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Study of mouse induced pluripotent stem cell transplantation into Wistar albino rat cochleae after hair cell damage*

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Background/aim: As the regeneration capacity of hair cells is limited, inner ear stem cell therapies hold promise. Effects of mouse induced pluripotent stem cells (IPSCs) on Wistar albino rats (WARs) with hearing impairment were investigated.

Materials and methods: Thirty-five adult WARs with normal hearing were divided into 4 groups. Excluding the study group (n = 15), the other three groups served as control groups for ototoxicity and IPSC injection models. IPSC injections were performed via cochleostomy after a retroauricular approach. Auditory functions were evaluated with auditory brainstem responses (ABRs) before and after the injections. After a final hearing assessment the WARs were sacrificed and cochleae were extracted to see the biologic behavior of IPSCs in the inner ear by light microscopy and immunohistochemistry.

Results: There were no significant differences in the click-ABR thresholds in the study group after IPSC transplantation. The mean hearing threshold in the study group after ototoxic agent injection was 53.2 dB (10–90 dB). There was no significant difference between groups ($P > 0.05$) and no differentiated stem cells were observed immunohistochemically.

Conclusion: Transplanted IPSCs did not show a therapeutic effect in this trial. We discuss potential pitfalls and factors affecting the therapeutic effect.

Key words: Hearing loss, induced pluripotent stem cell, therapy, transplantation

1. Introduction

The treatment of inner ear diseases causing hearing impairment is a problem as the regenerative ability of the mammalian cochlea is limited. Sensorineural hearing loss (SNHL) is the most common type of sensorial loss in humans. The loss of hair cells or spiral ganglion neurons in the cochlea compromises auditory function and causes SNHL. There are many factors that can lead to degeneration processes of these structures, such as ototoxic agents, inner ear trauma, congenital disorders, or aging (1,2). Recently clinicians have focused on studies that inhibit or compensate the pathology at the hair cell level.

Stem cell technologies started a new era in regenerative medicine and tissue engineering. Numerous papers have been published in the last decade evaluating the effects and roles of stem cells in the inner ear (3,4). Among these studies several stem cell lines have been used, such as embryogenic or neural stem cells (2,5,6). A new era began in 2006 as Yamanaka et al. generated induced pluripotent stem cells (IPSCs) from somatic fibroblastic cells using 4 transcription factors (Oct4, Sox2, Klf4, and c-myc) (7).

IPSCs are very similar to embryogenic stem cells (ESCs) in their molecular and functional features and are a new source for pluripotent stem cells. Being relatively simple in production and removed from ethical arguments, IPSCs have become a valuable method in regenerative medicine. The data from in vivo and in vitro studies with ESCs and neuronal induced stem cells and the use of IPSCs in the restoration of damaged spiral ganglions brought forward the idea of IPSC use in hair cell regeneration (8). In

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this manner, we planned to set up an ototoxicity model in Wistar albino rats (WARs), transplant IPSCs into the cochlea, and investigate the electrophysiological and histopathological changes after transplantation.

2. Materials and methods

2.1. Study set up, subjects, and clinical set up

All experimental protocols and surgical procedures were carried out in the Institute of Laboratory Animals and Department of Pathology Immunopathology Laboratories of the Ankara University Medical School. This study was approved by the institutional review board (IRB) of the ethics committee on animal experiments of Ankara University (IRB 2013-4-24). The stem cells were kindly donated from the Laboratory of Stem Cell and Developmental Biology, Konkuk University, South Korea.

This study was conducted on 35 adult (4 months old, 350–400 g) female WARs with normal hearing. Study subjects were divided into 4 groups. Excluding the study group (group 3, n = 15), the other three groups served as control groups for the method (group 1, n = 5), ototoxicity (group 4, n = 10), and sham operation (group 2, n = 5). The clinical set up and time schedule are summarized in Table 1.

Hearing evaluations and IPSC transplantation procedures were performed under anesthesia. A daily intramuscular amikacin injection (600 mg/kg) for 14 days was administered for the ototoxicity model. Groups 3 and 4 received amikacin while the other groups received IM saline solution (0.5 mL, 0.09% NaCl).

After 4 weeks auditory brainstem responses (ABRs) were tested for all animals to check hearing loss in groups 3 and 4. Afterwards, 10 µL of IPSC solution with Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific Inc., Waltham, MA, USA) was transplanted after thawing to groups 1 and 3 while 10 µL of DMEM was injected in groups 2 and 4 as a placebo. Four weeks later all study subjects underwent a control ABR test. Afterwards the cochleae were dissected out and cochlea morphology, hair cell damage, and the fates of implanted IPSCs were examined by light microscopy and immunofluorescence methods.

All hearing tests and surgeries were performed under anesthesia with ketamine (4 mg/100 g, intramuscular [IM]), and xylazine (1 mg/100 g, IM). All operations were performed under surgical microscope (Leica M400 E, Wetzlar, Germany).

2.2. Induced pluripotent stem cells

IPSCs are derived from mouse embryonic fibroblast (MEF) cells derived from day 13.5 embryos from the OG2 transgenic strain carrying green fluorescence protein (GFP) under control of the Oct4 promoter (Oct4-GFP), which were then transfected by retrovirus particles containing *Oct4*, *Sox2*, and *Klf4* genes (9) and cultured in MEF medium consisting of 1 mL of DMEM (Thermo Fisher Scientific). IPSCs were labeled genetically with GFP to follow cells. Stem cell cultures were generated as described before containing 30×10^5/mL of IPSCs. The solution containing IPSCs was placed in a 10-µL injector for the transplantation procedure.

2.3. Transplantation into the cochlea

Bullectomy and cochleostomy was performed on all rats via a retroauricular approach (10). Preoperative antibiotherapy (cefazolin at 500 mg/kg daily, IM) for prophylaxis and IM cyclosporine (15 mg/kg daily) from preoperative day 2 until postoperative day 7 to prevent immune reactions were administered to all animals (11).

After a right postauricular incision, a skin flap was elevated in the subplatysmal plane (Figure 1a). The angle of the mandible, masseter muscle, and parotid gland were identified. The trapezius muscle was identified and by caudal dissection the facial nerve was found. The otic bulla was exposed by retrograde tracing of the facial nerve (Figure 1b). After drilling of the right otic bulla, the stapedial artery and the basal turn of the cochlea were identified (Figure 1c). Cochleostomy was performed right

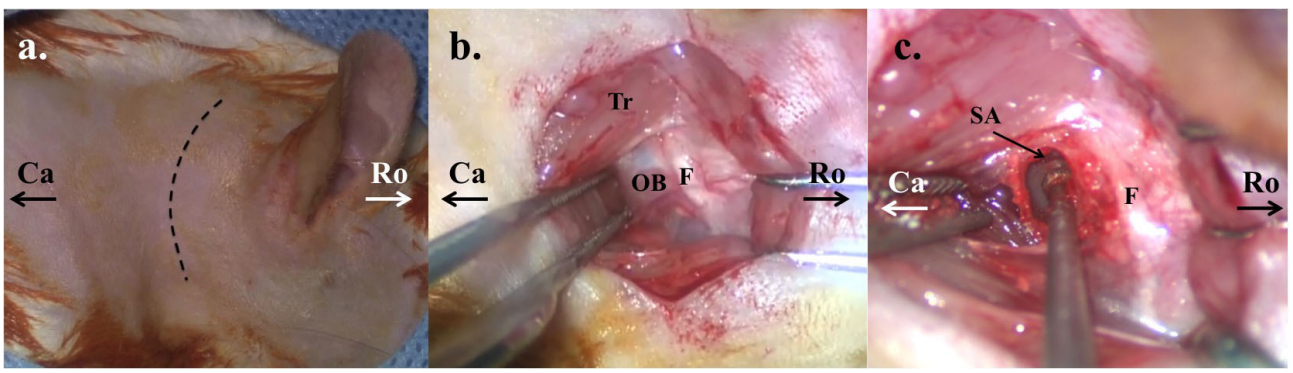

Figure 1. a) Retroauricular incision of the right ear, b) otic bulla (OB) and facial nerve (F) of the right ear, c) cochleostomy location according to stapedial artery (SA). Ro: Rostral side, Ca: caudal side, Tr: trapezius muscle.

afterwards (12). The solution containing IPSCs (30×10^5 cells, 10 µL DMEM) was injected at a speed of 1 µL/min. A micromanipulator and a 30-gauge Hamilton microsyringe (Hamilton Co., Bonaduz, Switzerland) were used in this process. A small piece of muscle tissue was placed over the cochleostomy and fibrin sealant was applied briefly.

2.4. Cochlea extraction

All the rats were sacrificed and decapitated 4–6 weeks after the operations. Extractions were made under the magnification of a surgical microscope (Leica M400 E). After the skin was excised, the mandible was dislocated and the skull base was exposed. The otic bulla was dissected in suture lines and the cochleostomy area was exposed. This region was evaluated for possible complications like erosion or infections. Afterwards, the skull was separated in the midline (Figures 2a and 2b). The right temporal bone of each rat was excised from the surrounding bony structures and cochleae were saved for histologic examination.

2.5. Light microscopy and immunohistochemistry

The cochlea was then isolated carefully, dissecting from the suture lines attached to the adjacent bones. After fixation and decalcification of the cochlea with 4% paraformaldehyde solution (0.1 M PBS, pH 7.4, 8 h) and 0.1 M 10% EDTA solution (20 days), specimens were aligned on a cryomatrix for sectioning and histopathological analyses. Cochleae were then frozen in OCT. compound media (Tissue-Tek, Sakura Finetek USA, Torrance, CA, USA). Alignment was adjusted so that the axial line between the apex of the cochlea and the cochlear nerve was parallel to the cutting plane. Transparency acquired after the decalcification along with the stapedial artery was used as a landmark for identifying the apex of the basal turn (Figure 2c). After that midmodiolar sections were achieved. H&E-stained sections were examined under light microscopy. For immunohistochemistry, the cryostat sections were thawed and rehydrated in PBS for 5 min and kept in acetone solution at 4 °C for 30 min. The sections were incubated at 4 °C overnight with anti-Nestin primary antibody (ab6142, Abcam, Clone:2Q178, 1:100) for neural stem cell differentiation. The slides were rinsed in PBS for 5 min at the end of the incubation. The secondary antibody cocktail was incubated at room temperature for 8 min. Negative control (normal inner ear) and positive control (mouse brain and normal inner ear) stainings were conducted.

2.6. Statistical analysis

Statistical analysis was carried out using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). The significance of the differences between groups in terms of mean values was investigated by Kruskal–Wallis test. $P < 0.05$ was considered significant.

3. Results

This study consisted of 35 adult WARs, which were operated on according to the study timeline (Table 1). Mean times to reach the bulla and appropriate cochleostomy were 4 min (2–13 min) and 10 min (4.5–22 min). The mean operation time was 31.1 min (11–205 min).

After the ototoxicity model, hearing thresholds between 30 and 60 dB were considered as moderate and those higher than 60 dB were considered as severe hearing loss (13). According to this, 5 rats (33.3%) had severe and 3

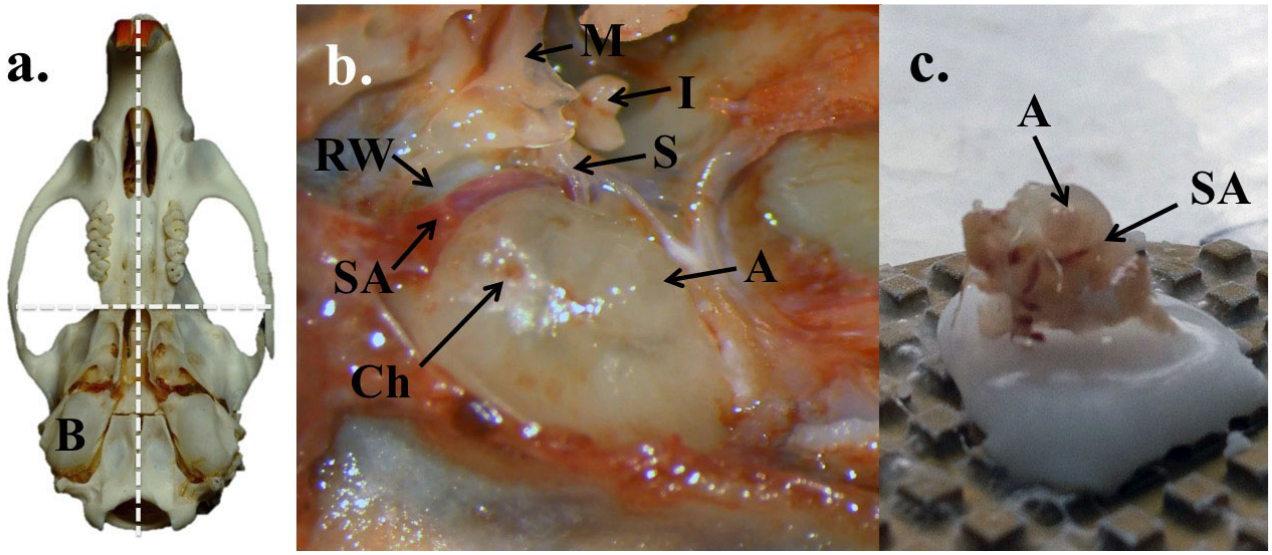

Figure 2. a) Ventral view of the skull, white dotted lines showing the cutting lines for extraction; b) middle ear structures critical for cochleostomy; c) alignment of the decalcified cochlea for sectioning. B: Right bulla, RW: round window, SA: stapedial artery, Ch: cochleostomy site, A: apex of the cochlea, S: stapes, I: incus, M: malleus, SA: stapedial artery.

Table 1. Clinical set up and timeline for groups.

	n	BAER	Ototoxic agent	BAER	Right ear surgery	Cochlear injection	BAER
Group 1	5	+	SF	-	+	IPS	+
Group 2	5	+	SF	-	+	DMEM	+
Group 3	15	+	Amikacin	+	+	IPS	+
Group 4	10	+	Amikacin	+	+	DMEM	+

rats (20%) had moderate hearing loss in group 3. In group 4, 2 rats (20%) had severe and 4 rats (44.4%) had moderate hearing loss. One rat died during the ototoxicity model (group 4).

Five rats (one each from groups 1, 2, and 4 and 2 rats from group 4) were excluded from the study due to intraoperative complications. Four of these intraoperative complications were large cochleostomies and one was major artery damage. Four rats were excluded from the study after postmortem examination of the otic bulla due to infection and wide erosion of the cochlea wall (two from group 3 and two from group 4).

The postoperative hearing thresholds of the method control (group 1) and sham operation (group 2) groups were 57.5 and 60 dB, respectively. There was no statistical significant difference in terms of hearing levels between these two groups.

The ABR results of groups 3 and 4 are shown in Table 2. The mean hearing level of the study group after ototoxic administration was 53.2 dB (range: 10–90). The mean hearing level of the rats to which IPSC and DMEM were applied were 86.8 dB and 80 dB, respectively. There was no significant difference between those two groups.

Cochlear sections were microscopically examined (Figures 3a–3f). Microscopically, a cochlear section from a normal rat showed hair cells (Figures 3a and 3b), whereas there were no hair cells in the cochleae of the IPSC-injected rats (Figures 3d and 3e). Immunohistochemical findings revealed that Nestin-positive hair cells were found within the cochleae of normal rats (Figure 3c); on the other hand, no hairy cells were seen in the cochleae of the IPSC-injected rats (Figure 3f).

4. Discussion

SNHL is a worldwide problem affecting more than 300 million people. As the regeneration capacity of inner ear structures is very limited, patients suffering from moderate to severe SNHL have limited treatment options. The possible rehabilitation methods to restore hearing in this situation seem to be conventional hearing aids or cochlear implants. However, there are huge amounts of patients whom we are unable to help. Besides this, these treatment methods are highly expensive and many countries do not refund the costs (1,2). Therefore, alternative modalities are needed for hearing restoration.

The transplantation of stem cells and progenitor cells into cochleae to regenerate the neural structures of the inner ear was found to be a potential to cure SNHL (14). The basic philosophy behind this idea was that the stem cells would replace the degenerated hair cells in vivo depending on the experience gathered from therapeutic stem cell applications in other degenerative diseases such as heart disease, diabetes, Parkinson disease, and other neurodegenerative disorders (14).

Numerous types of stem cell were used to show the possible therapeutic effect of stem cells after sensorial hearing loss. Corrales et al. studied ESCs in gerbils with damaged spiral ganglions and suggested that these stem cells help regeneration in neural tissue (5). Sharif et al. applied bone marrow-originated and stromal-marked stem cells into the perilymphatic space in the cochlea for the treatment of SNHL. They stated that these stem cells passed to the scala media within 2 weeks after the operation (15). Hakuba et al. applied neural stem cells via the round window in a cochlear ischemia model in guinea pigs and stated that the hearing results were better in subjects with stem cell transplantation (16). Cho et al. delivered mesenchymal stem cells to hearing-impaired guinea pigs via the round window. They reported an increase in the spiral ganglion cells and improvement in the hearing status in ABRs (17).

We designed this study to reveal the possible role of IPSCs in restoration of hair cells. We structured our hypothesis and methods based on previously published experiments. We reviewed the literature thoroughly during the study design, and we took necessary measures to minimize technical errors. The main strength of the current study comes from the utilization of distinct groups to reveal the possible therapeutic outcome of IPSC transplantation after hair cell damage (group 3) when compared to a placebo (group 4). Additionally, we set up two other groups to see the behavior of IPSCs in normal cochleae (Group 1) and to see the effect of inner ear surgery on normal-hearing cochleae (Group 2). We utilized a meticulous technique

Table 2. ABR results of groups 3 and 4.

Group	No.	BAER after ototoxicity (dB SP)		Deafness	Final BAER (dB SP)	
		Right	Left		Right	Left
Group 3	12	60	90	Advanced	100	100
Group 3	17	90	80	Advanced	95	90
Group 3	20	90	70	Advanced	100	70
Group 3	21	90	90	Advanced	100	90
Group 3	15	50	80	Moderate	90	60
Group 3	18	55	70	Moderate	70	70
Group 3	11	20	10	None	80	40
Group 3	14	60	20	None	100	40
Group 3	19	20	20	None	60	70
Group 3	24	20	20	None	90	50
Group 3	25	10	10	None	70	30
Group 4	29	90	70	Advanced	90	60
Group 4	32	100	100	Advanced	100	100
Group 4	26	50	45	Moderate	80	70
Group 4	27	35	60	Moderate	70	50
Group 4	31	45	50	Moderate	80	70
Group 4	30	20	20	None	60	50

and provided a sophisticated laboratory environment during each step of our experiments. However, we were unable to show restoration of hair cell damage, both electrophysiologically and histopathologically. Therefore, we would like to review our experiment and discuss the potential errors or shortcomings of the current study, for directing future studies.

4.1. Could there be a difference between using IPSCs or other types of stem cells?

IPSCs are very similar to ESCs in their molecular and functional features and are a new source for pluripotent stem cells. Being relatively simple in production and free of ethical arguments, IPSCs have become a valuable method in regenerative medicine. Nishimura et al. conducted an experiment to reveal the potential of IPSCs as a source of transplants for the restoration of auditory spiral ganglion neurons (8). They used mouse-derived IPSCs and cultured those cells with PA6 stromal cells (RCB1127; Riken Cell Bank, Kobe, Japan) for neuronal induction. Later, they cocultured with cochlear explants for forming projections to auditory hair cells. They transplanted induced IPSCs to the cochleae through the round window (scala tympani) and dissected cochleae 1 week after transplantation. They saw settlement of IPSCs to cochleae and some transplants expressed vesicular glutamate transporter 1, which is a marker for glutamatergic neurons. These findings indicate that IPSCs can be used as a source of transplants for the regeneration of spiral ganglion neurons (8). Similar results were also reported with IPSC use when compared to ESCs or neuronal stem cells (14,16,18,19). We also used mouse IPSCs, but we chose not to induce IPSCs before application based on former literature that showed migration and differentiation of mouse ESCs transplanted into cochleae after aminoglycoside-induced hearing loss (6). Therefore, further studies are needed to reveal the effect of neuronal-induced IPSCs on hair cell restoration.

One interesting finding in the later experiments of Nishimura et al. was formation of teratoma in some IPSC lines that were derived from mouse tail fibroblasts (20). We encountered teratoma formation neither in group 1 subjects (which had IPSC transplantation to normal cochleae) nor in group 3 subjects (which had IPSC transplantation after hair cell loss). One possible explanation for not encountering teratoma in our subjects is that we utilized IPSCs derived from embryonic fibroblasts (9). Additionally, the IPSCs that we used in our experiments were produced without the use of the c-myc oncogene, which might contribute to having more stable cells without teratoma formation (9).

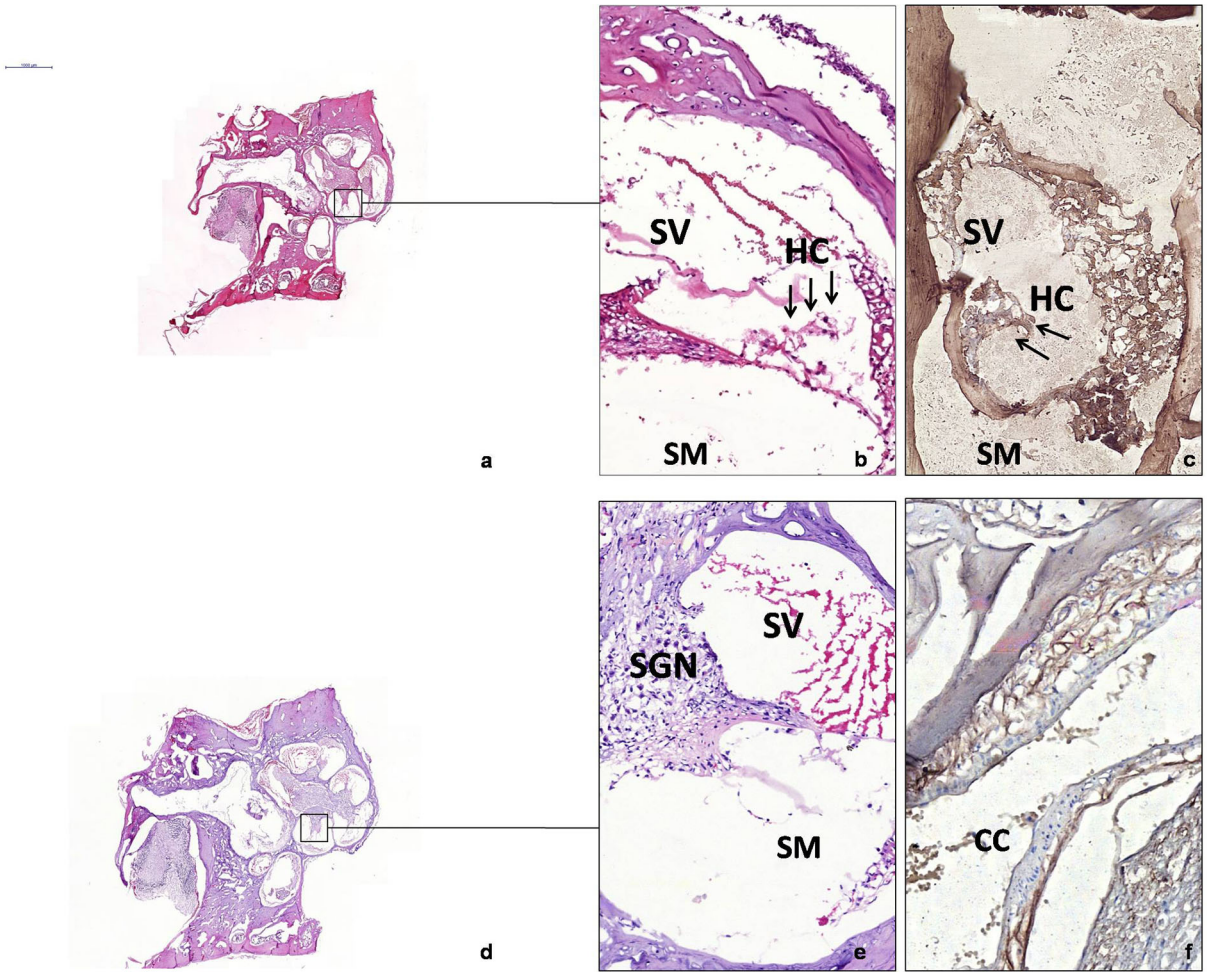

Figure 3. a, b) A cochlear section from a normal rat, organ of Corti stained by hematoxylin and eosin (H&E, 20×, 308×). c) Nestin-positive hair cells within the inner cells (278×). d, e) A cochlear section from a deaf rat, organ of Corti stained by hematoxylin and eosin (H&E, 23×, 251×). f) Immunohistochemical evaluation revealed no hairy cells in the cochlea of the IPSC-injected rats (455×). SV: Scala vestibuli, SM: scala media, HC: hair cell, SGN: spiral ganglion neurons, CC: Claudius cells.

4.2. Could there be any negative effects of transplanting mouse-derived IPSCs to rat cochleae?

The IPSCs used in this study was provided from mice. Therefore, we utilized immune suppression with cyclosporine after IPSC transplantation. However, cyclosporine itself is known to have ototoxic and cytotoxic features and could have inhibited the differentiation of IPSCs to hair cells in our experiments. This issue was met in previous studies: Zhao et al. transplanted mouse ESCs to rat cochleae after aminoglycoside-induced hearing loss (6). For preventing immune reaction, they also used intramuscular injection of cyclosporine at a similar dose and period to that in our study. They were able to show migration and differentiation of mouse ESCs in rat cochleae. Similarly, Okano et al. used mouse ESCs in guinea pigs for restoration of spiral ganglion neurons (18). Cho et al. used human mesenchymal stem cells in an auditory neuropathy guinea pig model (17). Therefore, use of homograft or xenograft stem cells does not seem to affect the experiment, at least not in murine models.

4.3. What would be the further directions for future studies?

The purpose of the current study was to show the possible therapeutic efficacy of IPSCs after hair cell damage. Therefore, we added ABR study to our design for being able to measure the final outcome and compare it between groups. Waiting 4 weeks before the final ABR test hindered us from following the behavior of IPSCs, with immunohistochemistry and immunofluorescent microscopy, at the early posttransplant period. Therefore, in future studies, we suggest to show the biological behavior of stem cells with histological exam of serially

sacrificed animals in the first place. Electrophysiological experiments would definitely contribute in showing the functionality of regenerated cells in following experiments.

The other issue that needs to be clarified in future studies is whether the neuronal induction of stem cells before transplantation is necessary or not. Some authors advocate that an acute injury environment in cochleae induces pluripotent stem cells to migrate to the injury area and differentiate into hair cells (6,21). Others utilized certain procedures for inducing stem cells to differentiate into neural cell lineages (2,14,17,18). Further experiments are needed to provide better evidence on this subject.

4.4. Conclusion

Stem cell use in restoration of sensorial hearing loss is still an evolving idea. There are fewer than 50 published articles in the English literature, including reviews, on this subject. Stem cell transplantation to the cochlea of an experimental animal is a meticulous procedure in every step. There are many technical issues, such as choosing the right animal and appropriate anesthesia technique, keeping the animal alive after the procedure, and ensuring immunosuppression when needed. Therefore, we hope that our experience and perspective will help researchers in future studies on this subject.

Acknowledgments

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