Cochlear-Bioelectrode: Auditory response to cochlear implant can be improved by stem cell delivery into inner ear in a pig model

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Research Article

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Abstract

Cochlear implant (CI) is the most successful auditory prosthesis and has changed the life for nearly half million people with profound hearing loss. The number and function of spiral ganglion neurons (SGNs) in the cochlea are crucial to CI performance. Clinically, some patients with SGNs degeneration fail to return to normal life due to unsatisfactory performance of their CIs. In this study, we show in a swine model that combined stem cell delivery and electrical stimulation (ES) via CI (defined as Cochlear-Bioelectrode) achieved stem cell enrichment near the SGNs and lowered CI stimulation thresholds. ES generated by the cochlear implant electrode array guided human induced pluripotent stem cells (hiPSCs) with a green fluorescent protein (GFP) marker to cross the osseous spiral lamina via naturally existing openings and congregate in the SGN region. In seven days, surprisingly efficient hiPSC migration into the SGN region was demonstrated, while hiPSC delivery and enrichment in the SGN region failed without ES. After two weeks of combined electric stimulation and stem cell delivery treatment, the number of SGNs increased significantly in the 60-day old pig model (mean number = 129.6/field), as compared to untreated deafness pigs of the same age (mean number = 6.2/field). With the replenished SGNs, CI stimulation thresholds were significantly reduced and hearing sensitivity and dynamic range extended, as demonstrated by electrically evoked auditory brainstem responses. Our findings provide the first evidence that in vivo Cochlear-Bioelectrode (CBe) may be feasible with broad promising clinical applications.

Introduction

Deafness is a sensory disability that can severely affect a patient’s quality of life. According to the latest data by the World Health Organization (WHO), the number of people with hearing impairment reached 466 million in 2018, and it is estimated that by 2050, the number will reach 933 million (nearly one in every ten people). Currently, cochlear implant (CI) is the only effective treatment for severe and profound sensorineural deafness (1). However, the performance of CI is not satisfactory in some patients with SGNs degeneration, such as in Waardenburg Syndrome (WS), preventing them from returning to normal life (2, 3). Improving CI performance in these patients has become a major clinical concern.

Previous studies have shown that the number and function of SGNs in the cochlea are crucial factors to CI performance (4). The unsatisfactory CI performance in patients with WS is also related to lack of sufficient SGNs as a result of secondary degeneration due to hair cell death (5). The most promising clinical strategy so far is to replenish the spiral ganglion neurons, hence to reconstruct the auditory conduction pathway, by stem cell transplantation (6). In previous studies, it has also been proved that replacement of SGNs by stem cells for sensorineural hearing loss was feasible (7). However, stem cell transplantation by direct injection into the cochlear modiolus or cochlear scala media (SM) can cause severe tissue damage and is thus impossible to be translated into the clinic (8).

Previous studies have confirmed that naturally existing tiny openings in the osseous spiral lamina (OSL) provide communication for dyes and neural growth factors to move between the scala tympani (ST) and the cochlear modiolus where sit the SGNs. Due to the small diameter of these openings (2-20 um), only a
single cell can pass through (9). When transplanted to the ST, stem cells will congregate and are unable to pass through the pores as single cells. We speculated that stem cells migration from ST to the SGN region might become possible if facilitated by an external force (such as electric stimulation) that separates the cells and drive them through the openings individually. In fact, there is plenty of existing evidence that the electric stimulation (ES) drives cells to move in a directional fashion, namely galvanotaxis(10, 11).

As already known, tremendous progresses have been made in CI technology and CI has become a routine clinical practice (12). A channel can be added in the electrode array for stem cells transplantation into the cochlea (making it a “Cochlear-Bioelectrode”). Electric stimulation (ES) via this Cochlear-Bioelectrode (CBe) can be used to guide stem cells crossing the cochlear partition and complete cell replacement therapy in the inner ear.

In this study, we investigated whether a cochlear implant electrode array could be used to generate electric stimulation (ES) to guide hiPSCs crossing the cochlear partition for enrichment in the SGN region and improvement of CI performance in a swine model of WS. We hope the findings can pave the way for integration of cell therapy into cochlear implant surgery, which can be translated for clinical application.

Results

In vitro Electric stimulation (ES) for hiPSC migration.

In this study, ES was used to facilitate transplantation of hiPSCs (an iPSC line derived from human skin) into the cochlear ganglion region in order to improve the outcome of CI in a swine model of genetic deafness. The hiPSCs can be labeled with multiple stem cell markers, including NANOG, OCT4, SOX2, SSEA4, TRA60 and TRA81, exhibit high alkaline phosphatase (AP) activity, and show similar growth curve as a human embryonic stem cell line in culture (Figure S1).

In vitro electric stimulation of cultured hiPSC resulted in relatively low rate of cell proliferation during the first 48 hours, which accelerated after 50 hours. As we believed that a time window between 24-48 hours was ideal for studying the effect of electric stimulation, we recorded images of hiPSC migration under ES from a CI device in culture continuously at a 10-minute interval for 24 hours using a Delta Vison Elite Live Cell Imaging System (Figure S2A). Using the system, we were able to simultaneously track 5 clones in one visual field with each clone containing 20 hiPSCs on average. In the presence of ES, hiPSCs were found to migrate towards the electrode array, with a clear migration path observed after 3 hours at a speed of 0.007 ± 0.002 µm/s at a current level (CL) of 50. Without ES, hiPSCs showed random movement at a mean speed of 0.004 µm/s (Figure S2, B and C). hiPSC migration speed peaked at 50 CL and slightly decreased as current level reached 100 CL, with average speed maintained at approximately 0.005 ± 0.002 µm/s at 100-225 CL. hiPSC migration speed was significantly higher under ES than without ES (one-way ANOVA, p=0.032), but there was no significant differences for electrical currents above 50 CL (Figure S2D) (one-way ANOVA, p=0.127). The results showed that electric stimulation derived from cochlear implant electrodes guided and accelerated hiPSC migration.
hiPSC traveling across cochlear partition to the SGN region under ES.

After in vitro observation of ES effects on hiPSC migration, we investigated its in vivo effects by placing a CI electrode array into the scala tympani via the round window membrane (RWM) in 16 deaf RongChang pigs (divided into a 3-day, a 7-day with 4 in each group, and a 14-day study group with 8), which extended about 3/4 of the length of the first cochlear turn, measuring approximately 18 mm from the RWM. The ES was generated between the stimulating electrode and the reference electrode (Figure 1A) and switched on for three hours twice in the 3-day group, and three times in the 7-day and 14-day groups (Figure 1B). In order to record hiPSC distribution in the cochlea after transplantation, seven positions were marked from the basal turn to the apical turn (positions 1-7) (Figure 1E).

After 7 days of ES, most hiPSCs were seen in the SGN region (Figure 1C), whereas without ES, hiPSCs only appeared in the SM and ST regions (Figure 1D). Cell counts in the cochlea ranged from 1342 to 2484/field (mean = 1911/field) after 3 days with ES, and from 50 to 311/field (mean = 161/field) after 7 days with ES (two-way ANOVA, P=0.012) (Figure 1G).

Innate pores as the channel for cell delivery.

Scanning electron microscopy (SEM) revealed numerous mesh-shaped pores in the mucosa in the scala tympani near the modiolus, as well as in the osseous spiral lamina (OSL) (Figure 2A), most dense in the area where the OSL and modiolus meet (Figure 2B). The diameter of pores varied from 1 to 20 μm in the mucosa (Figure S3), and about 10 μm in the OSL (Figure 2C). At high magnification, it was clearly shown that the pores in the scala tympani mucosa and OSL constituted a natural channel system leading from the scala tympani to the SGN region, providing a pathway for transplanted stem cells in the scala tympani to enter the SGN region (Figure 2D).

hiPSCs showing nerve cell characteristics two weeks after migrating into SGN region.

With ES, some hiPSCs in the 14-day group started to differentiate into neuronal phenotype as demonstrated by neurofilament 200 (NF200) labeling (Figure 3, A and B). The number of GFP-positive cells and double-stained cells were counted based on the region (n=4). The average per field total cell count in the SGN region was 708, and average GFP-positive cell count was 129 (18% of total cells) including 82 double-stained cells with nerve cell characteristics (63.5% of GFP-positive cells) (Figure 3C). SEM showed some SGN-like cells in the SGN region, although with small cell body, spherical in shape and showing protruding dendrite structures (Figure 3D).
hiPSC and Electric stimulation (ES) treatment resulted in partial SGNs recovery

In 60-days old normal hearing Rongchang pigs, 727.3 SGNs were counted per field on average, with large cell body and clearly defined nucleus (Figure 4, A and A'), while in mutant deafness Rongchang pigs of the same age, SGNs were almost completely absent (mean = 6.2 per field), with the few remnant SGNs showing loose structures (Figure 4, B and B').

Two weeks after hiPSC transplantation and ES treatment and at the age of 60 days, the number of SGNs in the treated right ear in mutant deafness pigs increased significantly (mean = 129.6 per field), with large cell body and clear cell nuclei, as well as well-defined boundaries between cells, a strong contrast to findings in untreated contralateral (left) ear (Figure 4C). However, compared with normal hearing pigs, this represented merely a 17.8% (129.6 vs 727.3/field) recovery. In the meantime, cell number in the untreated left ear in these pigs were not significantly different from that in those untreated mutant deafness pigs (Figure 4D). Additionally, the distribution of newly differentiated SGNs was balanced from position 1 at the basal turn to position 7 at the top of the cochlea (Figure 4E).

Replenished SGNs improved CI performance

To test whether the newly differentiated SGNS have function, electrically evoked auditory brainstem responses (EABR) were recorded from surface electrodes in both treated and non-treated pigs, using electrode array number 2, 6, 10, 16 and 22 for stimulation to represent neural responses from low to high frequencies, respectively (Figure 5A).

In deaf pigs after 2 weeks of hiPSC and ES treatment, well differentiated EABR waves III and V were recorded, especially at a 160-CL (Figure 5D), similar to wild-type pigs with normal hearing in whom EABRs were elicited at 100 CL (threshold) by No. 10 electrode array and showed well-differentiated waves III and V at 160-CL (Figure 5C). Whereas no EABRs could be elicited using the No. 10 electrode at maximum 200-CL in pigs treated with hiPSC injection without ES (Figure 5E). Using No. 10 electrode array for stimulation, EABR thresholds in pigs treated with both hiPSC transplantation and ES were significantly lower than in those treated with only hiPSC transplantation or only ES (p<0.05) (Figure 5B).

When analyzing EABR frequency characteristics, average EABR thresholds in ears treated with both ES and hiPSC transplantation were significantly lower than in those treated with only hiPSCc transplantation or only ES across all stimulating electrode arrays (Figure 5F), These results suggest enhancement of CI performance by ES mediated hiPSc therapy across all frequencies, with the best outcome at low frequencies.

Discussion

SGNs are crucial to the success of cochlear implant (13). Primary and secondary Loss of SGNs as a result of genetic diseases, drug related damage, secondary degeneration and other causes can severely
limit the efficacy and growth of cochlear implant application in clinical practice. Stem cell transplantation to supplement and replace damaged SGNs can effectively solve this problem (14). However, translation of stem cell therapy to clinical application for deafness treatment is hindered due to the deep location of SGNs in the cochlear modiolus and often presence of damage to delivery pathways secondary to cochlear pathologies (15-17). This study demonstrated application of the CBe concept, which integrates stem cell therapy with cochlear implant stimulation, in a swine model of WS (18, 19) to facilitate migration of hiPSCs to the SGN region by electrical stimulation generated by CI electrodes. The increased SGNs significantly reduced electrical stimulation auditory response thresholds and improved CI sensitivity and dynamic range (20).

In this study, cochlear implant electrodes widely used in clinical practice were skillfully applied to cell culture for electrical stimulation to affect hiPSC migration (20). Real-time image monitoring showed cell deformation and amoeba movement under CI generated electrical stimulation (supplementary Movies 1 and 2). Studies have shown polarized membrane proteins in many cell types, which can undergo irreversible rearrangement under electrical stimulation, leading to cell deformation (21, 22). Recent studies have also shown that direct current or pulsed electric fields can affect migration of neural precursor cells and iPSCs, similar to our in vitro findings which helped select the suitable hiPSCs for the next step in vivo research, as well as the suitable CI stimulation mode (23). Our work also confirms that the CI electrode array can be conveniently used as a tool to provide safe electrical stimulation in the cochlea.

There have been no data on in vivo cell motility induced by long-term electric stimulation, probably due to difficulties to establish such stimulation and potential electrolysis of the electrode and damage to surrounding tissues by long-term DC electric field stimulation (24). The cochlear implant, which has been widely used in clinical practice, can easily solve the above two problems.

Interestingly, although the scala tympani and SGN regions are separated by bone and mesothelial cells, with electric stimulation, hiPSCs transplanted into the ST could migrate toward the SGNs, which was not possible in the absence of electric stimulation (Figure 1, C and D). It is known from previous studies that communication exists between the cochlear perilymph and the SGNs in the cochlea, likely due to the fact that the thin bony sheet covering SGNs is porous (25, 26). The “pores” hereby formed are canaliculi perforates and were first discovered in cats by Schuknecht and Sei (27), and subsequently in other species including the guinea pig, chinchilla, squirrel monkey and rat (28). Our results also confirm that there are a plenty of natural openings connecting the ST and SGN regions in swine cochleae (29).

The question remains - why are hiPSCs unable to cross the cochlea to arrive at the SGN region without electric stimulation even in the presence of the openings for communication mentioned above? We hypothesized that electric stimulation can help overcome their adhesion to each other and to the cochlear wall, so they can move as single cells rather than to aggregate into a group of cells, which would provide hiPSCs with a chance to move through the natural openings with amoeba movement to enter the SGN region. Due to the limited diameter of these natural openings (up to 20 um), cells clustered in the
perilymph cannot pass through. In human cochlea, the modiolar wall of the ST in the first and second turn appear to be porous, forming a perilymphatic communication route to the perivascular and perineural spaces in the modiolus (30). Therefore, this cell delivery strategy may be successfully adopted in the clinic.

A cochlear implant electrically stimulates SGNs to provide auditory cues for patients. The survival of functioning SGNs are therefore critical to the performance of the cochlear implant (31, 32). In animals with extensive neural pathology, EABR thresholds are elevated with response amplitudes greatly reduced. With hearing loss, SGN may gradually degenerate following cochlear hair cell loss, leaving fewer neurons available for stimulation, which compromises the efficacy of cochlear implants (33-35). Compared to the normal cochlea, the cochlea of congenitally deaf Rongchang pigs showed far fewer SGNs and severely reduced EABR amplitudes after the cochlear implants. Significantly, we found that the density of SGNs increased significantly in response to electric stimulation after hiPSC transplantation, as compared to no stem cell transplantation and ES, with significantly lower EABR thresholds. Significant improvement in EABR thresholds is likely due to complicated interactions involving transplanted hiPSCs (with improved migration and differentiation), electric stimulation and existing SGNs (including better survival), ultimately leading to improved cochlear implants outcome.

Our work with the swine model of WS showed that similar amplitudes of nerve responses could be obtained in animals treated with stem cell transplantation and ES with less than half of the electrical stimulation intensity required in untreated control animals. Speech recognition performance in CI recipients is related to frequency spectrum coverage and dynamic range of the CI (36). A lower electrical stimulation response threshold means a larger pulse output dynamic range, and a lower electrical stimulation output also allows closer spacing of the electrode array, so more electrodes can be deployed within the 31mm implant electrode length limit for increased contact with SGN cell bodies and dendrites and best possible tone recognition and music appreciation (37). In addition, minimizing electrical stimulation output also extends battery replacement cycle, improving patient's experience with device use.

In this study, since the CI was upgraded with delivery channels for delivery of biological materials such as stem cells and nutrient factors, we call it “Cochlear-Bioelectrode” (Figure 6A). At the time of cochlear implantation, a device can be left in the middle ear for repeated delivery of biological materials such as stem cells, nutrient factors and gene editing vectors into the cochlea (Figure 6B) to achieve nerve regeneration, degenerative nerve repair, nerve function reconstruction and synergetic nerve prosthesis (Figure 6C). We believe that this study should open a novel approach for treatment of deafness with the CBe that can be promising for clinical use. Moreover, this strategy may be applicable not only to inner ear diseases, but also to other conditions where nerve regeneration is difficult or the effectiveness of a prosthesis is unsatisfactory, such as spinal cord injury, Alzheimer's disease and other neurodegenerative diseases.

Materials And Methods
**Culture and identification of hiPSCs**

Human induced pluripotent stem cells (hiPSCs) were derived from skin fibroblasts of a female adult (Beijing Cellapy Biotechnology Company). The hiPSCs were cultured in a feeder-independent and serum-free defined medium. After large quantity of hiPSCs were obtained, they were cultured in 12-well plates with the same density. Moreover, hiPSCs was counted at 24, 48, 72, 96 and 120 hours to generate a growth curve. Their pluripotency and stem cell nature were characterized with karyotype analysis, alkaline phosphatase staining, and the immunofluorescence detection of pluripotent markers expression, including NANOG, TRA-1-60, SOX2, OCT4, SSEA-4, and TRA-1-81.

**hiPSCs morphology**

hiPSC images were obtained using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Cells were stained with 0.1 μg/mL DAPI (4', 6-diamidino-2-phenyindole) for 30 minutes at 30 °C.

**Animals**

Adult Rongchang pigs (40-120 days old, 8-12 kg, with equal males and females) provided by Chongqing Academy of Animal Science, Key Laboratory of Pig Industry Sciences Ministry of Agriculture, were used in the study. The pigs with congenital deafness due to a MITF gene mutation were previously characterized (18). The sixteen deaf RongChang pigs (divided into a 3-day, a 7-day with 4 in each group, and a 14-day study group with 8) received cochlear implants with or without hiPSC injection. Ten Rongchang pigs with normal-hearing and ten deafness pigs were used as the controls. The cochlea was harvested as previously described and used for genotype identification by PCR.

**The cochlear implant and implantation surgery**

A 24-channel cochlear implant (CS-10A, Nurotron, Hanzhou China) was used in this work. Surgical techniques and implant testing protocols were described in previous articles (38).

**Electrical auditory brainstem response (EABR) testing**

EABR testing was performed using the software provided by Zhejiang Nurotron Biotechnology Co., LTD. (China) that was coupled with the implant. Electrodes 2, 6, 10, 16 and 22 were selected for EABR testing, starting at 200 CL and decreasing at 10 CL until the minimum intensity producing discernable EABR
waveforms on five repeats (threshold). Threshold measurement was repeated three times and the average was used as the final threshold.

**HE staining of frozen cochlear sections**

After removal of the implant, bilateral cochleae were dissected, fixed, and decalcified. They were then dehydrated, embedded at low-temperature, and sliced serially at a thickness of 8 µm parallel to the axis of modiolus with a cryotome. After placed at room temperature (25 °C) for approximately 10 minutes, the slices were dried with a thermostatic dryer at 60 °C. Once cooled down to room temperature, they were washed with distilled water and stained with hematoxylin to remove oxide film. Subsequently, a hydrochloric acid-alcohol-mixture (1%) was used for differentiation until tissues were dyed pale pink. This was followed by washing (until the color changed to blue) and counterstaining with eosin for 30 seconds after flushing with water. The slices were then treated with gradient dehydration and mounted with neutral gum for examination under the microscope.

**Spiral ganglion cell counts**

The number of spiral ganglion neurons was determined as previously described (Murillo-Cuesta, 2010).

**Statistics**

The SAS 9.2 (SAS Statistical Institute, Cary, NC) was used for statistical analysis. A P <0.05 were regarded as statistically significant. The data from the study groups were tested for normal distribution and homogeneity of variance, and, if failing these tests, transformed into ranking data. For within-group comparison between treated (left) and untreated (right) ears, a nonparametric analysis of Wilcoxon signed-rank test was undertaken. The multiple-way ANOVA was used to assess multi-variate comparisons. All figures were created with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego CA, USA), with error bars indicating standard error (SE).

**Declarations**

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**Author contributions:** WC, WWG and SMY designed the study; WC, FJ, JNL, LWX, LDZ, LC, TC, NY, HZ, LZ, SLY, YZ, ZH, LLR, and WS performed the experiments; WC, JNL and SMY analyzed the data, and wrote the manuscript.

**Competing interests:** The authors declare no competing financial interests.

**Data and materials availability:** All authors wish to share our data and materials. Moreover, we make readily reproducible materials described in the manuscript, including new databases and all relevant raw data, freely available to any scientist wishing to use them, without breaching participant confidentiality.

The experimental protocol in our manuscript was approved by the licensing committee of Chinese PLA General Hospital (Beijing, China). All experiments related to animals, the Adult Rongchang pigs used were utilized in this trial based on Ethical Committee of Chinese PLA General Hospital (Beijing, China) in accordance with ethics/animal carer guidelines in China.

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