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### Embryonic Stem Cell-Derived Neurons for Inner Ear Therapy

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#### 1. Introduction

Sensorineural hearing loss is a major public health problem affecting more than 278 million people worldwide. The primary cause of sensorineural hearing loss is loss or damage of sensory hair cells in the organ of Corti. However, approximately 10-15% of cases with profound hearing loss in children are caused by degeneration of the spiral ganglion neurons (SGNs) or neurons in the auditory brainstem. Moreover, SGNs gradually degenerate after the loss of hair cells due to a lack of excitatory stimulation. Since SGNs do not regenerate to any clinically significant extent, novel therapies for their preservation, regeneration or replacement are being heavily sought. Currently, no treatment option is available for peripheral auditory neuropathy.

Cell-based therapies offer a strategy to enhance auditory functions in the deaf patient and improve the benefits of cochlear implantation. There are three major areas for potential clinical applications relevant to this approach. First, for patients who have received cochlear implants, generation or preservation of SGNs via cell replacement therapy could significantly improve the quality of their sound perception. Another group of potential recipients who would benefit from cell replacement therapy are patients suffering from acoustic neuroma or neurofibromatosis. These patients generally exhibit significant loss of auditory or vestibular primary neurons with relatively intact sensory hair cells (Kaga et al., 1997; Evans et al., 2000; Sperfeld et al., 2002). Thus, replacing dead or damaged neurons with stem cells could be critical in restoring their hearing or balance sensation. Finally, auditory neurons generated from stem cells could be used in *in vitro* assays to test the effectiveness and safety of newly developed drugs before clinical trials.

Type I SGNs, comprising 95% of all neural populations in the SG, innervate inner hair cells in the organ of Corti and function as the primary auditory afferent neurons (Berglund and Ryugo, 1987; Liberman et al., 1990; Rusznak and Szucs, 2009). These SGNs predominantly express AMPA receptors (mainly GluR2-4) (Niedzielski and Wenthold, 1995; Parks, 2000; Dulon et al., 2006; Chen et al., 2007; Flores-Otero et al., 2007), which bind the neurotransmitter glutamate released from inner hair cells (Fig. 1). This subsequently triggers action potentials that propagate along the nerve fibers to the cochlear nucleus. The majority, if not all, of type I SGNs are glutamatergic and release glutamate from their presynaptic membrane in the cochlear nucleus (Rebillard et al., 2003; Reyes et al., 2008). Expression of AMPA receptors and glutamate transporters thus is the hallmark of mature type I SGNs. During embryonic development, SGNs arise from the otic placode and

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transiently express several key transcription factors, including Neurogenin 1 (Ngn1), NeuroD and Brn3a (Fig. 3). Inactivation of these transcription factors results in a significant reduction in the number of SGNs or severe retardation in the axon projections to the cochlea (Ma et al., 1998; Huang et al., 2001; Kim et al., 2001). Thus, Ngn1, NeuroD and Brn3a represent phenotypic markers for immature SGNs.

Over the past several years, progress has been made to generate *in vitro* functional neurons bearing a SGN phenotype from embryonic stem cells and different types of somatic stem cells (Martinez-Monedero et al., 2008; Reyes et al., 2008; Chen et al., 2009). Some of these stem cell-derived neurons were shown to establish synaptic contacts with sensory hair cells, the peripheral target for SGNs, *in vitro* (Matsumoto et al., 2008) and to survive in animals with selective loss of SGNs (Corrales et al., 2006; Matsuoka et al., 2007). However, little information is currently available how to promote pluripotent stem cells competent to give rise exclusively to glutamatergic sensory neurons.



## 2. Embryonic stem cells can be efficiently directed to differentiate into neurons *in vitro*

We have established an efficient feeder-cell free neural induction protocol based on a previous study (Watanabe et al., 2005) and, using this protocol, extensively characterized temporal changes in gene expression in ESCs undergoing neural differentiation. Briefly, undifferentiated mouse R1 ESCs were dissociated and cultured in bacteria plates to allow embryoid body (EB) formation, after which EBs were plated on tissue culture plates and cultured in a pre-induction medium (Step 1). After 2 days, the medium was changed to a neural induction medium and maintained for an additional 1-15 days (Step 2). During the first differentiation, EBs became larger in size, but there was no indication of neurite-like processes. Only one day after the start of the second step, cells in the outer edge emigrated

from the EB (Fig. 2A). Three days after the start of neural induction, numerous neurite processes emerged from the outer edge of EBs. At 7 days after the start of neural induction, much denser and longer processes than those seen at 3 days of neural induction were observed in EBs. At 15 days after the start of neural induction, processes remained dense and they appeared to extend to other EBs to make contacts. Immunohistochemical staining for HuC and TuJ1, specific neuronal markers, indicated that the cells bearing neurite-like processes were indeed neurons. Morphological changes in ES cells during neural induction, characterized by growth of neurite processes, were progressive in the first 7 days, after which they remained largely unchanged until the last day of 15 day neural induction.



Fig. 2. (A) Morphological changes in ESCs undergoing neural differentiation. TUJ1 and HuC, neuron-specific proteins, are detected in a subpopulation of cells suggesting a neuronal fate. TUJ1 accumulates in the axon-like processes, while HuC is restricted to the cell body consistent with its function as a translation factor. (B) RT-PCR analysis for ESC-and neural-marker genes in undifferentiated ESCs (ESC), ESCs at EB stage (day 5), 3d, 7d, 10d, and 15d after start of neural induction (NI-3, -7, -10 and -15, respectively)

To evaluate temporal changes in gene expression, qRT-PCR analyses were performed with RNAs collected from ESCs or ESC-derived cells at various time points during differentiation. The results demonstrated steep reductions of ESC-specific genes, including *Oct4, Rex1* and *Sox2,* in contrast with rapid (early neural genes: *Musashi1* and *HuC*) or gradual (mature neural genes: *TUJ1, NSE, Synaptophysin, Calretinin, TrkB* and *Tau*) increases of neural marker genes (Fig. 2B). Results from our Western blot analysis for the corresponding proteins were consistent with the qRT-PCR results with delayed peaks of about 3 days (Kondo et al., 2008). Down-regulation of Oct4 and Sox2 was accompanied by transient up-regulation of early neural marker proteins, including Musashi1 and HuC, which was followed by stable induction of mature neural proteins, including TUJ1, Calretinin, Synaptophysin, NSE and Tau. To evaluate the percentage of ESC-derived cells exhibiting neural cell surface markers during neural differentiation, we performed flow cytometric analyses for CD24 and Tau. Virtually none of undifferentiated ESCs express CD

24 or Tau, while over 90% of those are positive for both neural markers at neural induction day 7 (Kondo et al., 2008). To assess electrophysiological properties of induced ESC-derived cells, we performed single-cell current-clamp recordings. Robust action potentials and voltage-dependent inward sodium currents were recorded from  $\approx$ 70% of ESC-derived cells at neural induction day 7 (Kondo et al., 2008).

### 3. TIx3 promotes glutamatergic neuronal specification and is a direct target for Wnt signaling

We have previously demonstrated that T cell leukemia 3 (Tlx3; also known as Hox11-L2/Rnx) can be used as a potent intrinsic factor to generate, from mouse ESCs, excitatory neurons with a phenotype that resembles type I SGNs (Kondo et al., 2008). Tlx3 is a member of the Tlx family of homeobox transcription factors and is selectively expressed in cranial and dorsal root sensory ganglia, including SGNs, during early embryogenesis (Logan et al., 1998; Cheng et al., 2004; Cheng et al., 2005; Kondo et al., 2008). Tlx3 is a genetic switch for selection of a glutamatergic over a GABAergic transmitter phenotype during nervous system development (Cheng et al., 2004; Cheng et al., 2005; Xu et al., 2008). We have found that forced expression of Tlx3 in ESCs combined with directed neural induction leads to sequential up-regulation of genes (and proteins) that are expressed in transiently-amplifying neural progenitors (Mash1), immature (Ngn1, NeuroD, Brn3a) and mature (GluR2, GluR4, Vglut2) SGNs (Kondo et al., 2008). Furthermore, these Tlx3-expressing ESC-derived neurons exhibited robust action potentials and excitatory post-synaptic currents, indicative of functional excitatory neurons.



Fig. 3. (A) Sequential expression of Ngn1, NeuroD and Brn3a during SGN development. (B) Temporal changes in gene expression during inner ear development. C; cochlea, V; vestibule

Since Tlx3 is a transcription factor, we sought to identify a signaling molecule that controls Tlx3 expression with the ultimate goal of developing a means to extrinsically induce glutamatergic neuronal specification from ESCs (Kondo et al., submitted). Wnt/ $\beta$ -catenin signaling promotes neural differentiation by activation of the neuron-specific transcription factors, Ngn1, NeuroD and Brn3a, during neural development. The canonical Wnt pathway activation allows  $\beta$ -catenin to translocate to the nucleus, where it interacts with the T-cell factor (TCF) family of DNA-binding proteins and  $\beta$ -catenin/TCF complexes regulate transcription (Fig. 4). Based on this consensus signaling pathway, the  $\beta$ -catenin/TCF complexes are the prime nuclear effectors for canonical Wnt signaling. Since neurons in cranial sensory ganglia and dorsal root ganglia transiently express *Ngn1*, *NeuroD* and *Brn3a* during embryonic development, we hypothesized that Wnt(s) could instructively promote a sensory neuronal fate from stem cells undergoing neural induction. Consistent with our

hypothesis, Wnt1 induced expression of sensory neuron marker genes, including *Ngn1*, *NeuroD*, *Brn3a* and *P2X3*, as well as glutamatergic marker genes, such as *GluR2* and *GluR4*, in neurally-induced somatic stem cells in a dose-dependent manner (Fig. 4; Kondo et al., submitted). Additionally, Wnt1-induced up-regulation of these genes was suppressed by specific canonical Wnt antagonists, Dickkopf-related protein 1 (Dkk1) and Secreted frizzled-related protein 2 (sFRP2). The inhibitory effects of Dkk1 and sFRP2 were specific, as they had no effects on expression levels of *GATA3*, *Tau* and *TUJ1*, none of which is regulated by Wnt1. Furthermore, expression levels of Brn3a, GluR2, GluR4 and Vglut2 proteins in neurally induced stem cells grown in the presence of Wnt1 were significantly higher than those grown in the absence of Wnt1, indicating that Wnt1 up-regulates glutamatergic sensory neuron-specific proteins in neurally-competent stem cells.



Fig. 4. Schematic figure showing canonical Wnt signaling pathway and our results

Given the consensus function of Tlx3 as a glutamatergic selector gene, we postulated that the effects of canonical Wnt signaling on sensory neuron and glutamatergic marker expression in stem cells may be mediated by Tlx3. We first confirmed that Wnt1 can indeed up-regulate *Tlx3* expression in somatic stem cells and the Wnt1-induced *Tlx3* up-regulation was entirely suppressed by Dkk1 or sFRP2 (Fig. 4; Kondo et al., submitted). Next, we demonstrated that forced expression of Tlx3 induced sensory and glutamatergic neuron markers after neural induction. Moreover, our chromatin immunoprecipitation assays revealed that TCF3/4 directly bind a regulatory region of Tlx3 after neural induction (Kondo et al., submitted). To further characterize this binding site, we have cloned 2600 bp 5'- and 1700 bp 3'-non-coding regions flanking the Tlx3 coding region into a luciferase reporter vector and generated another reporter construct with a mutation in the TCF binding motif (Fig. 5A). Wild-type ESCs were transfected with a reporter construct containing the luciferase gene under the control of the wild-type or mutant *Tlx3* promoter and a constitutively expressing the Renilla luciferase construct for normalization of transfection efficiency. Transfected cells were incubated in neural induction medium in the presence or absence of 100 ng/mL Wnt1 for 2 days and luciferase activities were analyzed using a Dual Luciferase Reporter Assay System. Consistent with our hypothesis, the Wnt1-induced Tlx3 promoter activity was significantly reduced with a mutation in the region 3 TCF binding site, when compared to a wild-type (Fig. 5B). These results demonstrate that the TCF binding site in the Tlx3 promoter is required for Wnt-dependent transactivation of *Tlx3*.



Fig. 5. Luciferase reporter activity of ESCs transfected with the empty vector (pGL3), wild-type or mutant Tlx3 reporter construct

### 4. ESC-derived neurons expressing TIx3 form growth cones enriched with GAP43

We have characterized molecular and biochemical properties of Tlx3-expressing ESCs undergoing neural differentiation and found that Tlx3 instructively promotes these stem cells to acquire a glutamatergic phenotype, while suppressing a GABAergic phenotype (Kondo et al., 2008). To determine whether these Tlx3-expressing cells exhibit functional characteristics of excitatory glutamatergic neurons and make synaptic contacts with their targets after neural induction, we have performed subcellular fractionation and obtained evidence that ESC-derived neurons expressing Tlx3 form growth cones that are enriched with GAP43. Similar to primary neurons in vitro, GAP43 was co-localized with neurofilament 160 as well as F-actin in the distal tip of ESC-derived neurons at neural induction day 7 (Fig. 6A). Western blot analysis of whole cell lysates revealed that GAP43, Tau and the pan-sodium channel protein SP19 are abundant in ESC-derived neurons, but that none of these proteins was detectable in undifferentiated ESCs or EBs (Fig. 6B). To elucidate sub-cellular localization of GAP43, we isolated an enriched population of growth cone particles using a discontinuous density gradient sucrose (Fig. 6C). Our immunoprecipitation and western blot analyses detected a substantially higher GAP43 expression in the growth cone particle fraction (0.32/0.83M) when compared to whole lysate (before isolation) or the non-growth cone fraction (1.20/2.66M) (Fig. 6D). These results indicate that GAP43 is enriched in the growth cone of ESC-derived neurons expressing Tlx3 and suggest that the growth cone assembly, which is essential for axon guidance and target innervation, is already in place in these ESC-derived cells by neural induction day 7.



Fig. 6. GAP43 is enriched in growth cones of ESC-derived neurons expressing Tlx3. (A) Expression of GAP43 (red), Neurofilament 160 (blue) and F-actin (green) at neural induction day 7. (B) Western blot analysis for GAP43, Tau and SP19. ESC: undifferentiated ESCs; EB: embryoid bodies; NI: ESC-derived cells at neural induction day 7; B: brain (positive control). (C) Isolation of growth cone particle (GCP) fractions. (D) Immunoprecipitation and western blot analysis for GAP43 in GCP vs. non-GCP fractions

### 5. TIx3-expressing ESC-derived neurons are attracted to hair cells in the cochlea

To study interactions between Tlx3-expressing ESC-derived neurons and embryonic inner ear tissues, GFP-positive ESCs were co-cultured with an E18 mouse organ of Corti, the peripheral target tissue for SGNs (Fig. 7). Organ of Corti tissues were removed from E18 mice and embedded into rat type I collagen in 8-chamber slide wells. Cochlear tissues were removed from wild-type E18 mice and embedded into rat type I collagen. Four to five hours after the start of incubation, Tlx3-expressing ESCs forming spheres were placed into collagen approximately 100  $\mu$ m in the distance from a cochlear explant. On the following day, the medium was replaced with neural induction medium and incubation continued for an additional 5 days. In the presence of an organ of Corti explant, Tlx3-expressing ESCs survived and propagated vigorously, but upon exposure to the neural induction medium, withdrew from the cell cycle and differentiated into neurons. Additionally, these ESC-derived cells began expressing sensory neuron-specific antigens, such as GluR4 and calretinin, along with several pan-neuronal markers. Furthermore, ESC-derived neurons extended their processes towards the explant, formed ectopic synaptic contacts with cells in the organ of Corti.



Fig. 7. (A, B) Tlx3-expressing ESC-derived neurons are attracted to both embryonic cochlea and auditory brain stem. Low-magnification fluorescence photo micrographs of 3-D collagen cultures with GFP-positive ESC-derived neurons and an E18 cochlear explant (A) or a brainstem slice (B). (A) MyosinVII (Red) and TUJ1 (Purple) immunoflorescence. Note that GFP-positive processes from ESC-derived neurons are extended towards the cochlear explants. CO, cochlear explant. (B) Mafb (Red) and GAP43 (Purple) immunofluorescence

# 6. TIx3-expressing ESCs can survive and migrate towards endogenous SGNs in animal model of auditory neuropathy

We transplanted partially differentiated ESCs into the modiolus of the deafened gerbil cochlea that subsequently received neural induction medium for 3 days via osmotic pumps. Four-to-six month-old Mongolian gerbils were used as transplantation recipients. To deafen the animals, focal application of ouabain was performed based on a previously described procedure (Matsuoka et al., 2007) with minor modifications. Briefly, the animals, free of any signs of ear infection, were given atropine (0.2 mg/kg) to reduce secretion prior to the surgery. Following anesthesia with isoflurane, a small incision (less than 1.5 cm) was made to expose the right posterior aspect of the skull. A total volume of 5  $\mu$ L ouabain solution (1

mM in normal saline) was slowly infused into the round window niche using a siliconized glass micropipette attached to a 10  $\mu$ L Hamilton syringe. A small piece of gelform was placed in the round window niche to prevent leaking of excessive ouabain. The same amount of normal saline was infused into the left cochlea of the animal, which served as a control. Ouabain was washed off after 1 hour, the bulla will be closed with dental cement (Durelon, 3M ESPE), and a two-layer closure was made in the skin. The animals were allowed to recover for 4 weeks, during which progressive degeneration of spiral ganglion neurons took place. At the end of the recovery period, animals received a modiolar transplantation of ESCs stably expressing pBud-eGFP-cTIx3. Under anesthesia, a small fenestra was made in the wall of the basal turn scala tympani and a suspension of Tlx3-expressing stem cells (1x10<sup>6</sup> cells/ $\mu$ L) in 10  $\mu$ L PBS (left ear) through a 30-gauge needle that was inserted into the bony wall of the basal turn of the gerbil cochlea.



Fig. 8. Low (A) and high (B)-magnification micrographs of GFP-positive ESCs transplanted into the modiolus of the gerbil cochlea that has received neural induction medium. SG, spiral ganglion

We observed clusters of ESCs in the vicinity of SG of recipient animals 10 days after transplantation (Fig. 8). We were pleased with the extent of ESC migration as well as the number of engrafted ESCs. However, the percentage of engrafted ESCs expressing pan neural markers was low (less than 10%). We attributed this to (A) the short period in which neural induction medium was infused, and (B) the concentration of neural induction medium was not high enough to promote efficient neural induction, although we used a medium that contained 5-times higher concentrations of reagents used for our *in vitro* experiments. In order to increase the number of donor cells giving rise to neurons, we employ 3 approaches: first, we will increase the length of neural induction period from 3 days to 28 days; second, we will use a higher concentration of neural induction medium infused into the cochlea via osmotic pump; third, we will transplant stem cells that will have been incubated in neural induction medium for 2 days *in vitro*.

#### 7. Magnetic resonance imaging of ESC in the cochlea

Monitoring transplanted cell delivery, homing, and trafficking is of the utmost importance for developing translational strategies. Magnetic resonance imaging (MRI) has recently emerged as one of the most predominant imaging modalities for tracking stem cells in live animals in a noninvasive and repeated manner. To increase the resolution of images, specific contrast agents, such as iron-oxide nanoparticles, have been routinely used. In the present study, an alternative nontoxic agent, manganese chloride, was used to label only biologically active stem cells in host animals. Paramagnetic manganese ions (Mn<sup>2+</sup>) are calcium (Ca<sup>2+</sup>) analogs that are taken up by live cells through voltage-gated Ca<sup>2+</sup> channels (Yamada and Yang, 2008). Thus, only cells with active Ca<sup>2+</sup> channels are labeled with MnCl<sub>2</sub>. This property makes MnCl<sub>2</sub> a unique contrast agent for functional live cell imaging.

ESCs were labeled with 0.1 mM MnCl<sub>2</sub> as previously described (Yamada and Yang, 2008). Following injection of MnCl<sub>2</sub> labeled cells into the basal turn of the cochlea as described above, the animals were anesthetized with 2% isoflorane in an anesthetic chamber and maintained during imaging with 1-2% isoflorane delivered via face mask. The animals were placed with the head centered within a custom-made birdcage head coil. Axial T1-weighted images were acquired using a 3D asymmetric spin-echo pulse sequence with the following parameters: TR = 100msec, TE = 6.5msec, 16 mm FOV, 256 x 256 matrix (Lane et al. 2005) to yield a maximum resolution of approximately 62  $\mu$ m and minimum resolution of approximately 100  $\mu$ m. Images were imported into Analyze (v9.0, Biomedical Imaging Resource, Mayo Clinic, Rochester, MN) and volume registered with the long axis of the basal turn.

Using a 9.4T MR system, one of the highest resolution MR systems available in the USA, we have obtained serial coronal images of the gerbil cochlea that received transplantation of MnCl<sub>2</sub>-labeled ESCs. MnCl<sub>2</sub> is a strong T1 relaxation agent which, when imaged with T1 sensitive sequences, produces significant hyperintensity of the labeled cells. Images were taken 2 days after the animal received an intra-modiolar injection of 1 million MnCl<sub>2</sub>-labeled ESCs. As expected, the injected cochlea exhibited significantly hyperintense contrasts when compared to the uninjected cochlea in the same animal (Fig. 9). Furthermore, the surrounding non-labeled structure of the cochlea appeared hypointense relative to labeled cells. No noticeable differences in the contrast between the left and right cochleae were observed in the uninjected control animal. Further optimization of imaging parameters and cell labeling protocols is under development.



Resolution: 125 µm

Fig. 9. Contiguous trans-axial Proton density weighted images of gerbil cochleae obtained with the 9.4T MR system. Regions in post-transplantation cochlea (Injected Ear) exhibit hyperintense contrasts when compared to the control ear

#### 8. Translational approaches

Establishing a minimally-traumatic surgical technique to precisely place stem cells in the cochlea is a key for success in cell-based inner ear therapy. It addresses the unmet surgical challenge posed by the delicate cochlear structures being located deep in the hard temporal bone. Our previous studies using animal models demonstrated that transplanting stem cells directly into the modiolus of the gerbil basal cochlear turn yields a satisfactory degree of migration and engraftment (Matsuoka et al., 2006, 2007). However, our recent study using human cadaver temporal bones revealed that this approach would not be suitable for clinical applications due primarily to differences in anatomical structures between the rodent and human cochleae (Fritsch, 2009). For example, the human cochlea has a much larger scala tympani, scala vestibuli, and modiolus. Because of these anatomical differences, we recently started using cadaver human temporal bones to simulate surgeries for transplanting stem cells into the human cochlea. The large size of the human cochlear basal turn modiolus has actually made translation of gerbil studies into human temporal bone simulation surgeries less comparable. The reason is that the modiolus is less densely packed with cranial nerve VIII fibers in the human compared to the gerbil. Cerebro-spinal fluid (CSF) is present within the internal auditory canal in that area and is pulsatile in the living human due to brain pulsations. The net effect is that stem cells transplanted into the human basal turn modiolus are less apt to stay localized in the modiolus and more likely to either "squirt" into the larger internal auditory canal CSF during injection or be washed away by the pulsatile CSF. These two findings give rise to an incomplete or failed stem cell placement. For this reason, the investigators presently believe that the human middle turn modiolus will be the better target when translating gerbil studies to human subjects. It is much more likely to retain the stem cell dosage injected into the modiolus. This has been borne-out in early experiments on the gerbil and human cochleas with histological confirmation.

The human middle-turn cochlea and modiolus are comparable to the human basal turn anatomic structures except that they are smaller and the modiolus is solidly packed with nerve fibers. With injections into the middle-turn modiolus, the stem-cells are held in place by the gelatinous-consistency of the surrounding nerve fibers. From that placement position the stem cells are free to migrate under their own guidance rather than being mechanically washed-away by the CSF, as in the basal turn area. The exact mechanisms for how the stemcells find their final location as replacement cells for originally damaged inner ear cells is still unknown. However, it is felt that surgical placement directly into the modiolus, which is adjacent to the SG and other inner ear structures, gives them a "head start" to their final location; this is compared to simply injecting them into the bloodstream or CSF space. For this reason, the middle turn of the cochlea is especially targeted in this study relative to the other surgical sites.

Unlike the basal turn, the middle turn structures cannot be simply accessed surgically by drilling a hole into the large promontory of the middle ear space. That basal turn surgical technique is commonly used every day by cochlear implant surgeons. Rather, the middle turn is buried under several structures and has no "promontory" to declare it's presence. To this end, Fritsch has performed experimental surgery on the human cadaver temporal bone to delineate a new surgical approach to the middle turn structures: the middle turn scala tympani and scala vestibuli are uncovered by surgical dissection followed by injection into the modiolus. Ongoing studies are underway to refine the techniques; it is obvious to the surgeon that some manipulations of instruments are beyond the delicate touch of the

surgeon's own human hands and would be better served by micro-manipulators. For multiple simultaneous entry sites, a small-diameter endoscope with a micromanipulator is currently under development (Fritsch, 2009).

For humans undergoing implantation with a cochlear implant device, the aforementioned drilling into the basal turn promontory (ie: cochleostomy) is sealed using tissue autograft plugs to seal the cochleostomy (after device electrode placement into the cochlea). For the gerbil experiments so far, we have used a plastic compound to seal the needle puncture site in the cochlea after injection of stem cells. In humans, plastic compounds often result in foreign-body reaction responses resulting in giant cell granulomas. Though tissue plugs are used to good success in cochlear implants, they are too large for needle punctures within the cochlea modiolus after stem cell injections. Purified collagen-matrix graft materials are being tested in animals, for tissue healing, and in human cadaver temporal bones for mechanical effectiveness of delivery and function. These materials are placed into the cadaver human temporal bone to check for extravasation into the inner ear (with histological confirmation) and into the gerbil with follow-up histological to check for healing and sealing of the cochleostomy opening.

#### 9. Future directions

We plan on extending our research into 3 new directions. First, we will continue to fill in gaps of our understanding of molecular mechanisms underlying Tlx3-mediated glutamatergic neuronal cell-fate specification. Specifically, we aim to identify direct target genes for Tlx3 using a promoter array and elucidate their interactions with Tlx3 using chromatin immunoprecipitation and mutagenesis. We hypothesize that Tlx3, while activating Ngn1 transcription, triggers a counter-balance mechanism that suppresses GABAergic differentiation in ESC-derived neurons. Additionally, we aim to elucidate the epigenetic mechanisms underlying context-dependent transcriptional activation of target genes by Tlx3. Identifying and characterizing Tlx3-binding proteins that can alter chromatin structures via acetylation and/or methylation will greatly enhance our understanding of extrinsic modulation of neuronal cell-type specification. Second, we will extend our characterization of Tlx3-expressing ESC-derived neurons. To determine whether these Tlx3expressing ESC-derived cells exhibit functional properties similar to those of endogenous SGNs and make synaptic contacts with their targets after neural induction, we will evaluate (A) glutamate release, (B) synaptic transmission, (C) target innervation and (D) electrophysiological properties of Tlx3-expressing vs. non-expressing control ESC-derived neurons. These studies will reveal the extent of functional and synaptic maturation of these transgenic neurons and provide insights into further improvements of directed differentiation of ESC-derived neurons. Third, we plan to further refine our surgical/MRI techniques and record auditory brain stem responses (ABRs) when a considerable number of engrafted cells expressing neural antigens are detected in the modiolas of the gerbil cochlea. These in vivo experiments will be instrumental in establishing translational strategies for successful cell-based therapy in the inner ear.

#### 10. Conclusion

The present results reveal that Tlx3 confers ESCs undergoing neural differentiation with a glutamatergic neurotransmitter phenotype, which is accompanied by establishment of

proper synaptic assembly and axon outgrowth. Furthermore, Tlx3-expressing ESCs can migrate towards degenerating SGNs in the inner ear of host animals and these engrafted stem cells can be readily visualized by MRI. These results suggest that Tlx3-expressing ESCs can be used to replace damaged SGNs, which cause irreversible hearing loss in humans. Technical issues related to surgical approaches for safe and efficient transplantation of stem cells in the human cochlea, as well as, non-invasive monitoring of stem cell engraftment in the cochlea are discussed.

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes recent advances in the generation of tissue specific cell types for regenerative applications, as well as the obstacles that need to be overcome in order to recognize the potential of these cells.

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