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Restoration of auditory evoked responses by human ES cell-derived otic progenitors

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Abstract

Deafness is a condition with a high prevalence worldwide, produced primarily by the loss of the sensory hair cells and their associated spiral ganglion neurons (SGNs). Of all the forms of deafness, auditory neuropathy is of a particular concern. This condition, defined primarily by damage to the SGNs with relative preservation of the hair cells [1], is responsible for a substantial proportion of patients with hearing impairment [2]. While the loss of hair cells can be circumvented partially by a cochlear implant, no routine treatment is available for sensory neuron loss since poor innervation limits the prospective performance of an implant [3]. Using stem cells to recover the damaged sensory circuitry is a potential therapeutic strategy. Here, we present a protocol to induce differentiation from human embryonic stem cells (hESCs) using signals involved in the initial specification of the otic placode. We obtained two types of otic progenitors able to differentiate *in vitro* into hair cell-like cells and auditory neurons that display expected electrophysiological properties. Moreover, when transplanted into an auditory neuropathy model, otic neuroprogenitors engraft, differentiate and significantly improve auditory evoked response (ABR) thresholds. These results should stimulate further research into the development of a cell-based therapy for deafness.

Hair cell-like phenotypes and sensory neurons, with different degrees of functional maturation, have been obtained from mouse stem populations [4-10]. After transplantation, some cell types have showed engraftment but none have demonstrated evidence of functional recovery [10-15]. Although useful for research purposes, these products are unsuitable for a therapeutic application and to date appropriate cell types of human origin have remained elusive. Neuroprogenitors isolated from mature human cochleae display limited proliferative and differentiating potential [16] while hESCs-derived neural crest cells

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Supplementary Information, Methods and Spreadsheets are linked to the online version of the paper.

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may differentiate into sensory neurons by exposure to BMP but lack true otic characteristics [17,18]. Recently, we isolated a population of bipotent stem cells from the human fetal cochlea (hFASCs), with the ability to produce hair cell-like cells and neurons [19]. However, although hFASCs can be expanded *in vitro* for ~25 population doublings, they eventually undergo replicative senescence. Hence, there is a need for a reliable, renewable source of human otic progenitors, with the ability to produce both cell types for sensory replacement.

FGF signaling is necessary and sufficient for the induction *in vivo* of the otic placode, the primordium of the hearing organ [20,21]. Since in the mouse the ligands involved in placode signaling have been identified as FGF3 and FGF10 [22,23], we hypothesized that exposure to these factors would trigger otic differentiation of hESCs. Initial experiments with embryoid bodies (EBs) confirmed FGF3 and 10 induction of otic features (Supplementary Fig. 1a) therefore we focused on developing a method devoid of this initial cell-aggregation step, which is prone to high variability. Undifferentiated colonies of hESCs were dissociated for plating as a monolayer on laminin-coated flasks (see Supplementary Methods). Under these conditions, FGF3+10 treatment induced the placodal markers *PAX8* and *PAX2,* either in the presence of KOSR or under defined conditions using DFNB medium (Supplementary Methods, Supplementary Figs. 1b-2). Global analyses of gene expression was performed using Affymetrix GeneChip arrays and, after normalization (see Supplementary Methods), samples were mined in two different ways. In the first we used the Gene Set Enrichment Analysis (GSEA) tool [24] to look for genes that were enriched in the entire list of probe sets, without establishing a priori cut off of differential expression (Supplementary Table 1-2). This analysis showed that a set of otic markers was significantly enriched in the FGF-treated samples when compared with the undifferentiated hESCs (normalized enriched score, NES: 0.568, family-wise error rate *p*-value, FWER*p* 0.046) or cells grown in DFNB (NES: 0.707, FWER*p* 0.019) (Supplementary Table 1). A second type of analysis assessed genes differentially expressed using predefined criteria for fold change cut off and statistical significance (see Supplementary Methods). A total of 1,424 genes (represented by 2,124 probe sets) was differentially upregulated in the FGF-samples when compared to undifferentiated hESCs, while 423 genes (505 probe sets) were upregultaed in the FGF-treated vs. the DFNB controls (Supplementary spreadsheets 1-2). On the other hand, 2,368 genes (3,231 probe sets) were downregulated in the FGF-samples vs hESCs and 482 genes (607 probe sets) were downregulated vs DFNB (Supplementary spreadsheets 3-4). In a gene ontology (GO) analysis, the GO terms ‘sensory organ development’ (EASE p-value score in FGF vs hESC: 3.92×10^{-15}; FGF vs DFNB: 0.022); ‘ear development’ (FGF vs hESC: 4.47×10^{-8}; FGF vs DFNB: 0.014) and ‘ear morphogenesis’ (FGF vs hESC: 3.08×10^{-6}; FGF vs DFNB: 0.0497) were highly enriched in the FGF-treated cells in both comparisons, while ‘mechanoreceptor differentiation’ and ‘auditory receptor differentiation’ were up in FGF vs hESC (See Supplementary Spreadsheets 5-8). Both bioinformatics analyses therefore suggested that the FGF treatment was generating a global change of transcription compatible with the induction of otic progenitors.

We also used immunostaining to examine the co-expression of PAX8 and SOX2, to define the otic progenitors at a cellular level. Otic progenitors grew as colonies after the inductive phase. Initial immunolabelling showed a relatively large proportion of double positive cells in the FGF-treated condition (~78%), in contrast to the relatively moderate upregulation of otic transcripts detected with the arrays. However, a subset of cells expressed very high levels of PAX8 and SOX2, and these were assessed with an automated microscopy platform (InCell Analyzer 1000) that enabled quantification of the number of positive cells and their relative intensity (Fig. 1 and Supplementary Figure 3). When a stringent threshold was selected (75^{th} intensity percentile per cell line and antibody, see Supplementary Methods) 18.3%±0.8 of the cells expressed high levels of PAX8 and SOX2 ($PAX8^{hi}SOX2^{hi}$) after

FGF treatment (against 0% obtained without the growth factors, $p<0.001$) while 18%±2 cells were $PAX8^{hi}/FOXG1^{hi}$ (compared to 4%±4 obtained in the control, $p<0.001$). $PAX8^{hi}SOX2^{hi}FOXG1^{hi}$ cells also expressed the otic markers PAX2, NESTIN, SIX1 and GATA3 (Fig. 2h and Supplementary Figs. 4-5a). It is likely that this subset of $PAX8^{hi}SOX2^{hi}FOXG1^{hi}$ expressing cells represents the otic progenitors. The reproducibility of the protocol was tested across the hESC lines H7, H14 and Shef3, which all gave comparable results (see Fig. 1 and Supplementary Figure 3). FGF3 and 10 induced two morphologically distinct types of otic colonies (Fig. 2a-h). One cell population showed a flat phenotype, with large cytoplasm and formed epithelioid islands (Fig. 2a-d), while the second was small, with denser chromatin and presented cytoplasmic projections (Fig. 2e-f). Given their morphological appearance we have operationally named them otic epithelial progenitor (OEP) and otic neural progenitor (ONP), respectively. The relative proportion of these progenitors was dependent on the cell line, plating density and the degree of cell separation (single cells versus cell clusters) (Supplementary Figs. 5-6 and Supplementary Methods). Progenitor colonies were purified using sequential dissociation (see Supplementary Methods), yielding moderately homogenous cultures of the desired cell colony type and were expanded in OSCFM (Otic stem cell full media, Suppl. Methods).

The differentiation potential of OEPs and ONPs was tested in 'neuralizing' and 'hair cell' culture conditions developed previously using hFASCs [19] (see Supplementary Methods). OEPs produced hair cell-like cells as defined by the simultaneous expression of ATOH1 and BRN3C, or BRN3C and MYO7A (~45%) (Supplementary Fig. 7). A small subset differentiated a rudimentary apical bundle, expressing ESPIN (Supplemntary Fig 8). These hair cell-like cells also expressed an outward K^+ current, the inward rectifier K^+ current I_{K1} and an inward Ca^{2+} current (I_{Ca}) (Supplementary Fig. 9). Under 'neuralizing' conditions, they produced a small proportion (~9%) of sensory neurons (Supplementary Fig 7). On the other hand, ONPs were committed to produce neurons. Under 'neuralizing' conditions, almost all cells developed a bipolar morphology and were positive for BRN3A and β-tubulinIII, as well as for β-tubulinIII and NF200. They also expressed *NEUROD1*, *ISLET-1* and *TrkB*, a delayed-rectifier K^+ current (I_K), a Na^+ current (I_{Na}), and elicited single action potentials (Supplementary Fig. 9). No hair cell differentiation was obtained from ONPs under 'neuralizing' or 'hair cell' culture conditions. Detailed results are given in Supplementary Information.

The properties of ONPs *in vivo* were studied by transplanting them into ouabain-treated gerbils, a model of neuropathic deafness [25]. Application of ouabain directly to the round window selectively damages the type I SGNs, preserving the hair cells and the organ of Corti [26] (Supplementary Fig. 10). After ouabain application, only a small number of SGNs survived (6.4%, see Supplementary Table 3). Most of the surviving cells (~87%) were peripherin+, type II neurons therefore less than 1% of the original population of type I neurons remained (Supplementary Table 3 and Supplementary Fig. 13). Staining for myosin VIIa and the presence of distortion product otoacoustic emissions (DPOAEs) confirmed that the organ of Corti had not been damaged (Supplementary Figs. 10 and 11). DPOAEs are sounds produced as a consequence of electromechanical feedback from the outer hair cells and can be used to check their physiological integrity.

ONPs derived from Shef1 hESCs constitutively expressing either eGFP or Tomato fluorescent protein were expanded in OSCFM, dissociated with trypsin and delivered directly into the modiolus, approaching the cochlea through the round window. One set of animals was transplanted 3-5 days after ouabain application ($n=13$) while another was transplanted two weeks after the ototoxic drug ($n=5$). Since no functional or histological differences were encountered between the two groups ($p>0.05$; Supplementary Fig. 12), they were analyzed together. Two to three weeks after transplantation, five out of six animals had

surviving, transplanted cells grafted in the modiolus, forming an ectopic spiral ganglion (Fig. 3a-b). Cells in the marginal sides of the ectopic ganglion had undergone differentiation as judged by β-tubulin III staining (Fig. 3b) and displayed neural projections, targeting the organ of Corti (Fig. 3c-d). Animals were then followed up for 10 weeks posttranplantation (PT). Histological analysis after 10 weeks PT showed that the ectopic ganglion was still present and cells had also migrated into the Rosenthal's canal (Fig. 3e). Transplanted cells expressed the 3A10 neurofilament-associated antigen and NKAα3, a marker of type I neurons and afferent fibers in the inner ear [27] (Supplementary Fig. 14). Significantly, projections from the transplanted cells that reached the organ of Corti were targeting the hair cells, and fibers positive for NKAα3 and GluA2 were observed next to the basal pole of the inner hair cells suggesting the presence of synaptic connections (Fig. 3g). Moreover, fibers form the transplanted cells were visualized leaving the modiolus towards the brainstem (Fig. 3f). In the cochlear nucleus of three gerbils we found RFP-positive fibers also stained for synaptophysin, suggesting synaptic connections with the central auditory path (Fig. 3h-i). Transplanted ONPs contributed significantly to restore neuronal density (Fig. 3j, $p<0.01$). While 112.5±11.9 $TuJ1^+$ mm^{-2} cells were present in the ouabain-treated, untransplanted ears, 546.4±30.6 $TuJ1^+$ mm^{-2} were found after transplantation. From these, 94.9 ± 0.3% were also GFP (or Tomato) positive, confirming their exogenous nature (Supplementary Table 4). The number of projections detected in the brainstem was considerably lower than the number of transplanted cell bodies identified in the ganglion. While this could be explained by the limited sorting of fluorescent protein into the long afferent fibers, the pathfinding of the central innervations should require further future exploration. No tumors were detected in any of the transplanted animals at any stage throughout the experiment.

Functional performance was determined by measuring ABRs thresholds. These were established based on the wave ii-wave iii (P2-N3) amplitude [28]. These waves are generated by the cochlear nucleus and the superior olivary complex cells, and reflect neural connections with the central auditory pathway [29]. After ouabain application, auditory function was severely impaired, with thresholds rising from 20dBSPL to almost 80dbSPL, the maximum intensity tested. Frequency discrimination was also abolished. The amplitudes of wave ii-iii complexes were almost negligible at any of the frequencies explored at the maximum intensity of 80dBSPL (Fig. 4d). ABRs were recorded at 1-2 week intervals. Control animals ($n=8$) showed no sign of functional recovery throughout the experiment, with a mean auditory threshold after 10 weeks of 75.14±2.3 dB; similar to the 76.37±1.8 dB obtained after ouabain treatment. However in the transplanted animals ($n=18$), there was a detectable improvement in the ABR thresholds (Fig. 4a-b) starting approximately four weeks PT, with the mean auditory threshold lowered (improved) to 50.4±4.5 dB by 10 weeks PT. Furthermore, the mean auditory threshold shift, calculated as the difference between the threshold at 10 weeks PT versus the one before ouabain treatment, was of 53±1.7 dB in the control animals, compared to 28.6±3.6 dB in the transplanted cohort (p 0.0002, Fig. 4c). This represents an overall functional improvement of ~46%. The range of recovery went from modest to almost complete (see Supplementary Fig. 15), which is remarkable considering the technical challenges involved in the procedure. Tonotopical processing was also partially restored (Fig. 4d). A trend in the increment of wave ii-iii amplitudes was detected at each frequency explored, with amplitudes being significantly different at 22, 26 and 30 kHz, when compared to the untransplanted animals ($p<0.05$). When compared to the amplitudes before the ouabain application, the improvement was ~43%. Latencies were mostly similar to the ones before ouabain (Fig. 4e). The only significant difference was detected at 30 kHz (BO: 4.58±0.2 ms, $n=6$; PT: 5.9±0.4 ms, $n=5$; $p<0.05$) suggesting that some maturation was still taking place at this stage. Finally, there was a significant correlation between the increment of neural density by transplanted cells and the lowering of the ABR threshold ($R^2=0.3867$, $p<0.05$, Fig. 4f).

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Our developmentally-informed protocol produced hESC-derived auditory hair cells and neurons that closely resembled phenotypes obtained from hFASCs, providing validation of their cochlear characteristics. This was further supported by the restoration of ABR thresholds on transplantation of otic progenitors into a deaf adult mammal. The ability to reinstate auditory neuron functionality paves the way for a future cell-based treatment for auditory neuropathies. It may also, in combination with a cochlear implant, offer a therapeutic solution to a wider range of patients that currently remain without viable treatment.

Methods Summary

hESCs lines used (H7, H14, Shef1, Shef3, Shef1-EGFP and Shef1-Tomato), with a normal karyotype, were maintained on mouse embryonic fibroblast feeders (MEFs) under standard conditions. While EBs and initial monolayer experiments were performed in the presence of Knock Out Serum Replacement (KOSR), we later adopted a chemically-defined medium. This serum-free, chemically defined basal culture media included a 1:1 mixture of Dulbeco's Modified Eagle's Medium (DMEM):Ham's F12 and N2/B27 supplements (DFNB). In most experiments, FGF3 and FGF10 were used at 50ng ml^{-1}. Laminin (R&D Systems) was used at 5 $\mu g\ cm^{-2}$. Antibodies, PCR primers and microarray analysis are detailed in the supplementary methods. To induce hair cell differentiation, progenitors were transferred to gelatin-coated dishes and cultured with DFNB supplemented with all-*trans* retinoic acid (10^{-6} M, Sigma) and EGF (20ng/ml) for 2-4 weeks. To induce neuronal differentiation, cells dissociated with trypsin were plated on gelatin-coated dishes and incubated in DFNB with bFGF (20ng/ml) and Sonic Hedgehog (Shh-C24II, 500ng/ml, R&D Systems). On the third day, culture was supplemented with neurotrophin3 (NT-3, 10 ng/ml, Petropech) and brain-derived neurotrophic factor (BDNF, 10 ng/ml, Petropech). Shh-C24II was removed at the fourth-fifth day while the neurotrophins remained for the length of the incubation, normally between 7-14 days. Conditions for electrophysiological recordings are detailed in the supplementary methods. The auditory neuropathy model was generated by applying 1 mM ouabain directly into the RW niche of adult gerbils. Either three days or 2 weeks later, hONPs, expressing eGFP or Tomato fluorescent protein were injected into the modiolus. Functional recovery was monitored weekly by measuring ABRs and DPOAEs, for up to 10 weeks. Cochleae were taken, fixed and processed for analysis. Details for the hearing test and histological preparation are provided in the supplementary methods section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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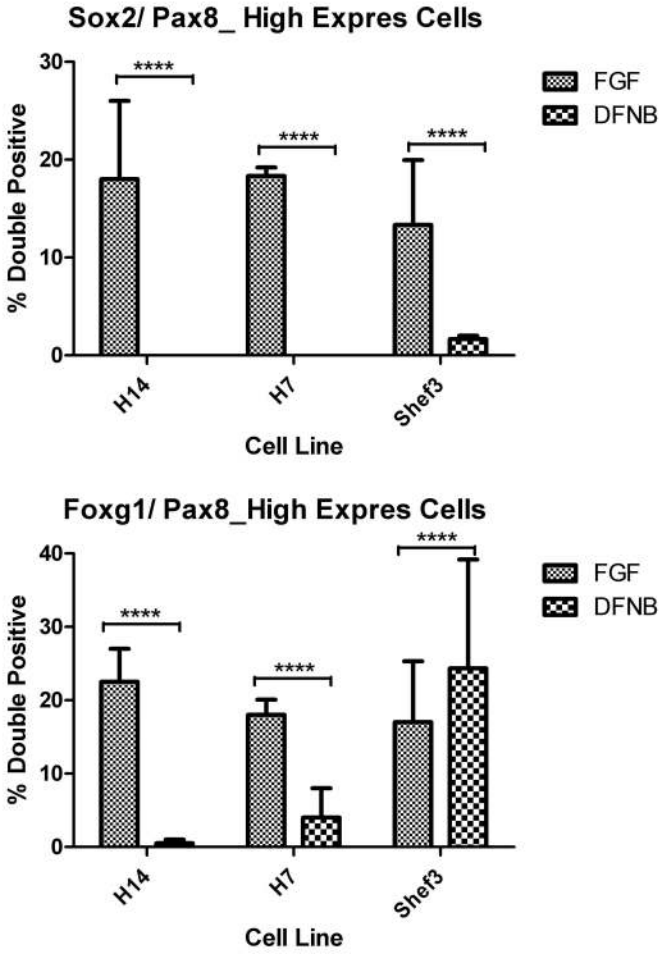

Figure 1. FGF3 and 10 generates otic progenitors

Bar chart showing the percentage of highly double positive cells at the FGF 75th percentile threshold (n=3; mean + s.e.m).

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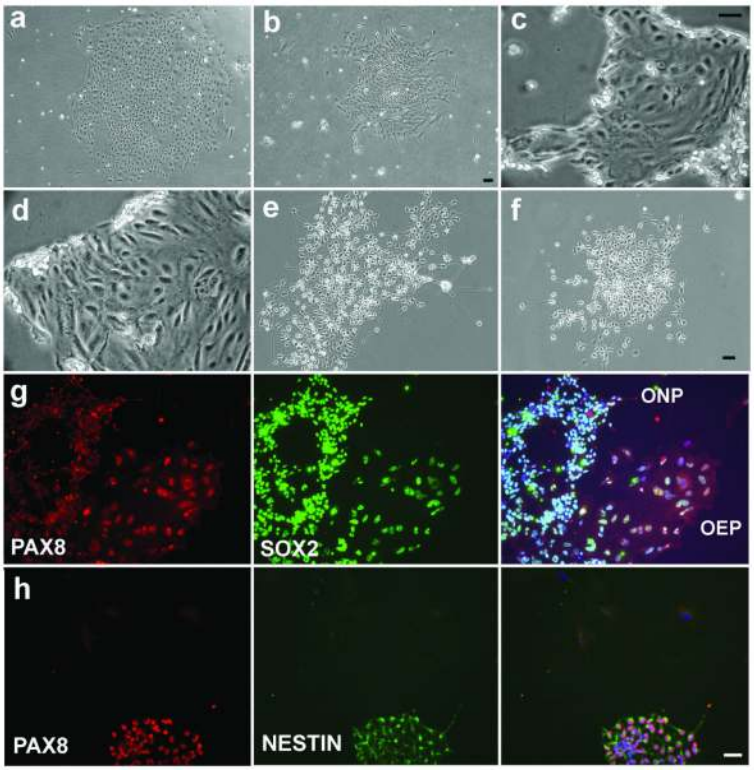

Figure 2. Otic epithelial (OEPs) and Otic Neuro progenitors (ONPs)

a and **b.** Morphology of an OEP colony. Bar is 100 μm. In all remaining panels, bar is 50 μm. **c** and **d**. Show the partial lifting of OEPs when treated with a short, mild trypsin incubation. **e** and **f.** Typical morphology of ONPs, showing cytoplasmic projections. **g.** Side-by-side ONP and OEP colonies, double-labeled for PAX8 and SOX2. **h.** ONP colony labeled for PAX8 and NESTIN.

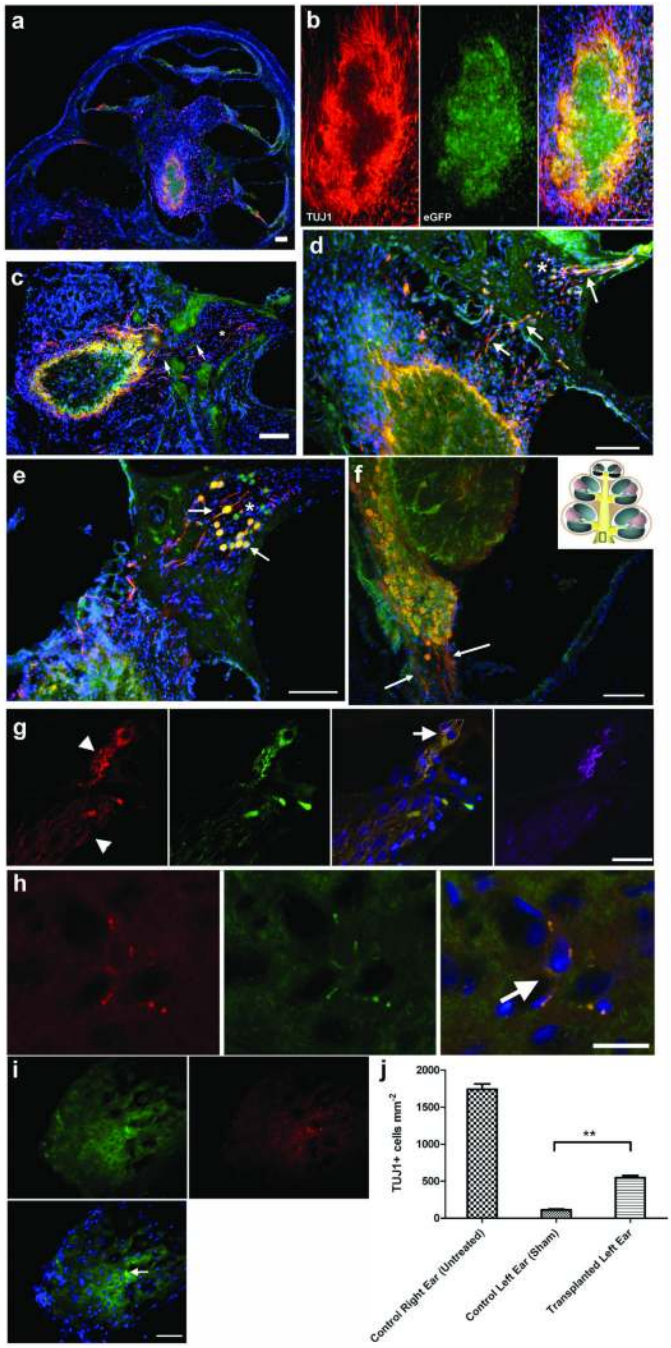

Figure 3. Transplantation of otic progenitors restores a population of spiral ganglion neurons

a. Mid-modiolar section of a transplanted cochlea showing the location of the newly formed, ectopic ganglion. **b**. Detail of the ganglion showing neuronal differentiation by TuJ1 staining (red). Neural fibers project from the ganglion towards the organ of Corti (**c** and **d**, arrows), passing through the Rosenthal's canal (**c** and **d**, asterisk). **e.** New neuronal bodies (arrows) are also found in the Rosenthal's canal (asterisk). **f.** Ectopic ganglion at the base of the modiolus, projecting TuJ1^{+} fibers centrally, towards the internal auditory meatus. **g.** RFP^{+} fibers (arrowheads) approaching the inner hair cells and expressing GluA2 (green), primarily concentrated in postsynaptic densities (PSDs) around the basal pole of IHCs

(arrow). Dotted lines show the positions of the IHCs. Fibers (including PSDs) were also positive for NKAα3 (purple), a marker of afferent terminals. Nine out of ten animals analyzed had fibers contacting the IHC, while the three animals labeled for GluA2, were positive. **h, i.** RFP^+ fibers in the cochlear nucleus, expressing synaptophysin (green, arrows). In **(h)**, the fiber branches and surrounds the cell, with morphology highly reminiscent of the maturing endbulb of Held. **j.** SGN density 10 weeks after transplantation. Conditions compared are cochleae treated with ouabain and sham operated versus those with ouabain and transplanted with ONPs. Density was significantly increased ($p<0.01$) from 112.5±11.9 ($n=3$; mean + s.e.m) to 546.4±30.6 ($n=8$). As a reference, the density of the control, untreated cochleae was 1,743±71.5 $TuJ1^+$ cells mm^{-2}. Scale bars for **a-f** are 100 μm and for **g-i** are 50 μm.

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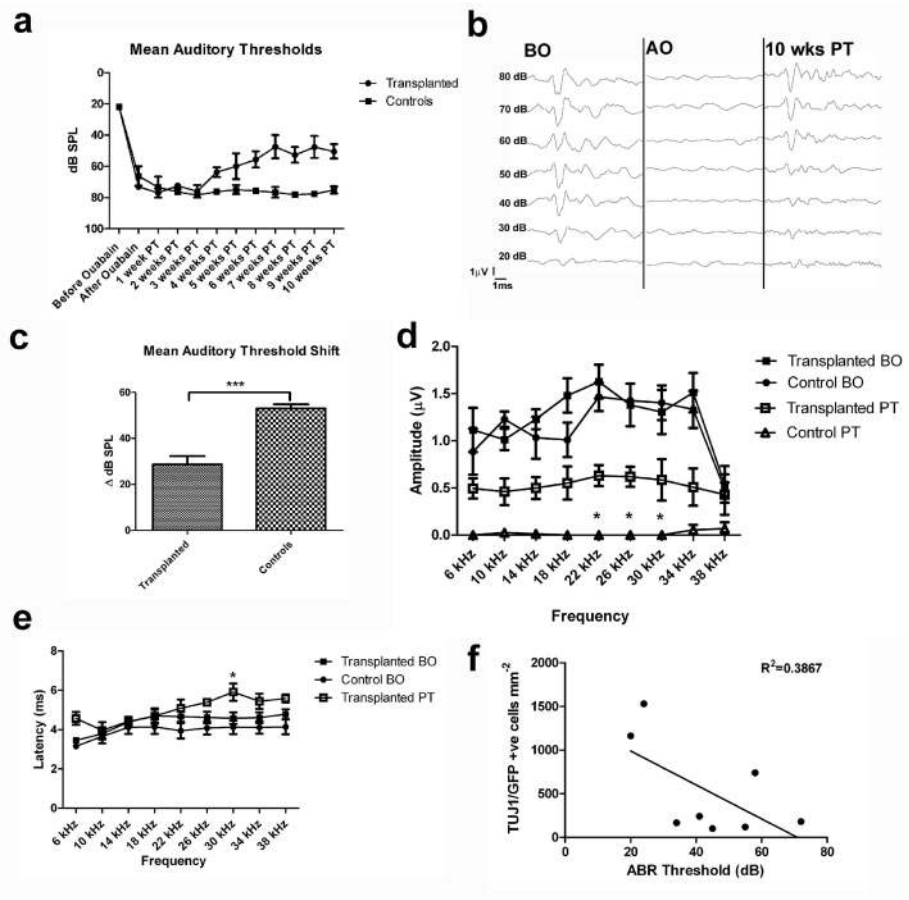

Figure 4. Transplanted cells provide a recovery of ABR thresholds

a. Evolution of the mean ABR thresholds (click) obtained in the transplanted animals (n=18; mean ± s.e.m) compared to the controls (n=8). **b.** Trace ABR showing the abolition of waves after ouabain treatment (AO) and the restoration of the complexes 10 weeks posttransplanation (PT). **c.** Graph showing the mean auditory threshold shift reduction obtained by the transplantation (transplanted 28.6±3.6 dB; n=18 vs 53±1.7 dB; n=8 in the control, p 0.0002; mean + s.e.m). **d.** Comparison of the wave ii-iii amplitudes obtained by tone ABRs. A general trend of enhanced amplitudes was obtained across all frequencies tested, being significantly different from the untransplanted controls at 22, 26 and 30 kHz. Amplitudes before ouabain (BO) were equivalent between the transplanted (n=6) and untransplanted animals (n=5; mean ± s.e.m). **e.** Latencies of wave ii-iii complexes were, in general, comparable before ouabain and after transplantation. Only at 30 kHz, a significant delay was observed (BO: 4.58±0.2 ms, n=6; PT: 5.9±0.4 ms, n=5; p<0.05; mean ± s.e.m). **f.** A significant correlation was observed between the mean density of TuJ1/GFP positive cells and the ABR thresholds (n=8; p<0.05).