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Investigating a Cell Replacement Therapy in the Inner Ear

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Abstract

The mammalian auditory system is sensitive to genetic disorders, aging and injuries caused by overstimulation, ototoxic drugs and viral infections. Since the sensory epithelium (the organ of Corti) and the spiral ganglion neurons (SGNs) in adult mammalian do not regenerate spontaneously when they are damaged, a cell substitution strategy was proposed to compensate the function of the degenerated SGNs. The current study investigated different aspects of cell transplantations in order to test the feasibility of a cell therapy approach in the adult auditory system.

Transplanted cells, including embryonic neuronal tissue (mouse embryonic dorsal root ganglion neurons, DRGs) and stem cells (adult neural stem cells, NSCs; embryonic stem cells, ES cells) were found to survive for up to ten weeks in the adult auditory system following transplantation. Supplementing exogenous neurotrophic factors significantly enhanced the survival of DRG neurons. Similarly, ES cell survival was remarkably enhanced by cografting embryonic neuronal tissue.

Further research was focused on identifying possible interactions between implanted cells and the host SGNs. The results showed that neurite projections were formed by implanted cells and seemed to contact the host SGNs. The supplement of exogenous nerve growth factor (NGF), chronic electrical stimulation or embryonic neuronal cograft promoted neurite formation. The implanted cells were observed to migrate through the bony modiolus to reach the SGN region in Rosenthal's canal. When tissue was transplanted along the auditory nerve (N. VIII), DRG neurons were found to migrate centrally to the internal meatus while ES cells migrated even further centrally and reached the cochlear nucleus in the brain stem. The results suggest that implanted cells have the potential to structurally integrate with the host auditory system, or even have the capability to replace degenerated SGNs and form connections between the peripheral auditory structures and the central nervous system.

Neuronal differentiation of the transplanted stem cells was also investigated. Adult NSCs did not seem to differentiate into a neuronal fate in a normal inner ear. Interestingly, NSCs differentiated into neuron-like cells when they were transduced with *neurogenin2* prior to transplantation. ES cells showed differentiation when cografted with embryonic neuronal tissue.

The possible function of implanted cells was evaluated in the DRG model by recording electrically-evoked auditory brain stem responses (EABRs). The results showed that exogenous NGF and/or chronic electrical stimulation enhanced neurite outgrowth from the DRG neurons. However, this did not translate to a functional change as measured by EABR.

In conclusion, the survival, neurite formation, migration and differentiation of cells implanted into the adult inner ear suggest that a cell replacement approach may provide an alternative for the development of an effective new treatment for hearing loss.

Keywords: Adult neural stem cell; Cell therapy; Differentiation; Dorsal root ganglion; Embryonic stem cell; Gene transfer; Hearing loss; Nerve growth factor; Neurogenin; Organ of Corti; Spiral ganglion; Transplantation

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To my family

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Hu Z**, Ulfendahl M, Olivius NP. Survival of neuronal tissue following xenografted implantation into the adult rat inner ear. Exp Neurol 2004, 185: 7-14

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IV. **Hu Z**, Wei D, Johansson CB, Holmström N, Duan M, Frisén J, Ulfendahl M. Survival and neural differentiation of adult neural stem cells transplanted into the mature inner ear. Exp Cell Res 2005, 302: 40-47

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CONTENTS

LIST OF ABBREVIATIONS

ABI	Auditory brain stem implant
ABR	Auditory brain stem response
BDNF	Brain-derived neurotrophic factor
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
DRG	Dorsal root ganglion
EABR	Electrically-evoked auditory brain stem response
EGFP	Enhanced green fluorescent protein
ES cells	Embryonic stem cells
GFAP	Glial fibrillary acidic protein
Ngn	Neurogenin
NGF	Nerve growth factor
NSC	Neural stem cell
NT-3	Neurotrophin 3
PNS	Peripheral nervous system
SGN	Spiral ganglion neuron
SP	Substance P

1 INTRODUCTION

1.1 EAR AND HEARING

Hearing is a key sense in mammalian which commences with the capture of the sound by the ear. The ear is composed of the external ear, the middle ear and the inner ear (Fig. 1). The most obvious component of the mammalian external ear is the auricle, which acts as a reflector to capture sound efficiently and to transfer it to the middle ear through the external auditory meatus.

The middle ear is an air-filled cavity. It comprises the tympanic membrane and auditory ossicles: the malleus, the incus, and the stapes. The main function of the middle ear is to transfer the vibrations of the tympanic membrane to the inner ear. The sound-induced increases and decreases in air pressure push and pull effectively upon the tympanic membrane, moving it inward or outward. Movements of the tympanic membrane displace the malleus and subsequently mobilize the incus. The incus then drives the stapes deeper into the inner ear and retracts it. The stapes's footplate serves as a piston that pushes and pulls cyclically upon the fluid in the inner ear. Thus the mechanical sound energy flows through the middle inner to the inner ear.

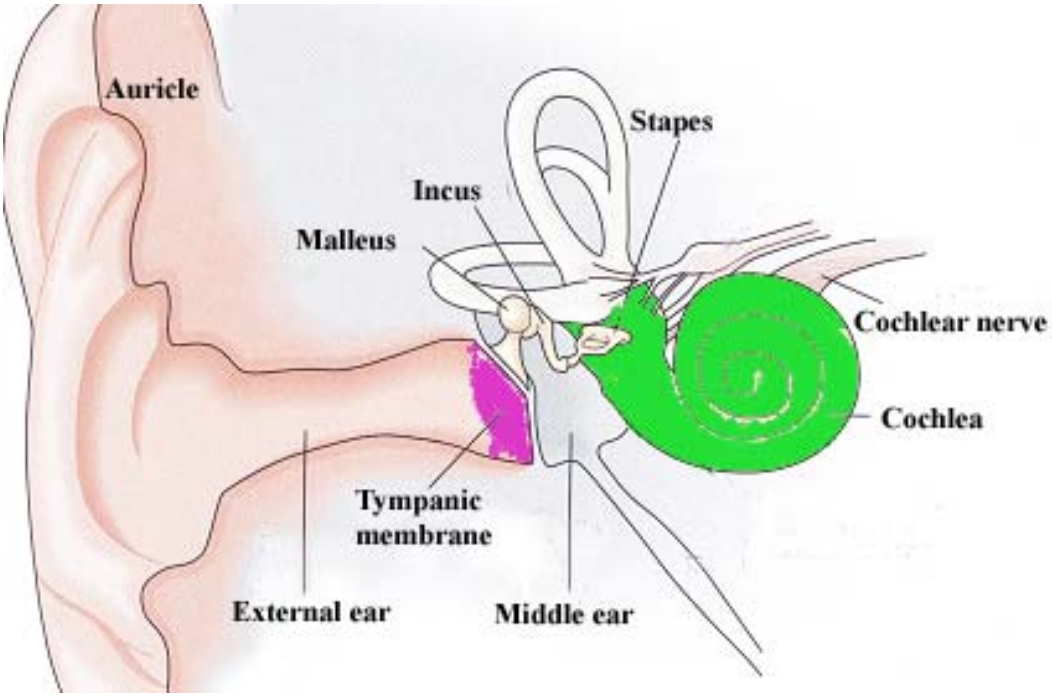

Figure 1. A coronal view of human ear illustrating the external ear, middle ear, and inner ear. The sound is captured by the auricle, focused into the external auditory canal, conveyed across the middle ear by ossicles. Vibration of the stapes stimulates the cochlea, the hearing organ in the ear. (Modified from Noback 1967).

The inner ear is covered with a thin layer of laminar bone and divided into two parts, cochlea (responsible for hearing) and vestibulum (responsible for balance). The entire cochlea is embedded within the dense structure of the temporal bone. The interior of the cochlea is divided into three fluid-filled compartments, scala vestibuli, scala media and scala tympani, wound helically around a conical bony core, the modiolus (Fig. 2). These three compartments are filled with two different fluids: perilymph (in scala tympani and scala vestibuli) and endolymph (in scala media). The vestibular membrane (Reissner's membrane) separates scala media from scala vestibuli. The basilar membrane, which forms the partition between the scala media and scala tympani, is a complex structure where the hearing organ (organ of Corti) is located. The basilar membrane is more broad, thin and rigid at the basal cochlear turn while it is narrow, thick and floppy at the apex of the cochlea. The hearing organ, organ of Corti, is located above the basilar membrane. There are four rows of hair cells in the organ of Corti: one row of inner hair cells and three rows of out hair cells. In human being, there are about 15000 hair cells, whereas 12500 in cat and 9500 in guinea pig. The hair cells lie within a matrix of supporting cells with their apical ends towards the endolymph of the scala media and their cilia projections into the gelatinous, collagen-containing tectorial membrane.

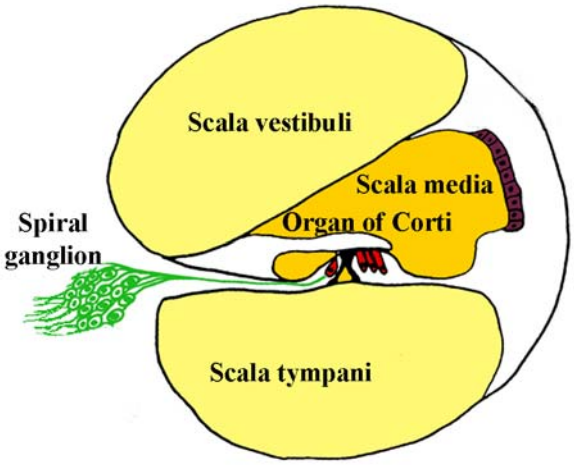

Figure 2. The cochlea is composed of three fluid-filled cavities. The organ of Corti is located above the basilar membrane. The spiral ganglion neurons localize in Rosenthal's canal within bony modiolus.

The space and fluid of the scala vestibuli and scala tympani are continuous and interconnect through a small opening at the apex of the cochlear called the helicotrema (Salt and Ma, 2001). The osseous spiral lamina, which separates scala tympani and Rosenthal's canal (where spiral ganglion located, Fig. 2), is a thin and highly porous bony lamina (Shepherd and Colreavy, 2004) with tiny canals called canaliculae perforantes of Schuknecht (Lim and Kim, 1983), which would appear to provide an open and extensive fluid communication channel between scala tympani and Rosenthal's canal. This is important for the design and application of cochlear implanted electrode arrays and may provide a potential route for a proper cell migration based on a cell replacement therapy delivered via the scala tympani.

The inwards and outwards of the stapes cause a cyclic increase and decrease in the pressure of the scala vestibuli, results in a downward and upward of the basilar membrane. The bowing movements of the basilar membrane create a shear force relative to the parallel sheet of the tectorial membrane. The mechanical sound waves thus are transduced into electrical signals in the organ of Corti by this sheer movement. It has been demonstrated by Georg von Békésy that the pure tone continuously traveling along the basilar membrane has different amplitudes at different point along

the base-apex axis. The low frequencies generate the maximal amplitudes near the apex while the high frequencies close to the basal membrane. The inner hair cells located above the basilar membrane transduce the vibration of sound into auditory afferent signals which result in the firing of the afferent nerve fibers. The out hair cells are mainly connected to the efferent nerve fibers and responsible for the high sensitivity and frequency turning properties of the hearing organ. Thus, hair cells close to the basal cochlear turn are sensitive to the high frequency sound while hair cells close to apex sensitive to the low frequency stimulation. Obviously, hearing organ in the adult mammalian inner ear is very delicate and complicated, including not only the existence of the functional sensory cells (hair cells) but also the proper contacts between the tectorial membrane and hair cells, as well as the appropriate position on the basilar membrane, which may lead to the difficulty in the replacement of organ of Corti based on a substitution strategy.

The afferent innervation of the hair cells is via the spiral ganglion neurons (SGNs) located in Rosenthal's canal (Fig. 2) at the center of bony cochlea (modiolus). The sensory hair cells activate the auditory nerve fibers, which emanate from the SGNs and projecting centrally to the cochlear nucleus in the brain stem. The sound intensity and frequency are considered to be encoded by the cochlear nerves. The neural pathway then leads to the higher auditory processing centers of the brain: superior olivary complex in the brainstem, the lateral leminiscal nuclei and inferior colliculus in the midbrain, medial geniculate body in the thalamus, and the primary auditory cortex.

1.2 NEUROTROPHIC FACTORS IN THE AUDITORY PERIPHERY

The neurotrophic factors are a family of growth factors critical for the development and functioning of the nervous system. There are at least four main neurotrophic factors in mammalian, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and NT-4/5. The first neurotrophic factor isolated was NGF, almost half a century ago (Levi-Montalcini, 1987; Levi-Montalcini and Booker, 1960), which was identified as a soluble factor required for the survival of specific groups of developing sensory and sympathetic neurons. The subsequent discovery of a family of such factors included BDNF, NT-3, and NT-4. All neurotrophic factors can bind to the low affinity receptor, p75 neurotrophin receptors (p75NTR), but tropomyosin-related kinases (Trks) show much greater specificity. Three Trk receptors have been identified, each specific for a different neurotrophin, TrkA is a high-affinity signaling receptor for NGF, while TrkB specific for BDNF and NT-4, and TrkC preferentially for NT-3, respectively. The functions of these neurotrophic factors extend well beyond survival (Crowley, et al., 1994; Minichiello, et al., 1995; Zimmermann, 1998) and include the retrograde interactions between neurons and their targets (DiStefano, et al., 1992; Snider and Wright, 1996), growth cone migration and axonal branching (Gallo, et al., 1997; Gallo and Letourneau, 1998), axons regeneration (Bloch, et al., 2001; Edstrom, et al., 1996; Terenghi, 1999), synapse formation, cell migration and proliferation (Segal, 2003), cell fate decision and expression of proteins crucial for normal neuronal function (Huang and Reichardt, 2001). In the adult nervous system, neurotrophic factors control

synapse function and plasticity, while continuing to modulate neuronal survival (Huang and Reichardt, 2001; Huang and Reichardt, 2003).

It is well accepted that neurotrophic factors are major factors involved not only in the inner ear development but also in the maintenance of the survival of SGNs in adults. It has been demonstrated that complete overlap of TrkB and TrkC, high affinity receptor of BDNF and NT-3, was identified in all SGNs during embryonic development, verified by *in situ* hybridization for neurotrophic factors and their receptors (Pirvola, et al., 1994; Wheeler, et al., 1994) , immunocytochemical investigations of neurotrophin receptors distribution (Farinas, et al., 2001), and analyses of neurotrophic factors using the sensitive LacZ reporter technique (Farinas, et al., 2001). The effect of NGF and its high-affinity receptor TrkA for the inner ear development is still controversial. It has been reported that NGF mRNA was not observed by *in situ* hybridization in the developing rat inner ear (Pirvola, et al., 1994; Pirvola, et al., 1992), whereas it was also shown that a transient expression of TrkA was detected in early embryo developing vestibulo-cochlear ganglion cells by *in situ* hybridization and PCR analyses (Bernd, et al., 1994; Schecterson and Bothwell, 1994). In the study of newborn mutant mice, no TrkA expression was detected in newborn TrkB $^{-/-}$ and TrkC $^{-/-}$ double mutants (Schimmang, et al., 1997). In general, it is well accepted that signaling of NGF via TrkA does not play a role for SGNs formation and innervation, which only appears to require TrkB and TrkC signaling (Fritzsch, et al., 1997b; Rubel and Fritzsch, 2002; Schimmang, et al., 1997) .

Survival of adult SGNs depends on the integrity of cells of the organ of Corti, particularly the hair cells with which SGNs make synaptic contact, probably through the roles of NT-3 and BDNF. It has been recently shown that adult cochlear supporting cells also provide critical trophic support to SGNs (Stankovic, et al., 2004). The data from the transgenic mice indicates that the survival of postnatal SGNs depends on reciprocal interactions between neurons and supporting cells, which are probably mediated by neuregulin and neurotrophins. It is reported *in vitro* model of early postnatal rat organ of Corti/spiral ganglion explants that BDNF affected early neuronal survival, whereas NT-3 was the most important survival factor for maturing SGNs (Staecker, et al., 1996). It is well documented that TrkA was transiently expressed in the early development and not detected in the SGNs of neonatal or adult mammalians (Malgrange, et al., 1998; Pirvola, et al., 1992; Qun, et al., 1999; Schecterson and Bothwell, 1994). P75NTR is involved in a proapoptotic signaling pathway but it is suppressed by Trk receptor initiated signaling (Huang and Reichardt, 2003). It has also been documented that P75NTR can enhance the ability of Trk receptors to bind and respond to neurotrophins and sharpen the discrimination of Trks for their specific neurotrophin ligands (Barker, 2004). It is reported that P75NTR was detected in the SGNs throughout development and in adulthood (Schecterson and Bothwell, 1994; Ylikoski, et al., 1993). In a situation where none of the known Trk receptors is expressed, promotion of neuronal survival via p75NTR on its own is rather unlikely (Lee, et al., 1992; Schimmang, et al., 1997).

1.3 HEARING LOSS AND REGENERATION OF SENSORY EPITHELIUM

Hearing loss is one of the most common disabilities in the world, affecting 1/3 adults over the age of 60 and half of the adults over the age of 75 (Marcincuk and Roland, 2002; Ruben, 1993). At birth or during early childhood (prelingual period), approximately 1/1000 individuals is affected by severe or profound deafness. A severe defect that is manifested in early childhood will have dramatic effects on speech acquisition and literacy. Later onset of severe hearing impairment seriously compromises the quality of life, as the affected individual becomes increasingly isolated socially. Finally, 0.3% and 2.3% of the population manifest a hearing loss greater than 65 dB between the ages of 30 and 50 and between 60 and 70, respectively. It could be due to head trauma, inflammatory diseases of the ear, overstimulation, ototoxic drugs during the other period of life. In general, in developed countries, at least 6-8% of the population suffers from hearing loss (Petit, et al., 2001).

Most hearing loss is due to the destruction of the sensory epithelium (organ of Corti) within cochlea, i.e. the degeneration and loss of hair cells. In the auditory system, as in other afferent nervous systems, degeneration of the SGNs and auditory nerve fibers occurs secondary to the loss of the cochlear sensory epithelium, thereby aggravating the impairment of the auditory system (Ernfors, et al., 1995; Marzella and Clark, 1999; Nadol, et al., 1989; Ryals, et al., 1989). The sequence of pathological alterations in SGNs appears to be: swelling, demyelination and degeneration of the peripheral dendrities; demyelination and shrikage of the cell soma with preservation of the dentral axon: and demyelination of the central axon and degeneration of the cell perikayon (Leake and Hradek, 1988). The chromatin condensation, cell shrinkage, membrane blebbing also manifested in the case of apoptosis.

There is evidence that the cochlea in lower vertebrates and avian possesses a self-repair mechanism that can be activated following damage to the sensory epithelium (Corwin and Cotanche, 1988; Cotanche, 1999; Ryals and Rubel, 1988; Stone and Rubel, 2000; Woolley, et al., 2001). However, in mammalians, the cochlear sensory epithelium and its neural components do not regenerate spontaneously, and there is currently no effective intervention for their repair. Recent observation demonstrates that the adult utricular sensory epithelium contain pluripotent cells (Li, et al., 2003). The situation in the cochlea is complex and it has been suggested that cochlear precursor cells cannot be reactivated to differentiate into sensorineural cells in adult mammals.

It is reported by Raphael and associates that *in vivo* inoculation of adenovirus with Math1 gene infused into the scala media of the mature guinea pig cochlea resulted in Math1 overexpression in nonsensory cochlear cells. The Math1 protein was found in supporting cells of the organ of Corti and in adjacent nonsensory epithelial cells. The study further demonstrated that Math1 overexpression leaded to the appearance of immature hair cells in the organ of Corti and new hair cells adjacent to the organ of Corti in the interdental cell, inner sulcus, and Hensen cell regions. Axons were found to extend from the bundle of auditory nerve toward some of the new hair cells, suggesting that the new cells appeared to attract auditory neurons (Kawamoto, et al., 2003).

However, due to the retrograde changes of the SGNs and auditory nerve fibers (Hardie and Shepherd, 1999; Leake and Hradek, 1988), regeneration of hairs cell alone

may not be sufficient to restore profound sensorineural hearing loss. A re-construction of functional structure of the organ of Corti, a proper location on the basilar membrane, as well as a re-establishment of appropriate relationship to the tectorial membrane are essential. Moreover, in order for the sensory epithelium to convey auditory signal to the central auditory system and restore hearing, the primary auditory neurons must also form functional synapses with the hair cells.

1.4 PREVENTING THE DEGENERATION OF SENSORINEURAL CELLS

One consequence of sensory epithelium loss and neuronal degeneration in the cochlea is the loss of neurotrophic factors that can lead to a change in oxidative state (formation of free radicals), changes in the concentration of intracellular Ca^{2+}, and up-regulation of apoptotic genes (e.g., JNK, c-Jun) (Mattson, 2000; Miller, et al., 2002). Interventions by means of neurotrophic factor supplement as well as the enhancement of their receptor expression may preserve the cochlear sensory epithelium and SGNs following inner ear insults. Extensive studies have provided strong evidence that neurotrophic factors (e.g. BDNF, NT-3), anti-apoptotic agents (e.g., Z-VAD-FMK), antioxidants, and chronic electrical stimulation can prevent the cochlear sensory epithelium from death as well as the secondary damage to the SGNs shortly after the cochlear sensory epithelium is damaged (Liu, et al., 1998; Miller, et al., 2003; Miller, 1991; Miller, et al., 2002; Mitchell, et al., 1997; Schindler, et al., 1995; Shah, et al., 1995; Shinohara, et al., 2002).

1.4.1 Neurotrophic factors

It is well known that the neurotrophic factors are a family of growth factors critical for the functioning of the nervous system. In the auditory system, NT-3 is the major survival factor for the SGNs. NT-3 mutant mice showed a paucity of afferents and lost 87% of SGNs (Ernfors, et al., 1995; Farinas, et al., 1996; Fritzsch, et al., 2002; Fritzsch, et al., 1997a). It is reported that the loss of target innervation and the degeneration of approximately 90% of the adult SGNs caused by aminoglycoside toxicity can be prevented by infusion of NT-3 in the membranous labyrinth in guinea pigs (Ernfors, et al., 1996). The potency of NT-3 in protecting SGNs from degenerating suggests that neurotrophic factors may be essential for the treatment of hearing disorders. BDNF is another major neurotrophic factor in the maintenance of SGNs and shows similar function as NT-3 (Ernfors, et al., 1995; Farinas, et al., 2001; Fritzsch, et al., 1997b). The role of NGF in prevention of SGNs degeneration is controversial. However, an *in vitro* study indicates that NGF did not have any effect on the survival of SGNs throughout the development and adulthood (Qun, et al., 1999).

1.4.2 Caspase inhibitors

In vitro studies have tested the efficacy of different caspase inhibitors, Ac-VAD-cmk (caspase-1 inhibitor), z-DEVD-fmk (caspase-3 inhibitor) and B-D-fmk (BOCDFK, a general inhibitor), for protecting auditory sensorineural cells from cisplatin-damage

induced hearing loss. Treatment of 3-day-old rat organ of Corti explants with these caspase inhibitors protected >80% of the auditory hair cells from cisplatin-damage initiated apoptosis. Dissociated cell cultures of 3-day-old rat spiral ganglia treated with any of these three caspase inhibitors in addition to exogenous neurotrophic factors have highly significant increases in SGNs survival following cisplatin exposure. These results indicate that loss of auditory sensory cells as a result of cisplatin-induced damage involves apoptosis and that blocking of this cell death pathway at the caspase level may rescue hair cells and SGNs (Liu, et al., 1998).

1.4.3 Gene therapy

In order to prevent the secondary degeneration of cochlear neurons that ensues from withdrawal of neurotrophic factors, adeno-associated virus (AAV) containing *bdnf* gene was administrated to the rats following ototoxic exposure. Neuronal survival was significantly promoted in AAV-*bdnf*-transduced cochlear explants compared with control samples following exposure to aminoglycoside. Following deafening with aminoglycoside and loop duretics and introduction of AAV-*bdnf* gene through an osmotic minipump, the animals infused with neurotrophic factor gene (*bdnf*) showed enhanced SGNs survival in the basal cochlear turn of the cochlea when compared with the control group (Lalwani, et al., 2002).

1.4.4 Electrical stimulation

It has been reported that early electrical stimulation can reduce the progress of SGN degeneration secondary to ototoxic-induced hair cell loss in ototoxically deafened animals (Miller, 1991). In order to investigate whether the high rate stimulation would affect the SGNs survival, Mitchell and co-authors treated the deaf animals with chronic electrical stimulation at different frequency and found that high-rate stimulation provided no additional risks and the same benefits to SGNs survival as low-rate stimulation (Mitchell, et al., 1997). However, it has also been reported that electrical stimulation was not always protective (Araki, et al., 1998; Li, et al., 1999; Shepherd, et al., 1994). The mechanisms through which electrical stimulation would enhance SGN survival are not completely understood. In vitro study, the SGN explants have shown a depolarization-induced enhancement of SGN survival that is mediated by L-type voltage gated Ca^{2+} channels (Hegarty, et al., 1997). This was subsequently verified by the experiments *in vivo* (Miller, 2001; Miller, et al., 2003). These observations suggest that electrical stimulation may promote SGNs survival by maintaining intracellular Ca^{2+} levels with in a physiological range.

1.4.5 Combined therapy

Due to the complicacy of the inner ear damage mechanism, a combination of the different interventions may be more helpful in order to prevent the degeneration of cochlear cells. It is documented that a combination of agents directed against both hair cells and SGNs degeneration can provide significant functional protection against noise or drug-induced damage (Duan, et al., 2000). Inner hair cells release glutamate, which

excites N-methyl--aspartate (NMDA) receptors on the dendrites of the SGNs. NMDA receptors have been implicated in excitotoxic cell death and may be key mediators of damage induced both by noise or by aminoglycosides (Basile, et al., 1996). It has been shown that NT-3 protects SGNs during aminoglycoside treatment in guinea pigs (Ernfors, et al., 1996) and an NMDA antagonist helps to preserve hair cell morphology, the Preyer reflex and distortion product oto-acoustic emissions (DPOAEs) following similar treatment in the same animal. Applied together, shortly before noise damage or infusion of amikacin into the perilymph, the NMDA antagonist MK801 and NT-3 prevent dendritic swelling, preserve the morphology of hair cells and SGNs, and significantly decrease threshold shifts of auditory brain stem responses.

In order to test whether the absence of both excitatory activity and chemical survival factors is implicated in the loss of auditory neurons, Kanzaki and co-authors treated the deaf animals with electrical stimulation, GDNF, and a combination of electrical stimulation and GDNF. The results showed that treated animals showed obviously greater SGNs survival than untreated animals. The combination of electrical stimulation and GDNF showed significantly better SGN survival than either treatment alone (Kanzaki, et al., 2002).

In general, neurotrophic factors, anti-apoptotic agents, antioxidant and chronic electrical stimulation are able to prevent the degeneration of hair cells and SGNs. However, for patients with profound sensorineural hearing loss who have already had an extensive loss of hair cells and SGNs, it is less interesting to prevent the degeneration of the SGNs in the inner ear, suggesting that new approaches are needed in the treatment of these cases.

1.5 THE COCHLEAR PROSTHESIS

The cochlear prosthesis is now a standard treatment to profound sensorineural hearing loss cases in clinics for decades. It is well accepted that the efficacy of cochlear implant depends not only on the efficacy of cochlear implant device but also on the integrity and the function of surviving SGNs.

1.5.1 Cochlear implant and its clinical application

In normal hearing sounds are perceived in the inner ear through external ear and middle ear. The different frequencies of incoming sounds create an appropriate displacement at different points along the basilar membrane within the cochlea. With this way the cochlea acts like a spectrum analyzer, which decomposes complex sounds into their frequency components. In cochlear implants, the speech processor and the implanted electrodes are expected to take over this task.

The cochlear implant system contains three external components (directional microphone, speech processor, and transmitting part) and one surgically implanted internal device. The external part perceives sound wave from the outside environment, converts the analog signal to digital output and transfers the digital information across the recipient’s skin to the surgically implanted receiver/stimulator. The internal part of

the device is anchored to the postauricular skull and is composed of a magnet and a multichannel electrode array that is placed into the scala tympani through round window. The multichannel electrode array stimulates the SGNs together with their central processes (auditory nerve fibers), thereby bypassing the damaged cochlear sensory epithelium (Copeland and Pillsbury, 2004). Thus, a minimal number of functional SGNs are needed for the cochlear implant to work properly.

Now cochlear implant has become the standard treatment to the profound hearing loss cases and more than 40,000 patients have received the implantation (Rauschecker and Shannon, 2002). It is reported that cochlear implant recipients can not only regain the speech recognition (at least enhance the lip-reading) but also the primary ability to discriminate the rhythm, timbre, and songs by verbal cues (Leal, et al., 2003). However, there are still a huge number of patients who are not suitable for a cochlear implant therapy, i.e. acoustic neurofibromatosis and other cases who lack enough SGNs to response to the cochlear implant.

1.5.2 Cochlear implant and spiral ganglion neurons

In order to have a high speech perception, cochlear implant device needs a certain number of functional SGNs to stimulate. However, degeneration of SGNs and auditory nerve fibers is an ongoing process, eventually resulting in extensive loss of surviving SGNs in animals deafened for a long period of time (Hardie and Shepherd, 1999; Leake and Hradek, 1988). Based on a limit number of histological observations there was a debate on the relationship between the number of SGNs and postoperative implant performance (Fayad, et al., 1991; Nadol, et al., 2001). It is commonly believed that degeneration of SGNs will reduce the efficiency of the cochlear neurons in response to an electrical stimulation (Hardie and Shepherd, 1999; Shepherd and Javel, 1997; Zhou, et al., 1995). Also a minimum number of functioning SGNs is required to achieve a high level of speech perception in cochlear implant patients (Blamey, 1997). However, profound sensorineural hearing loss cases will ultimately have an extensive loss of the number and function of SGNs. As a consequence, a cochlear prosthesis may not have the minimum number of functioning SGNs to stimulate, leading to a suboptimal function of the device.

1.5.3 Auditory brain stem implant

Cochlear implant is not an option for the patients whose deafness is caused by lesions beyond the cochlea. It includes acoustic neurofibromatosis, a genetic disorder with the occurrence of 1/40,000 births (Evans, et al., 1992a; Evans, et al., 1992b). Acoustic neuroma is often located along afferent nerve tracts as they enter the brain stem. Dissection of the neuroma almost always necessitates a transection and removal of the auditory nerve and thus results in total deafness. The auditory brainstem implant (ABI) has been applied in the rehabilitation of hearing for more than ten years and it does provide auditory sensations, recognition of environmental sounds and aid in spoken communication in about 300 patients worldwide (Kuchta, 2004). ABI has been well accepted as the treatment of patients who have lost hearing due to bilateral tumors of the cochlear nerve who transmits the acoustic information from the cochlea to the

brain. Most of the implanted patients are completely deaf when the implant is switched off. In contrast to cochlear implants, only few of the implanted patients achieve open-set speech recognition without the help of visual cues. On average, the ABI improves communicative functions like speech recognition at about 30% when compared to lip-reading only. The task for the next step is to improve the outcome of ABI by developing new less invasive operative approaches as well as new hardware and software for the ABI device (Federspil and Plinkert, 2004; Kuchta, 2004; Lenarz, et al., 2002; Otto, et al., 2004).

1.6 CELL REPLACEMENT STRATEGIES

The cell transplants have been used clinically in human beings to repair the articular cartilage of the knee (Brittberg, et al., 1994) and as therapy for Parkinson's disease (Lindvall, et al., 1990). Due to the poor regenerative ability of adult auditory system, exploration of novel approaches to restore auditory function is needed (Dazert, et al., 2003; Holley, 2002; Hu, et al., 2004a; Hu, et al., 2004b; Hu, et al., 2005). A new concept of restoring hearing function of the inner ear would be to use a biological implant with the potential to replace the degenerated or absent sensorineural cells in the inner ear, to become integrated with the auditory system, and to develop into a functional auditory unit (Paper I).

1.6.1 Target cells in the inner ear

The targeted cell for replacing is a key issue in the cell replacement therapy in the inner ear. The selection of donor candidates and transplantation approach mainly depends on the target cells to be substituted. It is apparent that the sensory epithelium (organ of Corti) in auditory system is a highly structurally characterized organ compared to the other tissues in the afferent system. The replacement of hair cells alone is not enough, since an intact organ of Corti including the functional supporting cells, the appropriate contact between the hair cells and tectorial membrane, and a proper location on the basilar membrane are crucial to transduce the mechanical sound wave to the electrical auditory input signals. Also the hair cells need to form the appropriate synaptic connections with the afferent auditory nerve fibers and SGNs to transfer the auditory signals centrally to the cochlear nuclei in the brain stem. It is suggested that SGN may be a more feasible and immediate target for the cells replacement at the present level of the field.

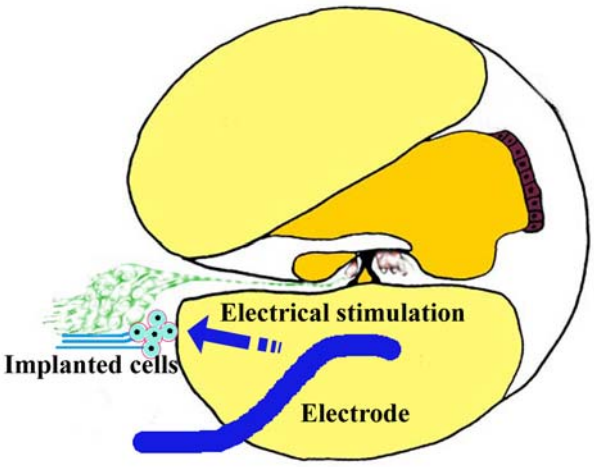

Figure 3. The implanted cells receive the electrical stimulation from the implanted electrode directly, thus bypassing the degenerated hair cells and spiral ganglion neurons.

Combined with a cochlear prosthesis, the cochlear electrode processed electrical signals could stimulate the implanted cells directly, thus bypass the degenerated or absent sensory epithelium and SGNs (Fig. 3). In the present research, we focused on the study of substitution for SGNs. It is also noted that a regeneration or rehabilitation of the hair cells and the organ of Corti is very interesting in the future research.

1.6.2 Approaches for replacement therapy

The inner ear in mammalian is a very complex organ. The complicacy of the auditory periphery may lead to a difficulty in selecting a proper transplantation approach. Theoretically, the transplantation location is expected to be close to SGNs and the surgical approach lead to the minimal damages to the host tissues.

An approach of opening the bony modiolus to Rosenthal's canal is obviously more direct in terms of the SGNs replacement. However, it is also apparent that this direct modiolus approach may lead to severe hearing and balance damage and disorder following surgery in future clinical applications, i.e. vertigo, tinnitus. A scala vestibuli approach is feasible but this location is possibly a bit far from the SGNs while a scala media approach will destroy the stria vascularis and result in a severe inner ear blood supply disorder. Structurally, the SGNs are close to the scala tympani, only separated by the highly porous bony spiral lamina. The porous spiral lamina (Shepherd and Colreavy, 2004) and Schuknecht's canaliculae perforantes (Lim and Kim, 1983) may be also beneficial to the cell migration. Further, the long-term aim of the study is to introduce a cochlear implant electrode into the scala tympani, convert sound and stimulate the implanted cells directly (Fig. 3). Thus, a scala tympani approach appears more safe and feasible. This approach has been widely used in cochlear implant surgery in clinics and has been well accepted. Thus, in the present study we selected the scala tympani as the transplantation location (Paper I, II, IV-VI). In order to test whether implanted cells can survive along the auditory nerve, we also transected the auditory nerve fibers through the modiolus at the basal cochlear turn and transplanted the exogenous cells (Paper III).

1.6.3 Candidates for a cell replacement therapy

Since the exogenous cells are expected to replace the degenerated SGNs, a neuronal tissue or the cells with the potential to differentiate into neurons are potential donor candidates. In the present study, embryonic neurons (e.g., embryonic dorsal root ganglion, DRGs), pluripotent embryonic stem (ES) cells and multipotent neural stem cells (NSCs) were transplanted into the adult inner ear.

1.6.3.1 Embryonic neuronal tissue

DRG neurons are psedo-unipolar neurons which has a central process to the spinal cord and a peripheral process to the sensory receptors, i.e., mechanoreceptors, thermoreceptors, chemoreceptors. According to the size, the DRG neurons can be divided into three main subtypes, small size (diameter <25 μm), medium size (diameter 25-40 μm), and large size (diameter >40 μm). The DRG neurons also synthesize

neuropeptides, i.e., substance P (SP) and calcitonin gene-related protein (CGRP) (Hökfelt, et al., 1975; Jessell, et al., 1979; Keen, et al., 1982; Kessler and Black, 1981), which may play a role in the axonal transport and inflammatory response (Brimijoin, et al., 1980; Fernandez and Hodges-Savola, 1994; Galeazza, et al., 1995; Hiruma, et al., 2000; Hökfelt, et al., 1975; Kashihara, et al., 1989; Keen, et al., 1982). It is reported that the immunoreactivity for SP is present mainly in small and medium size DRG neurons (Gibson, et al., 1984), whereas CGRP immunoreactivity distributes mainly in medium size cells (Hökfelt, et al., 1976; Ishida-Yamamoto and Senba, 1990; Ju, et al., 1987; Kruger, et al., 1988; Lee, et al., 1985). It is also demonstrated that during development, similar to inner ear development mentioned above, DRG neurons depend for their survival on neurotrophic factors synthesized by the tissues that they innervate (Davies, 1994). Thus the mutant mice deficient in either Trk receptors (e.g. TrkA, TrkB, TrkC) or its high affinity ligand neurotrophic factors (NGF, BDNF, NT-3) suffer from loss of DRG neurons (Crowley, et al., 1994; Ernfors, et al., 1994a; Ernfors, et al., 1994b; Jones, et al., 1994; Klein, 1994; Klein, et al., 1993; Smeyne, et al., 1994). The axons of DRG neurons include unmyelinated C fibers, the smallest-diameter myelinated Aδ fibers and other myelinated fibers. NGF dependant DRG neurons are mostly associated with the C fibers and Aδ fibers. Aδ fibers have a similar conduction velocity as the cochlear nerve (Moller, et al., 1994; Nguyen, et al., 1999), suggesting the possibility for replacing the auditory input function.

Previous studies have shown that embryonic rat (Rosario, et al., 1993) or human (Kozlova, et al., 1997) DRG cells survived in the dorsal root cavity following extirpation of native adult rat DRG neurons. It has also been reported that implanted DRG neurons were able to send axons to peripheral tissues (Bauchet, et al., 2001; Rosario, et al., 1995) as well as centrally into the spinal cord (Bauchet, et al., 2001; Kozlova, et al., 1997) where they made functional synaptic contacts (Levinsson, et al., 2000). Recently, it was also shown that DRG neurons from guinea pig embryos survived for three weeks following allograft transplantation into the cochleas of guinea pigs (Olivius, et al., 2003). In the present project, we aimed at testing whether xenografted (mouse to rat and mouse to guinea pig) DRGs have the capability to survive in the adult auditory system. It is also noted that DRG neurons may not be the most appropriate donor candidate for the cell replacement therapy in the inner ear. However, the results from DRG implantation experiment will prove the principle of whether xenografted cells possess the ability to survive in the adult inner ear. It will also provide important information for how to implant other types of neurons or cells, i.e. SGNs and stem cells.

1.6.3.2 Stem cells

Another interesting candidate for a cell replacement therapy in the inner ear would be stem cells. In the present study, our selections were multipotent adult neural stem cell (NSC) and pluripotent embryonic stem (ES) cells. For ethical considerations, adult stem cells may be a more appropriate candidate. Also, using adult NSCs rather than ES cells would possibly facilitate differentiation into cell types of functional interest for the auditory system. NSCs have been reported to possess the capacity to self-renew and differentiate into the major cell types of the neural tissue, i.e. neurons, astrocytes and

oligodendrocytes (Clarke, et al., 2000; Gage, 2000). It has been documented that adult NSCs could generate olfactory bulb neurons and their progeny could migrate and differentiate into astrocytes at the site of the lesion (Johansson, et al., 1999). Further, NSCs have been tested in animal models of several severe nervous system disorders, i.e. Parkinson's disease, Huntington's disease, Alzheimer's disease and spinal cord injuries (Storch and Schwarz, 2002; Sugaya and Brannen, 2001). Following transplantation into the damaged retina, neuronal differentiation and morphological integration of adult neural progenitor cells have also been demonstrated (Pressmar, et al., 2001; Warfvinge, et al., 2001; Young, et al., 2000). These observations suggest the potential of adult NSCs to differentiate into neuronal cell types within the injured mature neural system. It also indicates that adult NSCs may be a proper candidate for a cell replacement therapy in the cochlea (Paper IV).

ES cells are self-renewing pluripotent cells that can differentiate into many cell types, including ectoderm, mesoderm, and endoderm (Burdon, et al., 2002; Henningson, et al., 2003). Because of their pluripotency, ES cells would be a logical candidate, though the destruction of the embryos during ES cells harvesting may lead to ethical concerns about using these cells for a scientific purpose. In the recent years embryonic stem cells-derived neurons and neural precursors (Liu, et al., 2000; McDonald, et al., 1999), cardiomyocytes (Klug, et al., 1996), mast cells (Tsai, et al., 2000) have been implanted into appropriate recipient sites and shown to survive, integrate and to some measurable extent function within host tissue. In the nervous system transplantation of ES cells-derived neural cells has resulted in functional improvement in rodent with Parkinson's disease (Björklund, et al., 2002; Kim, et al., 2002; Lee, et al., 2000) and spinal cord injury (Liu, et al., 2000; McDonald, et al., 1999). These observations suggest that ES cells may be an interesting candidate for a cell replacement therapy in the inner ear.

1.6.4 Survival of exogenous cells in the inner ear

Apart from the selection of the donor candidates, one important issue regarding cell replacement therapy in the inner ear is to investigate whether the implanted exogenous cells are able to survive in the adult auditory system. In order to prove this principle, we attempted to transplant embryonic DRGs, adult NSCs and ES cells into the inner ear (Paper I, IV, V). Different exogenous supplements were also simultaneously supplied into the inner ear with the aim to promoting the survival of implanted cells (Paper II, IV, VI). These approaches included exogenous neurotrophic factors, chronic electrical stimulation, or an appropriate extracellular microenvironment.

1.6.4.1 Neurotrophic factors

It is well accepted that neurotrophic factors play important roles in the development and survival of neurons. It is reported that during vertebrate development, the survival of many peripheral neurons, i.e. DRG neurons and SGNs, depends on neurotrophic factors released by the peripheral target that they are innervated (Davies, 1994). Extensive loss of peripheral neurons was identified in the mutant mice deficient in either Trk receptors (e.g. TrkA, TrkB, TrkC) or their high affinity ligands (e.g. NGF,

BDNF, NT-3) (Crowley, et al., 1994; Ernfors, et al., 1994a; Ernfors, et al., 1994b; Farinas, et al., 1994; Klein, 1994; Klein, et al., 1993; Smeyne, et al., 1994). It is also shown that neurotrophic factors have a neuroprotective role to the auditory neurons (Ernfors, et al., 1996; Lalwani, et al., 2002). The function of the exogenous cells transplanted into the inner ear needs to be evaluated in the study. In order to clarify whether the implanted cells contribute to the hearing improvement, it is necessary to distinguish the effect of neurotrophic factors on the host SGNs and on the implanted cells. Thus, at least to some extent, a neurotrophic factor with less neuroprotective effect on SGNs will be an optimal choice. It has been reported that the mRNA for TrkC and TrkB, the high-affinity receptors for NT-3 and BDNF respectively, are strongly expressed in the SGNs throughout life (Qun, et al., 1999). It has also been well documented that the supplement of exogenous NT-3 and BDNF have a significant neuroprotective role to SGNs (Ernfors, et al., 1996; Lalwani, et al., 2002). The neuroprotective role of NGF to SGNs in the inner ear is still controversial at least in the NGF mRNA detecting (Ylikoski, et al., 1993), TrkA mRNA detecting (Malgrange, et al., 1998) and *in vitro* supplement studies (Qun, et al., 1999). Compared to NT-3 and BDNF, NGF may be a more appropriate candidate. Accordingly NGF was selected as a neurotrophin candidate simultaneously infused into the cochlea together with a DRG implantation in Paper II and VI.

1.6.4.2 Electrical stimulation

Chronic electrical stimulation may be another approach to enhance and maintain the survival of neurons together with their neurite outgrowth. It has been reported that survival of primary auditory afferents is enhanced by electrical stimulation (Miller, et al., 2003; Miller, 1991; Mitchell, et al., 1997). In order to promote the survival of implanted cells, to stimulate the neurite outgrowth, and to enhance potential therapeutic aspect of the exogenous cell implants, chronic electrical stimulation was applied to the inner ear of adult guinea pigs transplanted with embryonic mouse DRG (Paper VI).

1.6.4.3 Extracellular microenvironment

It is known that neighboring cells play an important role in cell survival. In embryos, the death and removal of one cell will normally result in a compensatory change of fate of neighboring cells (Gurdon, 1999). It is reported that an autocrine loop in the sensory neurons has the potential to prevent cell death by the release of neurotrophic factors from neighboring cells (Acheson, et al., 1995; Davies and Wright, 1995). Thus embryonic neuronal tissue may be a good candidate to support the survival of implanted stem cells. Further, the implantation of cograft may provide a cell matrix for cell growth in the fluid-filled inner ear. In the present study, in order to investigate the mechanism of the stem cells survival in the adult inner ear, we transplanted the stem cells into scala tympani together with a simultaneously implantation of embryonic DRGs.

1.6.4.4 Injured inner ear

It is proposed that the sensorineural cells in an injured inner ear are expected to release growth factors, i.e. neurotrophic factors, through a reciprocal interaction between neurons and hair cells to provide support to the damaged neighboring cells. In order to investigate whether the survival of implanted cells will be enhanced by the growth factors released in the injured inner ears, the inner ears were chemically damaged by injecting of neomycin prior to receiving the exogenous cells implantation in the study (Paper I, IV, V).

1.6.5 The cell fate of implanted cells in the inner ear

The fate of the implanted stem cells in the auditory system is one of the most important issues we have to explore in the study. As for embryonic neuronal tissues, i.e. DRGs, the cell fate has been determined. However, as for stem cells, i.e. pluripotent ES cells or multipotent adult NSCs, there are potential problems that need to be addressed. First problem we need to know is the risk of un-controlled proliferation *in vivo*. Second issue is malignant transformation. A third concerning is that the transplanted stem cells may differentiate into an inappropriate cell fate. The un-controlled proliferation and the malignant transformation are not uncommon in ES cells transplantations (Asano, et al., 2003; Damjanov, 1993) but not reported in the NSCs transplantation. In the transplantation of ES cells into the inner ear, the survival of implanted cells remains a major issue in the cell replacement therapy in the inner ear. So it is less likely for the ES cells to develop into an un-controlled proliferation or tumor formation fate in the inner ear. As for the third consideration, the differentiation of implanted cells, there are several theories considered to be involved in the cell linage differentiation of the implanted cells, including intrinsic determinants (e.g. transcriptional factors), the extrinsic factors (e.g. growth factors, extracellular matrix), or both. In this study we tried to investigate the mechanism of the differentiation of stem cells by providing different intrinsic and extrinsic factors.

1.6.5.1 Intrinsic determinant

The selection of gene/transcriptional factor is a key issue with the aim for transducing stem cells prior to implantation and driving the stem cells to an appropriate neuronal cell fate in the auditory system. It has been demonstrated in the fate-mapping experiments in avian embryos that the neuronal precursors that generate cranial sensory ganglia derive from both the cranial neural crest and placodal ectoderm. According to the origin of their constituent neurons these ganglia can be roughly separated into two groups: distal ganglia which are deprived from epibranchial placodes (including the geniculate ganglia, distal VIIIth; petrosal ganglia, IXth; and nodose ganglia, Xth) and proximal ganglia which are deprive from either the neural crest and/or the trigeminal or otic placodes (including trigemal ganglia, Vth; superior, jugular, accessory and vestibulo-cochlear ganglia, VIIIth) (Ma, et al., 1998). Experiment in developmental neurobiology reveals that certain transcript factors may be involved in the determination of neural precursors. Several basic helix-loop-helix (bHLH) genes are

expressed in epibranchial placodes and have been identified as important transcriptional regulators of cell-type determination in this process, suggesting that a similar regulatory cascade may underlie the development of sensory neurons in cranial ganglia. Among the bHLH genes examined so far, *ngn2* and *ngn1* are the only ones that could function as neuronal determinants, as they are expressed prior to delamination, an early stage in the development of these lineages (Fode, et al., 1998). Interestingly, the expression of *ngn1* and *ngn2* appeared to define the precursors of proximal and distal ganglia, respectively, in an essentially complementary manner from the earliest stages of development analyses (Ma, et al., 1998). It is further showed that the otoic placodes-deprived vestibulo-cochlear ganglion was missing in *ngn1* mutant E10.5 embryos (Ma, et al., 1998). However, it is also revealed that *ngn1* is not activated in *ngn2* mutant placodes. This result clearly identifies *ngn2* as the first known regulator to operate in this bHLH cascade and *ngn1* as a potential transcriptional target of *ngn2* in the placodes. The activation of *ngn1* by *ngn2* probably reflects cross-activation between neuronal determination genes, similar to the cross-regulatory interactions that exists between proneural genes in Drosophila. The lack of distal cranial ganglia defects in *ngn1* mutant mice is consistent with the hypothesis that *ngn1* does not have an essential role downstream of *ngn2* (Fode, et al., 1998). It has recently been shown that transducing NSCs with *ngn2* results in around 95% of the transduced cells differentiating into a neuronal fate *in vitro* (Falk, et al., 2002). In order to drive the implanted stem cells into an appropriate cell fate, i.e. neuronal fate, we selected *ngn2* as a candidate transcriptional factor transduced to NSCs prior to the inner ear implantation (Paper IV). However, it should be noted that other genes/transcriptional factors, i.e. NeuroD, GATA3, TrkB, TrkC, are also interesting alternatives in the future research.

1.6.5.2 Extrinsic factors

Extrinsic factors, i.e. growth factors, are also expected to essential in the cell fate determination in transplantation into the adult auditory system. During development, cell fate determination depends on the genes expressed by the cell. The expression of the gene relies on the inducing factors. There are two main kinds of inducing factors involved in neural induction. The first one is the transcriptional factors released by the neighboring cells. The second one is the protein on the committed cell surface to which the first transcriptional factor acts. During development, cell fate is mainly determined by the location of the cell. Thus, the differentiation of embryonic cells is determined primarily by its neighbors and not by its ancestry (Gurdon, 1999). Consequently, a specific microenvironment must be established if implanted stem cells are expected to differentiate into sensorineural cells in the inner ear. Interestingly, a recent study have shown that medium from embryonic rat retina contains factors that are able to induce the stem cells to differentiate into a neuronal fate (Kaneko, et al., 2003). Moreover, neuronal differentiation of stem cells transplanted into rat retina explants is more efficient when using embryonic retinas as compared to postnatal host tissue (Akita, et al., 2002). It suggests that an embryonic tissue may play a role in cell differentiation *in vitro*. All these observations appear to lead to a notion that it may be an interesting approach to provide an embryonic neuronal microenvironment in the transplantation location in the inner ear (Paper V). In the present study, we selected embryonic DRGs as cograft, since it is documented that embryonic DRG neurons has an autocrine loop

which could release neurotrophic factors, i.e., BDNF, NT-3 (Acheson, et al., 1995; Davies and Wright, 1995), that are expected to be involved not only in the promotion of the cell survival but also in inducing a proper differentiation of the implanted cells.

1.7 FUNCTIONAL EVALUATION OF THE CELL REPLACEMENT THERAPY

The long-term aim of the cell replacement therapy is to compensate the function of damaged cells in the hosts. Thus, it is vital to investigate the function of implanted cells. When transplanting mouse ES cells into an animal model of Parkinson's disease, it was found that ES cell-derived dopamine neurons caused gradual and sustained behavioral restoration of dopamine-mediated motor asymmetry (Björklund, et al., 2002). Behavioral recovery was observed to be paralleled *in vivo* by positron emission tomography and functional magnetic resonance imaging data. The results may suggest that ES cell-derived dopamine neurons can restore cerebral function and behavior in an animal model of Parkinson's disease.

Similarly, a functional evaluation of implanted cells is essential in a cell replacement therapy in the inner ear. In the present study we aimed at replacing the host SGNs with exogenous cells. The hypothesis was that outside sound, converted into electrical signals by an implanted electrode, would act on the surviving exogenous cells. The cell would transfer the electrical signals either to the host SGNs or directly to the cochlear nucleus in the brain stem, thereby improving auditory function (c.f. Fig. 3). In order to prove this, mouse DRGs were transplanted into the scala tympani of adult guinea pigs while an electrode was simultaneously introduced into scala tympani. Electrically-evoked auditory brain stem responses (EABRs) were used to evaluate a possible functional integration of the implanted DRG neurons transplanted into the inner ear (Paper VI).

2 AIMS OF THE STUDY

The long-term goal of the current study was to explore novel means for an effective treatment for hearing loss caused by irreversible degeneration of sensorineural cells in the mammalian inner ear. The aim of the current project was to investigate:

1. Are exogenous cells able to survive in the adult mammalian auditory system?
2. Will implanted stem cells differentiate into an appropriate cell fate in the cochlea?
3. Can grafted cells migrate to functionally relevant regions in the inner ear?
4. Do transplanted cells have the potential to become functionally integrated with the host auditory system?

3 MATERIALS AND METHODS

3.1 ETHICAL PERMISSIONS AND ANIMAL CARE

The experimental procedures and animal care in this thesis were approved by Northern Stockholm Animal Ethics Committee. The approval was also obtained from the University Committee on Use and Care Of Animals, University of Michigan, while the experiments in Paper VI were performed at Kresge Hearing Research Institute (KHRI).

Animals were housed in facilities with free access to food and water throughout the experiment. All surgical procedures and functional measurements were performed under deep anesthesia (ketamine, 4 mg/100 g body weight, and xylazine, 1 mg/100 g body weight, i.m.). A considerable effort was made to minimize the number of animals used and the suffering of animals involved in the study.

3.2 DONORS FOR A REPLACEMENT THERAPY IN THE INNER EAR

3.2.1 Embryonic dorsal root ganglion

The donor DRG neurons were dissected out from mouse embryos at embryonic days 13-14 (E13-14). In order to analyze the morphology and number of the non-transplanted DRG neurons, a total of 12 lower lumbar DRGs were dissected out from 3 embryos and treated as in Paper II.

3.2.2 Embryonic stem cell line

Two ES cell line were used in the study, tau-GFP ES cell line in Paper III (Pratt, et al., 2000), and GSI-1 ES cell line in Paper V (Harkany, et al., 2004). Tau is one of the microtubule, which is important for maintenance of the cytoskeleton in nervous tissues. A potential benefit of using tau-GFP ES cells is that this procedure may affect the cytoskeleton in the implanted cells A further potential benefit of tau-GFP over the untagged donor is that the tagged donor is anchored to the cytoskeleton thereby reducing the risk of GFP protein diffusion out of the cells during the processes of transplantation and histochemical tissue preparations.

3.2.3 Adult neural stem cells and viral transduction

Adult NSCs were prepared from the lateral wall of the lateral ventricle of adult mice (Clarke, et al., 2000). For viral transduction, two retroviral constructs were used (Falk, et al., 2002). Briefly, one construct encoded myc-tagged *ngn2* followed by an internal ribosomal entry site and GFP. The control construct was identical except it did not include the *ngn2* gene. By delivering retroviral particles to the cell medium, the retroviral genome was transduced into the cells and inserted in the cell genome, encoding both *ngn2* and GFP (*ngn2*-transduced cells). The neurospheres were transduced by *ngn2* for 48 h prior to transplantation.

3.3 HOST ANIMALS

There were totally 154 pigmented guinea pigs (body weight 300-450 g), 108 Sprague–Dawley rats (body weight 250–300 g) involved in the experiments.

3.4 INNER EAR DAMAGING PROCEDURES

3.4.1 Neomycin injection

Under deep anesthesia, the animals received an injection of 10% neomycin through the tympanic membrane. The middle ear cavity was filled with the neomycin solution and the head of the animal was kept for 15 minutes in the appropriate position for the drug to diffuse via the round window into the cochlea. Neomycin-treatment was performed two days prior to implantation.

3.4.2 Kanamycin and ethacrynic acid injection

Kanamycin (450mg/kg) was administered subcutaneously two hours prior to ethacrynic acid (50mg/kg) injection, via aseptic jugular vein cannulation while under deep anesthesia.

3.5 TRANSPLANTATION PROCEDURES

The DRGs or stem cells were transplanted into scala tympani in Paper I, II, IV-VI. Under deep anesthesia, the left post-auricular region of the animal was shaved and cleaned with 70% ethanol. The animal was then placed on a heating pad (37°C), and using a postauricular approach, the left bulla was exposed and opened to visualize the basal cochlea. A small hole was made into the scala tympani at the basal cochlear turn. The DRGs or stem cells were transplanted into the scala tympani. A small piece of fascia was placed over the cochleostomy. The defect on bulla was sealed using carboxylate cement. The incision was then approximated with sutures.

In Paper III, the auditory nerve was transected at the first cochlear turn and the DRGs or ES cells were transplanted directly at the transection site.

3.6 ELECTRODE IMPLANTATION PROCEDURES

Under deep anesthesia, the animal was given local anesthesia (1% lidocaine) and placed on a water-circulating heating pad for surgery utilizing aseptic procedures. Beginning approximately 2.5 cm anterior to bregma (intersection of sagittal and coronal suture lines of the skull) a midline skin incision was made on the dorsal surface of the head and continued behind the left ear (postauricular), ending at the base of the pinna. The periosteum was removed. Three holes were drilled through the skull using a 1.5 mm-diameter cutting burr and 3 screws (J - mx-172 - 5B, 5/16”) were placed, using

bregma as reference: 1 cm posterior at midline (active), 2 cm anterior (reference) at midline and 1 cm lateral towards implanted ear (ground). A restraint bolt for securing the stimulator was secured by 3 small anchor screws around bregma. All screws and electrode were secured with methyl methacrylate. The bulla was opened and provided the access to the round window and basal cochlear turn via a surgical microscope. The round window was penetrated with a 30g. needle to ease insertion of a KHRI designed catheter and electrode combination device. The ball electrode was inserted approximately 3mm with the cannula terminating at a depth of 2mm. The reference electrode was placed inside the bulla contacting the wall. The defect was sealed using carboxylate cement. The catheter was connected to a mini-osmotic pump (Alzet Model 2002, 0.5 µl/h, duration 2 weeks). The pump was inserted subcutaneously on the back of the animal between the scapulae. Thirteen days following implantation, the pump was replaced under aseptic surgical conditions to assure a continuous infusion.

3.7 IMMUNOSUPPRESSANT AND ANTIBIOTICS ADMINISTRATION

In order to reduce the risk of postoperative immunological rejection and infection, the DRG-transplanted host animals in paper I-III, VI received daily injections of cyclosporin (0.56 mg/100 g body weight) and doxycycline (0.24 mg/100 g body weight) intraperitoneally until the day of sacrifice. We didn't use immunosuppressant and antibiotics in the NSCs- and ES cells-transplanted animals.

3.8 ABR AND EABR MEASUREMENTS

3.8.1 ABR measurement

The auditory brain stem response (ABR) measurement was performed on a Tucky David system. Animals were anesthetized using the same drugs mentioned above. Responses were recorded with subdermal recording needle electrodes placed at the vertex (active) against a reference placed at the midline of the skull approximately 2 cm anterior to bregma. A subcutaneous electrode in the thigh provided the ground. In a soundproof room, computer generated alternating polarity voltage pulses (160 µs duration, 50 pps) were delivered to a transducer positioned at the opening of the ear canal. A mean of 1024 samples of 7.7 ms electrophysiological activity following stimulation were recorded. Stimuli were provided at various intensities to determine threshold, which was defined as the lowest stimulus intensity that evoked at least a 0.2 µV replicable waveform. Post-deafening ABR must show at least a 60 dB SPL threshold shift or the animals would be eliminated from the study.

3.8.2 EABR measurement

The animals were anesthetized as previously described. Prior to EABR collection, the impedance of the electrode was measured using an impedance monitor (sinusoid waveform at 1000 Hz). The animal was placed in a sound proof booth. Stimulus current ranged from approximately 10 to 1000 µA and the response was collected from

epidural recording screws connected at the following sites: active recording site - vertex, reference site - midline recording screw and ground - left screw. Using Hall's method (Hall, 1990) the EABR responses were summed to alternate polarity current pulses, where each pair provides charge balancing. Two thousand forty-eight responses to 50 μs computer-generated monophasic current pulses, presented at 50 pps with an alternating polarity on each presentation were collected for analysis. Intensity of stimuli varied from P3 threshold to P1 saturation, where saturation was defined as the intensity that evokes less than a 5% increase in response amplitude from lower stimulus intensity. The P3 thresholds (amplitude ≥ 0.2 μV) and N2-P3 amplitude input/output function were determined.

3.9 CHRONIC ELECTRICAL STIMULATION

The subjects received continuous pulsatile, biphasic, charge-balanced electrical stimulation from a battery-powered, wearable stimulator designed at KHRI by Chris Ellinger. The stimulator plugs into the electrode connector and was secured by the restraint bolt. Stimuli were provided at a 40% duty cycle: 400μs on, 600μs off.

3.10 HISTOLOGY

Following an overdose of pentobarbital (i.p.), the animals were transcardially perfused. The specimens were taken out and keep in 0.1 M phosphate buffer for further treatment.

3.10.1 X-Gal staining and JB4 embedding

An X-gal-based histochemical method was used to detect β-galactosidase activity in the cochleas transplanted with DRGs or stem cells expressing LacZ. The specimens then were embedded in JB4 and sectioned at a thickness of 5 μm.

3.10.2 Cryo-sectioning and EGFP detection.

Following trimming the bone around the inner ear, the cochleas transplanted with EGFP/GFP-expressing-cells were cryo-protected by 30% sucrose and cryo-sectioned at a thickness of 12 μm (Paper I-VI).

3.11 IMMUNOHISTOCHEMISTRY

The primary antibodies used in the study included:

1. Polyclonal rabbit anti-GFP antibody (1:500, Abcam Ltd, Cat No: ab6556-25) and secondary antibody (fluorescein (FITC)-conjugated affiniPure Donkey anti-rabbit IgG (H+L), 1:500, Jackson Immunoresearch Laboratories)

2. Neurofilament antibody (NF-L, 1:100; Santa Cruz Biotechnology, Inc, Cat No: sc-20012) and secondary antibody goat anti-mouse IgG_1-Texas red (1:200; Santa Cruz Biotechnology, Inc, Cat No: sc-2979)

3. TUJ1 antibody (1:500; Nortic Biosite) and secondary antibody cy3-conjugated affiniPure $F(ab')_2$ fragment goat anti-mouse IgG (1: 400; Jackson Immunoresearch Laboratories, Inc. Code No. 115-165-071)

4. Rat anti-mouse Thy 1.2 (1:100; Serotec, Cat No: MCA1474) and secondary antibody TRITC-conjugated affiniPure $F(ab')_2$ fragment donkey anti-rat IgG (H+L) (1:400, Jackson Immunoresearch Laboratories, Inc. Code No. 712-026-153)

5. Polyclonal rabbit anit-GFAP antibody (1:250; DAKO, Code No: Z 0334) and secondary antibody amca affinity purified goat anti-rabbit IgG (H+L) (1:400, Vector Laboratories, Cat No: CI-1000).

6. Affinity purified rabbit anti-myosin-VIIa antibody (1: 100, gift from Dr. Tama W. Hasson, Division of Biological Sciences, University of California, San Diego, USA) and amca affinity purified anti-rabbit IgG (H+L) (1: 100, Vector Laboratories, Cat No: CI-1000)

7. Goat polyclonal anti-CGRP antibody (1:50, Santa Cruz Biotechnology, Inc, Cat No: sc-8857) and secondary antibody rabbit anti-goat IgG-TR (1: 100; Santa Cruz Biotechnology, Inc, Cat No: sc-3919)

8. Goat polyclonal anti-SP antibody (1:50; Santa Cruz Biotechnology, Inc, Cat No: sc-9758) and secondary antibody rabbit anti-goat IgG-TR (1: 100; Santa Cruz Biotechnology, Inc, Cat No: sc-3919)

The proper secondary antibodies conjugated with cy3, texas-red, FITC, amca were used in the study.

The brain, DRG and cochlea sections of normal rats or guinea pigs were used as positive controls. As for negative controls, the primary antibodies were replaced by 1% BSA-PBS.

3.12 MICROSCOPY AND IMAGING

Tissue sections were observed using either a Zeiss fluorescent microscope equipped with a digital camera (Spot RT, Diagnostic Instrument, Polaroid DMC le, or Nikon Coolpix 990, 5000), or a confocal microscope (Zeiss LSM 510). The appropriate filters were used to detect GFP/EGFP fluorescence, cy3, texas-red, and amca.

3.13 STATISTICS

Student *t*-test and ANOVA were used in the study.

4 RESULTS

4.1 TRANSPLANTATION OF EMBRYONIC NEURONAL TISSUE

4.1.1 Survival of DRG neurons in the inner ear

4.1.1.1 Identification of DRG neurons (Paper I-III, VI)

The implanted DRG cells were identified by either X-gal positive staining or EGFP/GFP fluorescence. The implanted mouse DRG neurons were found surviving in the adult inner ears for up to ten weeks and labeled with neuronal markers (neurofilament and Thy 1.2 antibodies). The implanted DRG neurons were further labeled with SP antibody or CGRP antibody suggesting the small (< 25μm) and medium (25-40 μm) size population of DRG neurons survived in the inner ear. Morphologically, the surviving DRG cells appeared normal and showed no sign of degeneration. Most DRG neurons were located in clusters and solitary neurons were rarely seen. Outgrowth of axon from survival DRG neurons was also observed, suggesting the surviving DRG cells in a rather good metabolic state.

4.1.1.2 The number of animals showing survived DRG implants

It was found that the number of animals showing surviving DRG cells decrease with the time, relatively high at three week survival (nearly 60-70%) and less than 40% at a 6-week survival. At nine-ten weeks survival 2/8-3/8 of animals still showed the surviving DRG cells.

4.1.2 Enhance the survival of implants in the inner ear

In the present observation, the total number of DRG neurons surviving in the inner ear was quite poor. In order to enhance the survival of DRG cells in the inner ear, different approaches had been tested. To investigate whether an injured inner ear would affect the survival of implanted neurons, DRGs were transplanted into the inner ears chemically deafened by the injection of neomycin prior to implantation (Paper I). There was no significant difference in the survival of DRGs between animals with normal inner ears and animals with injured inner ears.

In order to explore whether neurotrophic factors possess the ability to promote the survival of DRG neurons transplanted in the inner ear, NGF was infused into the scala tympani of adult rat inner ears (Paper II). At 3-week survival, there was higher percentage (6/6) of animals showing surviving DRG cells in the animals supplied with NGF than when only artificial perilymph was infused (4/6). At 6-week survival, there were still 2/6 of the NGF-group animals showing DRG survival whereas none of the animals in the non-NGF-group (0/6). Cell counting revealed a significantly higher number of surviving DRG cells in the NGF group (332 ± 36) than in the non-NGF group (135 ± 14; $p<0.01$, two tailed paired *t* test). These results clearly indicate that the supplement of exogenous neurotrophic factors possess the ability to enhance the survival of neurons transplanted into the inner ear. However, it is also notable that the overall percentage of surviving DRG cells was quite low, around 15% in the NGF group while 6% in the non-NGF group.

4.1.3 Location and migration of surviving cells

In the present studies, mouse DRG implants were transplanted into scala tympani of adult inner ears. We found that most of the surviving DRG cells were located in scala tympani and attached close to the bony spiral lamina (in the vicinity of Rosenthal's canal, Paper I, II, VI). Interestingly, we also found that the surviving DRG cells appeared to pass through the bone and reach the spiral ganglion region and the organ of Corti region, thus locating outside the transplantation site of scala tympani. It suggests that the surviving cells have the capability to migrate to a functionally relevant region in the adult inner ear. The mechanism for this migration is still unknown, but possibly due to the growth factors released by the cochlear sensorineural cells, i.e. SGNs or hair cells.

However, a more central migration, i.e. further centrally towards the brain stem, was not observed for up to ten weeks. In order to test whether these embryonic neurons have the potential to migrate centrally towards the brain stem, we tried a different approach, i.e. to open the modiolus, transect the auditory nerve at the level of the first cochlear turn and transplant the DRGs directly to the transection site (Paper III). We found the DRG cells migrating along the auditory nerve fibers centrally to the internal meatus at a 6-week survival, suggesting DRG neurons may possess the capability to migrate centrally in a mature auditory system. However, we found the DRG cells did not migrate further centrally at a 9-week survival.

4.1.4 Neurite contacts

One of the most exciting findings in the present study was the observation of neurite outgrowth from the surviving DRG cells (Paper I, II, III, VI). It was observed that neurites projected from the surviving DRG neurons towards the SGNs located in Rosenthal's canal. The processes of the DRG cells thus reached the bone of spiral lamina, even passed the bone and enter the spiral ganglion region.

Neurite outgrowth was further stimulated and enhanced by the supplement of exogenous NGF and/or chronic electrical stimulation. The effect of NGF and chronic electrical stimulation was not additive. Interestingly, the neurite projections were shown not only among the surviving DRG cells but also between the implanted DRG neurons and host SGNs, illustrating the possibility for the implanted cells to become both structurally and functionally integrated with the host auditory system.

4.2 TRANSPLANTATION OF STEM CELLS INTO THE AUDITORY SYSTEM

4.2.1 Survival of implanted stem cells

4.2.1.1 Identification of stem cells

Implanted stem cells were identified by either X-gal positive staining or EGFP/GFP fluorescence. Surviving NSCs and ES cells were found outside the lateral wall of

cochlea but within the fascia covered the cochleostomy. We did not find extensive ES cells proliferation or tumor formation (e.g. teratomas) within or outside the cochlea.

4.2.1.2 Survival of adult NSCs (Paper IV)

The survival of NSC was poor compared to that of the DRG cells. In about 50% of the animals, NSCs were found at one and two weeks following transplantation (2/4 and 11/20, respectively). However, the survival went down dramatically at later stages: 1/4 animals had remaining NSCs at three weeks and none out of eight animals at four weeks following transplantation.

In order to promote the survival of NSCs transplanted into the inner ear, the deafened host animals and gene-transduced-NSCs were tested in the study. The number of host animals showing surviving NSCs increased when the animals were chemically deafened prior to the transplantation. Here, surviving cells were found in 5/6 animals at two weeks and in 2/6 hosts at four weeks. Similar survival rates were seen in the host animals transplanted with *ngn2*-transduced-NSCs where implanted cells were found in 3/6 animals at two weeks and in 2/6 hosts at four weeks. However the cell counting, at 2-week survival, revealed that only 0.4‰ of the cells survived in the normal hearing group, 0.7‰ in the deafened group and 0.6‰ in the neurogenin group, suggesting that the absolute number of survived NSC was less influenced by these procedures. It should be noted that the survival of adult NSCs was nearly 100-fold lower than DRGs.

4.2.1.3 Promotion of ES cells survival in the inner ear

Due to the poor survival of NSCs in the inner ear, mouse GSI-1 ES cells were transplanted into the scala tympani of adult guinea pigs in order to investigate whether a pluripotent ES cell line would show a better survival (Paper V). In the transplantation to the animal with a normal inner ear, it was found that all the animals had surviving ES cells for up to four weeks. Cell counting revealed a better survival (6.5‰, 10-fold higher) compared to the NSCs. It decreased dramatically (7 fold) at 4-week survival, dropping to 0.9‰. The survival of ES cells was nearly 10-fold lower than that of DRGs. Two approaches were used to enhance the survival of ES cells in the study.

Firstly, an embryonic neuronal tissue (DRG) was simultaneously cografted into the inner ears. The number of animals showing surviving ES cells and the cell counting at 2-week survival were similar to non-cografted group. Interestingly, at 4-week survival, the number of surviving ES cells was significantly higher ($P<0.01$, ANOVA) in the co-grafted animals (>2.5‰) than in non-cografted animals (0.9‰). It suggests that an embryonic neuronal cograft could enhance the survival of ES cells in the inner ear.

Secondly, the ES cells were also transplanted into the inner ears chemically damaged by neomycin prior to implantation. There was no significant difference in the ES cell survival between normal and neomycin-treated animals (neither in the DRG cograft nor in the non-DRG-cograft animals) at any of the two time points, indicating that the survival of ES cells was less affected by the chemical damage procedures to the inner ear prior to the implantation.

In the transplantation of tau ES cells along the auditory nerve fibers (Paper III), the cell survival rate was better, around 1.1-1.5% at 3-9 weeks observation. This survival rate was still lower than DRGs (around 5-fold).

4.2.2 Differentiation of surviving stem cells

Implanted NSCs and ES cells were found to be labeled with glial cell marker (GFAP antibody) and neuronal marker (TUJ1 antibody) but not with the hair cell marker (myosin VIIa antibody). In the present study, we aimed at exploring the possibility for implanted cells to replace the host SGNs. Here, we focused on investigating the neuronal differentiation of the transplanted stem cells.

In order to obtain a higher percentage of implanted stem cell differentiation into neurons, NSCs were used in the study (Paper IV). However, neuronal differentiation of the transplanted NSCs was not identified in the normal hearing inner ears. It appeared that NSCs could not differentiate into a neuronal fate spontaneously in a normal inner ear. Several approaches were tested in our experiment in order to drive implanted stem cells into a neuronal cell fate, i.e. altering the intrinsic genetic programs regulated by the transcriptional factors or providing an appropriate extracellular microenvironment.

4.2.2.1 Intrinsic factors

It has been previously shown that transducing NSCs with *ngn2* results in around 95% of the transduced cells differentiating into a neuronal fate *in vitro* (Falk, et al., 2002). In the present study, the NSCs were transduced with *ngn2* prior to transplantation (Paper IV). It is shown that neuronal marker positive NSCs were found in 5/12 animals transplanted with the *ngn2*-transduced-NSCs. It suggests the possibility of single gene transduction to drive a neuronal differentiation of stem cells transplanted into the adult inner ear.

4.2.2.2 Extrinsic factors

In order to test whether an extrinsic factor, i.e. a neuronal microenvironment, would affect the differentiation of stem cells transplanted in the inner ear, stem cells were transplanted into the scala tympani together with a simultaneously implantation of embryonic neuronal (DRG) cograft (Paper V). We found that the embryonic neuronal cografts appeared to stimulate and promote the neuronal differentiation of ES cells in the adult auditory system. At 2-week survival, nearly 12% of surviving ES cells was found to be labeled with neuronal marker in the animals both in the normal and damaged inner ears without DRG cografts. Interestingly, a significant higher percentage (nearly 83%) of surviving ES cells was found to be expressing neuronal marker (TUJ1) in the cografted animals at the same survival. At 4-week survival, nearly 36% and 45% of the surviving cells were labeled with neuronal marker in the normal and injured inner ears without DRG cografts, respectively. A significantly higher number of surviving ES cells were labeled with the neuronal marker in animals with DRG cografts at the same survival (nearly 90%).

Further, in order to test whether the neuronal differentiation would be stimulated in an injured inner ear, adult NSCs and ES cells were transplanted into the inner ears chemically damaged prior to implantation. The results revealed that a neuronal differentiation of NSCs was shown in the injured inner ears. Here, two deafened animals (2/12) showed the NSCs labeled with neuronal marker, suggesting that the deafened inner ears may release factors which could induce the neuronal differentiation of NSCs in the inner ear. In the transplantation of ES cells, we found that neuronal

differentiation was promoted in the injured inner ears without an embryonic neuronal cograft ($P<0.05$, ANOVA). However, there was no such difference in the animals transplanted an embryonic neuronal cograft.

4.2.3 Location and migration of implanted stem cells

In the transplantation to scala tympani, the stem cells were found not only within the transplantation location in the scala tympani at the first cochlear turn but also in the other part of the inner ear, i.e. scala tympani of the other cochlear turn and scala vestibuli (Paper IV, V). Interestingly, the implanted NSCs and ES cells were found to have migrated towards functionally relevant region in the inner ear, through the osseous spiral lamina and located along the auditory nerve fibers leading from the organ of Corti, and close to SGNs.

In the transplantation along the auditory nerve, implanted ES cells migrated more centrally than that of DRGs (Paper III). Here, ES cells were found along the central processes of the auditory nerve fibers in the internal meatus (6-week survival) as well as within the brain stem and close to the ventral cochlear nucleus (9-week survival).

4.2.4 Neurite contacts

Similar to DRG transplantation, neurite outgrowth and contacts were also observed in the implantation of ES cells into the inner ear, but not in NSCs transplantation. The neurite outgrowth and apparent contacts with the host auditory system appeared to be stimulated by the existence of an embryonic neuronal cograft. In the DRG cograft groups the implanted ES cells were found not only to differentiate into neurons but also to generate extensive neurite outgrowth towards the osseous modiolus. Furthermore, regions of apparent proximity or contacts were observed between projections from the differentiated (TUJI positive) ES cells and SGNs or cochlear afferent nerve fibers in eleven out of 24 host animals in the DRG cograft groups (Paper V). In the non-DRG-cograft groups neither neurite outgrowth nor regions of structural proximity were observed.

4.3 FUNCTIONAL STUDY

The survival and migration of DRG neurons as well as the neurite contacts formed between the implanted DRG neurons and host SGNs suggested the possibility to explore a potential functional improvement in a cell replacement therapy in the inner ear. The EABR thresholds and input/output functions were obtained at experimental days 7, 11, 17, 24 and 31 from animals receiving DRG implantation with or without NGF and/or CES supplement as well as from un-transplanted control animals (Paper VI).

Extensive neurite outgrowth was found to project from DRGs, penetrate the bony modiolus and reach the spiral ganglion region in the animals supplied with exogenous NGF, chronic electrical stimulation or both at four weeks following transplantation. There was no significant difference in the mean EABR P3 thresholds between any of these groups ($P>0.05$, ANOVA). Thus, there appeared to be no positive effect on

EABR thresholds, neither in DRG-transplanted animals nor in animals receiving NGF and/or chronic electrical stimulation supplements. There were slight but not significant changes in the input/output functions. In the non-NGF supplement group, a better input/output function was observed on experimental day 31 compared to day 7, but only in the transplanted-animals supplied with chronic electrical stimulation. In the animals supplied with NGF, there was slightly improvement in the input/output function of the transplanted animals at the stimulation levels of 800 μA, 900 μA and 1000μA.

5 DISCUSSION

5.1 HEARING LOSS AND CELL REPLACEMENT THERAPY

It is well known that mammalian cochlea is vulnerable to aging, genetic disorder and different kinds of insults, i.e. sound overstimulation, ototoxic agents, viral infections. All these insults usually result in a sensorineural hearing loss, which is characterized by selective damage to the sensory epithelium (organ of Corti). Pathologically, as in the other afferent nervous system, degeneration of the SGNs often occurs secondarily, thus aggravate the hearing impairment and reduce the possibilities for rehabilitation (Marzella and Clark, 1999; Ryals, et al., 1989).

It has been demonstrated that the hearing organ in the lower vertebrates and avian have the ability to regenerate following damage (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). However the hearing organ in adult mammalians, the organ of Corti, is considered not capable of regenerating spontaneously after degeneration. Recent observations both *in vitro* and *in vivo* have suggested that transduction of an appropriate gene to the hearing organ may give a possibility for generation of new sensory cells either by activating cochlear progenitor cells, or by conversion of supporting cells (Kawamoto, et al., 2003; Löwenheim, et al., 1999; Raphael, 2002; Zheng and Gao, 2000).

Cochlear implant has been a standard treatment for the patients suffering from sensorineural hearing loss. It is also well accepted that the efficacy of cochlear implant depends not only on the efficiency of the cochlear implant device but also on the integration and function of SGNs. Thus any interventions which can prevent the degeneration of SGNs or replace the degenerated SGNs are essential. A number of studies have provided strong evidence that neurotrophic factors (e.g., BDNF, NT-3), anti-apoptotic agents (e.g., Z-VAD-FMK), and electrical stimulation are capable of preventing or arresting the cochlear hair cell death as well as the secondary damage to the SGNs following the injure to the sensory epithelium (Duan, et al., 2000; Ernfors, et al., 1996; Lalwani, et al., 2002; Liu, et al., 1998; Miller, et al., 2002; Shah, et al., 1995; Shinohara, et al., 2002). In order to replace the degenerated or missing SGNs a novel approach, a cell replacement therapy, was proposed and investigated in the present research.

The hypothesis is that the implanted cells are expected not only to take the position of degenerated SGNs but also to become functionally integrated into the auditory system. However, there are a number of issues regarding the implantation of exogenous cells that have to be considered before any speculation of a use of cell therapy in the inner ear. Can exogenous cells survive in the adult mammalian auditory system? Do implanted stem cells differentiate into an appropriate cell fate in the cochlea? Will grafted cells migrate into a functionally relevant region in adult auditory system? And finally, will transplanted cells have function in the new environment and even have the capability to become functionally integrated into the host auditory system? In the present study, a number of experiments were designed and accomplished to address these issues.

5.2 SURVIVAL OF THE IMPLANTED CELLS IN THE AUDITORY SYSTEM

The first issue we have to address in a cell replacement therapy in the inner ear is whether the implanted exogenous cells have the capability to survive in the adult auditory system. To answer this question, the selection of donor cell is one of the key factors which we should consider. Similarly, methods to enhance the survival of implanted cells are vital.

5.2.1 The selection of candidate

The major aim of the study was to replace degenerated SGNs and it followed that neuronal tissues and undifferentiated cells having the potential to develop into a neuronal fate were appropriate candidate donors. In the present study, embryonic neurons (DRGs) and undifferentiated stem cells, including multipotent adult NSCs and pluripotent ES cells, were selected as donors and transplanted into the adult auditory system.

5.2.1.1 Embryonic tissue

Previous studies have shown that it is feasible to restore spinal central sensory input from the periphery via transplantation of neuronal tissue. It has been demonstrated that allogenic and xenogenic grafts of DRG neurons can survive and reinnervate denervated host targets in a variety of locations in the gangliectomized rat (Rosario, et al., 1995). Xenografted human fetal DRGs have been shown to extend axons through the peripheral nervous system-central nervous system (PNS-CNS) boundary, to migrate into the CNS and to form functional connections in the rats (Kozlova, et al., 1997; Levinsson, et al., 2000). It has also previously been shown that guinea pig DRGs survived in the guinea pig cochlea (allograft implantation). In the present study we selected embryonic mouse DRGs as a candidate donor tissue and found that xenografted (mouse to rat and mouse to guinea pig) DRG neurons survived in the adult cochlea for up to ten week, suggesting that embryonic neuronal tissue may be a possible donor candidate in a cell replacement therapy in the auditory system. However, it should also be noted that other neuronal tissue, i.e. SGNs, probably a more promising candidate tissue could be investigated in a similar transplantation study.

5.2.1.2 Stem cells

Stem cells may be another interesting candidate alternative in a cell replacement therapy in the auditory system. During development NSCs will differentiate into neurons, astrocytes, and glia. It has been shown by a number of observations that NSCs differentiate into neural cell types in the transplantation of NSC or its progeny to the nervous system in animals models (Armstrong, et al., 2000; Blesch, et al., 2002; Storch and Schwarz, 2002; Sugaya and Brannen, 2001). In order to replace the degenerated SGNs, it is logical to use NSC as a candidate since it has the potential to differentiate into neurons. In the present study, we transplanted adult NSCs into the inner ear and found that adult NSCs survived for up to four weeks following transplantation. It

indicates that multipotent adult NSC may be a potential candidate donor in the cell replacement therapy in the auditory system.

Due to the pluripotency, ES cell is an also logical candidate donor in an inner ear replacement therapy, though the ethical and potential tumorigenic issues remain a challenge. However, a number of studies have demonstrated that cells derived from ES cells survive well and have, to some extent, functional improvement when implanted to appropriate sites in the animal models (Klug, et al., 1996; Liu, et al., 2000; McDonald, et al., 1999; Tsai, et al., 2000). In the present study we transplanted mouse ES cells into adult auditory system and found that ES cells survived in the inner ear for at least four weeks and along the auditory nerve fiber for up to nine weeks, suggesting the ES cell transplantation is feasible in the adult auditory system.

5.2.2 Promoting the survival of implanted cells

In order to replace the degenerated SGNs, a considerable number of surviving cells is essential. However, it was found that the survival of implanted cells was very poor in the present study: around 6% for DRGs cells whereas 10-fold lower for ES cells and 100-fold lower for adult NSCs at 2-3 weeks survival. The survival of implanted cells was further observed to decrease with time. We speculated that the microenvironment of the transplantation location in the inner ear, the scala tympani, may be one of the major problems. It is likely that nutrition and factors necessary for the survival of implanted cells are missing in the perilymph of the scala tympani. Moreover, a structural matrix for cell growth is essentially absent in the fluid compartments. It seems that a supplement of appropriate growth factors and proper structural matrix may be needed to enhance the survival of implanted cells.

5.2.2.1 Neurotrophic factors

Neurotrophic factors are a group of vital agents in neuronal development, including regulation of neuronal survival, affecting nerve fiber elongation and outgrowth (Nosrat, et al., 2001; Snider, 1994; Tuttle and O'Leary, 1998). Moreover, supplement of exogenous NGF has been shown to enhance the survival and neurite outgrowth of a population of transplanted DRG neurons *in vivo* (Miller, et al., 1999). It is thus logical to speculate that neurotrophins may have the potential to enhance the survival of implanted DRG neurons in the inner ear. Since NT-3 and BDNF have a significant neuroprotective effect on SGNs, NGF is thus a better alternative in order to distinguish the effect of neurotrophins on SGNs and on implanted cells. It was found that the implanted DRG neurons had a significantly higher survival in the NGF group than in the non-NGF group (Paper II) at 3 weeks survival. At 6 weeks postoperatively, surviving DRG implants were found only in the NGF group while no surviving DRG implants were found in the non-NGF group. These results clearly indicate that exogenously supplemented neurotrophic factors enhance the survival of implanted neurons following transplantation into the adult inner ear.

5.2.2.2 Embryonic neuronal cograft

In order to test whether an embryonic neuronal cograft has the ability to provide an extracellular microenvironment for implanted cell growth, ES cells were transplanted into the scala tympani with a simultaneously implantation of embryonic DRG neuronal cograft. Here, embryonic DRG neuronal cografts were expected not only to release growth factors to support ES cells survival but also to provide a structural matrix for cell growth in the fluid-filled scala tympani. In the cograft groups the cell counts were significantly higher as compared to non-cograft groups. These results clearly showed the ability of the embryonic neuronal cografts to promote the survival of stem cells transplanted into the inner ear, suggesting a proper extracellular environment may be essential for the survival of exogenous cells transplanted in the fluid-filled inner ear.

5.2.2.3 Injured inner ear

It is speculated that certain growth factors would be released by cells in the cochlea in response to damage. In order to investigate whether the growth factors released by an injured inner ear would affect the survival of implanted cells, DRGs, adult NSCs and ES cells were transplanted into the inner ears chemically damaged by neomycin prior to implantation. There was no difference in the number of animals showing DRG cell survival between the normal inner ears and chemically damaged inner ears (Paper I), suggesting that the survival of embryonic neuronal tissues is less affected by the damage procedures to the inner ear. In the implantation of adult NSCs, no surviving NSCs were found in normal inner ears at 4-week survival, while surviving NSCs were observed in 2/6 inner ears chemically damaged prior to the surgery. The results may suggest that the survival of NSCs is influenced by the damage procedures to the inner ear. The mechanism for this enhancement has not been fully understood. In the transplantation of ES cells, all the animals showed ES cells survival up to four weeks following implantation. There was, however, no significant difference in the ES cell survival between normal and neomycin-treated animals (neither in the DRG cograft nor in the non-DRG-cograft animals), indicating that the survival of ES cells was less affected by the chemical damages to the inner ear prior to the implantation.

5.2.2.4 Immunosuppressant and antibiotics

In order to prevent the possible immunological rejection and inflammation, an immunosuppressant and antibiotics were administrated to the animals transplanted with mouse DRGs. There were no obvious signs of immunological rejection or inflammation at least in the histological sections of the cochleas. Similar results were also observed in the animals transplanted with stem cells without the administration of immunosuppressant and antibiotics. However, it cannot exclude the possible immunological rejection and inflammation at a sub-cellular level. The relatively high survival of the DRGs implants may support this notion. For the concern of future clinical application, a systematic administration of a life time immunosuppressant is not suggested. This is also the reason why we tried to transplant stem cells into the inner ear without immunosuppressant in the present study. However, in order to enhance the

survival of implanted cells, a proper selection of an immunosuppressant with minimal side-effects is still worth to try in the future study.

5.2.3 Un-controlled proliferation and tumor formation

One of the major concerns regarding ES cell implantation is un-controlled proliferation of the transplanted stem cells, i.e. the formation of benign teratomas and/or malignant teratocarcinomas. Transplantation of human ES cells into the testes of mice showed the teratoma formation (Hovatta, et al., 2003). The teratomas were analyzed 18 weeks following injection. They contained macroscopically well circumscribed tumor-like structures, containing cystic cavities and solid areas. Some of the cysts were filled with a gelatinous substance. The tissues with endodermal, mesodermal, and ectodermal differentiation were observed. The transplantation of human ES cells into the thoracic cavity also demonstrated the formation of teratoma (Asano, et al., 2003). In the nervous system, out of 25 rats receiving ES cell injection into the brain, five rats were found the teratoma like tumors (Björklund, et al., 2002). The mechanism for the teratoma formation in ES cells transplantation is still un-known. However, it is speculated by Björlund and co-authors that the differentiating ES cells were sending conflicting signals to each other that promoted the growth and formation of the teratoma. The diluting of the cells may, thus lessen the chance that the cells would interact with each other, thereby encouraging development along the possible default pathway for neuronal differentiation. This hypothesis was further identified by Harkany et al. (Harkany, et al., 2004).

On the other hand, it has also been speculated that the environment at transplantation location may affect the un-controlled proliferation of ES cells. Transplantation of ES cells into the knee joints and in the subcutaneous spaces in mice reveals that the growth of tumor in the knee joint was slower than in the subcutaneous space, suggesting that the joint environment is not optimal for the ES cells to grow and form a tumor (Wakitani, et al., 2003). It is also conceivable that the confined space in the knee joint may have interfered with cell growth. In the present study, the stem cells were transplanted into scala tympani, which is a confined cavity surrounded by the bone. The environment at the transplantation location appears not optimal for tumor formation. In the meantime, the scala tympani is full-filled with perilymph. Obviously, this location lacks the essential factors necessary for stem cell survival. Further, the poor survival of ES cells in the present study lessened the possibility for the cells to interact with each other, thus preventing the tumor formation. It is logical to speculate that it is less possible for the stem cells to form teratoma in scala tympani. The finding of no formation of the three layers of germ cells and the low percentage of stem cells survival in the present study seems to support the notion. However, it is also necessary to apply proper teratoma markers in the future study to exclude the possibility of teratoma formation in the inner ear.

5.3 INDUCING TRANSPLANTED STEM CELLS INTO AN APPROPRIATE CELL FATE

The fate of implanted stem cells in the auditory system needs to be explored. There are several theories that are considered to be involved in the cell linage differentiation of implanted cell, including intrinsic determinants (e.g. transcriptional factors), the extrinsic factors (e.g. growth factors, extracellular matrix), or both. In the implantation of DRGs, the cell fate of embryonic DRG neurons has already been determined while the cell fate of adult NSCs and ES cells is less clear. In the transplantation to the inner ear, it is unclear whether the adult cochlea is able to provide the extracellular microenvironment and transcriptional factors that are needed to lead stem cells through the appropriate developmental stages required to generate functional sensorineural cells. In order to identify the nature of the cellular and molecular mechanism involved in the stem cells differentiation in the inner ear, it is necessary to challenge the stem cells by altering both their intrinsic genetic programs regulated by transcriptional factors as well as extracellular microenvironment.

5.3.1 Intrinsic determinants

In order to test whether intrinsic factors will affect the differentiation of stem cells transplanted in the inner ear, adult NSCs were transduced with transcriptional factor prior to the implantation. There are a number of candidates for transcriptional factor, i.e. neurogenin, NeuroD, GATA3, lunatic fringe (Fgn), FGFs. It is reported that *ngn1* is required for the differentiation of sensory neurons in the inner ear. However, it is demonstrated that *ngn1* is not activated in *ngn2* mutant placodes clearly identifies *ngn2* as the first regulator and *ngn1* as a potential transcriptional target of *ngn2* in the placodes (Fode, et al., 1998). It is also known that transducing NSCs with *ngn2* results in around 95% of the transduced cells differentiating into a neuronal fate *in vitro* (Falk, et al., 2002). Thus, in the present study in order to drive NSCs differentiation into a neuronal fate, NSCs were transduced by *ngn2* prior to the implantation. NSCs expressing neuronal marker were found in the animals transplanted with *ngn2*-transduced-NSC, suggesting that a single gene have the potential to drive adult stem cells into an appropriate cell fate in a cell replacement therapy in the auditory system. It should, however, be noted that for the differentiation of sensory neurons in the cochlear, it is rather *ngn1* that is required (Ma, et al., 1998). Since the implanted cells are expected to differentiate into sensory neurons to restore hearing function, it cannot exclude that *ngn1* would provide more promising results.

It would be interesting to transduce the stem cells, prior to the transplantation, with the other candidate genes/transcriptional factors which are essential in the SGNs development. During development, TrkB and TrkC are expressed in the embryonic SGNs (Farinas, et al., 2001; Pirvola, et al., 1994). In addition, these delamination SGNs all express the neuronal developmental marker, NeuroD (Kim, et al., 2001; Ma, et al., 1998), GATA-3, a zinc finger gene (Karis, et al., 2001; Rivolta and Holley, 1998), and Fgn (Morsli, et al., 1998). On the other hand, in order to generate new hair cells in the adult cochlea, Brn 3a and Brn 3c (POU family of genes), and Math1 gene may be interesting candidate genes. It has been reported that Math1 has the capability to stimulate hair cell regeneration both *in vitro* (Zheng and Gao, 2000) and *in vivo*

(Kawamoto, et al., 2003). Combined with an appropriate extracellular matrix, the stem cells would be expected to differentiate into an appropriate sensorineural cell fate in the adult auditory system.

5.3.2 Extrinsic factors

In order to investigate the mechanism of stem cells differentiation in the inner ear, it is interesting to alter the microenvironment at the transplantation location. The microenvironment is a subset of tissues, cells and extracellular substrates. Interaction with other cell types and the component of the extracellular matrix are believed to influence the survival and development of the committed cells (Preston, et al., 2003) . The strategies of extrinsic signaling in cell fate specification are rather complicated, including inductive signaling, gradient signaling, antagonist signaling, cascade signaling, combinatorial signaling and lateral signaling (Edlund and Jessell, 1999). It has been illustrated that the microenvironment at the transplantation site plays an important role in the differentiation of stem cells into site-specific lineage (Akita, et al., 2002; Brustle, et al., 1995; Harkany, et al., 2004; Kaneko, et al., 2003; Renfranz, et al., 1991; Snyder, et al., 1997; Suhonen, et al., 1996; Vicario-Abejon, et al., 1995). Here, we attempted to provide an embryonic neuronal microenvironment into the transplantation location with the aim to providing factors for implanted cell survival and differentiation as well as an extracellular matrix for cell growth. There was significant difference in not only the survival but also, and more importantly, in the neuronal differentiation of ES cells between the neuronal cograft groups and the non-DRG-cograft groups. The embryonic neuronal tissue appeared to release factors beneficial to a neuronal differentiation of stem cells. It indicates that an embryonic neuronal microenvironment may be one of the key factors for stimulating the differentiation of ES cells in the inner ear.

In the present study, stem cells were not only transplanted into the normal inner ears, but also into the inner ears chemically damaged by the injection of neomycin prior to implantation. The cochlear cells in a chemically injured inner ear are expected to release certain growth factors for protection and regeneration. Two weeks following transplantation of NSCs into the normal inner ears, none of surviving cells was found to express neuronal marker (out of 20 inner ears). However, implanted cells were found to be labeled with neuronal marker in the injured inner ears at the same survival (2 out of 12 inner ears). In the transplantation of ES cells, we found that the number of surviving cells expressing neuron specific marker was promoted in the injured inner ears without an embryonic neuronal cograft. However, there was no such difference in the animals cografted with an embryonic neuronal tissue, suggesting that the beneficial effect of the neuronal cograft plays a more important, or at least more direct, role in the induction of ES cell differentiation compared to that of inner ear injury caused by neomycin administration.

5.4 LOCATION AND MIGRATION OF IMPLANTED CELLS

The mammalian inner ear is a very complex organ. The hearing organ, organ of Corti, and SGNs are surrounded by bony spiral structure (Fig. 2). The SGNs located in the center of the cochlea, in Rosenthal's canal within the bony modiolus. In order to minimize the trauma to the inner ear and for the concern of integrating with the cochlear implant electrodes in the future application, exogenous cells were transplanted into scala tympani in the present studies. As illustrated previously, the bony tissue, spiral lamina, separates scala tympani and the SGNs. In order to replace the function of the degenerated or absent SGNs, a cell migration through the bony spiral lamina, from scala tympani to spiral ganglion region, is thus essential.

In the present study the transplanted cells were shown to survive not only at the transplantation location in scala tympani but also outside the fluid-filled cavity. Implanted cells, both DRGs and stem cells, were seen along auditory peripheral nerve fibers. Interestingly, implanted stem cells were found also in scala vestibuli of the same cochlear turn. For implanted cells to reach this location, they may have followed the perilymphatic fluid to the apex, through helicotrema entering scala vestibuli and then downwards to the basal cochlear turn.

One of the most exciting findings in the study is that the surviving cells, more frequently observed in DRG transplantation, were also observed to have migrated through the bony tissues towards spiral ganglion region and located within Rosenthal's canal. The figures in Paper I provide strong evidence to show a cell migration through spiral lamina, entering Rosenthal's canal and close to SGNs. The mechanism for this migration is still unknown but we speculate the highly porous bony lamina (Shepherd and Colreavy, 2004) and Schuknecht's canaliculae perforantes (Lim and Kim, 1983) may provide a path for implanted cells to penetrate the bone and reach the spiral ganglion region. Secondly, the implanted cells may also be directed by the growth factors released by the SGNs, i.e. FGFs. Thirdly, the implanted cells themselves may have some degenerative effect on the osseous modiolus, i.e. by release of osteolytic factors. Irrespective of how the implanted cells reached their final locations, it is apparent that cells implanted into the fluid-filled scala tympani have the potential to reach functional relevant regions also outside of the transplantation site.

However, the implanted cells didn't show a further central migration at a later stage. The reason is still unknown but we speculate that the migration activity of implanted cells may be reduced after migration through the bony spiral lamina. The host auditory system may also have some restrict factors (i.e. PNS-CNS boundary) which may prevent the implanted cells from a central migration. In order to test whether the implanted cells have the capability to migrate through the PNS-CNS boundary towards cochlear nuclei, both DRGs and ES cells were transplanted along the auditory nerve fibers in the modiolus at the first cochlear turn. We found that the implanted cells not only survived along the auditory nerve but also migrated centrally towards the cochlear nucleus in the brain stem. Here, ES cells appear to have migrated more centrally, into the brain stem and close to cochlear nucleus, than DRG cells at the same survival. It indicates that pluripotent cells possess more potential to migrate than the differentiated neurons. Interestingly, surviving ES cells were also found to express neuronal marker along the auditory nerve fibers. Thus, we believe that implanted cells,

including embryonic neurons and stem cells, are able to survive and migrate through the PNS-CNS boundary centrally towards the brain stem.

It was also observed that implanted embryonic neurons migrated through basilar membrane and located in the region of organ of Corti (Paper II). The reason for the migration is obscure but probably directed by the growth factors released by the organ of Corti (e.g. NT-3, BDNF) and also possible through the tiny holes (Habenula perforata) which permits the communication between the scala tympani and the organ of Corti. Though the mechanism for cell migration into the organ of Corti region is still unknown, this finding may suggest that sensory epithelium replacement is also feasible.

5.5 HOST-GRAFT INTEGRATION

For a successful cell replacement therapy of the inner ear, apart from survival, appropriate differentiation of stem cells, the implanted cells need to form neural connections with the host auditory system in order to replace the function of degenerated or absent SGNs. There are several possible ways to reach this. One possibility is that the implanted cells migrate to the spiral ganglion region and make cell-cell connections with the limited number of surviving SGNs. These implanted neurons are expected not only to be stimulated by the outside signal but also to transfer the auditory inputs to the SGNs, thus increase the intensity of the auditory inputs at the SGNs level. Alternatively, these implanted neurons would also be speculated to generate axons centrally towards the cochlear nucleus and make synaptic connections with the cochlear nucleus. Thus, the implanted cells could conduct the signal centrally towards cochlear nucleus in the brain stem directly, bypassing the degenerated SGNs and the organ of Corti. The results in the present study suggest that both ways are possible.

It was observed in the study that the implanted cells have migrated close to the host SGNs. Whether there exists a cell-cell contact or communication still need to be shown. Perhaps the most interesting finding was that neurite projections were found between the implanted cells and auditory nervous system, i.e. SGNs and the nerve fibers leading from the organ of Corti. The underlying mechanism of how this projection formed has not been identified, most likely related to the growth factors released by the target cells. It was found that neurite outgrowth proximal to implanted cells was labeled with EGFP/GFP fluorescence, suggesting the axonal sprouting may be initiated from the implanted cells and directed by the host auditory system. However, the neurite outgrowth proximal to the auditory system was EGFP/GFP negative. This phenomenon suggests that part of the neurite projection may be originated from the host auditory system and directed by the implanted cells. The other possibility is that neurite projections may be originated from the implanted cells but there was a down-regulated-expression/weak-expression of the EGFP/GFP in axon distal to the cell body. The later explanation is less possible since the EGFP/GFP fluorescence was not enhanced after the application of EGFP/GFP antibody.

The finding of neurite contacts between implanted cells and host auditory system is very important since it indicates the possibility for implanted cells to improve or even replace the signal input function of the host auditory system. In order to investigate the synapse between the implanted cells and host SGNs, pre-synaptic marker and post-synaptic markers will be used in our future studies.

Current transplantation research focused mainly on the potential of the neuronal tissue and stem cells to replace the degenerated or absent cell populations in the inner ear. In the mean time, we should also know that it is essential to investigate the effect of the graft on the host auditory system, i.e. graft induced host plasticity. One obvious group of molecules that is likely to play a key role in this graft-host effect is the group of neurotrophic factors, i.e. NT-3 and BDNF, which have important neuroprotective role to the auditory system. Laminin, cell adhesion molecules may be also involved in the axonal outgrowth, postsynaptic partners targeting and the stabilization of the connections once they are established. Another group of active molecules would be injury-induced cytokines, which is released during the immunological response of the host tissue after transplantation. There is evidence that a suppression of the immunological response by medication can interfere substantially with the regenerative capacity evoked in the recipient (Bresjanac, et al., 1997).

5.6 FUNCTIONAL EVALUATION OF IMPLANTED CELLS

The survival and migration of the implanted cells as well as the neurite projections between the implanted cells and host auditory system lead to the possibility to assess the physiological function of the exogenous cells transplanted into the inner ear. Due to the better survival rate, frequently observed neurite contacts and mutuality of the animal model, DRG neurons were selected as a donor to transplant into the scala tympani of the chemically damaged inner ears. It was found that extensive neurite projections from implanted cells were observed to penetrate through the bony modiolus and extended towards the host SGNs in the animals supplied with exogenous NGF, chronic electrical stimulation or both. However, there was no significant difference in the EABR thresholds and input/output functions between the animals with and without DRG transplantation. The same result was also found in DRG-transplanted-animals with and without supplement of NGF and/or chronic electrical stimulation. It suggests that NGF and/or chronic electrical stimulation can stimulate the neurite outgrowth from the implanted neurons, although based on EABR measurement this enhancement did not translate into a functional significance.

The lack of positive findings in the functional EABR measurements is disappointing but may have several explanations. It is possible that the implanted cells failed to form *functional* contacts with the host auditory nervous system, or that the implanted cells were not electrically excitable. However, it is very likely that, even if surviving cells were both functional and structurally well integrated, they were too few to modify the electrical responsiveness of the inner ear. Probably the

population of implanted cells must be much larger. It is obvious that further experiments are required to optimize the implantation procedures so that more cells survive and become integrated with the host auditory system. The negative finding in the EABR measurements may also be related to the metabolic status of implanted cells and the neurite contacts between the implanted cells and the host SGNs. The technical limitation and small sample size may be one of the reasons. The interaction between the implanted cells and host neurons thus needs to be investigated at a molecular and protein level in the future studies. In the meantime, exploring a more sensitive method other than EABR measurement may be helpful to detect the potential functional improvements provided by the implanted cells.

6 SUMMARY

It is well accepted that the sensorineural cells in the inner ear of adult mammalian do not regenerate spontaneously when they are damaged or degenerated. In order to restore the auditory function of these cells, the hypothesis of replacing the degenerated sensorineural cells based on a cell substitution strategy was tested in the present study. The spiral ganglion neurons (SGNs) were selected as the target cell type to be replaced and the scala tympani as the transplantation location in the adult inner ear.

Cell fate. The results demonstrated that exogenous cells, including embryonic neuronal tissues (DRG), pluripotent adult neural stem cells (NSCs) and multipotent embryonic stem (ES) cells were able to survive in the adult inner ear. The supplement of exogenous neurotrophic factors could enhance the survival of DRG neurons while an embryonic neuronal cograft was able to promote the survival of implanted ES cells. Transduction with the gene *neurogenin2* not only enhanced survival but also induced a neuronal differentiation of adult NSCs transplanted in the inner ear. In addition, neuronal differentiation of ES cells was significantly enhanced by an embryonic neuronal cograft.

Graft-host interaction. In order to replace the degenerated SGNs by implanted cells, proper cell migration and neural contact formation must be established between the implanted cells and the target SGNs. In the present study, implanted cells were found to migrate towards functional relevant areas in the inner ear, close to the host SGNs. Moreover, neurite connection was shown between implanted cells and the SGNs. The supplement of an exogenous neurotrophic factor, chronic electrical stimulation or embryonic neuronal cograft stimulated the formation of neural projections.

Functional evaluation. The positive structural findings, especially the neurite projections from implanted DRG cells toward the host auditory system, promoted an evaluation of the possible function of the exogenous cells. However, recordings of electrically evoked auditory brainstem responses (EABRs) did not reveal any significant changes in the responsiveness of the host auditory system. This negative finding is possibly caused by the low survival rate of implanted cells: the exogenous cell population was simply too small to be of significance. Further research is clearly needed to characterize the interaction between implanted cells and the host tissue, and to what extent this can be used to modulate the responsiveness of the host auditory system.

7 PERSPECTIVES

This study has investigated the feasibility of a cell replacement therapy in the inner ear. The hypothesis is that the implanted cells would not only take the place of absent cochlear cells but also compensate the auditory deficiency caused by the degenerated SGNs. Different donor candidates, including embryonic neuronal tissue, ES cells and adult NSCs, have been identified as the potential donors in the inner ear substitution strategies. Efforts have also been directed to enhance the survival of implanted cells, stimulate the implanted cells differentiation into an appropriate cell fate, evaluate the function of surviving cells and the neurite projections between the implanted cells and host SGNs. Although the preliminary EABR measurement did not show any significance, it leads to the extensive works that go further into the molecular and protein level in the future studies (Fig. 4).

Firstly, the test for a more optimal donor candidate for transplantation is still worthwhile. It includes testing different sources of tissues or cells, i.e. other ganglion tissues (SGNs), and hematopoietic stem cells. Because of the pluripotency, ES cells and derived progenies would be logical alternatives. It has been shown that transplantation of ES cells-derived neural cells has resulted in functional improvement in the animal models of Parkinson's disease (Björklund, et al., 2002; Kim, et al., 2002; Lee, et al., 2000) and spinal cord injury (Liu, et al., 2000; McDonald, et al., 1999). It is thus interesting to induce ES cells differentiation into an appropriate cell fate, i.e. neural cells, and then transplant into the inner ear. For the concerns of ethics and the neural differentiation, adult NSC is still an exciting alternative. The stem cells from the inner ear, i.e. vestibular and cochlear stem cells, would probably be even more appropriate for the transplantation in the inner ear.

Secondly, the survival of implanted cells remains the major issue in a cell replacement therapy of the inner ear. It is noted that the total number of the implanted cells survived in the inner ear is actually rather poor. Approaches including a supplement of essential growth factors and appropriate extracellular microenvironment for cell growth are feasible. The immunological response needs to be investigated and suppressed.

Thirdly, the cell differentiation needs to be investigated. Identification of proper intrinsic and extrinsic factors capable of inducing an appropriate cell differentiation should be studied. In the meantime, techniques regarding the proper application of these factors, including the gene transduction procedures, need to be optimized.

Finally, one of the most exciting aspects of the study is to evaluate the function of the implanted cell. Exploring an appropriate deafened animal model and a more sensitive method to detect the auditory input function as well as detecting the interaction between the implanted cells and the host auditory cells at a molecular and protein level probably will be the major challenges for the future study.

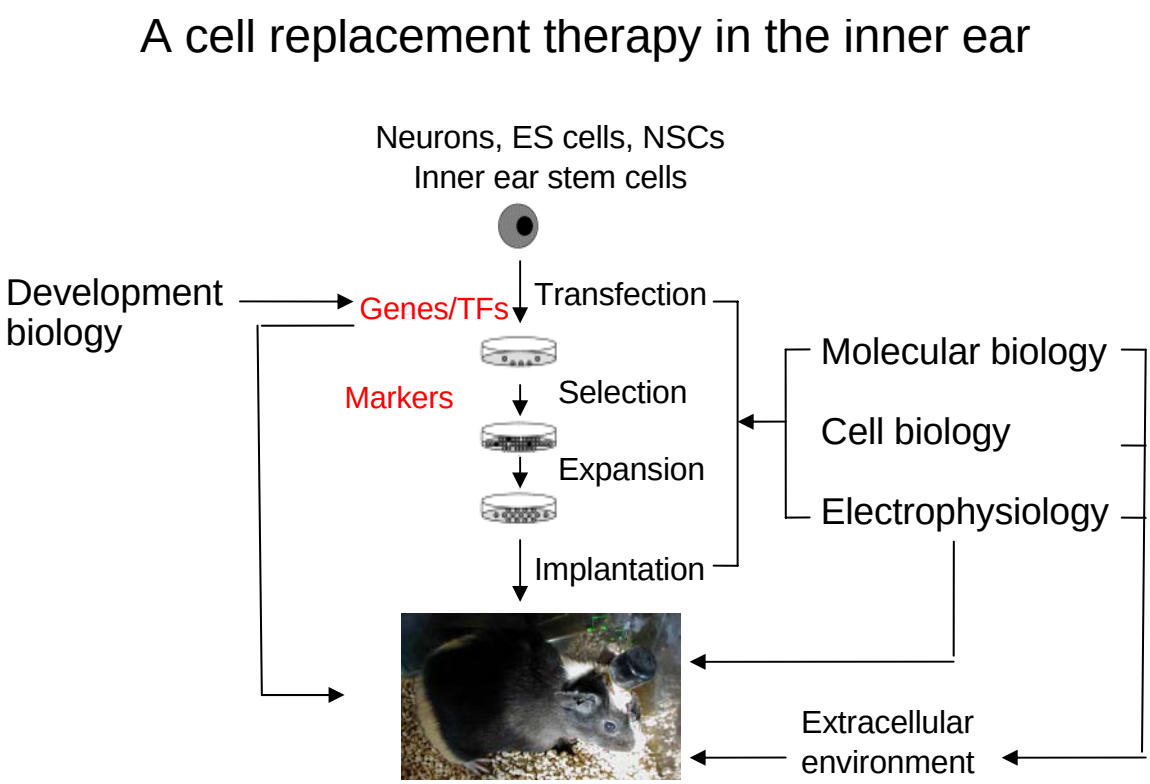

Figure 4. Future perspectives of a cell replacement therapy in the inner ear.

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