes with electronically dense material (Fig. 8D) showing the existence of

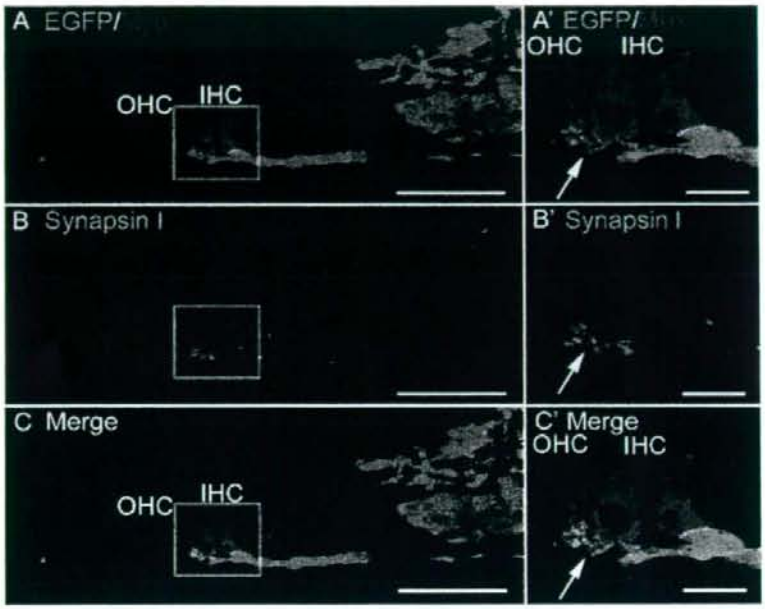

Fig. 4. Synapsin 1 expression in the nerve ending of embryonic stem cell-derived neurons. A–C: Low-magnification photographs of coculture with ESC-derived neurons and auditory epithelia on 7 days in vitro (DIV). A′–C′: High magnification of the boxes shown in A–C, respectively. **A:** ESC-derived neurons labeled with EGFP (green) elongate their neurites toward IHCs (red). **A′:** ESC-derived nerve endings attach to the base of IHCs (arrow). **B,B′:** The expression of synapsin 1 (blue) is accumulated in the nerve endings (arrow), not nerve fibers and soma of ESC-derived neurons. **C,C′:** Merged image. The expressions of EGFP (green) and synapsin 1 (blue) overlap in the nerve endings of ESC-derived neurons (arrow). EGFP, enhanced green fluorescent protein; Myo, myosin VIIa; IHCs, inner hair cells; OHCs, outer hair cells. Scale bars = 50 μm in A–C; 10 μm in A′–C′.

synaptic contacts. However, synapse ribbons, which are included in characteristics for afferent synaptic contacts between IHCs and SGN dendrites (Sobkowicz, 1992; Sobkowicz et al., 1997), were not observed in the base of IHCs attaching to ESC-derived nerve endings.

DISCUSSION

Our ultimate goal is to regenerate auditory HCs and SGNs and improve functional auditory systems after the degeneration of inner ears. Previous studies on human temporal bones have indicated that the loss of SGNs can cause SNHL (Schuknecht, 1993; Nelson and Hinojosa, 2001), and other studies have shown that cell transplantation is a realistic potential strategy for the replacement of SGNs (Naito et al., 2004; Tamura et al., 2004; Hu et al., 2005a,b; Okano et al., 2005; Corrales et al., 2006; Matsuoka et al., 2006).

In regeneration of the auditory afferent system, the formation of new synaptic contacts between IHCs and regenerated SGNs is crucial for its functionality. To investigate this, we have established an explant culture model in which SGNs are selectively eliminated by using fine forceps and HCs are well maintained (Matsumoto et al., 2005). The procedure causes no damage to auditory epithelia and does not disrupt the arrangement of HC rows. By contrast, alternative elimination methods, such as the use of β-bungarotoxin that binds the potassium channels, have the potential to destroy HCs (Fuchs, 1996; Herkert et al., 2001; Shakhman et al., 2003; Martinez-Monedero et al., 2006). Our mechanical elimination method is preferable for in vitro experiments that examine the activity of engrafted cells for neurite outgrowth and the formation of new synaptic contacts with HCs.

The present findings demonstrate that ESC-derived neurons located at the corresponding position of SGNs used appropriate routes to extend their neurites, which were correctly terminated at the base of HCs. In the current study, ESC-derived neural progenitors were cocultured with explants of auditory epithelia just after dennervation. Thus, the routes in which nerve fibers of SGNs had existed may not be scarred at the time point for starting coculture. In addition, previous studies have indicated that HCs have the potential to induce neurite outgrowth from neurons (Kawamoto et al., 2003;

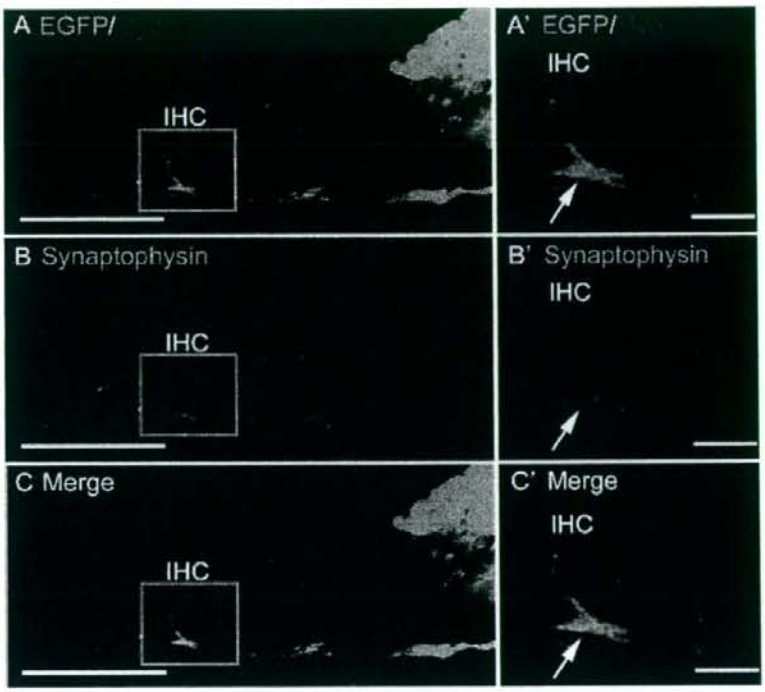

Fig. 5. Synaptophysin expression in the nerve ending of embryonic stem cell-derived neurons. A–C: Low-magnification photographs of coculture with ESC-derived neurons and auditory epithelia on 7 days in vitro (DIV). A′–C′: High magnification of the boxed shown in A–C, respectively. **A:** ESC-derived neurons labeled with EGFP (green) elongate their neurites toward IHCs (red). **A′:** ESC-derived nerve endings attach to the base of IHCs (arrow). **B,B′:** Synaptophysin immunoreactivity (blue) is observed in the nerve endings of ESC-derived neurons adjacent to the base of IHCs (arrow) and in the cytoplasm of IHCs, not nerve fibers and soma of ESC-derived neurons. **C,C′:** Merged image. The expressions of EGFP (green) and synaptophysin (blue) overlap in the nerve endings of ESC-derived neurons (arrow). EGFP, enhanced green fluorescent protein; Myo, myosin VIIa. Scale bars = 50 μm in A–C; 10 μm in A′–C′.

Kondo et al., 2005; Matsumoto et al., 2005; Martinez-Monedero et al., 2006). Therefore, ESC-derived neurons may elongate neurites through the route taken by host auditory nerves under the guidance of HCs. However, the details of HC guidance cues are still unclear and will have to be elucidated prior to the development of cell therapy for functional restoration of the auditory system.

Immunohistochemical analyses in the present study revealed the active formation of synaptic contacts between ESC-derived neurons and IHCs. The distribution of synapsin 1 immunoreactivity was identical to that in the auditory epithelia of P12 mice, at the onset of hearing, and showed an accumulation in the nerve endings of ESC-derived neurons adjacent to IHCs. Moreover, the distribution of synaptophysin immunoreactivity in cocultured specimens was similar to that of P4 mice. In addition, the nerve endings and neurites of ESC-derived neurons exhibited GAP43 immunoreactivity, indicating active neurite outgrowth and synaptic sprouting of ESC-derived neurons (Knipper et al., 1995). Immunohistochemistry for ctbp2/ribeye indicated the presence of synaptic ribbons in the basal pole of IHCs adjacent to ESC-derived nerve endings, similar to immature synaptic contacts between IHCs and afferent nerve fibers (Sobkowicz, 1992). Such immunohistochemical findings suggest active synapse formation between ESC-derived neurons and IHCs.

In the present study, TEM analysis revealed contacts between vesiculated nerve endings from ESC-derived neurons and IHC membranes, as well as synaptic densities in the membrane between IHCs and nerve endings of ESC-derived neurons. This represents the first evidence of synapse formation between ESC-derived neurons and IHCs. In normal cochleae of mature mice, afferent synapse contacts at the base of IHCs are characterized by the presence of synaptic ribbons and by a postsynaptic membrane density on the dendrite (Sobkowicz, 1992; Sobkowicz et al., 1997). However, in TEM analysis, no synaptic ribbons were found in the base of

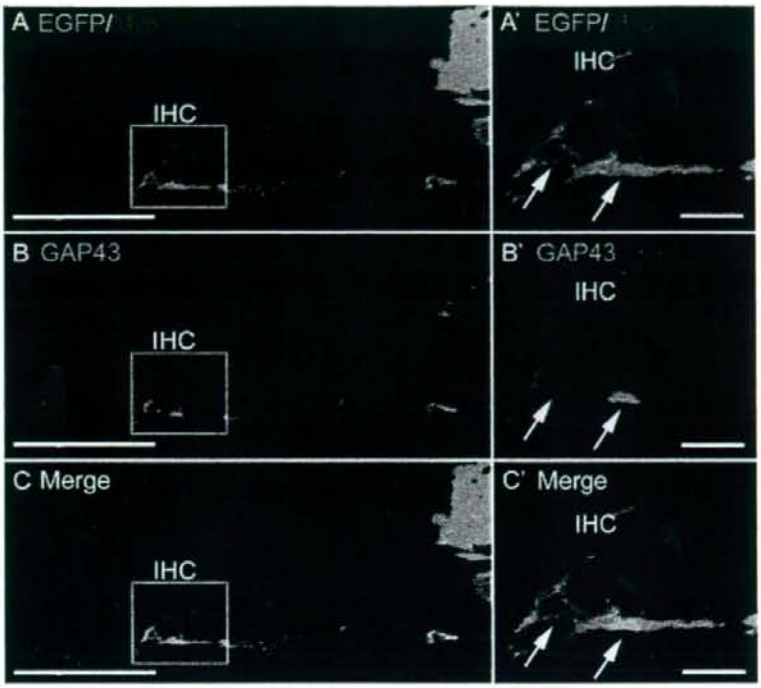

Fig. 6. GAP43 expression in the nerve ending of embryonic stem cell-derived neurons. **A–C**: Low-magnification photographs of coculture with ESC-derived neurons and auditory epithelia on 7 days in vitro (DIV). A′–C′: High magnification of the boxes shown in A–C, respectively. **A**: ESC-derived neurons labeled with EGFP (green) elongate their neurites toward IHCs (red). **A′**: ESC-derived nerve endings attach to the base of IHCs (arrows). **B,B′**: GAP43 immunoreactivity (blue) is found in the nerve endings of ESC-derived neurons adjacent to the base of IHCs (arrows) and the nerve fibers. **C,C′**: Merged image. The expressions of EGFP (green) and GAP43 (blue) overlap in the nerve endings of ESC-derived neurons (arrows) and the nerve fibers. EGFP, enhanced green fluorescent protein; Myo, myosin VIIa. Scale bars = 50 μm in A–C; 10 μm in A′–C′.

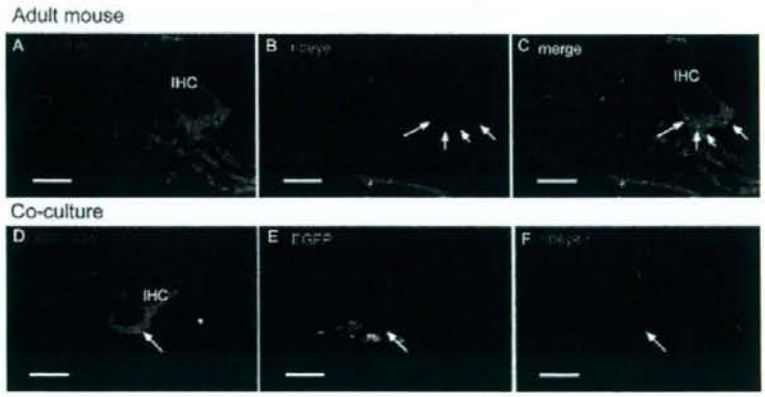

Fig. 7. Expression of ctbp2/ribeye and calretinin in adult mouse cochleae and cocultured specimens. **A–C**: In the adult mouse cochlea, ctbp2/ribeye expression (blue) is found in the nuclei and the base pole of the IHC (arrows), and calretinin expression (red) is observed in hair cells and afferent nerve fibers. **D–F**: In cocultured specimens, immunoreactivity for ctbp2/ribeye is found in the nucleus and in the basal pole of the IHC (arrow), and ESC-derived neurites expressing EGFP (green) are negative for calretinin. On the other hand, the IHC is positive for calretinin. Scale bars = 10 μm.

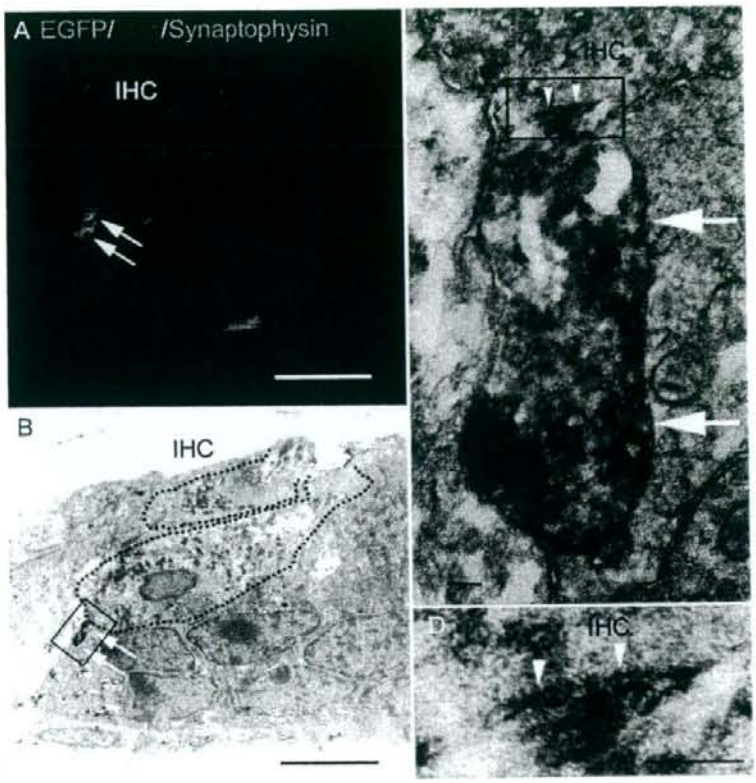

Fig. 8. Ultramicrostructure of contacts between inner hair cells and nerve endings of embryonic stem cell-derived neurons. **A:** Synaptophysin-positive nerve endings (blue) of ESC-derived neurons expressing EGFP (green) attach to the basal portion of the IHC (red; arrows). **B:** An EGFP-positive nerve ending visualized by DAB staining, which is identified as electronically dense regions, is found at the base of the IHC (arrows). **C:** A high magnification of the box shown in B demonstrates the nerve ending of ESC-derived neurons as a vesiculated nerve ending (arrows) and their contact with the membrane of the IHC (arrowheads). **D:** In a higher magnification of the box shown in C, this region exhibits a parallel apposition of the two membranes, with an electronically dense material between the nerve endings of ESC-derived neurons and the IHC (arrowheads). EGFP, enhanced green fluorescent protein; Myo, myosin VIIa; IHC, inner hair cell. Scale bars = 10 μm in A,B; 0.1 μm in C,D.

IHCs adjacent to ESC-derived nerve endings. Therefore, the demonstration that the ECS-derived neurons may be used for replacement of SGNs requires additional experiments.

The neurites of ESC-derived neurons adjacent to IHCs share some properties of presynaptic fibers, because synapsin 1 and synaptophysin are markers for small synaptic vesicles, which are usually located in the presynaptic compartments. In addition, the neurites of ESC-derived neurons were negative for calretinin, which is expressed in mature SGN and their neurites (Dechesne et al., 1994). However, previous studies have observed expression of synapsin 1 and synaptophysin in afferent growth cones (De Camilli et al., 1988). Immature afferent dendrites of SGNs are lack of calretinin expression (Dechesne et al., 1994). Moreover, recent findings using cocultures of auditory epithelia explants and primary SGN cultures found that the processes from SGNs adjacent to HCs express markers for presynaptic fibers (Martinez-Monedero et al., 2006). In addition, coincident with synaptophysin and GAP-43 immunoreactivity in SGNs, outer spiral bundle fibers (OSB) exhibit immunoreactivity for synaptophysin on P2 but not P4 (Knipper et al., 1995), suggesting the localization of synaptophysin in the afferent growth cones of immature SGNs. Morphologically, immature efferent fibers are practically indistinguishable from those of afferent processes (Sobkowicz, 1992). Therefore, determining the type of synaptic contacts between ESC-derived neurons and IHCs will require further examinations.

CONCLUSIONS

The present findings demonstrate that ESC-derived neurons project neurites toward HCs in the auditory epithelium and have the ability to form new synaptic contacts with IHCs. In future experiments, we will investigate the maturation process of ESC-derived neurons after the formation of synaptic contacts with HCs and elucidate HC-derived guidance cues for neurite projection.

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Surgical Invasiveness of Cell Transplantation into the Guinea Pig Cochlear Modiolus

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Key Words
Cell transfer · Surgery · Regeneration · Spiral ganglion neuron · Cochlear function

Abstract
Objective: Previous studies have demonstrated the potential of cell transplantation for regeneration of spiral ganglion neurons (SGNs). However, the effect of surgical invasion on host cochleae has yet to be evaluated. The present study investigated the efficiency and invasiveness of our surgical procedure using a fine glass pipette for injections into the cochlear modiolus. ***Methods:*** We examined the survival of transplanted embryonic stem cell-derived neurons in the cochlear modiolus of guinea pigs. Surgical invasiveness was assessed by measurements of electrically evoked auditory brainstem responses (eABRs) and SGN densities after an injection of 5 μl of saline into the cochlear modiolus. ***Results:*** All of the transplanted animals exhibited localization of transplanted cells in the cochlear modiolus. No significant alterations in the eABR thresholds or SGN densities were found following surgery. ***Conclusion:*** These findings indicate that our procedure is a viable method for testing the potential of transplants for SGN replacement.

Introduction

The mammalian inner ear was originally believed to have no regenerative ability [1]. Therefore, in order to try and induce the regeneration of inner ear cells, novel strategies including cell and/or gene therapy have been investigated. Several studies have shown the efficacy of cell transplantation for the replacement of spiral ganglion neurons (SGNs), auditory primary neurons [2–8]. However, surgical procedures for cell transplantation involve a risk of damaging the host's auditory system. It is therefore crucial to determine the level of surgical invasiveness and evaluate the efficacy of cell transplantation on functionality, although as of the present, these issues have yet to be well documented.

We previously reported that transplantation of embryonic stem (ES) cell-derived neurons into the cochlear modiolus rescued impaired auditory function in guinea pigs [9]. In this previous study, we used a 30-gauge needle for the injection of cell suspensions into the cochlear modiolus. More recently, we have revised our surgical procedure in order to reduce the surgical invasiveness to the host SGNs. In the revised procedure, we use a fine glass pipette to introduce the cell suspensions into the cochlear modiolus of guinea pigs. In the present study, we investigated the efficiency of our revised procedure, and

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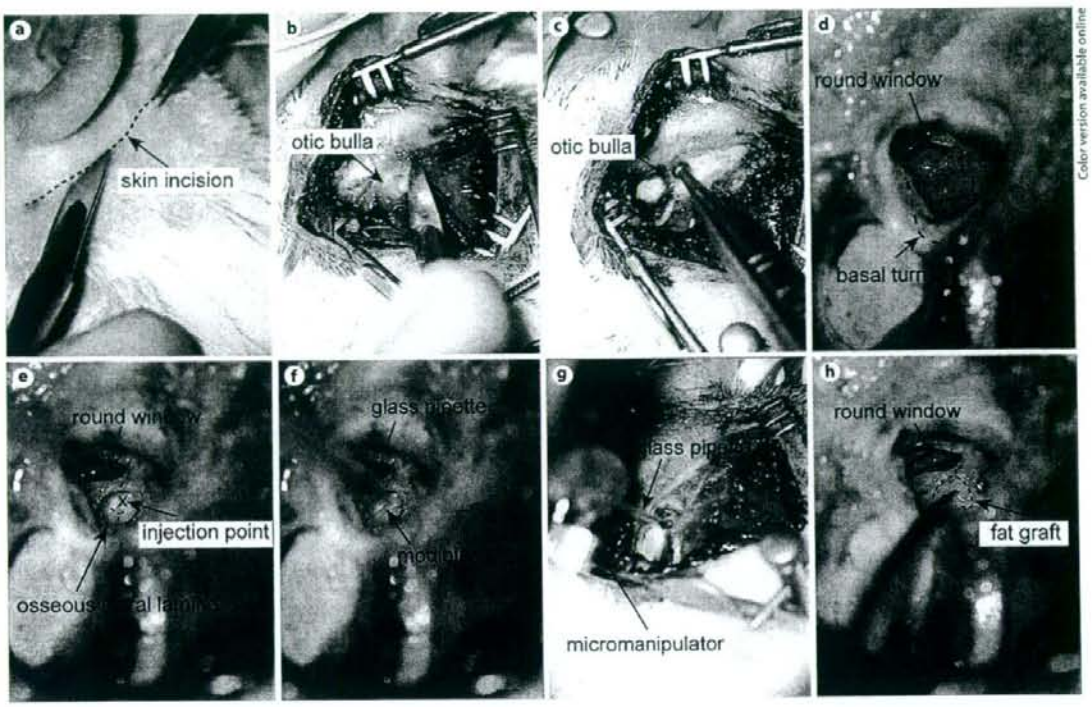

Fig. 1. Surgical procedure for cell transplantation into the cochlear modiolus. **a** Skin incision is made in the retroauricular region. **b** An otic bulla is exposed. **c** A small hole on the bulla is made using a drill. **d** The round window and the basal turn of the cochlea are observed through an opening on the bulla. An interrupted line indicates the location of the basal turn. **e** Cochleostomy is performed on the basal portion of the cochlea corresponding to the location of the scala tympani. A dotted line indicates the location of the osseous spiral lamina. A cross indicates the injection point. **f**, **g** A glass pipette is inserted into the cochlear modiolus using a micromanipulator. **h** The cochleostomy site is closed with a fat graft (dotted line).

evaluated the functional and histological damage to the host SGNs. To evaluate cell transplantation efficiency, we examined the survival of the transplanted ES cell-derived neurons in the cochlear modiolus of guinea pigs. The level of surgical invasiveness was estimated by measuring electrically evoked auditory brainstem responses (eABRs) and SGN densities in Rosenthal's canal following an injection of 5 μl of saline.

Materials and Methods

Animals

A total of 12 Hartley strain guinea pigs weighing 350–400 g were purchased from Japan SLC Inc. (Hamamatsu, Japan). All of the animals had otoscopically normal tympanic membranes and normal hearing, as determined by tone-burst ABR. The Animal Research Committee of the Graduate School of Medicine, Kyoto University, Japan, approved all of the experimental protocols. Animal care was carried out under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. All of the experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Surgical Procedure

The animals were anesthetized with an intramuscular injection of ketamine (75 mg/kg) and xylazine (9 mg/kg), and body temperature was maintained at 37 ± 1°C using a heating pad. We used a conventional retroauricular approach in the lateral recumbent position (fig. 1a). After exposure of the otic bulla (fig. 1b), a small hole was made on the otic bulla to expose the round window niche and the basal turn of the cochlea (fig. 1c, d). After fixation of the head position using clay and surgical tape, cochleostomy was performed

on the basal portion of the cochlea to visualize the cochlear modiolus through the scala tympani (fig. 1e). A fine glass pipette was made with a Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, Calif., USA), with the tip cut with a fine blade to make an outer diameter of approximately 100 μm. A micromanipulator was used to insert the glass pipette, which was connected to a microsyringe (Hamilton, Reno, Nev., USA), into the cochlear modiolus of the basal portion of the cochlea (fig. 1f, g). After removal of the perilymph with filter paper, we directly inserted the glass pipette into the bony wall of the cochlear modiolus from a point slightly basal to the junction of the osseous spiral lamina and the modiolus (fig. 1f). In this particular region, a glass pipette is capable of penetrating the bony wall of the cochlear modiolus due to the thickness of the bony wall. Subsequently, we infused 5 μl of the substrate through the glass pipette using a microinfusion pump set at a rate of 1 μl/min. The glass pipette was removed 1 min after stopping the infusion. Finally, the cochleostomy site was closed with a fat graft and then covered with fibrin glue (fig. 1h).

Efficiency of the Cell Transplantation Procedure

We estimated the transplant survival of mouse ES cell-derived neural progenitors in the cochlear modiolus using histological analysis. Mouse G4-2 ES cells (provided by H. Niwa of Riken CDB, Kobe, Japan) that were derived from the E14tg2ab ES cell line and which carried the EGFP gene driven by the CAG promoter were used as the transplant source. Neural induction of ES cells was performed by stromal cell-inducing activity [10] before transplantation. Briefly, undifferentiated ES cells were cultured on a feeder layer of PA6 stromal cells (RCB1127; Riken Cell Bank, Tsukuba, Japan) in Glasgow's modified Eagle's medium (GMEM; Invitrogen, Carlsbad, Calif., USA) supplemented with 5% knockout serum replacement (Invitrogen), 1 mM pyruvate (Sigma, St. Louis, Mo., USA), 0.1 mM nonessential amino acids (Invitrogen), and 0.2 mM 2-mercaptoethanol (Wako, Osaka, Japan). Colonies that formed on the PA6 monolayer after 6 days of culture were isolated and prepared as suspensions of 10^4 cells/μl GMEM.

In 4 guinea pigs, 5 μl of the cell suspensions were injected into the cochlear modiolus in each animal using the procedure described above. At 1 week after cell transplantation, the otic bullae of the experimental animals were opened under ketamine and xylazine anesthesia, and then 4% paraformaldehyde in 0.01 M phosphate-buffered saline at pH 7.4 was perfused into the perilymph from the round window. The animals were deeply anesthetized with a lethal dose of ketamine and xylazine that was perfused intracardially with physiological saline, followed by infusion of the same fixative. The temporal bones were then collected and immersed in the same fixative for 4 h at 4°C. Specimens (10 μm thick) were prepared using a cryostat after decalcification with 0.1 M ethylenediaminetetraacetic acid in phosphate-buffered saline for 3 weeks at 4°C. From each cochlea, three mid-modiolus sections were stained with 2 μg/ml 4',6-diamino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, Oreg., USA), and then viewed with a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan). Settlement of transplants was determined by the existence of both EGFP- and DAPI-positive cells in Rosenthal's canal or the cochlear modiolus.

Functional Estimation of Surgical Invasiveness

We injected 5 μl of physiological saline into the modiolus of the left cochlea of each of 8 guinea pigs. Functional damage was

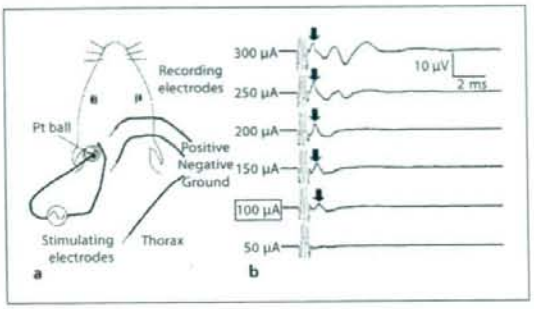

Fig. 2. Measurement of eABRs. **a** A platinum ball electrode is inserted into the scala tympani in the basal portion of the cochlea. Another wire serves as extracochlear ground for monopolar stimulation, and is fixed to the temporal bone. For recording, three electrodes are used (positive; vertex, negative; neck, ground; thorax). **b** The threshold is defined as the smallest current amplitude required to evoke a response within a latency of 4 ms after stimulus onset. Arrows indicate the first positive wave after stimulus onset. The threshold of this sample is 100 μA.

evaluated by determining eABR thresholds immediately before and after surgical treatment, and on days 1 and 14 after surgical treatment. eABR measurements were performed in a sound-attenuated and electrically shielded room. One platinum/iridium ball electrode (Nihon Koden, Tokyo, Japan) was inserted into the scala tympani through the cochleostomy site and was then placed approximately 1.5 mm deep from the cochleostomy site. Another electrode was fixed to the temporal bone where it served as an extracochlear ground for monopolar stimulation (fig. 2a).

Biphasic current pulses were generated under computer control using a real-time processor (Tucker-Davis Technologies, Alachua, Fla., USA). Stimuli were altered in 50-μA steps, with the stimulus current levels calibrated by measuring the voltage with an oscilloscope from 1,000 to 10,000 Ω. The responses against electrical stimuli were recorded separately using stainless steel needle electrodes (fig. 2a, vertex positive, neck negative, thorax ground). The electrical stimulus consisted of charge-balanced biphasic current pulses, which occurred 50 times per second. The scalp-recorded response was amplified by 3×10^3 and band-pass-filtered (200–1,500 Hz). The filter output was fed to an analog-to-digital converter and sampled for 10 ms following stimulus onset. For each recording, 500 responses were averaged and then stored for subsequent analysis. Two sets of recordings were made at each current level, and the current amplitude was reduced to levels below threshold. The thresholds for eABRs were determined as described previously [11–15]. The threshold was defined as the smallest current amplitude that was required to evoke a response with a latency of 4 ms following the stimulus onset for both responses (fig. 2b).

Histological Estimation of Surgical Invasiveness

After saline injection into the cochlear modiolus, the temporal bones were collected on days 1 (n = 4) and 14 (n = 4). Right co-

Fig. 3. Locations of transplanted cells in the cochlea. EGFP-expressing cells (green; light gray in the printed version) are found in the cochlear modiolus and Rosenthal's canal from the basal to the middle portion of cochleae (**a**, **b**, arrows). One specimen exhibits settlement of transplanted cells in the scala vestibuli (**c**, arrowhead). Blue fluorescence (rendered dark gray in the printed version) shows nuclear staining with DAPI.

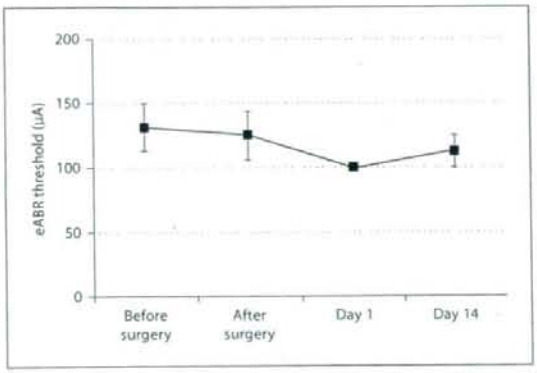

Fig. 4. eABR thresholds immediately before and after surgical treatment, and on days 1 and 14 after surgical treatment. The y-axis shows the mean of eABR thresholds. There are no significant differences in eABR thresholds among experimental groups. Bars represent standard errors.

chleae, which underwent no surgical treatments, were used as controls. After eABR recordings, cochlear specimens were collected and prepared as cryostat sections using a similar procedure as for the transplanted ES cell-derived neurons. Sections were stained with hematoxylin and eosin (HE). SGN counting in Rosenthal's canals was performed in accordance with a method that has previously been described [13–15]. SGN numbers were determined in the basal, mid-basal or second turn from 5 randomly selected mid-modiolar sections of the cochleae that underwent surgery, and from the contralateral cochleae that received no surgical treatment. The cross-sectional areas of Rosenthal's canals were measured using Image/J software (http://www.rsb.info.nist.gov/ij). SGN densities were then calculated by dividing the number of SGNs by the area. This value was used to reduce the variance caused by differences in the cutting directions among the cochlear specimens.

We also evaluated cell infiltration into the cochlea on days 1 and 14 after surgery. The mean numbers of nucleated cells in the scala tympani, scala vestibuli and scala media were determined in the basal, mid-basal and second turns from 5 randomly selected mid-modiolar cochlear sections.

Statistics

The overall effect on the eABR threshold shifts during the surgical procedure was examined by one-way factorial analysis of variance (ANOVA). The values of SGN densities in Rosenthal's canals that were obtained on days 1 or 14 after surgical treatment were compared with those of the contralateral cochleae by an unpaired t test. A p value less than 0.05 was considered statistically significant. All data are presented as the mean ± standard error.

Results

Efficiency of Cell Transplantation

We examined the survival and the location of engrafted ES cell-derived neurons in order to determine the efficiency of cell transplantation with our surgical procedure. All of the cochlear specimens exhibited settlement of the EGFP-expressing cells in Rosenthal's canals or the modioli in the basal portions of the cochleae, indicating that our transplantation procedure using a fine glass pipette was efficient for introducing transplants into the cochlear modiolus. EGFP-positive cells were also identified in the middle portion of the cochlear modiolus in 2 out of 4 experimental animals (fig. 3a, b). EGFP-positive

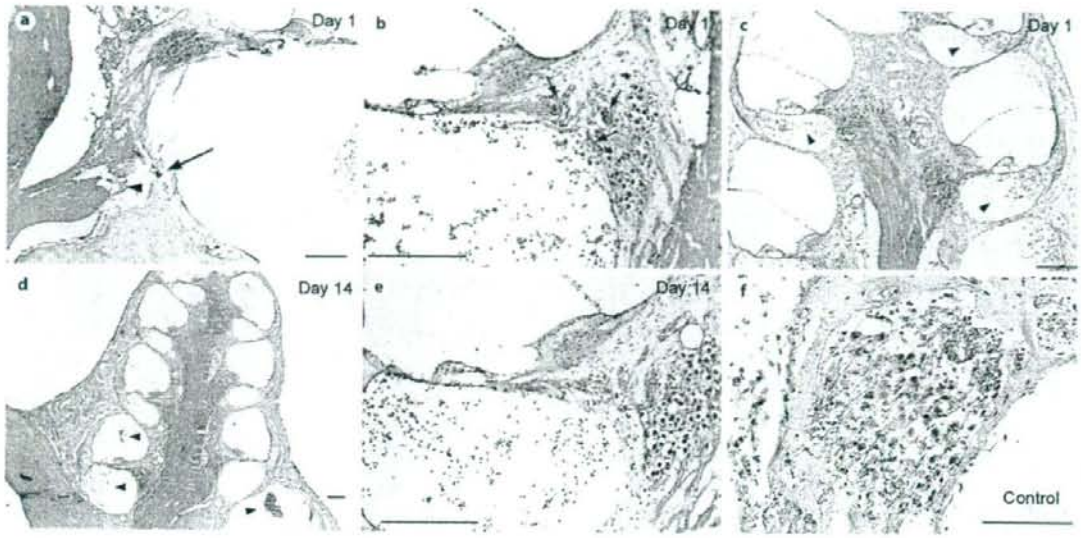

Fig. 5. Histological damage in a cochlea due to surgical procedures. **a** At the injection site, bone fracture of the cochlear modiolus (arrow) and injury of nerve fibers (arrowhead) is found on day 1 after surgery. **b** In the mid-basal portion of the cochlea on day 1 after surgery, red blood cells are observed in Rosenthal's canal (arrows). **c** From the second to the apical turn of the cochlea, no degenerative changes in the organ of Corti and spiral ganglions are identified, although a number of red blood cells are found in the scala tympani (arrowheads). **d** Cell infiltration (arrowheads) is observed in the scala tympani and scala vestibuli of the basal portion of the cochlea on day 14 after surgery. **e** In the basal turn of the cochlea on day 14 after surgery, the organ of Corti and the spiral ganglion exhibited normal morphology, despite cell infiltration in the scala tympani. **f** Spiral ganglion in the basal turn of the control cochlea. Bars = 20 μm.

cells were also observed in the scala vestibuli in 1 of the experimental animals (fig. 3c), indicating leakage of injected transplants from the cochlear modiolus.

Functional Damage due to Surgical Procedures

To evaluate the functional damage to SGNs and nerve fibers, we measured the eABRs, which are frequently used for evaluation of SGN function [11–15]. The mean values of the eABR thresholds immediately before and after surgery were 131.3 ± 18.6 and 125.0 ± 18.9 μA, respectively. On days 1 and 14 after surgery, the mean values were 100.0 ± 0.0 and 112.5 ± 12.5 μA, respectively. There were no significant differences in the eABR thresholds among the experimental groups (fig. 4), indicating that our procedure caused no severe functional damage of the SGNs.

Histological Damage due to Surgical Procedures

HE staining of mid-modiolar sections revealed actual points of injection in the cochlear modiolus. In all specimens, the injected points were located in the cochlear modiolus of the basal turn from a point slightly inferior to Rosenthal's canal (fig. 5a). On day 1 after surgery, infiltration of nucleated cells in the modiolus and injury in the nerve fibers was observed in the injected site (fig. 5a). From the basal to the second turn, a number of red blood cells were found in the scala tympani, scala vestibuli and scala media. Red blood cells were also found in Rosenthal's canal of the basal and mid-basal portions of cochleae (fig. 5b). However, organs of Corti and SGNs were well preserved in these portions of cochleae. In the apical portion of cochleae, no apparent histological damage was identified, although cell infiltration into the scala tympani and scala vestibuli was observed (fig. 5c). On day 14, red blood cells in the cochlear fluid space decreased (fig. 5d); however, cell infiltration in the scala tympani of the basal and mid-basal portions were still observed (fig. 5d, e). Remarkable degeneration of SGNs was not observed in the cochleae on day 14 in comparison with normal cochleae (fig. 5f). We quantified the histological damage to SGNs

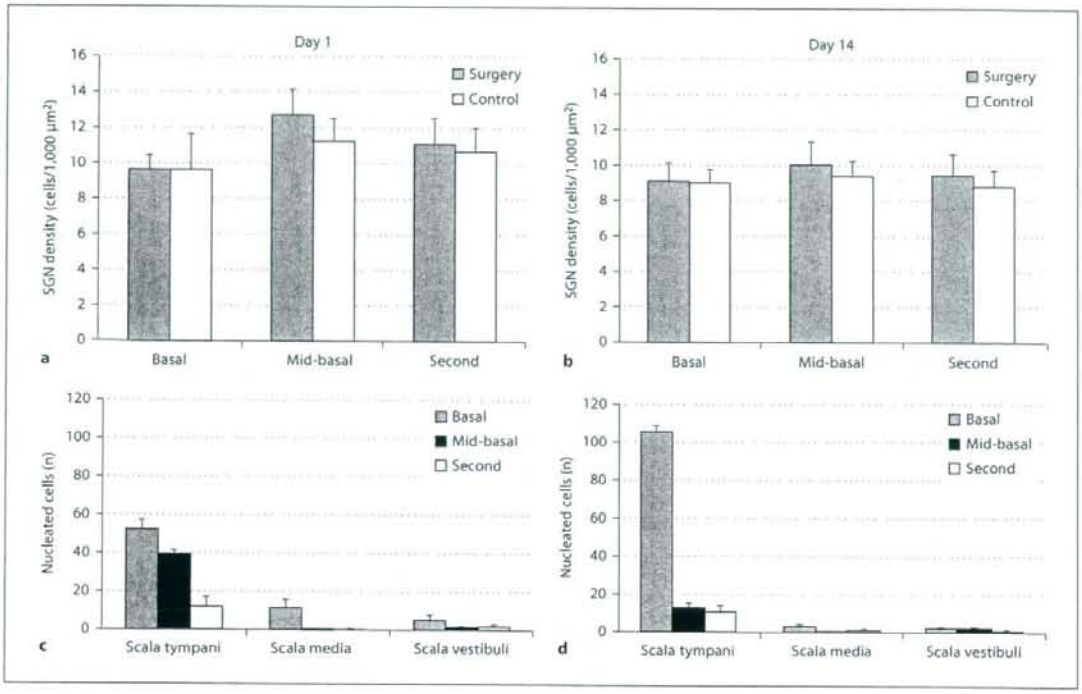

Fig. 6. Mean density of SGNs (**a**, day 1; **b**, day 14) and mean number of nucleated cells (**c**, day 1; **d**, day 14) in the basal, mid-basal and second turns of cochleae. No significant differences in the density of SGNs (SGN density) are found in each turn of cochleae between specimens after surgery and control specimens (**a**, **b**; unpaired t test). Bars show standard errors.

by measuring SGN densities in Rosenthal's canals. There were no significant differences in the SGN densities in every portion between the cochleae with surgical treatment and the cochleae without surgical treatment on days 1 or 14 after surgery (fig. 6a, b). We also quantified the number of nucleated cells in the scala tympani, scala media and scala vestibuli. A number of nucleated cells were found in the basal and mid-basal turns of the cochleae, notably in the scala tympani, on day 1 after surgery (fig. 6c). On day 14, there was a trend for the numbers of nucleated cells to decrease in the mid-basal and second turn of cochleae, while in the scala tympani of the basal turn, an increase in the number of nucleated cells was observed (fig. 6d). These findings indicate that our surgical procedure causes no significant loss of SGNs, although certain injury in the injected points and consecutive cell infiltration occurred in the basal turn of cochleae.

Discussion

The primary aim of this study was to establish a minimally invasive procedure for cell transplantation into the cochlear modiolus, which would allow for successful introduction of the transplants into the cochlear modiolus. Previously, Brown [16] established a method for recording single efferent and afferent auditory nerve cells. In this method, after cochleostomy in the basal turn of the cochlea, a small opening on the osseous spiral lamina is made using a fine insect pin followed by insertion of a fine glass electrode through the opening. This method is actually a very low invasive and efficient procedure for accessing the auditory nerves from the scala tympani. The aim of the present study was to establish a method for cell transplantation into the cochlear modioli of guinea pigs by introducing cell spheres through a glass pi-

pette. To achieve this primary aim, it is necessary to use glass pipettes with larger tips. However, the comparatively thick glass pipettes that were used in the present study were too large for insertion into the osseous spiral lamina. Therefore, we inserted a glass pipette into the modiolus of the basal portion of the cochlea.

The present findings demonstrated that there was no significant elevation of eABR thresholds after surgery, indicating that no severe functional damage of the SGNs due to surgical procedures had occurred. Measurements of eABR thresholds have frequently been used for evaluation of SGN function in animal experiments [9, 11–15]. In such experiments, animal models for severe SGN degeneration are used. Although eABR thresholds are actually effective for evaluating severe SGN degeneration, it has been reported that the eABR is of limited value when it comes to detecting only minimal SGN loss [17]. Therefore, our present findings for eABRs did not indicate that there was a complete preservation of host SGNs. We recorded eABRs immediately before and after surgery, and on days 1 and 14 after surgery in order to evaluate acute and chronic effects of our surgical procedure on ABR thresholds. The results demonstrated that there were no significant alterations in eABR thresholds between the time points for which the data were collected, indicating that the surgical procedure itself and the inflammatory responses that follow surgery do not affect eABR thresholds.

Histological analysis of the SGNs also revealed that there was no significant loss of SGNs due to our surgical procedure, which supports our present findings for eABR measurements. Although there was no significant decrease in SGNs after surgery, infiltration of nucleated cells and the presence of bleeding were found in the cochleae, mainly in the scala tympani. On day 1, nucleated cells were predominantly found in the basal and mid-basal portions of cochleae, while on day 14, the distribution of nucleated cells was limited in the basal portion of cochleae. However, the number of nucleated cells in the basal portion of cochleae on day 14 increased from that on day 1. These findings indicate that immediate inflammatory responses due to surgery occur in the basal and mid-basal portions of cochleae, and chronic inflammation continues in the basal portion until day 14. However, such inflammatory responses caused no significant SGN degeneration.

In our previous studies [9, 18], we used a 30-gauge needle for introducing transplants into the cochlear modiolus, which resulted in efficient settlement of the transplants in the cochlear modiolus. In the present study, in order to reduce the surgical invasiveness to host SGNs, a glass pipette was used for the injection of substrates into the cochlear modiolus instead of a needle. The outer diameter of a 30-gauge needle is approximately 500 μm, while that of a glass pipette is only 100 μm, which is likely to reduce the extent of surgical damage. Although the use of a glass pipette could result in an insufficient introduction of ES cell-derived neurons into the cochlear modiolus, the present histological findings demonstrated that there was an efficient settlement of transplanted cells in the cochlear modiolus. We therefore believe that the use of a glass pipette is an efficient and safe method for the introduction of cells into the cochlear modiolus of guinea pigs.

Several previous studies have documented the secretion of trophic factors from transplanted stem cells in the cochlea [19–21]. Culture media can also include various trophic factors, and thus, an injection of cell suspensions or culture media has the potential to protect SGNs against surgical invasiveness. Therefore, to evaluate surgery-related functional and histological damage to host SGNs, instead of cell suspensions, we injected saline into the cochlear modiolus.

In conclusion, our present findings demonstrate that a surgical procedure that uses a glass pipette can efficiently introduce transplants into the cochlear modiolus with no significant alteration in eABR thresholds and in SGN densities in normal adult guinea pigs. Our revised procedure for cell transplantation into the cochlear modiolus can be used to investigate the potential of various transplants for functional regeneration of SGNs in the guinea pig.

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