Review (unsolicited)

Cell Transplantation to Restore Lost Auditory Nerve Function is a Realistic Clinical Opportunity

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Abstract
Hearing is one of our most important means of communication. Disabling hearing loss (DHL) is a long-standing, unmet problem in medicine, and in many elderly people, it leads to social isolation, depression, and even dementia. Traditionally, major efforts to cure DHL have focused on hair cells (HCs). However, the auditory nerve is also important because it transmits electrical signals generated by HCs to the brainstem. Its function is critical for the success of cochlear implants as well as for future therapies for HC regeneration. Over the past two decades, cell transplantation has emerged as a promising therapeutic option for restoring lost auditory nerve function, and two independent studies on animal models show that cell transplantation can lead to functional recovery. In this article, we consider the approaches most likely to achieve success in the clinic. We conclude that the structure and biochemical integrity of the auditory nerve is critical and that it is important to preserve the remaining neural scaffold, and in particular the glial scar, for the functional integration of donor cells. To exploit the natural, autologous cell scaffold and to minimize the deleterious effects of surgery, donor cells can be placed relatively easily on the surface of the nerve endoscopically. In this context, the selection of donor cells is a critical issue. Nevertheless, there is now a very realistic possibility for clinical application of cell transplantation for several different types of hearing loss.

Keywords
auditory nerve, cell transplantation, glial scar, nerve regeneration, scaffold.

Introduction
Over 450 million people suffer disabling hearing loss (DHL), equivalent to 6.1% of the world’s population (https://www.who.int/deafness/estimates/en/). Hearing loss affects our most important means of communication, and it may lead to social isolation, depression, and even dementia in the elderly1.

Traditionally, significant efforts to cure DHL have focused on hair cells (HCs). No less important, however, is the auditory nerve, which contains the sensory neurons that transmit electrical signals generated by HCs to the brainstem2,3.

Auditory nerve damage may occur as a result of various types of insult. These include internal causes, such as neuropathies and intracranial mass lesion, and head trauma, which is a representative external cause1. Over several decades, cell transplantation has emerged as a promising therapeutic option to rebuild lost auditory nerve function. Numerous studies in vitro and in vivo have explored different combinations of cells and delivery methods, and two successful studies have provided proof of principle that cell transplantation can lead to functional recovery. The challenge now is to focus on how the human auditory system can be approached in the clinic, including the selection of donor cells and how auditory nerve function can be restored with surgically acceptable techniques involving minimal intervention.

Degeneration Pattern of Auditory Neurons Following Insult
Insight into why cell transplantation works comes from the nature of tissue degeneration (Fig. 1). When auditory nerve
Auditory neurons express the tyrosine receptor kinase B (TrkB) and tyrosine kinase receptor C (TrkC) (Fig. 1A) for brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3), which are produced mainly by HCs\textsuperscript{3,5}. Thus, damage to HCs can lead to degeneration of auditory neurons and transneuronal degeneration of the cochlear nucleus cells and upper relay neurons (Fig. 1, \textcircled{1}, \textcircled{2})\textsuperscript{3}. It is well known clinically that degeneration of the HCs is triggered by systemic use of pharmacological agents such as aminoglycoside antibiotics and platinum-based drugs\textsuperscript{6} and also exposure to intense noise\textsuperscript{7}.

Hearing levels can deteriorate progressively in closed head injury patients\textsuperscript{8}. Similarly, in MiS and RT for vestibular schwannoma (VS), the hearing preservation rates measured within a few years of treatment can be misleading because hearing loss that is unrelated to tumor recurrence continues to progress even after 7 to 8 years\textsuperscript{9–14}. Various mechanisms are responsible for such delayed hearing loss, but one contributing factor is likely to be the unusually slow speed of auditory nerve degeneration, which can be protracted for years\textsuperscript{15}. There are several reasons for the slow degeneration of the auditory nerve. First, the soma of human SGCs contact each other and can provide mutual trophic support\textsuperscript{16}. Second, non-myelinated Schwann cells (SCs) and satellite glial cells surrounding the soma prevent the SGCs from dying even after HCs are damaged\textsuperscript{17}. Third, SGCs depend on neurotrophins provided mainly by HCs but supporting cells are also a source of neurotrophins\textsuperscript{18} even after the HCs degenerate. Cochlear implants (CIs) stimulate auditory neurons directly and they exploit the protracted course of auditory nerve degeneration\textsuperscript{12}. Cell transplantation is more likely to succeed for the same reason because degenerated auditory neurons can be replenished progressively by donor cell-derived neurites that seem to regenerate over several months\textsuperscript{2}.

Figure 1. Auditory neurons and their degeneration patterns. (A) The auditory nerve is a bundle of bipolar auditory neurons. The peripheral processes of auditory neurons form synapses with HCs and the central processes with CNs in the brainstem. HCs provide much of the trophic support required for the maintenance and survival of auditory neurons, including BDNF and NT-3. Auditory neurons synthesize the high-affinity tyrosine receptor kinases, TrkB and TrkC. The interface between the PNS and CNS is called the TZ, which is distal to the IAM. Myelin sheaths are formed by oligodendrocytes centrally from the TZ, and the surrounding milieu is astrocytic. Peripheral to the TZ, the myelin sheaths are formed by Schwann cells that are enveloped in endoneurium. The interface is penetrated only by axons. (B) The onset of anterograde (Wallerian) (\textcircled{1}), trans-neuronal (\textcircled{2}), and retrograde degeneration (\textcircled{3}) of the auditory nerve depends on the initial site of injury (\textcircled{x}). In HC damage, neurodegeneration involves the auditory neuron entirely (\textcircled{3}) and neurodegeneration proceeds to higher-level neurons including the CNs (\textcircled{2}). Shaded arrows indicate the progression of degeneration, and dotted arrows indicate transneuronal degeneration. BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CNs, cochlear nucleus cells; HC, hair cells; IAM, internal auditory meatus; NT-3, neurotrophin 3; PNS, peripheral nervous system; TZ, transitional zone.

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Clinical Issues

Causes of Auditory Nerve Degeneration and Related Clinical Issues

Neuropathies. The classical description of auditory neuropathy (AN) is that auditory nerve function is impaired but outer HCs in the cochlea are functional\textsuperscript{19}. In AN, speech comprehension is compromised although pure tone audiograms are disproportionately well maintained so patients can hear but cannot understand\textsuperscript{19}. This type of hearing loss is observed in various diseases including a subset of neuropathic and presbycusis patients\textsuperscript{20,21}. Nowadays, the causative sites for AN include not only the auditory nerve and outer HCs but also the inner HCs and inner HCs ribbon synapses (auditory synaptopathy)\textsuperscript{19}. Nevertheless, AN due to auditory nerve dysfunction and auditory neuropathic hearing loss is a potential candidate for cell transplantation\textsuperscript{2,3,22}. Some patients with genetic disorders have polyneuropathy disorders, such as auditory neuropathic hearing loss and optic neuropathy with bilateral blindness\textsuperscript{20}, and their anguish would be alleviated remarkably even if only their hearing was restored. For patients with pathologies in both the auditory nerve and
HCs, auditory nerve regeneration would most effectively be coupled with HC regeneration, should that eventually prove to be successful in mammals.

**Tumors.** VS develops from the vestibular nerve, but the surgical removal of VS inevitably imposes direct mechanical stress to the auditory nerve, potentially leading to the severance of continuity of auditory neurons or to the initiation of auditory nerve degeneration. VS surgery can also have far-reaching effects on the cochlea through the vasculature (the internal auditory artery or labyrinthine artery), leading to cochlear ischemia and reflow phenomena that are inevitably repeated during surgery, eventually leading to HC death. The latter presumption is supported by recordings of distortion product otoacoustic emissions (DPOAEs), which are sounds generated within the cochlea recorded by a microphone fitted into the ear canal. The amplitude of DPOAEs reflects the blood flow to the cochlea, and an intraoperative decrease in DPOAEs indicates cochlear ischemia due to mechanical pressure upon the vasculature. Several minutes of cochlear ischemia are sufficient to cause morphological changes of the distal ends of the auditory neurons, and longer periods can cause cessation of internal auditory artery blood flow leading to HC death. Postmortem histological examinations of VS patients without surgery reveal structural changes within the cochlea, including degeneration of HCs and the stria vascularis in the outer wall of the scala media.

**Radiation.** Radiotherapy (RT) for the central nervous system (CNS) and peripheral nervous system (PNS) lesions incur multiple pathological processes, including vascular endothelial damage, neuroinflammation, genetic/epigenetic alterations, apoptosis/necrosis of neurons and glial cells, reactive gliosis, and demyelination and deterioration of stem cell and progenitor cell proliferation. It is extremely difficult to avoid radiation injury to the auditory nerve in RT for VS. To make matters worse, not only the cochleovestibular nerve but also the facial nerve and other vital structures, such as HCs and the stria vascularis, are packed in a confined space of the cochlea (Fig. 2). The horizontal diameter of the internal auditory canal is only about 4.5 mm. Within this narrow canal, the cochleovestibular and facial nerves are compressed by the tumor and take a tortuous course. Reports revealed that radiation doses in the cochlea and cochlear nucleus during RT are correlated with patients’ hearing outcome, implying radiation injury to auditory neurons is responsible for hearing deterioration in RT in addition to that to HCs and the stria vascularis, both vital to hearing. Patients with small VS in which auditory neurons degenerate but HCs are still functional are an ideal candidate for auditory nerve replacement, and this is the case in a subset of presbycusis or auditory neuropathic patients as mentioned above.

**Head Injury.** In patients with a closed head injury even without temporal bone fractures, damage to auditory neurons is observed primarily and/or secondarily following HC
have too few remaining functional auditory neurons\textsuperscript{45,46}. In fact, the minimum number of functional auditory neurons needed for the successful performance of a CI is astonishingly few and estimated to be 5\% to 10\% of the normal number\textsuperscript{15}. CIs are beneficial to neurofibromatosis type 2 (NF2) patients with bilateral VS\textsuperscript{47}, but a significant number of patients experience a decline in performance as the VS grows\textsuperscript{49}. Hence, replenishing auditory neurons would potentially benefit NF2 patients.

**Surgical Options for Cell Delivery**

There is extensive literature on cell transplantation to the auditory system, but in this context, the relevant studies are those done in vivo on deafened animals and on deaf humans (Table 1). To establish proof of principle, two main conditions must be met in the analysis of cell integration and functional recovery\textsuperscript{49}. First, there must be an electrophysiological analysis of the restoration of nerve function with an objective method such as the auditory brainstem response (ABR). Second, to link recovery to the transplanted cells, it is important to provide morphological evidence for synaptic connections, not only with HCs in the cochlea but also with neurons of the cochlear nuclei within the brainstem. In other parts of the nervous system, functional improvements have been recorded without morphological integration of the donor cells\textsuperscript{50–52} by indirect mechanisms, including trophic effects, immunomodulation, and other bystander effects\textsuperscript{53–55}.

Equally important from the experimental aspect are clinical relevance, which is reflected in the animal model used to replicate human clinical pathology, and clinical feasibility, which relates to whether or not the surgical techniques can be used in the clinic. In the following sections, we consider a number of in vivo studies in these terms. We focus on local delivery of cells to parts of the inner ear because trials with systemic cell delivery have not led to successful migration of donor cells to the auditory system\textsuperscript{56–64} (Table 1).

**Cell Delivery into the Cochlea with Injury to the Membranous Labyrinth.** The soma of spiral ganglion neurons (SGNs) are located within the cochlea, and it is worth knowing whether or not cells delivered into the cochlear fluid spaces or cochlear wall are able to find their way into Rosenthal’s canal in which the SGN soma are housed (Fig. 2A). This does not seem to be the case, and none of the relevant studies have led to functional recovery (Table 1). With the exception of two studies\textsuperscript{65,66}, the membrane that seals intracochlear fluid-containing spaces (the membranous labyrinth = the scala tympani, scala vestibuli, the scala media, and posterior semicircular canal) was breached and/or trespassed (membranous labyrinth injured [MLI])\textsuperscript{67–92} (Table 1). Importantly, invasion into the membranous labyrinth is clinically unacceptable because it leads to hearing loss\textsuperscript{83}. Furthermore, the cochlea in small experimental animals is easily accessible as it is conspicuous within the hollow dome-like bulla, but it is not as accessible in humans as it is deeply buried in

**Cochlear Implants (CIs)**

Cell transplantation could potentially enhance the performance and candidacy for CI in patients, who generally
Table 1. In vivo studies to restore auditory nerve function.

| Study            | Site of donor cell delivery | Host animal Deafening procedure | Verification of functional restoration and synaptogenesis | Donor cell* |
|------------------|-----------------------------|----------------------------------|----------------------------------------------------------|-------------|
| Hu et al. (2004) | ScT (MLI)                   | Rat Pharmacol, local             | No DRGC                                                  | Mouse DRGC  |
| Hu et al. (2005) | ditto                       | Guinea pig Pharmacol, local      | No Mouse ESC and DRGC                                   |             |
| Hu et al. (2005) | ditto                       | Guinea pig Pharmacol, local      | No Mouse NSC                                            |             |
| Coleman et al. (2006) | ditto              | Guinea pig Pharmacol, systemic   | No Mouse ESC                                            |             |
| Matsuoka et al. (2007) | ditto          | Gerbil Pharmacol, local          | No Mouse ESC                                            |             |
| Parker et al. (2007) | ditto         | Mouse/guinea pig Sound exposure | No Mouse NSC                                            |             |
| Altschuler et al. (2008) | ditto    | Guinea pig Pharmacol, systemic   | No Mouse ESC                                            |             |
| Lang et al. (2009) | ditto                       | Gerbil Pharmacol, local          | No Mouse ESC                                            |             |
| Hu et al. (2009) | ditto                       | Guinea pig Pharmacol, systemic   | No Mouse DRGC                                            |             |
| Cho et al. (2011) | ditto                       | Guinea pig Pharmacol, local      | No Human MSC                                            |             |
| Pettingill et al. (2011) | ditto    | Guinea pig Pharmacol, systemic   | No Schwann cells                                         |             |
| Warnecke et al. (2012) | ditto      | Guinea pig Pharmacol, systemic   | No BDNF-secreting cells                                  |             |
| He et al. (2014) | ditto                       | Guinea pig Pharmacol, local      | No Mouse NSC                                            |             |
| Jang et al. (2015) | ditto                       | Guinea pig Pharmacol, local      | No Human MSC                                            |             |
| Fetoni et al. (2014) | ditto       | Guinea pig Noise exposure        | No Guinea pig ADSC                                       |             |
| Gillespie et al. (2015) | ditto     | Guinea pig Pharmacol, systemic   | No BDNF-expressing fibroblast                            |             |
| Jang et al. (2016) | ditto                       | Guinea pig Pharmacol, local      | No Human ADSC                                            |             |
| Xu et al. (2016) | ditto                       | Rat Noise exposure               | No olfactory epithelium NSC                             |             |
| Dai et al. (2016) | ditto                       | Rat Pharmacol, systemic          | No Rat OEC                                              |             |
| Wise et al. (2016) | ditto                       | Guinea pig Pharmacol, systemic   | No Human ESC                                            |             |
| Chen et al. (2017) | ditto                       | Mouse Pharmacol, systemic        | No Mouse iPSC                                            |             |
| Schendzielorz et al. (2017) | ditto   | Guinea pig Pharmacol, local      | No Guinea pig ADSC                                       |             |
| Huang et al. (2019) | ditto                      | Gerbil Pharmacol, local          | No Mouse NSC                                            |             |
| Hildebrand et al. (2005) | ScM (MLI)  | Guinea pig Pharmacol, systemic   | No Mouse ESC                                            |             |
| Hu et al. (2005) | ditto                       | Guinea pig Pharmacol, local      | No Mouse ESC mouse DRGC                                  |             |
| Lang et al. (2008) | ScM, RC (MLI)              | Gerbil Pharmacol, local          | No Mouse ESC                                            |             |
| Ahn et al. (2008) | PSCC (MLI)                  | Mouse Pharmacol, systemic        | No Mouse ESC                                            |             |

(continued)
| Study                      | Site of donor cell delivery | Host animal | Deafening procedure | Verification of functional restoration and synaptogenesis | Donor cell* |
|---------------------------|----------------------------|-------------|---------------------|----------------------------------------------------------|-------------|
| Zhang et al. (2013)       | Cochlea wall (MLP)         | Rat         | Pharmacol, local    | No Mouse NSC                                             |             |
| Hackelberg et al. (2017)  | Scaffold in IAC (MLI)      | Guinea pit  | Pharmacol, local    | No Human ESC                                             |             |
| Tamura et al. (2004)      | AuN                        | Mouse       | Pharmacol, local    | No Mouse NSC                                             |             |
| Naito et al. (2004)       | ditto                      | Chinchilla  | Pharmacol, systemic | No Bone marrow cell                                      |             |
| Hu et al. (2004)          | ditto                      | Rat         | Pharmacol, systemic | No Mouse DRGC, ESC                                       |             |
| Okano et al. (2005)       | ditto                      | Guinea pig  | Pharmacol, systemic | No Mouse ESC                                             |             |
| Regala et al. (2005)      | ditto                      | Guinea pig  | Pharmacol, systemic | No Mouse DRGC                                            |             |
| Corrales et al. (2006)    | ditto                      | Gerbil      | Pharmacol, local    | No Mouse ESC                                             |             |
| Matsuoka et al. (2007)    | ditto                      | Gerbil      | Pharmacol, local    | No Mouse MSC                                             |             |
| Shi et al. (2007)         | ditto                      | Gerbil      | Pharmacol, local    | No Human ESC                                             |             |
| Altschuler et al. (2008)  | ditto                      | Guinea pig  | Pharmacol, systemic | No Mouse ESC                                             |             |
| Reyes et al. (2008)       | ditto                      | Guinea pig  | Pharmacol, systemic | No Mouse ESC                                             |             |
| Ogita et al. (2010)       | ditto                      | Guinea pig  | Pharmacol, local    | No Guinea pig MSC-derived spheres                        |             |
| Chen et al. (2012)        | ditto                      | Gerbil      | Pharmacol, local    | Yes Human ESC                                            |             |

**Direct cell injection into the auditory nerve (MLP)**

| Study                      | Site of donor cell delivery | Host animal | Deafening procedure | Verification of functional restoration and synaptogenesis | Donor cell* |
|---------------------------|----------------------------|-------------|---------------------|----------------------------------------------------------|-------------|
| Sekiya et al. (2006)      | AuN                        | Rat         | Compression of AuN  | No Mouse ESC                                             |             |
| Sekiya et al. (2007)      | ditto                      | ditto       | Rat Pharmacol, local | No Mouse auditory neuroblast                              |             |
| Palmgren et al. (2012)    | ditto                      | Rat         | Pharmacol, local    | No Mouse ESC                                             |             |
| Jiao et al. (2014)        | ditto                      | Rat         | Pharmacol, local    | No Human neural precursors                                |             |
| Chen et al. (2019)        | ditto                      | Mouse       | Pharmacol, local    | No Human limbus-derived MSC                               |             |

**Cell transplantation onto the auditory nerve (MLP)**

| Study                      | Site of donor cell delivery | Host animal | Deafening procedure | Verification of functional restoration and synaptogenesis | Donor cell* |
|---------------------------|----------------------------|-------------|---------------------|----------------------------------------------------------|-------------|
| Sekiya et al. (2015)      | AuN                        | Rat         | Compression of AuN  | Yes Mouse auditory neuroblast                             |             |

**Systemic delivery (MLP)**

| Study                      | Route | Host animal | Deafening procedure | Verification of functional restoration and synaptogenesis | Donor cell* |
|---------------------------|-------|-------------|---------------------|----------------------------------------------------------|-------------|
| Revoltella et al. (2008)  | i.v.  | Mouse       | Pharmacol, systemic | No Human cord blood stem cells                           |             |
| Choi et al. (2012)        | i.v.  | Rat         | Noise               | No Human MSC                                             |             |
the temporal bone. Thus, this method is not suitable for human patients.

**Direct Cell Injection Into Auditory Nerve With Injury to the Membranous Labyrinth.** For targeting donor cells to the auditory nerve, this method seems to be more dependable. Unlike injection into the cochlear fluids, the cells are located into the appropriate neural tract with morphological continuity with the relevant target cells. Nevertheless, the membranous labyrinth is injured as with direct injection into the cochlea. Moreover, intraneural injection with a syringe needle can damage the morbid, fragile auditory nerve and trigger an inflammatory reaction along with reactive gliosis around the needle and transplant (see the following sections for further discussions). This method has proved successful in one study and offers important proof of principle for clinical translation, especially from the viewpoint of donor cell selection (Table 1; Fig. 2A). However, it remains possible that leakage of the donor cells outside the cochlea might have played a predominant role (see below for the details) in addition to indirect bystander effects that could account for the observed improvement of the ABRs.

**Direct Cell Injection Into or Onto Auditory Nerve With Preservation of the Membranous Labyrinth.** Even without damaging cochlear structures, cell injection into the cerebellopontine angle portion of the auditory nerve trunk through a hole posterior to the mastoid process (the retro mastoid region) has not restored auditory nerve function. Surprisingly, functional restoration was observed if donor cells were simply placed onto the surface of the auditory nerve via the retromastoid route, thus preserving the integrity of both the nerve and the membranous labyrinth (membranous labyrinth preserved [MLP]) (Fig. 2A, B) (Table 1). This “surface transplantation” method can be regarded as a more promising option for cell transplantation, and it is, thus, considered in more detail in the following sections.

**What is the Nature of Nerve injury and Degeneration and How Could Cell Transplantation Work for Specific Clinical Conditions?**

The success of in vivo experiments with animal models is encouraging, but it is important to understand the biology that underlies the pathology of nerve degeneration and the subsequent structural and biochemical environment that underlies the successful integration of transplanted cells. This not only informs the optimal technique for cell delivery but also the selection and possibly the conditioning of donor cells.

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**Table 1. (continued)**

| Study                        | Site of donor cell delivery | Host animal            | Deafening procedure                            | Verification of functional restoration and synaptogenesis | Donor cell*                   |
|------------------------------|-----------------------------|-------------------------|------------------------------------------------|----------------------------------------------------------|-------------------------------|
| Choi et al. (2012)           | i.v.                        | Guinea pig              | Pharmacol, local                               | No                                                       | Human blood MSC              |
| Yoo et al. (2015)            | i.v.                        | Mouse                   | Autoimmune hearing loss                         | No                                                       | Human ADSC                   |
| Lang et al. (2016)           | i.v.                        | Mouse                   | Pharmacol, local                               | No                                                       | Mouse and human blood cell   |
| Kil et al. (2016)            | i.v.                        | Guinea pig              | Pharmacol, local                               | No                                                       | MSC from human placenta      |
| Ma et al. (2016)             | i.t.                        | Congenital deaf albino pig | Pharmacol, local                                    | No                                                       | Human umbilical cord MSC     |
| Lee et al. (2018)            | i.v.                        | Human cases             | Pharmacol, local                               | No                                                       | MSC                           |
| Abd El Raouf et al. (2019)   | i.v.                        | Guinea pig              | Pharmacol, systemic                             | No                                                       | Guinea pig Harderian gland stem cells |

*ABR, auditory brainstem responses; ADSC, adipose tissue-derived stem cell; AuN, auditory nerve; BDNF, brain-derived neurotrophic factor; CPA, cerebellopontine angle; DPOAE, distortion product otoacoustic emissions; DRGC, dorsal root ganglion cell; ESC, embryonic stem cell; IAM, internal auditory meatus; iPSC, induced pluripotent stem cell; i.t., intrathecal injection; i.v., intravenous injection; MLI, membranous labyrinth injured; MLP, membranous labyrinth preserved; MSC, mesenchymal stem cell; NSC, neural stem cell; OEC, olfactory ensheathing cell; RC, Rosenthal’s canal; Ref, reference number; ScM, the scala media; ScT, the scala tympani; ScV, the scala vestibuli.

* "Donor cell" indicates the provenance of donor cell. Donor cells may have been preconditioned in vitro before transplantation. For example, application of neural induction for ESC.

* "Pharmacol, local" indicates that pharmacological agents were applied locally to the auditory system. For example, ouabain applied to the round window of the cochlea.

**"Pharmacol, systemic" indicates application intravenously. For example, ototoxic antibiotics such as kanamycin injected in the tail vein of the host.

Note: Studies using more than one cell delivery routes are repeatedly listed in Table 1. The references in the text and table are listed basically in chronological order.
Structural and Biochemical Cues for Cell Transplantation

Protective Addition. In principle, regenerative medicine should add new functional elements without causing further damage, following the principle of “protective addition”. This principle is most effectively met for the auditory nerve by placing donor cells on the tissue surface\(^1\). As discussed above, all other delivery techniques involve significant tissue damage.

The Scaffold. The scaffold is an indispensable element for the formation of the nervous system. For example, radial glia plays a crucial role as the scaffold for cell migration from the ventricular zone toward the brain surface\(^108,109\). Hence, various artificial scaffolds such as collagen-rich acellular matrices and matrices such as hydrogel with in vitro expanded donor cells attached have been intensively investigated in many neurodegenerative disorders, including spinal cord injury (SCI) with efforts to overcome various obstacles including provocation of host immune responses\(^110–114\). Currently, another practical issue to be solved aiming at clinical translation is the surgical maneuverability of artificial materials in the delicate and confined space of the CNS.

The Scaffold Within: A Natural Autologous cell Scaffold. One of two successful studies was serendipitous but demonstrated that an autologous cell scaffold had been spontaneously formed in collaboration with SCs during the progression of auditory nerve degeneration. This naturally occurring autologous cell scaffold plays key roles in cell integration as described below. A number of donor cells incidentally spilled onto the nerve surface from a hole through which a syringe needle had been inserted for traditional intra-neural injection of donor cells. These “leaked” donor cells autonomously entered the nerve, gradually transformed into the bipolar shape characteristic of auditory SGCs in the nerve, and finally formed synapses with target HCs and cochlear nucleus cells (Fig. 2B and 4). Intriguingly, donor cell migration and axon elongation apparently recapitulated processes observed during development. These processes include glia-guided migration\(^115\) and migration within GFAP-positive astrocytic, tube-like structures in the rostral migratory stream\(^116,117\). Even residual neurons appeared to be used as a migration guide\(^118\) (Fig. 4D).

Natural Autologous Cell Scaffold—the Astrocyte Outgrowth and SCs Form a Bridge Between the CNS and PNS. When neurons in the CNS die, astrocytes react to form the glial scar (the astrocyte scar), irrespective of the cause, which may be ischemia, mechanical trauma, irradiation, or genetic disorder\(^119–122\). In mouse SCI, reactive astrocytes of the gliotic auditory nerve (see Sekiya et al., 2015 for original images). Scale bars: (1), 200 \(\mu\)m; (2) 50 \(\mu\)m. Cited from Sekiya et al. (2015) with publisher’s permission. ABR, auditory evoked brainstem responses; AuN, auditory nerve; BS, brainstem; CSF, cerebrospinal fluid; DCs, donor cells; GFAP, glial fibrillary acidic protein.

were apparently longer than in sham rats in the penumbra even 30 days after ischemic and hemorrhagic stroke\(^124\). After a stab lesion in the cerebral cortex of mice, one subset of astrocytes directed their processes toward the lesion\(^125\). After injection of iron into mice, reactive astrocytes around the lesion core extended long and overlapped processes\(^126\).

![Figure 4](image-url)
Similarly, at the cranial and peripheral nerve roots, such elongated processes of reactive astrocytes are observed as a conspicuous tongue-like protrusion toward the periphery, the astrocyte outgrowth (the AO) (Fig. 3). One clinical study demonstrated that auditory nerve specimens taken during VS surgery were gliotic, indicating that reactive astrocytes had invaded the auditory nerve\(^\text{127}\). Other than damaged auditory nerve, the AO has been reported in a plethora of diseases in which motor and sensory neurons die, including amyotrophic lateral sclerosis\(^\text{128–133}\). Electron microscopy shows that the AO comprises processes of reactive astrocytes of the glial scar, which have been known as “glial bundles”, extending from the spinal cord/brainstem\(^\text{128,130,133}\). It should be noted that the polarity of the AO plays pivotal roles in cell migration and axon elongation\(^\text{134}\).

Normally, astrocytes in the CNS and SCs in the PNS are apart and mutually exclusive but their mutual repulsion decreases following motor and sensory neuron death in the brainstem/spinal cord\(^\text{135}\). As a result, the distal tip of the AO extensively apposes with SCs or is directly wrapped by SC cytoplasm within a common basal lamina\(^\text{133,135}\). Distally, SCs form structures called SC columns or bands of Bungner that can guide regenerating axons back to their targets\(^\text{136}\). Thus, a continuous structure, the AO–SC complex, forms autonomously and can act as an anatomical bridging scaffold connecting the CNS and the PNS\(^\text{137}\) (Fig. 2B). In fact, in one study on the auditory nerve, the AO–SC complex appeared to be the only continuous scaffold between the PNS and CNS\(^2\).

Furthermore, upon injury, both astrocytes and SCs become rich sources of pro-regenerative molecules, including laminin, N-cadherin neural cell adhesion molecule, nerve growth factor, BDNF, NT-3, and fibroblast growth factor, glial cell line-derived neurotrophic factor, artemin, and vascular endothelial growth factor\(^\text{136,138,139}\).

### Intraneural Injection and Surface Transplantation

It is difficult to compare the different cell transplantation experiments in the auditory system because the donor cells, surgical techniques, and animal models are so varied. However, when intraneural injection and surface transplantation were compared under the same parameters\(^2\), surface transplantation was clearly more successful. There was no ABR improvement with intraneural transplantation, and the transmission of electrical activity failed to pass the transplantation site\(^\text{140}\) (Fig. 4A). Morphological examination revealed a failure of cell migration with cell debris mainly at the site of cell transplantation with a few cells stuck in the midst of the gliotic auditory nerve (Fig. 4C,1). Another finding was cavity formation in the nerve, apparently due to infusion pressure during injection and the large volume of the infused cell mass that might also have damaged residual host neurons and vascular networks (Fig. 4C, 2). In contrast, the animals in which cells were delivered by surface transplantation demonstrated statistically significant improvement of the ABRs measured 3 months after cell transplantation (Fig. 4B). Morphologically, various modes of cell migration were observed as mentioned above (Fig. 4D, 1–4), and synaptic connections with HCs and the cochlear nucleus cells were morphologically confirmed (refer to ref. 2 for the original images).

### The Glial Scar, is it Friend or foe?

Emerging evidence challenges the traditional belief that the glial scar is a physical and molecular barrier to neural regeneration\(^\text{141–143}\). An in vivo experimental study on SCI showed that regenerating axons skirted around the surface of the glial scar\(^\text{144}\), indicating that they can negotiate its surface and benefit from the structural and chemical cues that it contains. In unilateral cerebral stroke of the motor cortex in mouse, axons of the contralesional corticospinal tract normally sprout into the denervated spinal cord and contribute to motor functional recovery. In a double knockout of GFAP and vimentin (the principal genes responsible for glial scar formation), corticospinal axons only rarely crossed the midline and the reduced astrocytic reactivity led to impaired neurological recovery\(^\text{142}\). Another study showed that scar-forming reactive astrocytes do not only have a protective function but also promote axonal regeneration after SCI. In two different transgenic mouse models to either prevent or inhibit glial scar formation, the study showed that there is a failure in axonal regrowth following removal of reactive astrocytes in both acute and chronic glial scars\(^\text{141}\). Reactive astrocytes in cerebral infarct play a crucial source of a pro-regenerative molecule, the stromal cell-derived factor 1 (SDF-1)\(^\text{145}\). Blocking SDF-1 action with a neutralizing antibody against a receptor for SDF-1, CXCR chemokine receptor 4 (CXCR4), strongly attenuated progenitor migration\(^\text{146}\), indicating that SDF-1/CXCR4 promotes migration of stem/progenitor cells toward the lesion\(^\text{147}\).

### Pro- and Anti-Regenerative Astrocytes

Astrocytes are not homogenous and are composed of at least five distinct subpopulations, although it is not clear how each subpopulation responds to different insults in different locations\(^\text{148–150}\). Astrocytes not only conform to different environmental niches but also show different transcriptional changes induced by different types of injuries\(^\text{149,151}\). In a non-penetrating lateral fluid percussion brain injury model in adult rats, the morphology of reactive astrocytes is regionally distinct; those in the injured cortex, subcortical white matter tracts, and CA3 region of the hippocampus show a distinct morphology with an enlarged cell body and long intertwined processes, but those in the thalamic nuclei have thicker shorter processes\(^\text{152}\). Following experimental occlusion of the middle cerebral artery, reactive “A2” astrocytes are likely to be protective as they lead to increased expression of neurotrophic factors and cytokines, transferring mitochondria to injured neurons\(^\text{143,148,153}\). In
contrast, neuroinflammation with systemic endotoxin lipopolysaccharide injection induces neurotoxic “A1” astrocytes143,148,153. A recent study reported that such molecular and functional diversity of astrocytes in the healthy adult brain depends on cues from neurons through neuron-derived sonic hedgehog (Shh)154. This is also another example of glia–neuron interaction (see above).

Thus, it is more likely that there are pro- and anti-regenerative reactive astrocytes, and further research is required to identify those subsets of reactive astrocytes that can aid and contribute to axon elongation efficiently for auditory nerve regeneration.

What is the Ideal Animal Experimental Model?

Studies of the auditory nerve require animal models in which the auditory nerve is selectively, quantifiably, and reproducibly damaged without confounding factors such as concomitant HC damage.

In pharmacological models, to induce auditory nerve degeneration, ouabain is most commonly used22,57,59,60,65,66,70,75,76,82,85,86,93,96,100,103. However, with this approach, SGNs are hard to damage reproducibly to avoid “sudden and all-or-none type cell death”. It is technically difficult to titrate the dose, so ouabain treatments destroy nearly all SGNs in most of the studies60,85,86,93,96,155–158. This makes it hard to assess any further damage that may occur through surgical intervention.

Instead, clinically relevant animal experimental models of neurodegenerative disorders, including hearing loss, should ideally involve a reproducible, “intermediate” degree of stable injury to reflect the gradual progression of tissue degeneration and a suitable opportunity to systematically test potential therapies. In fact, this critical issue has long been discussed when creating animal models of SCI159.

Ouabain is usually applied to the round window in the middle ear. It enters the cochlea across the round window membrane and is diluted in the perilymph of the scala tympani before reaching the SGN through Schuknecht’s canalicular perforantes3,160. Pharmacological agents, including ouabain, that are applied even locally to the cochlea generally diffuse throughout the cochlear fluid space in an uncontrollable manner and tend to affect not only auditory neurons but also HCs158,161. Moreover, the effect of ouabain is different between species; ouabain selectively destroys SGNs in gerbils and mice, whereas in guinea pigs, it preferentially damages HCs158. In rats, if high doses applied to the round window are not sufficiently diluted, then both HCs and SGN can be damaged158.

Ouabain is a potent inhibitor of the ubiquitous Na+–K+ pump162, which maintains a low Na+ and high K+ concentration within most cells to ensure their excitability and to provide the driving force for the transport of glucose, amino acids, and other nutrients into the cell162,163. Thus, a caveat with ouabain is that it may affect not only neurons but also cells in the surrounding epithelial, connective, and muscle tissues. This also applies in systemic administration of pharmacological agents164. Thus, the majority of animal models are not ideal for clinical translation.

In contrast, if mechanical compression is applied to the CNS portion of the auditory nerve, it can produce selective, “intermediate” degree of degeneration of auditory neurons with HCs preserved2,165,166 (Fig. 1B, 4A, B). This leads to transneuronal death of CNS cells (cochlear nucleus cells) and formation of a protruded bundle of reactive astrocytes of the glial scar (the AO), which plays a crucial role with distal Schwann cell columns for auditory nerve regeneration as elucidated above (Figs. 2B, 3B, 4). Unique to this model, the transneuronal degeneration of cochlear nucleus cells (Fig. 1B, 4) can be quantitatively analyzed165,167,168. Mechanical compression is thus likely to be the most realistic model for the clinical conditions that lead to auditory nerve degeneration.

Donor Cells for Auditory Nerve Regeneration

Cell Source. Selection and preparation of donor cells are not the focus of this review, but they are critical issues because the cells must be competent to respond to regenerative cues within the damaged tissue.

As depicted in Table 1, embryonic stem cells (ESC) and neural stem cells (NSC) were most frequently used as xenografts or allografts after preconditioning in vitro with diverse factors such as bFGF, BMP4, and the bHLH transcription factor neurogenin 268,80,93,100,169. These approaches carry a greater risk of immune rejection compared with autologous transplantation170. Even using autologous-induced pluripotent stem cells (iPSCs) as donor cells, immune rejection can be an issue170–172, despite major histocompatibility complex matching173. Furthermore, the phenotypes of individual iPSCs are not entirely predictable, and preconditioning can be complex, time-consuming, and expensive174–177.

Human cells, particularly autologous human cells, are the most likely candidates for clinical translation and those derived from mesenchymal stem cells and adipose tissue-derived stem cells are being studied intensively as donor cells in human disease178–184. Tissue-specific autologous stem cells are naturally strong candidates because they are more closely adapted to the host environment185,186. The human inner ear contains endogenous adult stem cells, as has been shown in other organs187,188,189, although their potential at the clinical level is not yet clear.

Bipolarity, a Key Requisite as Donor Cells for Auditory Nerve Regeneration. Auditory neurons are bipolar, and donor cells must connect both peripherally with HCs and centrally with cochlear nucleus cells. Table 1 shows that functional recovery of the auditory nerve has been achieved only in two studies, one with human ESCs22 and the other with a mouse cell line2. In both cases, the donor cells adopted a bipolar phenotype2,22. The ESCs were conditioned as otic
progenitors by simulating the initial, developmental specification of the otic placode with sequential application of selected factors, including NT-3, BDNF, bFGF, and Shh. The mouse cells were from a conditionally immortal mouse otic neuroblast cell line, US/VOT-N33, derived from a mouse otocyst (inner ear anlage). They show that ontogenetic-stage/region-restricted precursors can be successfully integrated into the host tissue, which has also been shown in a study of retinal regeneration.

Ultimately, the selection of appropriate donor cells must be made in the context of the animal model most closely allied to the clinical application. It cannot be assumed that a given cell type would be equally effective with both intraneural injection and cell surface delivery because the biochemical cues encountered from the damaged tissue may be different. Thus, there is a need for more systematic research with a number of potential donor cell types in carefully controlled animal models. This is recognized more generally in cell transplantation to address a number of technical hurdles, not least those of phenotype instability, cost versus benefit, and ethical issues.

A Minimally Invasive Technique for Cell Transplantation

Endoscopic surface transplantation. Minimal invasiveness of cell delivery is an indispensable requisite for clinical translation. Endoscopy may fulfill this requirement most efficiently (Fig. 5). It has long been used in clinical otorhinolaryngology, has been reported in neurosurgical procedures since the 1970s, and its safe maneuverability in the CPA has been established.

Surface transplantation of donor cells to the auditory nerve can be done with an endoscope introduced intracranially through a single keyhole in the retromastoid area. It could be applied to diseases such as auditory neuropathic hearing loss in neuropathies and head trauma (Fig. 5A), VS immediately after tumor removal (Fig. 5B), and VS following RT (Fig. 5C). This simple procedure requires only local anesthesia under sedation, and it is, thus, applicable to physically more sensitive patients, including the elderly.

Surface transplantation has several other advantages. Excessive numbers of cells are not required to compensate for the very high rates of donor cell death as observed in...
intraparenchymal injection\textsuperscript{197} because donor cells apparently autonomously enter the host tissue in proportion to the demand and capacity of the host environment\textsuperscript{2}. Moreover, in contrast to intraneural injection, donor cells transplanted onto the nerve are immediately nourished by cerebrospinal fluid, which is a very rich source of nutrients including proteins, ions, lipids, hormones, cholesterol, glucose and metabolites, and pro-regenerative molecules such as BDNF and IGF-2\textsuperscript{198,199}, before they establish a link to the blood supply. In transplantation experiments of Parkinson’s disease, most dopamine neurons injected into the brain died due to apoptosis within the first 24 h of transplantation\textsuperscript{200}, and subsequently, more than 90\% of transplanted neurons died by the end of a typical several week transplantation study\textsuperscript{201,202}. In rats, more than 1 week is required after transplantation before sufficient neovascularization is established between the host and transplants\textsuperscript{203,204}. Until then, the intraparenchymally transplanted cells suffer insufficient nutrients diffusing from host vessels located outside the graft perimeter, resulting in apoptotic cell death (see the sections above).

It is worth noting that “surface transplantation” of donor cells is distinct from “stem cell sheet technology” such as that explored in heart, kidney, and liver disorders\textsuperscript{205,206}. In surface transplantation for auditory nerve regeneration, a uniformly molded cell sheet manufactured before cell transplantation cannot be applied to the target area. On the contrary, to facilitate the integration of donor cells into the host, it is important to drop them freely into the narrow spaces within the irregular and complex contours of the tissue surface.

**Conclusion**

We conclude that there is great potential for clinical translation of cell transplantation in the auditory nerve. Proof of principle has been established; appropriate clinical techniques are available, and there is considerable theoretical support from wide-ranging studies on neurodegeneration and tissue repair. Auditory nerve damage may occur as a result of neuropathies, intracranial mass lesions, head trauma, and even therapeutic intervention. The finding that donor cells placed on the surface of the gliotic auditory nerve autonomously entered the nerve tissue, migrated, and functionally integrated into the host neural circuit makes clinical surgery much more realistic\textsuperscript{2}. For clinical translation, endoscopy provides the best way to deliver viable cells to the tissue surface with minimal damage to residual functional elements in the nerve.

Whilst proof of principle is an important step, there is clearly a need for focused animal experiments that recreate the combination of approaches necessary for clinical application. “From the bench to the clinic” is a slogan that has been repeated also in regenerative medicine. Now it can be accomplished if an appropriate cell transplantation method is applied to humans, choosing potent human stem cells or human cochlear precursors\textsuperscript{207,208} that may or may not be conditioned to achieve integration.

Notably, the auditory nerve holds an advantageous and suitable position for cell transplantation therapy because HCs, auditory neurons, and cochlear nucleus cells are aligned over a relatively short distance\textsuperscript{209,210}. This contrasts with the recovery of the injured pyramidal tract in SCI, which involves not only axon sprouting but also recruiting endogenous relay neurons\textsuperscript{211,212}.

Finally, transdisciplinary combinations with regenerative studies for both auditory nerve and HCs would pave a new path for more widespread treatment of DHL and even for a number of other neurodegenerative conditions.

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