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STEM CELL *IN-VITRO* STRATEGIES FOR THE INDUCTION OF SENSORY NEURONS FOR INNER EAR CELL THERAPY

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Stockholm 2016

Cover photo: Colony of *in vitro* cultured of bi-polar BRN3a+/TUJ1+ neurons derived from HS181 human embryonic stem cells. BRN3a (green), TUJ1 (purple) and DAPI (blue).

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STEM CELL *IN-VITRO* STRATEGIES FOR THE INDUCTION OF SENSORY NEURONS FOR INNER EAR CELL THERAPY

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Stockholm 2016

To my beloved family

ABSTRACT

The cochlea, a part of the auditory system, is a complex structure consisting of several different types of cells including hair cells and spiral ganglion neurons. In mammals, the regenerative potential of hair cells and spiral ganglion neurons is lost soon after the birth and damage to any of these cells causes sensorineural hearing loss. Cochlear implant is presently the only available treatment for sensorineuronal hearing loss, bypassing the malfunctioning hair cells and directly simulating the spiral ganglion neurons. Cochlear implant functionality depends on the activation of remaining spiral ganglion neurons. A possibility for a stem cell approach replacing auditory neurons in the cochlea has attracted great interest. The aim of this thesis was to identify methods to induce progenitors for sensory neurons from human pluripotent stem cells, with the prospective use in a cell therapy for the inner ear.

Paper I focused on differentiation of sensory neural types from precursors at different stages of neural rosette formation derived from human embryonic stem cells (day 4, 7, or 11-rosettes). Neural and sensory neural phenotype differentiation was examined by immunocytochemistry. Cells positive for *NESTIN* and *TUJ1* were present at all tested time points, indicating presence of the earliest stages of neural differentiation. Starting from the 11-days neural rosettes resulted in decreased potential for neural differentiation, compared to starting from the 4-and 7-days protocols which more effectively could be driven into differentiation towards cells with sensory neuron marker phenotype.

In paper II an approach using the SB431542 small molecule for blocking of the TGFß/Activin/Nodal signaling pathway was evaluated for effects on sensory neural differentiation, examined by gene expression and immunocytochemistry. Blocking of this pathway significantly facilitated the induction of markers present on sensory neurons; *NGN1*, *NEUROD1* and *BRN3A*. Consecutive treatment with bFGF further enhanced the expression of these markers, as well as an increase in expression of *GATA3*. This protocol also enhanced the presence of colonies positive for *TUJ1* as well as *BRN3A* or *ISL1*. Notably, addition of a third culture step, with exposure to the neurotrophic factors BDNF + NT3 resulted in > 90% presence of colonies with a phenotype compatible with sensory neurons.

In paper III, we evaluated the capacity of additional small molecules (Isoxazole-9 and Metformin) to induced sensory neurons, starting from long-term neuroepithelial stem (lt-NES) cells. Here, Isoxazole 9 (ISX9), but not Metformin or SB431542, significantly induced sensory neural genes (*GATA3*, *BRN3A* and *PERIPHERIN*) after a 4-day treatment. Exposure to ISX9 significantly increased the number of *BRN3A/TUJ1* positive cells. Further treatment with BDNF7NT3 resulted in increased levels of cells with *GATA3* expression and also *BRN3A/TUJ1* positive cells in ISX9-exposed populations.

In conclusion, this thesis has explored and demonstrated cultural conditions for *in vitro* induction of sensory neural phenotypes, starting from *in vitro* cultured human pluripotent stem cells or lt-NES cells. The presented approaches may provide appropriate strategies to develop an effective treatment for sensory neural hearing loss.

LIST OF SCIENTIFIC PAPERS

I. **Rouknuddin Qasim Ali**, Yen-Fu Cheng, Beata Kostyszyn, Lars Ährlund-Richter, Mats Ulfendahl.
Differentiation of human embryonic stem cells towards a sensory neural phenotype.
Advances in stem cells. Vol. 2014. Article ID 252491, Page 1-12, (2014).

II. **Rouknuddin Qasim Ali**, Lars Ährlund-Richter, Mats Ulfendahl.
The TGFß/Activin/Nodal pathway inhibitor SB431542 is a potent inducer of sensory neural markers when applied to neural differentiation from HS181 embryonic stem cells.
Manuscript.

III. **Rouknuddin Qasim Ali**, Evelina Blomberg, Anna Falk, Lars Ährlund-Richter, Mats Ulfendahl.
Induction of sensory neurons from neuroepithelial stem cells by the ISX9 small molecule.
American journal of stem cells. Accepted for publication (2015).

CONTENTS

LIST OF ABBREVIATIONS

bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
BMP4	bone morphogenic protein 4
BDNF	brain derived neurotrophic factor
CNS	central nervous system
CNF	ciliary neurotrophic factor
CI	cochlear implant
CVG	cochleovestibular ganglion
DRG	dorsal root ganglion
DPHS	Dulbecco's phosphate buffer saline
EB	embryoid body
ES	embryonic stem
EGF	epidermal growth factor
FGF	fibroblast growth factor
FGF10	fibroblast growth factor 10
FGF3	fibroblast growth factor 3
GDNF	glia cells derived neruotrophic factor
GSK3	glycogen synthase kinase 3
hESC	human embryonic stem cells
iPS	induced pluripoten stem cell
IHC	inner hair cells
IGF-1	insulin like growth factor-1
ISX9	isoxazole 9
lt-NES	long-term neuroepithelial stem
NGF	nerve growth factor
NCSC	neural crest stem cell
NSC	neural stem cells
NGN1	neurogenin 1
NT-3	neurotrophin-3
OCH	outer hair cells
PNS	peripheral nervous system
PSC	pluripotent stem cells
SB	SB431542
SG	spiral ganglion
SGNs	spiral ganglion neurons
SIDA	stromal-derived inducing activity
TrkB	tyrosine kinase B

1 INTRODUCTION

1.1 HEARING LOSS

Hearing impairment is one of the most common disabilities. It is associated with poor quality of life of the affected individuals, which can only be partly restored using traditional hearing aids. Therefore, it is of great importance for an individual to maintain intact hearing throughout life. According to WHO, it is estimated that 360 million people have disabling hearing loss. Hearing impairment is broadly classified as conductive or sensorineural hearing loss, based on the affect part of the ear (Figure 1).

Conductive hearing loss is a condition when sound is not conducted efficiently to the inner ear through the outer or/and middle ear. It involves reduction of the loudness of sound or ability to hear faint sounds. Conductive hearing loss is usually a result of anatomical deformations of the outer or middle ear, diseases of the outer or middle ear like tumors, ear infections, earwax or damage to the tympanic membrane and the presence of fluid in the middle ear. Conductive impairment can be treated either by hearing aids to normalize the sound range, or antibiotics or surgery to overcome defects in the outer or middle ear.

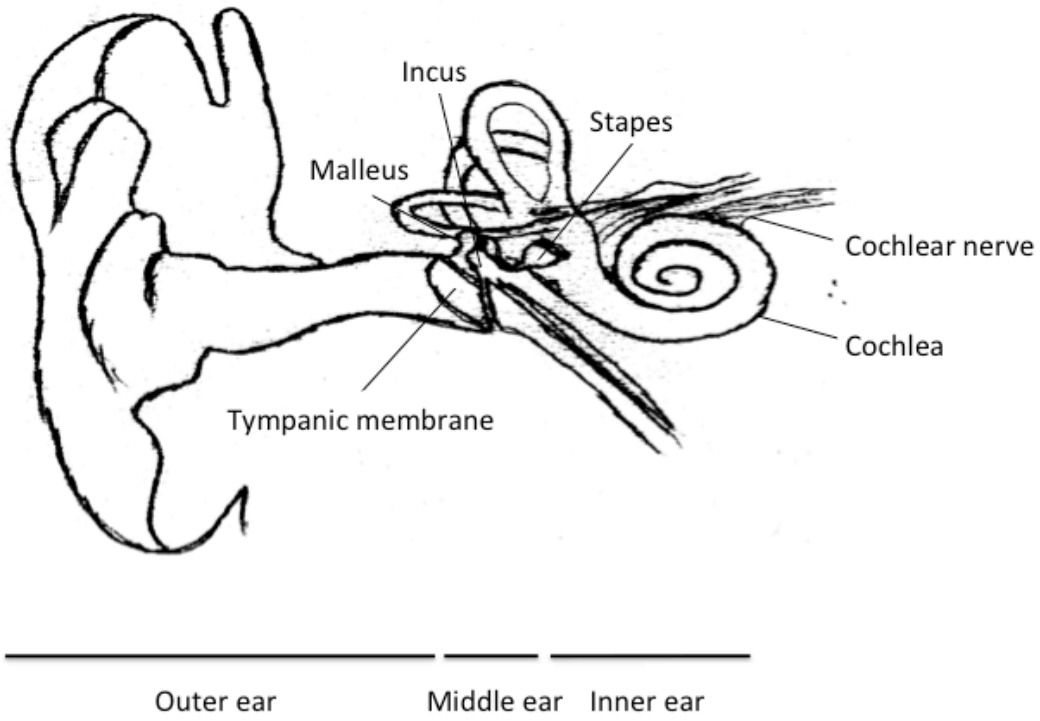

Figure 1: Anatomy of human ear. Modified from Noback, 2005.

Sensorineural hearing loss is the most common type of hearing disability. It is a condition caused by damage to cells in inner ear (cochlea) or the cochlear nerve, which connects the

cochlea to the brainstem. Sensorineural hearing loss, caused by malfunctioning hair cells or spiral ganglion neurons in the cochlea, involves the lack of ability to convert mechanical sound waves to electrical signals, or the electrical signals to activate cell in the auditory brainstem. The loss of hair cells and/or spiral ganglion neurons is permanent in mammals. The most common cause is age-related changes, ototoxic drugs, noise exposure, genetic factors, infections or neuropathy. Unlike conductive hearing loss, treatment options are very limited in sensorineural hearing loss. Cochlear implants are currently the only available option to treat, at least partially, sensorineural hearing loss. The cochlear implant provides electrical stimulation that acts directly on the spiral ganglion cells, thus bypassing the damaged or lost hair cells.

1.1.1 Inner ear

The inner ear is fluid-filled structure located within the temporal bone of the skull. It is composed of the cochlea and the vestibular system, the sensory organs for hearing and balance, respectively. The two systems are separate but enclosed within the same bony capsule and sharing the same fluid system. The hearing organ is located in the cochlea in the inner ear. The cochlea is spiral-shaped structure divided into three parallel fluid-filled compartments: scala vestibulo, scala media (the cochlear duct) and scala tympani (Figure 2). The two outer compartments, scala tympani and scala vestibuli are filled with a fluid called perilymph. Scala vestibuli and scala tympani are interconnected though a small opening at the apex of the cochlea called helicotrema allowing free movement of perilymph. The middle compartment, scala media, is filled with endolymph and is separated from scala vestibuli by Reissner's membrane and from scala tympani by the basilar membrane. The hearing organ, the organ of Corti, rests on the basilar membrane. The organ of Corti consists of one row of inner hair cells (IHC) and three rows of outer hair cells (OHC) and different supporting cells. In one human cochlea, there are approximately 3,500 inner hair cells and 12,000 outer hair cells. The hair cells lie within a matrix of supporting cells on the basilar membrane. The stereocilia of the hair cells project into the overlaying tectorial membrane. Sound vibrations, entering into the cochlea through the oval window, creates pressure waves in the cochlear fluid that displace the basilar membrane, thus causing a relatively motion with respect to the tectorial membrane. The sensory hair cells detect the motion via their stereocilia. The hair cells are depolarized and following transmitter release and activation of the fibers of the spiral ganglion neurons, nerve impulses are generated.

1.1.2 Spiral ganglion neurons

The spiral ganglion (SG), a collection of the cells bodies of the auditory neurons, is located in a bony canal of the cochlea called Rosenthal's canal. The bipolar SG neurons extend processes towards the hair cells in the organ of Corti and auditory nuclei in the brain stem. There are two types of SG neurons, type I and type II, based on their different morphology, synaptic connection and function. Type I neurons, representing 90-95% of the neuronal population, are large and bipolar. They innervate the IHC in the organ of Corti. Their main function is to convey signaling from the IHC to the auditory brain stem. Type II neurons are small, bipolar or pseudomonopoler and represent 5-10% of the neuronal population. They innervate OHC in the organ of Corti. The exact function of type II neurons is not well established.

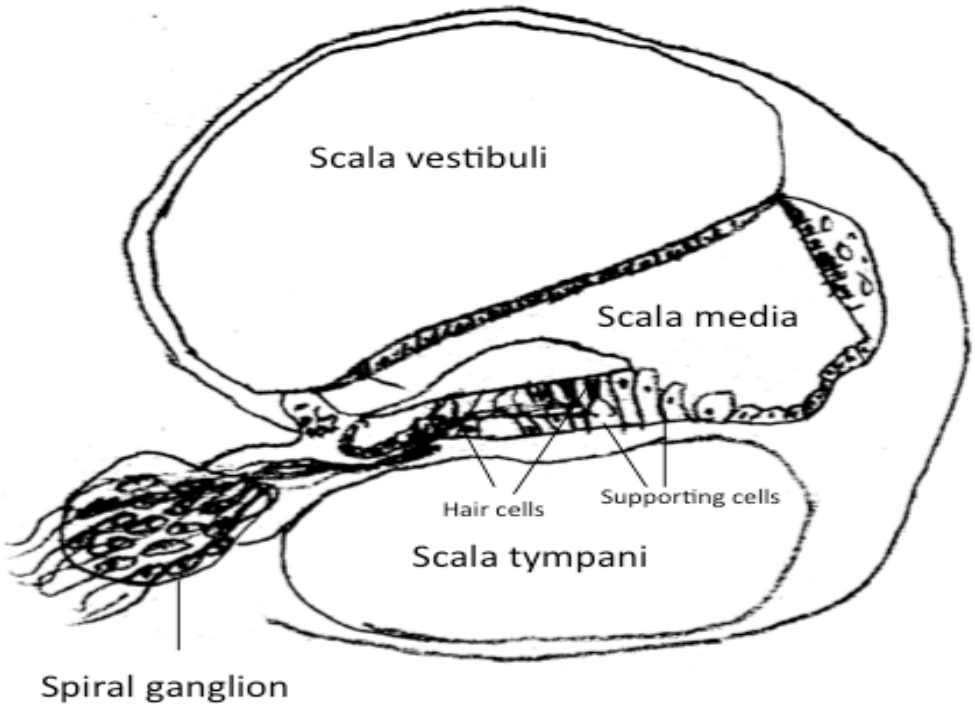

Figure 2: Cross section of the cochlea.

1.2 DEVELOPMENT OF SPIRAL GANGLION NEURONS

The development of the embryonic inner ear arises from a thickening of the ectoderm in the head region of the vertebrate called the otic placode. The otic placode develops from the non-neural ectoderm adjacent to the developing neural tube, which further invaginates to form the otocyst or otic vesicle. The pro-neurosensory domain of the otic vesicle gives rise to the neurons and sensory epithelial cells in the inner ear (Figure 3).

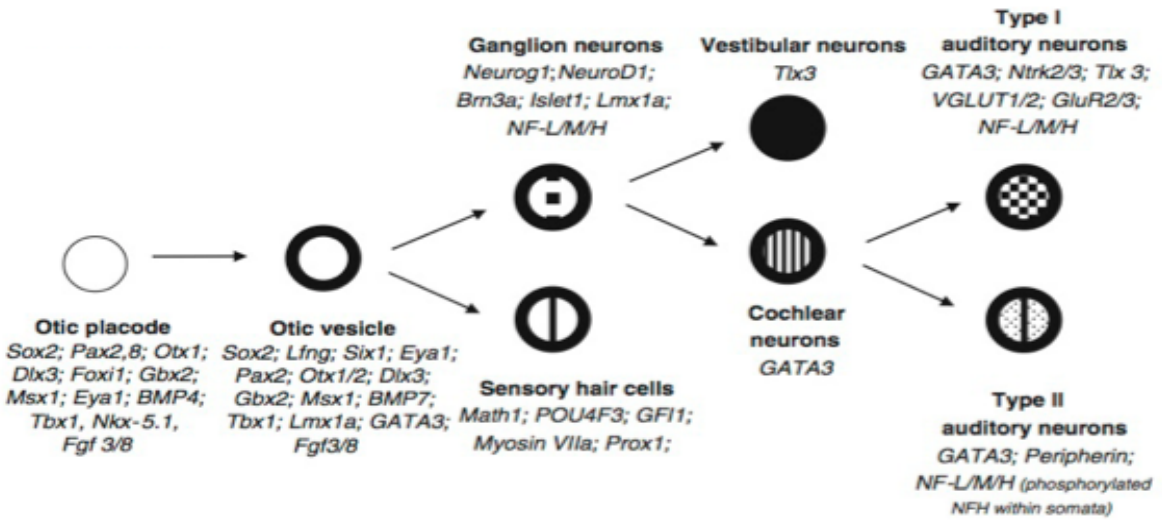

Figure 3: Development of auditory neurons. Modified from Gunewardene et al. 2012

During development, the fate of the spiral ganglion neurons and sensory hair cells is specified by the proneural basic helix-loop-helix (bHLH) transcription factor *Neurogenin 1* (*NGN1*) and the transcription factor *ATOH1,* also known as *Math1* (Ma et al., 1998, Fritzsch et al., 2010). Expression of *NGN1* is necessary for the generation of the spiral ganglion neurons in the inner ear. In the mouse, deletion of *NGN1* has been shown to result in a complete loss of spiral ganglion neurons (Ma et al., 1998, Ma et al., 2000). Specification of the neuroblasts takes place in otic vesicle by expression of *NGN1*, which in turn induces the expression of the neural differentiation bHLH transcription factor *NEUROD1* (Ma et al., 1998). Loss of *NGN1* has been shown to result in the absence of *NEUROD1* positive neuroblasts in the otic vesicles (Ma et al., 1998). Following the induction of *NEUROD1*, the neuroblasts start to delaminate from the otic vesicle and migrate out to from the cochleovestibular ganglion (CVG), which in turn further differentiates into spiral and vestibular ganglion neurons. *NEUROD1* has also been shown to be of importance for spiral ganglion neuron differentiation and survival (Liu et al., 2000, Kim et al., 2001). Deletion of *NEUROD1* results in the complete disappearance of CVG neurons after the delamination from the otic vesicles (Kim et al., 2001).

The LIM-HD gene *ISLET1* is restricted to cells in the neuronal lineage and is another important player in the development and differentiation of the proneurosenory domain. During sensory epithelium development, expression of *ISLET1* is induced in parallel to induction of *NGN1* and *NEUROD1,* but downregulated in the entire cochlear epithelium during sensory epithelium differentiation (Radde-Gallwitz et al., 2004). Thus, *ISLET1* is an early marker for sensory epithelium and later marker for neural development.

Parallel to the otic development, the POU domain transcription factor *BRN3A* is expressed in facial-stato-acoustic ganglions prior to the differentiation of sensory neurons and otocyst innervation (Huang et al., 2001). *BRN3A* expression is important for proper growth and migration of neuroblasts in the innear ear and also involved in targeting innervation and axon guidance by the spiral and vestibular ganglion neurons (Huang et al., 2001). Loss of *BRN3A* results in a significant reduction and defective migration of spiral ganglion neurons (McEvilly et al., 1996).

The zinc finger transcription factor *GATA3* is another important transcription factor in spiral ganglion neurons. *GATA3* is one of the earliest genes expressed in the spiral ganglion neuroblasts as well as in post-mitotic spiral ganglion neurons (Lawoko-Kerali et al., 2004, Karis et al., 2001, Duncan et al., 2011, Jones and Warchol, 2009). *GATA3* is expressed throughout the early otic placode and the early otic vesicle, and with time limited to the future proneurosensory domain (Lillevali et al., 2006). Mouse studies have shown that *GATA3* is co-expressed with *NEUROD1* throughout during initial proneurosensory domain but downregulated or even absent in the delaminating *NEUROD1* positive neuroblast and the early CVG neurons (Lawoko-Kerali et al., 2004). In chicken, *GATA3* expression is absent in all *NEUROD1* positive neuroblasts in the otic vesicle (Jones and Warchol, 2009). In contrast, the post-mitotic spiral ganglion neurons in chicken and mouse express *GATA3* (Jones and Warchol, 2009, Lawoko-Kerali et al., 2004). Conditional knockout mice have shown that *GATA3* is important for the spiral ganglion survival and a proper axon projection in the inner ear at later stages of development (Luo et al., 2013).

1.3 STRATEGIES FOR RESTORING SENSORINEURAL HEARING LOSS

Degeneration of sensory hair cells and/or spiral ganglion neurons in the cochlea results in a sensorineural hearing loss. In mammals, neither of these cells can regenerate. Sensory hair cell regeneration has been a central target for therapeutic strategies for sensorineural hearing loss because of their crucial importance in conversion of sound stimuli to neural signals. An alternative approach is used by the cochlear implant, in which hair cell function is bypassed by directly stimulating spiral ganglion neurons. However, following loss of hair cells, secondary degeneration of spiral ganglions follows due to a lack of trophic support. Therefore, loss of spiral ganglion neurons challenges the clinical benefits of cochlear implants, and for this reason also spiral ganglion regeneration has been an important target for therapeutic strategies. Besides hair cells and spiral ganglion neurons, there are other

components in cochlea, which lack a regenerative capacity, e.g. the stria vascularis and spiral ligament at the later wall of cochlea.

1.3.1 Cochlear implant

A cochlear implant (CI) is an electronic device designed to stimulate SG neurons in the cochlea of patients with sensorineural hearing loss. The CI bypasses the affected hair cells and electrically stimulates the SG neurons. To date, more than 300,000 patients have received cochlear implants. This estimation represents small number of individuals who may potentially benefit from the implants. The CI has improved the quality of life of thousands of people since it was taken into clinical use. Based on the results reported by Djourno and Eyries, William House and John Doyle implanted the first CI in an adult in 1961. They inserted a gold wire electrode into the scala tympani in two deaf patients and electrical stimulation of this single-channel electrode provided some auditory sensation in these patients. In 1984, Graeme Clark developed a multichannel implant with bipolar stimuli. After that, the American Food and Drug Administration approved the use of CI in 1985.

The cochlear implant device is composed of an external and an internal part. The external part includes the microphone, a cable and a sound processor. The microphone gathers sound from the environment, which is then analyzed by the sound processor and converted into electrical current. The internal part is surgically implanted under the skin behind the ear auricle and is comprised of a receiver/stimulator, a magnet, and a bundle of fine wire constituting the electrode array. The electrical current is delivered across the skin by radio waves to a receiver/stimulator, which convert them into electrical impulses. The current is transferred to the electrode array, which is implanted in the cochlea and activates the neurons connected to the cochlea resulting in the perception of sound. The whole concept of CI is to bypass the malfunctioning or absent hair cells and directly stimulate the remaining spiral ganglion neurons in the cochlea.

1.3.2 Growth factor base survival

Neurotrophic factors are secreted proteins, which play important roles in neuronal survival, neurite outgrowth and neuronal cell differentiation. Neurotrophins are synthesized by the targeted tissue or neurons at considerable distance from the neuronal cell body (Thoenen, 1995). These factors are divided into different families; e.g., neurotrophins (brain derived neurotrophic factor, neurotrophin-3, nerve growth factor), growth factor family (e.g.,

fibroblast growth factor), gila cells derived neurotrophic factor family and the neurokine family (e.g., ciliary neurotrophic factor).

Since the discovery of neurotrophic factors there has been expectations of clinical applications for effective therapies for degenerative diseases. Neurotrophic factors are important in inner ear development and innervation during development (Fritzsch et al., 1999). In addition they play important roles in survival and maintenance of spiral ganglion neurons in adulthood. Treatments with neurotrophic factors protect and prevent degeneration of cells in cochlea, triggered by drugs, noise or age. The two most commonly applied neurotrophic factors for treatment of the auditory system are the brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). In situ hybridization studies have shown that BDNF and NT-3 are synthesized by sensory hair cells and that their targets, receptor tyrosine kinase B and C (TrkB and TrkC), are synthesized in the ganglion neurons during development (Pirvola et al., 1994, Pirvola et al., 1992, Schecterson and Bothwell, 1994, Wheeler et al., 1994, Ylikoski et al., 1993). Deletion of either BDNF, NT3, TrkB or TrkC results in loss of ganglion neurons in the cochlea (Minichiello et al., 1995, Fritzsch et al., 1997, Farinas et al., 1994, Ernfors et al., 1995). This illustrates that the neurotrophins are essential for the survival of sensory neurons during development. In addition, BDNF and NT-3 dramatically increase the survival of neurons in the adult rat spiral ganglion (SGNs).

Degeneration of adult SGNs, normally seen after loss of hair cells in the cochlea, can be prevented by the exogenous neurotrophic factor NT-3 (Staecker et al., 1996, Ernfors et al., 1996). Infusion of NT-3 in ototoxically deafened animals resulted in significant survival of SGN after 2 (Miller et al., 1997), 4 (Gillespie et al., 2003), or 8 weeks of treatment (Staecker et al., 1996). Shepherd et al. showed that the infusion of intracochlear BDNF with patterned electrical stimulation resulted in significant increase in survival of SGN in deaf animals compared to treatment of BDNF treatment alone (Shepherd et al., 2005). A combined infusion of BDNF and CNTF resulted in not only an increased survival of spiral ganglion neurons in the deafened animals but also a significantly enhanced electrical responsiveness (Shinohara et al., 2002). Glia cell derived neurotrophic factor (GDNF) has been shown to provide protective effects on SGN survival and function (e.g. (Fransson et al., 2010, Maruyama et al., 2008)). A combination of GDNF with antioxidants significantly improved the electrically evoked auditory brain stem response (Maruyama et al., 2008). A positive intervention effect of intracochlear neurotrophic factor treatment has been shown for up to 6 weeks post deafening (Yamagata et al., 2004).

A major challenge with neurotrophic treatment is the delivery method to the cochlea. Different methods have been used in different studies e.g., mini osmotic pumps, carrier based delivery, or encapsulated cells (Pettingill et al., 2011, Noushi et al., 2005, Havenith et al., 2011, Agterberg et al., 2009, Shinohara et al., 2002). Although positive effects have been observed, these methods need further development. For example, commercially available mini-osmotic pumps have a limited life span, up to 4 weeks. Another alternative is to use an implantable pump that can be used for 8 months (Praetorius et al., 2001) but these pumps need refilling and with that an increased risk of infections in the inner ear. Another alternative is to deliver neurotrophic factors by local administration via the middle ear, e.g. using geloform or alginate (Noushi et al., 2005, Havenith et al., 2011). However, the effect of degradation of these carriers needs to be elucidated. An interesting approach could be to implant cells producing neurotrophic factors in order to provide intracochlear treatment for longer periods of time. For safety, these cells could be inserted encapsulated in a material allowing release into the cochlea. Using this technique with encapsulated BDNF overexpressing Schwann cells in animal experiments an improved survival of SGN has been shown (Pettingill et al., 2011). However, long term effect of these encapsulated cells need further research.

1.3.3 Gene manipulation based regeneration

Applying a gene therapy approach to induce upregulation or downregulation of specific genes is another option for inner ear treatment. For effective gene manipulation the gene in question must be delivered appropriately to the target cells. One method is to use viral vectors for the gene delivery. Till now, large numbers of vectors have been tested, from different families of viruses e.g., adenovirus, adeno-associated virus or lentivirus. Non-viral vectors, like plasmids or lipid-covered packages are also alternatives for gene delivery. Although they have advantages over viral vectors by reducing cell toxicity, these methods have shown low efficiency in terms of transduction of the gene and gene expression patterns. Electroporation has also been effective for gene delivery in cochlear explants (Zheng and Gao, 2000).

The *ATOH1* transcription factor was the first gene reported for specification of hair cells in the proneurosensory domain during development (for review (Fritzsch et al., 2010)). Targeted deletions of *ATOH1* showed complete loss of hair cells in the mouse inner ear (Bermingham et al., 1999). Based on these findings, overexpression of *ATOH1* gene was done *in vitro* by means of electroporation in the postnatal mammalian cochlea (Zheng and Gao, 2000) and *in vivo* by adenovirus vector transfer (Kawamoto et al., 2003, Izumikawa et al., 2005). Cochlear

explant cultures revealed induction of new hair cells in the epithelia ridge suggesting that epithelial cells in this region can transdifferentiate into hair cells (Zheng and Gao, 2000). Similarly, *in vivo* overexpression of *ATOH1* resulted in the differentiation of new hair cells. Izumikawa et al. showed that new hair cells were connecting to the basal membrane suggesting a transdifferentiation from supporting cells (Izumikawa et al., 2005). In addition to new hair cells regeneration, this study also showed an improved hearing threshold in deaf mice.

Down-regulation of cell cycle inhibitors by gene manipulation is another approach for new hair cells regeneration. Cell cycle regulators play important roles in the maintenance of the post mitotic cell state of supporting cells in the cochlea. During development, *p27Kip1*, a cyclin-depended kinase inhibitor, is upregulated in sensory epithelium and remains high in supporting cells until adulthood (Chen and Segil, 1999). Down-regulation of *p27Kip1* resulted in production of supporting cells as well as hair cells in the postnatal and adult organ of Corti (Lowenheim et al., 1999). A potential drawback with this approach is tumorigenesis due to uncontrollable cell proliferation in organ of Corti.

Gene manipulation could be an effective approach for survival of spiral ganglion neurons in the cochlea. As it has been discussed earlier, neurotrophins are important for SGNs survival after loss of hair cells. Several gene therapy studies have reported introduction of neurotrophins to the mammalian inner ear (Rejali et al., 2007, Okano et al., 2006, Nakaizumi et al., 2004). Adenovirus based delivery of mouse BDNF into scala tympani of the guinea pig inner ear showed robust survival of SGNs compared to control (Nakaizumi et al., 2004). In another study, a protective effect of BDNF in SGNs survival was observed when cochlear implant electrodes were coated with BDNF secreting fibroblasts and inserted into scala tympani of deafened guinea pigs (Rejali et al., 2007). Wise et al studied prevention of spiral ganglion neuron degeneration as a result of aminoglycoside ototoxicity from adeno-associated virus (Wise et al., 2011). Animals transfected with BDNF and NT3 showed a significant increase in survival of SGNs in the cochlea of 4 weeks old deafened animals. However, in prolonged observations (up to 8 weeks) the effect was diminished. The results illustrate the potential of this therapy but that the long-term benefits may be limited.

1.3.4 Stem cell based cell replacement

Cell replacement therapy has attracted great interest in regenerative medicine. The approach has also been proposed for the replacement or restoration of damaged hair cell and/or SGNs.

Stem cells are defined as having ability to self-renew and to differentiate into several cell types. Different types of stem cells have been used in differentiation and transplantation studies for hair cells and/or SGNs and some of them are discussed below.

1.3.4.1 Auditory stem/progenitor cells

Tissue specific stem cells are regarded as the best choice for cell therapy because of reduced risk for tumour development. Although the inner ear has limited ability to regenerate it is evident that stem cells are present. Several research groups have reported stem or progenitor cells in the animal cochlea, however their ability to regenerate is limited and the majority of these cells can differentiate only into a given phenotype such as hair cells (Zhai et al., 2005, Savary et al., 2007, Oshima et al., 2007, Malgrange et al., 2002) or SGNs (Rask-Andersen et al., 2005).

Stem cells has been isolated in 9-11 weeks old human foetal cochlea and grown in culture in using medium containing bFGF, EGF, and IGF-1 (Chen et al., 2007, Chen et al., 2009). These stem cells express otic progenitor markers *SOX2* and *GATA3* as well as stem cell markers *OCT-4*, *NANOG* and *REX-1*. Human feotal audtitory stem cells can differentiate into hair cells when cultured in hair cell conditioned medium, and have been shown to express the hair cells markers *ATOH1*, *BRN3C* and *MYO7A*. When cultured in neural conditioned medium, they expressed SGNs markers *NGN1*, *BRN3A*, *TUJ1* and *NEUROFILAMENT 200 (Chen et al., 2009)*. These human cells are promising candidates for future hair cell therapies, however, awaiting confirming results from transplantation in animals.

1.3.4.2 Neural stem cells and neural crest stem cells

Neural stem cells (NSC) have been reported to self-renew and differentiate into different types such as neurons, astrocytes and oligodendrocytes (Gage, 2000, Clarke et al., 2000). Previously, NSC have also been used for an inner ear cell therapy approach (Parker et al., 2007, Hu et al., 2005b, Fu et al., 2009). Adult neural stem cells, transduced with *neurogenin 2*, have been transplanted into normal and deafened animal cochlea (Hu et al., 2005b). This study showed poor survival of NSC in all groups even in a neurogenin-transduced group. Parker et al. reported interesting result when they transplanted an NSC cell line derived from immortalized male murine foetal cerebellum, from sound damaged animals (Parker et al., 2007). NSC were found with characteristics of both cell of neural tissue and organ of Corti.

Neural crest cells arise just after the closure of neural tube between interface of neural tube and dorsal ectoderm. Neural crest cells undergo an epithelial to mesenchymal transition and delaminate from the neural tube and differentiate into different types of cells including peripheral sensory neurons. Although it has been suggested that all cell types of the inner ear are derived from the otic placodal cells, it has been shown that neural crest cells also contribute as progenitors of glial Schwann cells of the cochleovestibular nerve and in small numbers in vestibular ganglions as well as melanocytes in the stria vascularis of cochlea (Hilding and Ginzberg, 1977).

Recently, molecular comparisons of sensory neuron development from neural crest and otic placode have been made (Huisman and Rivolta, 2012). It was proposed that neural crest and otic placode share similar molecular events during the specification and differentiation of sensory neurons. Hence, both sources are important candidates for cell therapy approaches for the inner ear.

1.4 PLURIPOTENT STEM CELLS

Pluripotent stem cells (PSC) have the capacity to sustain an undifferentiated state, to self renew for long time as well differentiate (Thomson et al., 1998) (Figure 4). Human diploid bonafide embryonic stem cells (hESC) and induced pluripotent stem (iPS) cells are classified as pluripotent.

The first embryonic stem cell lines were developed from inner cell mass of a mouse embryo and reported in 1981 by Evans and Kaufman (Evans and Kaufman, 1981), This was followed by the report in 1998 of the first long term culture of human embryonic stem cells by Thomson (Thomson et al., 1998). Takahashi and Yamanaka 2006 reported the first mouse iPS cell line in 2006 (Takahashi and Yamanaka, 2006) and later also human iPS cell lines (Takahashi et al., 2007).

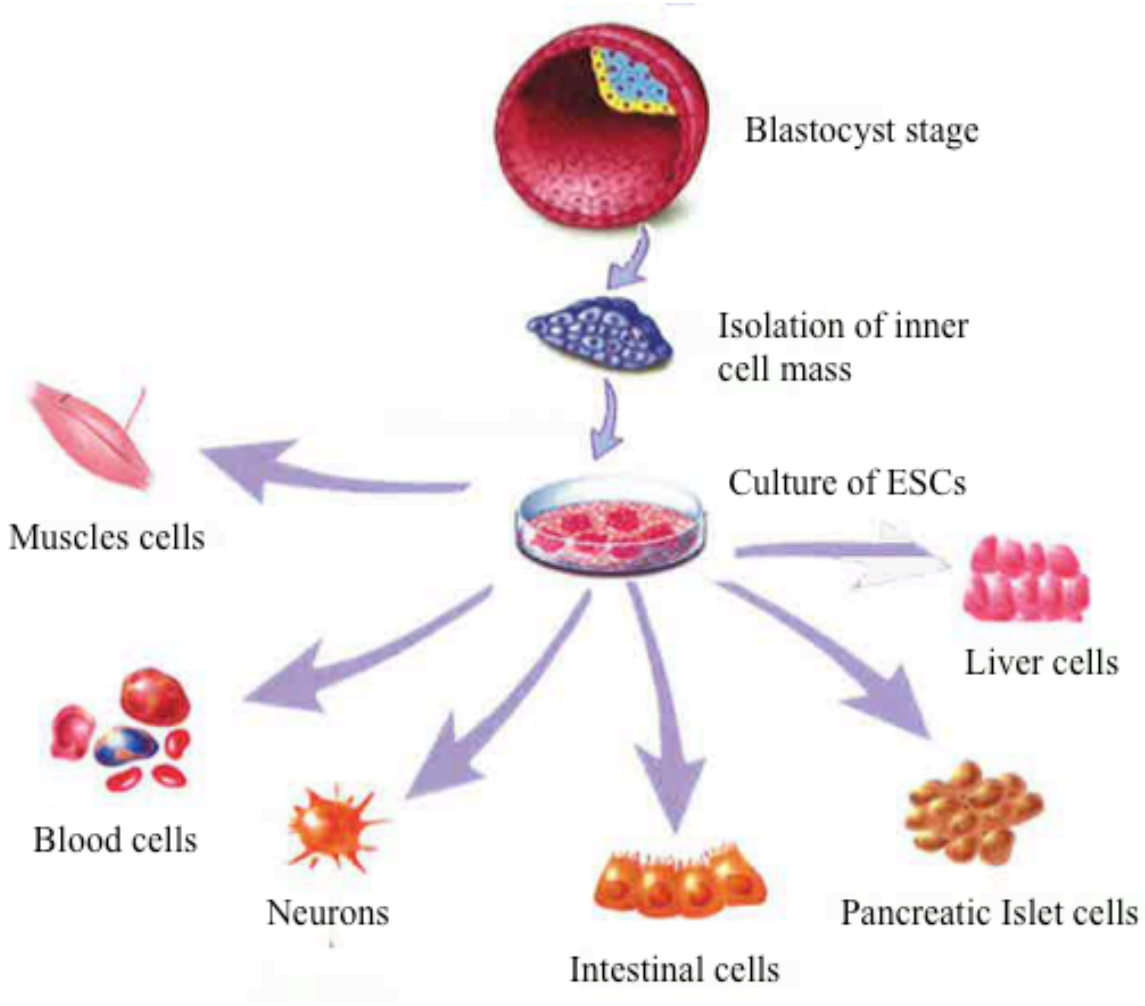

Figure 4: Differentiation of stem cell into different cell types. Modified with permission from © 2011 Meregalli M, Farini A, Torrente Y. Published in Stem cells in Clinic and Research, Chapter 17 under CC BY-NC-SA 3.0 license. Available from: http://dx.doi.org/10.5772/24013, http://dx.doi.org/10.5772/23660

During the last decade, ES cells have been studied to replace hair cells and spiral ganglion neurons for inner ear regeneration. In one early study mouse ES cells were tagged with green fluorescent protein and delivered into the cochlea (Hildebrand et al., 2005). Survival of ES cells was observed at the site of transplantation as well as in the stria vascularis, endolymph fluid of scala media, and in the spiral ligament for up till nine weeks. However, cells failed to differentiate. In another study, transplanted cells showed extensive migration in cochlea and were observed not only in the cochlear nerve but also found present in the Rosenthal's canal and the scala media (Sekiya et al., 2006). These cells showed tropism mostly toward the damaged cochlear nerve compared to undamaged nerve. However, neuronal differentiation was seen more in the undamaged nerve. In a separate study, ES cells were transplanted together with dorsal root ganglion tissue into the scala tympani and revealed migration towards the Rosenthal's canal and close to the cochlear spiral ganglion neurons (Hu et al., 2004a). Reyes et al. performed experiment with mouse ES cells conditionally expressing

neurogenin-1. After transplantation, neurogennin-1 was induced by doxycycline and continuous supplement of GDNF was provided for three weeks. This resulted in presence of transplanted cells in several areas of cochlea, with surviving cells with more neuronal like appearance and expressing the neuronal marker *TUJ1* and the vesicular glutamatergic neuron marker *vGLUT* (Reyes et al., 2008). Although these studies have shown survival and differentiation of embryonic stem cells, the high risk of teratoma formation is evident. Due to this reason, it is important to differentiate ES cells into appropriate cell lineage before the cell implantation. In next section, different methods to induce neural differentiation from ES cells are discussed.

1.4.1 Methods to induce neural differentiation in pluripotent stem cells

Due to a very limited capacity of regeneration of inner ear hair cells and/or spiral ganglion neurons, treatment of sensorineural hearing leoss is one of the major challenges in field of medicine. Clinically, cochlear implants have been used to treat sensorinerual hearing loss for about three decades. As the spiral ganglion neurons are the target for the electrical stimulation by the CI, several studies have been conducted to improve survival of SGN cells, e.g. using different growth factors. It has also been attempted to replace SGN cells by exogenous cell types transplanted into the cochlea. However, the outcome has so far not been convincing, primarily due to a low survival rate of these exogenous cells. However, the development of iPS cells may make it possible to treat with patient their own specific cell types to reduce the risk of an immune and subsequent tissue rejection It is important to differentiate pluripotent stem cells into appropriate candidate of neural progenitors that can function like SGN. Different methods have been used to induce neural differentiation from pluripotent stem cells.

1.4.1.1 Embryoid body (EB) formation and monolayer cultures

The formation of embryoid body form ES cell is a common method for producing different cell lineages for further applications. The simple method of EB formation is achieved by culturing ES aggregated in suspension without any growth factors on non-adherent dishes. This EB formation method results in multilayer 3D structures containing cells from all three germ layers: ectoderm, endoderm and mesoderm (Schuldiner et al., 2001). Previously, many groups have used this method to induce neural differentiation from ES cells (Zhou et al., 2008, Schuldiner et al., 2001, Reubinoff et al., 2000, Mohr et al., 2010). This is a straightforward method but have some drawbacks, including long-term cell culture, development of cyst and required high concentration of extrinsic factors to penetrate deep inside the EBs (Wichterle et al., 2002, Schuldiner et al., 2001, Carpenter et al., 2001, Bain et

al., 1995). Embryoid bodies can be formed by the hanging drop method (Cerdan et al., 2007), floating small aggregates of ES cells (Schuldiner et al., 2001), and multiwall plates (Koike et al., 2005, Mohr et al., 2010). Another method of neural differentiation is achieved by prolonging culturing of ES cells as monolayer without splitting for 3 to 4 weeks (Reubinoff et al., 2000, Shin et al., 2006). This method permitted ES cell to grow in two dimension and ES colonies pile up to each other and start to differentiate. A wide range of cell types have been observed in these flat cultures, including ectodermal neuronal cells, mesodermal muscle and endodermal organ tissue types (Reubinoff et al., 2000). These two methods can be combined to induce neuronal differentiation (Zhang et al., 2001, Ali et al., 2014). First EB can be formed and allowed to attach on coated plates to form a monolayer culture.

1.4.1.2 Stromal-derived inducing activity (SIDA)

Co-culture techniques with stromal cells have provided efficient method to promote neuronal differentiation. The first reported methods used PA6 stromal cell line (Kawasaki et al., 2000), isolated from skull bone marrow (Kodama et al., 1986), to induce neural differentiation from mouse ES cells. Another cell line MS5 from stromal feeder cells has also been successfully used to promote neuronal differentiation of mouse and human ES cells (Perrier et al., 2004, Barberi et al., 2003). A variety of neuronal phenotypes have been generated by using SIDA methods e.g., midbrain dopaminergic neurons (Zeng et al., 2004), peripheral sensory neurons (Brokhman et al., 2008), neural crest cells (Pomp et al., 2005, Lee et al., 2007a) and motor neurons (Lee et al., 2007b). The exact mechanism of SIDA on inducing neuronal differentiation is unknown. Several studies have reported that fixed PA6 cells also induced neuronal differentiation when co-cultured with ES cells, indicating that differentiation was induced by interaction with membrane bound molecules (Vazin et al., 2008, Kawasaki et al., 2000). However, others have showed condition medium obtained from PA6 cells was enough to induce neuronal differentiation, suggesting the involvement of secreted factors (Hayashi et al., 2008, Vazin et al., 2008, Schwartz et al., 2005). This method is also an efficient method for neuronal differentiation but it may introduce unknown factors thus limiting it for clinical use.

1.4.1.3 Growth factors and media supplements

In the beginning, neural differentiation was considered as a default pathway, suggested by different animal studies (reviewed in (Stern, 2006)). Different growth factors have shown to be important for neuronal differentiation such as bFGF, Noggin, retinoic acid. Ying et al. cultured ES cells as a monolayer in serum free condition and showed that bFGF is crucial for

proper neuronal differentiation (Ying et al., 2003). Interestingly, mouse ES cells were differentiated into primitive neural stem cells fate in the absence of exogenous or paracrine bFGF but under this conditions, cells were unable to survive and proliferate further without bFGF (Smukler et al., 2006). These studies show the dispensable role of bFGF during initial steps but that it later is essential for cell survival, proliferation and differentiation. bFGF is also used to isolate and maintain neural precursors derived from ES cells (Elkabetz et al., 2008, Dhara et al., 2008) as well as to generate neural differentiation (Benzing et al., 2006, Axell et al., 2009). Noggin, a BMP inhibitor, is another protein that has been used by different groups to induce neural differentiation of ES cells grown adherently (Pera et al., 2004, Gerrard et al., 2005, Baharvand et al., 2007), co-cultured with MS5 stromal cells (Elkabetz et al., 2008, Lee et al., 2007b), or as EB in suspension (Itsykson et al., 2005). Besides bFGF and noggin, retinoic acid is also most commonly used factor to induce neural differentiation. Different concentration of retinoic acid results in wide range of neuronal fate (Baharvand et al., 2007, Carpenter et al., 2001, Zhang et al., 2001, Erceg et al., 2008) at various stages of differentiation (Carpenter et al., 2001, Schuldiner et al., 2001).

Media supplements have also impact on neural differentiation when differentiation is done on serum free conditions. Two most common supplements used as media supplements are N2 and B27. An N2 supplement is used in most neuronal differentiation protocol and contains five components, transferrin, insulin, putrescine, progesterone and selenite. These components are shown to induce neural differentiation (Savaskan et al., 2003, Cai et al., 2002, Aizenman and de Vellis, 1987). On the other hand, B27 supplement contains the same five components as N2 and some additional components, e.g. vitamins, hormones etc., and used in protocols for neuronal differentiation of ES cells (Gerrard et al., 2005, Itsykson et al., 2005, Nat et al., 2007). The exact composition of B27 has not been disclosed.

1.4.1.4 Small molecules

Chemical approaches have been particularly useful for direct conversion of stem cell into any specific cell lineages and maintenance of stem cell pluripotency (detail reviewed in (Li et al., 2013, Xu et al., 2013)). Small molecules are very useful in differentiation of stem cells. They have a number of advantages, e.g. high target specificity, ease of use, and the effect can be tuned by varying concentration and combinations and can modulate their effect at any time. Small molecules are widely using in neural induction protocol in recent times. Studies have shown that the SB431542 molecule, inhibitor of Activin/Nodal signaling pathway by blocking phosphorylation of ALK4, 5, and 7 receptors, can induce neuronal differentiation in

EB based or adherent based differentiation protocol (Patani et al., 2009, Smith et al., 2008). Besides SB431542, some other small molecules such as LND193189 (inhibitor of type1 receptor ALK2/3), CHIR99021 (GSK3β inhibitor), dorsomorphin (inhibitor of BMP type 1 receptors ALK2/3/6) have been shown to promote neuronal induction either alone or in combination with other small molecules (Morizane et al., 2011, Li et al., 2011, Chambers et al., 2012). Chambers et al. showed that the combination of SB431542 and noggin protein was sufficient to enhance neural induction from ES cells (Chambers et al., 2009). Recently, they also showed that pluripotent stem cells can be accelerated developmental timing and converted into nociceptors neurons (Chambers et al., 2012). In this study, SB431542 and LND193189 were first used to induce neuronal differentiation and then three small molecules, SU5402 (inhibitor of FGF, VEGF and PDGF signaling), CHIR99021 and DAPT (γ-secretase inhibitor), were added. Combining these small molecules induces more than 75% of neuron in 10 days of time.

1.5 INDUCTION OF SENSORY NEURONS FROM PLURIPOTENT STEM CELLS

Neural crest derived sensory neurons have been generated by culturing hES with PA6 stromal cell lines (Pomp et al., 2008, Jiang et al., 2009, Pomp et al., 2005, Brokhman et al., 2008). When human ES cells were cultured on a PA6 cell line, differentiation of sensory neurons was seen but yield was very low (less then 1%) (Pomp et al., 2005). Later, they combined two techniques; first they generated neurospheres by treating with noggin in suspension and then these neurospheres were dissociated and cultured on a PA6 cell line. This methods produced a 10-fold increase of sensory neurons (Brokhman et al., 2008). Further improvement was achieved when human ES cell were cultured with PA6 cell line for two weeks. Colonies were then detached from PA6 cultures and cultured in petri dishes to form neurospheres. After forming neurospheres, dissociated cells were again cultured on fresh PA6 cells to differentiate into sensory neurons. This method not only increased the yield of sensory neurons (more the 25%) but also the purity of sensory neurons (Pomp et al., 2008). Jiang et al. showed that the efficiency of neural crest stem cells (NCSC) differentiation from human ES cell can be increased by combining SIDA culture and FACS based enrichment (Jiang et al., 2009). In their study, they cultured human ES cells with PA6 cells for 1 week and identified that half of colonies were positive for *p75* receptor protein and vast majority of colonies were also positive for *HNK1* (a general NCSC marker). Later they sorted out *p75* positive cells by FACS and showed not only differentiation into sensory neuron but also showed neural crest derivatives in developing chick embryo *in vivo*. Recently, human ES

cells were differentiated into NCSC by growing human ES in suspension culture to form EBs (Liu et al., 2012) . EBs were cultured in medium containing 50% neurobasal medium with b27 and bFGF and 50% PA6 conditioned medium for 10 days and then cultured on geltrex-coated dishes for 4 days. NCSC were sorted with FACS by *p75* marker. Differentiation of NCSC showed 25% sensory neurons in the cultures. Small molecules have shown that ES cells can be induced to particular lineages. Different small molecules have been shown to induce neural differentiation (discussed earlier). Recently, Denham et al. showed that inhibition of glycogen synthase kinase 3ß and Activin/Nodal pathways by small molecules differentiated human ES into preneuroepithelial progenitors. Exposure to bFGF on these cells directed them towards NCSC and they further differentiated into sensory neurons (Denham et al., 2015). Sensory neural fate from stem cells has been compared from three different *in vitro* treatments (Nayagam and Minter, 2012). Mouse stem cells were first differentiated in suspension culture to form EBs for days and then treated with retinoic acid to induce neural differentiation for 4 days. These EBs were then cultured in three different media; neurotrophin medium (BDNF and NT3), conditioned medium (from sensory explant cultures) and BMP4 containing medium. This study showed that BMP4 showed highest number of *PERIPHERIN* and *TrkB* positive cells (9 to 10%) compared to other treatment.

Human embryonic stem cell has been differentiated into auditory like sensory neurons. Human ES cell were cultured as suspension to formed EBs in N2 medium and then treated with BMP4 (Shi et al., 2007). This treatment induced ES cell into sensory like neurons, expressing *BRN3A*, *PERIPHERIN*, *NGN1*, *GATA3* and *TUJ1*. Animal studies from these cells also showed the survival and engraftment in animal cochlea. In recent study, otic neural progenitor cells were derived from human ES and iPS cell (Chen et al., 2012). Cell were cultured as monolayer in the presence of FGF3 and FGF10 and further cultured in bFGF and sonic hedgehog, followed by NT-3 and BDNF supplementation. Cells were expressing otic marker *PAX2*, *PAX8* as well as *SOX2* and upon differentiation neurons expressed *BRN3A* and *TUJ1*. Transplantation studies also showed the survival of these cells in the cochlea, and that the implanted animals also gained auditory brain response. Gunewardene et al. also showed the stepwise differentiation of auditory like neurons from human ES and iPS cells (Gunewardene et al., 2014). Monolayer culture of stem cells in bFGF, noggin, N2 and B27 for 14 days to induced *PAX2* and *PAX7* positive cells. Colonies then cultured as suspension in bFGF and EGF and then attached on day 18 on human fibroblast and cultured for 35 days. During this differentiation step the expression of key makers for auditory development on cells was analyzed, e.g., *NEUROD1, BRN3A, ISLET1, GATA3, vGLUT1, TUJ1* and

NEUROFILAMENT. It has been also shown that sensory neurons derive from this protocol exhibited electrophysiological properties (Needham et al., 2014). Recently, Dincer et al. showed that combination of SB431542 and noggin at different time point could induce placodal cell types (Dincer et al., 2013). Removal of noggin after three days and culturing human ES in SB an additional 11 days, significantly induced the expression of markers for developing placodal. They also showed that these placodal cells differentiated into sensory neural types expressing *BRN3A, ISL1, GLUTAMATE* and *PERIPHERIN*.

2 AIMS

The overall aim of the thesis was to establish and analyze differentiation of auditory like sensory neurons from human embryonic stem cell for inner ear cell therapy approach.

Specific aims

- To design and evaluate cell culture conditions for the generation of sensory neurons
- To identify and modulate genes or signaling pathways by which *in vitro* differentiation towards sensory neurons can be enforced
- To investigate gene expression patterns during the course of differentaition towards sensory neurons from PSC cells

3 MATERIALS AND METHODS

3.1 CELL CULTURES

3.1.1 Human foreskin fibroblasts cells (Paper I)

Commercially available human foreskin fibroblast cell (CRL-2429 from ATCC) were cultured in Isocov's Modified Dulbecco's Medium with 10% fetal bovine serum and 1% penicillin/streptomycin (all reagents from Invitrogen) and incubated at 37°C, 6.8% CO2 and 95% humidity. Cells were mitotically inactivated by treatment with mitomycin C (10µg/ml) for 3 hours. After 3 hours, cells were plated at density of 2x104 cells/cm2 on 6 well plates (BD Falcon). Fibroblasts were allowed to attach for 2-5 days prior to used as feeder layer.

3.1.2 Human embryonic stem cells (Paper I and II)

The human embryonic stem cell line HS181 were used to performed all the experiments. HS181 were cultured (Paper I) in knock-Out Dulbecco's Modified Eagle Meduim (KO-DMEM) containing 20% Knock-Out Serum Replacement (KO-SR), 2 mM L-glutamine, 1% nonessential amino acids, 0.1mM ß-mecaptoethanol and 4ng/ml basic fibroblast growth factor (bFGF) (all reagents from Invitrogen). Cells were passaged every 3-5 days depending on the seeding by using Dispase (10mg/ml) (Invitrogen) for 5-7 min at 37°C with mild mechanical separation. Cell were then washed twice, plated on feeder layer (at ration of 1:3 to 1:4 well) and incubated at 37°C, 6.8% CO_2 and 95% humidity with daily change of medium.

For Paper II, HS181 were cultured as feeder free system. HS181 cell were cultured in Essential 8 medium (DMEM/F-12 (HAM) 1:1) and Essential 8 supplement (50X) (Life technologies). Prior to splitting, 6 well plates were coated with human recombinant vitronectin (0.5µg/cm^2) (Life technologies) for 1 hour at room temperature. Confluent HS181 cells were washed twice with Dulbecco's phosphate buffer saline (DPBS) without calcium and magnesium and incubated with 0.5mM EDTA in DPBS in each well for 4-5 minutes at 37°C. EDTA was removed and pre warmed medium was added in each well and gently pipetting colonies up by 5 ml pipette and transferred to 6 well plated pre-coated with vitronectin at ration of 1:4 to 1:6 well. Plates were gently tapped to disperse cell in each well and incubated at 37°C, 6.8% CO_2 and 95% humidity with daily change of medium.

3.1.3 Human neuroepithelial stem cells (Paper III)

Long-term neuroepithelial stem cell lines (AF22 and C1GFP) were derived from iPS cells and were cultured in DMEM/F-12 with l-glutamine, N2 (1:100), B27 (1:1000), Penicillin/Streptomycin (1:100), bFGF (10ng/ml) and hrEGF (10ng/ml) (Life technologies). Prior to splitting, flasks were coated with 0.1mg/ml polyornithine (Sigma) for 30-60 min at 37°C, washed twice with DPBS and immediately added 2µg/ml laminin (Sigma) and incubated for 4 hours at 37°C. Flasks were incubated with TrypLE express (Life technologies) for 3-4 min at 37°C and then detached cells by gentle tapping and spin with medium. Split ration was 1:3 if cells were 100% confluent otherwise 1:2 to maintain high cell density. Flasks were split every 2-3 days.

3.2 EMBRYOID BODIES FORMATION AND NEURAL DIFFERENTIATION (PAPER I AND II)

In paper I, confluent hES cells were treated with Dispase as described above and hES colonies were manually picked and detached from feeder layers. These colonies were transferred to 60-mm petri dishes (BD Falcon) in embryoid medium (Knock-Out Dulbecco's Modified Eagle Medium (KO-DMEM) containing 20% Knock-Out Serum Replacement (KO-SR), 2 mM l-glutamine, 1% nonessential amino acids and 0.1mM ß-mecaptoethanol (all reagents from Invitrogen). Next day, EBs were transferred to new petri dish to remove unattached feeder cells. Embryoid bodies were cultured in suspension for 4 days with daily change of complete medium. After 4 days, embryoid bodies were cultured in NIM medium supplemented with bFGF (20ng/ml) for 2 days. The composition of NIM medium is KO-DMEM, 2 mM l-glutamine, 1% nonessential amino acids and N2 supplements (1:100). Embryoid bodies after 6-days in suspension cultured, were cultured as adherent on 4-well plates (Nuclon). 4-well plates were pre-coated with 20µg/ml laminin (Sigma) for over night at 37°C (Paper I). EBs were transferred 4-well plates (4-5 EBs per well) and cultured in NIM medium supplemented with bFGF (20ng/ml) for 4, 7, 11 days. Cells were treated with mild Dispase (0.5 mg/ml) for 15-20 minutes to dissociate neural rosettes from surrounding non-neural structures. The dissociated neural rosettes were transferred to 6-well plates to adhered to non neural cells for 1 hour while floating clumps of neural cells were plated again on laminin coated 4-well plates in NIM medium for 1 week or 3 weeks. Half of the medium was replaced every second day.

In paper II, AggreWell plates (Stem Cells Technologies) were used to form EBs. Confluent hES cells were treated with Accutase (Sigma) for 5-6 min at 37°C. cells were detached and

dissociated cells with gentle pipetting the cell suspension for 2-3 times. Cell were transferred to 15 ml tube and washed well with DMEM/F-12 medium and transferred to same 15 ml tube. Cells were spin down and resuspended in TeSR E6 medium (DMEM/F-12 with TeSR-E6 20X supplements from Stem Cells Technologies) with 10µm Rho-Kinase inhibitor (Y-27632) (Calbiochem). Ten thousand cells per microwell were added to each well of AggreWell plate in TeSR-E6 medium with Rho-Kinase inhibitor and cultured for 24 hours at 37°C, 6.8% CO_2 and 95% humidity. Next day, EBs were dislodged from AggreWell plate by gentle pipetting and transferred to petri dish with TeSR-E6 medium. EBs were transferred to 35-mm petri dishes (BD Falcon) and cultured for 6 days as suspension culture in TeSR-E6 medium supplement with either TGFß/Activin/Nodal pathway inhibitor SB431542 at concentration of 20µm or IGF-1 (Life Technologies) at final concentration of 10ng/ml or a combination of these two supplements. Control cultures were treated with 0.1% DMSO (Sigma) and all the medium was changed daily. After 6 days treatment, EBs were transferred to 4-well plates (Nuclon), pre-coated with 0.1mg/ml polyornithine (Sigma) for 30-60 min at 37°C, washed twice with DPBS and immediately added 20µg/ml laminin (Sigma) and incubated for over night at 37°C in DMEM/F-12 medium with l-glutamine, N2 supplements (1:100), nonessential amino acids (1%), penicillin/streptomycin (1:100) and bFGF (20ng/ml (all reagents from Life technologies) for 7 days with complete change of medium every second day. In the next step, cell were further cultured for 7 more days in DMEM/F-12 medium with l-glutamine, N2 supplements (1:100), B27 (1:100) nonessential amino acids (1%), penicillin/streptomycin (1:100) and BDNF/NT-3 at concentration of 10ng/ml. Half medium was changed every second day.

3.3 NEURAL DIFFERENTIATION (PAPER III)

3.3.1 Dose response experiments

Dose response experiments were performed with Isoxazole 9 (ISX9) molecule (Torcis). Prior to experiment, cover slips were coated with 0.1mg/ml polyornithine (Sigma) for 30-60 min at 37°C, washed twice with DPBS and immediately added 2µg/ml laminin (Sigma) and incubated over night at 37°C. Cell were plated at density of 50,000 cell/ cm2 in NES differentiation medium (1:1 ratio of DMEM/F-12 and Neurobasal medium, N2 (1:100), B27 (1:100)) for 2 days to attached on cover slip. After 2 days, NES differentiation medium were added with ISX9 at concentration of 5, 10, 20 or 40µm for 4 days.

3.3.2 Differentiation experiments

For differentiation experiments, cells were plated on polyornithine/laminin-coated plates for 2 days in NES differentiation medium as described above. ISX9 and SB431542 were dissolved in DMSO and Metformin (Tocris) was dissolved in distilled water. After 2 days, cells were treated with small molecules ISX9 (20µm; identified by dose response experiments), SB431542 (10µm) and Metformin (500µm) in NES differentiation medium for 4 days with daily changed for medium. In following step, cells were cultured in NES differentiation medium with BDNF (10ng/ml) and NT-3 (10ng/ml) for another 7 days and half medium was changed every second day.

3.4 EXPRESSION ANALYSIS

3.4.1 RNA extraction and reverse transcription (RT) PCR (Paper II and III)

Total RNA was extracted by using Trizole reagent (Life Technologies) according to manufacturer's instructions. RNA was dissolved in 20µl water (Sigma, Molecular biology grade). The concentration of RNA was measured by NanoDrop1000 spectrophotometer (Thermo Scientific). One microgram RNA, pre-treated with RNase free DNase according to manufacturer's protocol (Invitrogen), was used for RT-PCR. High capacity RNA to DNA kit was use for RT-PCR according to supplier's recommendation (Life Technologies).

3.4.2 Polymerase chain reaction (PCR) (Paper II)

Polymerase chain reaction was performed in 25µl reaction mix by using following components: 1µl of cDNA (50ng), 0.5ml o each primer (0.4µM each), 0.5µl MgCl2 (1mM), 0.5ml dNTPs mix, (0.2mM each), 2.5µl 10X PCR mix (1X) 0.2µl Platinum Taq DNA polymerase (4 units) and DNas/RNase free water (all reagents from Invitrogen). The thermal cycling parameters for PCR reaction were done as follow: initial denaturation at 95°C for 10 min followed by denaturation at 94°C for 50 sec, annealing at 55°C (for *OCT-4* and *GAPDH*) and 56°C (for *NODAL*) for 50 sec, extension at 72°C for 50 sec in 30 cycles and final extension at 72°C for 5 min. The primer sequences were as follow: *GAPDH*, GCT CAG ACA CCA TGG GGA AGG T/GTG GTG CAG GAG GCA TTG CTG A (F/R, size 470bp); *OCT-4*, AGG ATC ACC CTG GGA TAT ACA CA/AAG CTA AGC TGC AGA GCC TCA (F/R, size 113bp); *NODAL*, CAT GAA AGC TAT AGG TGA CTT CAT/TGT AAA TGA AGG GCT CAG TGG A (F/R, size 250bp).

3.4.3 Real time PCR (Paper II and III)

Gene expression was quantified in Applied Biosystems 7500 real time PCR system using SYBR Green master mix (Applied Biosystems). Primers were purchased from commercially available KiCqStart from Sigma for following genes; *GATA3, BRN3A, NEUROD1* and *18S* (for Paper II and III), *NESTIN* and *NGN1* (Paper II only) and *PERIPHERIN* (Paper III only). Amplification was carried out in 96 well optical plates using 4µl diluted cDNA (20ng), 10µl master mix, 0.5µl of each primer and 5µl of water. All reactions were performed according to standard protocol of 40 cycles of denaturation-annealing. The ribosomal mRNA *18S* was used to normalized and relative expression levels were reported as $2^{-\Delta\Delta CT}$ for fold changes. All reactions were performed in triplicate and repeated three times from with RNA samples from separate cell cultures.

3.4.4 Immunocytochemistry

Cells were fixed in 4% paraformaldehyde solution for 15-20 min at room temperature. After fixing, cells were washed twice with DPBS and permeabilized with TTPBS (0.5% triton X-100 + 1% Tween 20 in PBS) in Paper II and III. No permeabilization was done in Paper I. Cells were incubated with blocking buffer (10% fetal bovine serum + 0.1% Tween 20 in PBS) for 30 min in Paper I and (3% bovine serum albumin in TTPBS) for 1 hour in Paper II and III at room temperature. Primary antibodies were diluted in respective blocking buffer and incubated at 4°C for overnight. Next day cells were washed three times with PBS (Paper I) or TTPBS (Paper II and III) for 5 min each. Secondary antibodies were diluted in PBS and cells were incubated for 2 hours at room temperature. Cell were again washed three times with PBS or TTPBS for 5 min each and mounted with Vectashield DAPI (Vector labs Inc) (Paper I) or conterstained with DAPI.

Following primary antibodies were as followed: *Ki67* (Abcam, 1:200), *CLEAVED CASPASE 3* (Cell Signaling, 1:200), *ACTIVE CASPASE 3* (BD Pharmingen, 1:200), *NESTIN* (Abcam, 1:500), *NESTIN* (Chemicon, 1:200), *TUJ1* (Covance, 1:500 and 1:1000), *TUJ1* (Millipore, 1:700), *ISLET 1* (DSHB, 1:50), *BRN3A* (Millipore, 1:200), *PERIPHERIN* (Chemicon, 1:200), *TrkB* (Santa Cruz, 1:200) and *TrkC* (Santa Cruz, 1:200). Respective secondary antibodies were as followed: goat anti mouse and rabbit Alexa 488 or 594 (Invitrogen, 1:200), donkey anti mouse cy3 (Jackson Immunoresearch, 1:400), donkey anti chicken cy5 (Jackson Immunoresearch, 1:400), donkey anti rabbit cy5 (Jackson Immunoresearch, 1:400).

In paper I, all fluorescent microscopic images were captured using Axiovision (Carl Zeiss). In paper II and III, an LSM 700 confocal microscope (Carl Zeiss) was used to capture images.

3.5 STATISTICS

GraphPad Prism statistical software was used to perform all the analysis. Statistical differences were measured by One-Way ANOVA (Paper I-III) and unpaired t-Test was done (Paper I) to determine level of significance among different groups and between two groups respectively.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Differentiation of human embryonic stem cells towards a sensory neural phenotype

Human embryonic stem cells (hESC) have been shown to have the potential to differentiate into specific cells types, leading to great attention in regenerative medicine for treatment of neurodegenerative diseases. In this study, we differentiated hESC into sensory neurons phenotypes from different time stages of *in vitro* generated neural rosettes. With this approach, we could expect the bulk of cells to be differentiated neurons but also some level of possible contamination with heterogeneous cells populations derived from primitive neural ectoderm during early stages of differentiation.

We first generated embryonic bodies (EBs) from the hESC line HS181. After transfer of EBs to neural induction medium, neural rosettes started to appear from day 4. These rosette structures persisted until our last tested stage day 11. Neural rosettes are elongated columnar cells, which has previously been resembled to primitive neural ectoderm (Wilson and Stice, 2006), and express many of the protein presents in neuroepithelial cells of the neural tube (Wilson and Stice, 2006, Curchoe et al., 2012). The neural rosettes were cultured for 4, 7 and 11 days in the presence of bFGF (the cultures were denoted NS4, NS7 and NS11, respectively). Studies have shown that bFGF is important in the differentiation of neural progenitors from hESC (Zhang et al., 2001, Stern, 2006). Immunostaining showed that the rosettes were positive for *NESTIN* and *TUJ1* at all stages where NS11 cultured showed more mature neuronal population compared to NS4 and NS11. These results, in line with previous studies, suggest the presence of neural cells (Erceg et al., 2009, Zhang et al., 2001). We then further investigated the proliferation (by *Ki67*) and apoptosis (by *CLEAVED CASPASE* 3) in the NS4, NS7 and NS11 cultures. Proliferation capacity was similar in all the cultures after one week (NS4; 33.6%, NS7; 32.4 and NS11; 30.5). However, apoptosis was significantly higher in NS11 cultures (15.3%) compared to NS4 (10.2%) and NS7 (11.4%). Presence of more mature neuronal population in NS11, which undergo degeneration could explain the higher number of apoptotic cells.

Neural rosettes, from NS4, NS7 and NS11, were re-plated and differentiated for a one- or three-week period of culture. After one week, we observed high number of cells positive for *NESTIN* in all cultures (97%, 92% and 88%, respectively). Three-week cultures showed a

significant decrease in *NESTIN* positive cells in NS4 (48%) and NS7 (49%) cultures but not in NS11 (89%) cultures. *TUJ1* staining showed higher number of positive cells in cultures after one week in NS4 (84%) and NS11 (79%) compared to three weeks in NS4 (40%) and NS11 (57%). On the other hand, NS7 cultured showed similar number of cells positive for *TUJ1* after one week (72%) and three weeks (69%).

We next investigated that neural rosettes from different stages has effect on differentiation towards sensory neural phenotypes. For this we used three markers, *PERIPHERIN*, *TrkB* and *TrkC*. *PERIPHERIN* is an intermediate filament protein expressed in axons of peripheral sensory neurons in combination with *TUJ1* (Troy et al., 1990, Schimmang et al., 1995, Postigo et al., 2002), but can also be detected in some sympathetic ganglion and motor neurons (Escurat et al., 1990). *PERIPHERIN/TUJ1* exhibited a similar expression range (9-12%) in NS4 and NS7 cultures at the one- or three-weeks time points, but the NS11 cultures showed less than 1% *PERIPHERIN/TUJ1* positive cells at both time points.

Trk receptors (TrkB and TrkC) are important for neuronal survival of sensory neurons of spiral ganglion and dorsal root ganglion neurons (Ernfors et al., 1993, Schimmang et al., 1995). *TrkB/ TUJ1* staining did not revile any differences at the three different time points (NS4, NS7 and NS11) after one or three weeks. However, *TrkC/TUJ1* staining in NS4 cultures showed an increased in the number of positive cells (17%) at three weeks compared to one-week cultures (<1%) whereas the NS11 cultures did not show much difference between one week (<1%) and three weeks (3%). NS7 cultures showed higher number of *TrkC/TUJ1* positive cells (20%) in both, one week and three weeks cultures.

Previously, it has been shown that the neurogenic potential of rosettes cultures decreases with time and that GFAP positive glia cells appear more frequently (Abranches et al., 2009). This indicated a switch from neurogenic to gliogenic identity of the progenitor cells. Although we did not test markers for other progenitor types, our data for NS11 also suggests that NS11 rosettes displayed a reduced potential to differentiate into sensory neurons compared to the NS4 and NS7 cultures. However, NS4 and NS7 showed similar or higher number of *PERIPHERIN* positive cells compared to published protocols (Pomp et al., 2005, Brokhman et al., 2008) but our result did not match the results of the recently developed protocol showing 40% to 70% of *PERIPHERIN* positive neurons (Lee et al., 2010, Chambers et al., 2012).

In conclusion, we could show that the NS7+1week protocol was the most favorable protocol in comparison to other tested protocol, based on *PERIPHERIN* and *TrkC* expression. This protocol showed induction of upto11% of *PERIPHERIN* and 20% of *TrkC* positive cells.

4.2 PAPER II

The TGFß/Activin/Nodal pathway inhibitor SB431542 is a potent inducer of sensory neural markers when applied to neural differentiation from HS181 embryonic stem cells

The development of a stem cell based cell therapy will require an efficacious differentiation of stem cells into the appropriate neurosensory lineages. Signaling pathways have major impact on the maintenance of pluripotency and differentiation into particular lineages. In this study we showed that blocking of Activin/Nodal pathway by small molecule, exit hES cells from pluripotency and differentiate them towards neural and sensory neural phenotypes. With this approach, we directed the HS181 cells to mainly differentiate into neuroectodermal lineage and blocked/reduced the mesoderm and endoderm differentiation. We could assume that this approach could also generate other types of neurons as well in the cultures.

Small molecule, SB431542 (SB) induced a downregulation of the pluripotency marker *OCT-4* in 6-day treated embryoid bodies (EBs) compared to control. We also showed that SB effectively downregulated *NODAL* gene expression in EBs. Further analysis showed a significant upregulation of *NESTIN* (3.7 fold) in SB treated EBs compared to control. Activin/Nodal pathway is important for maintenance of pluripotency and blocking this pathway lead to neuroectoderm differentiation (Vallier et al., 2005, Smith et al., 2008). Downregulation of the *NODAL* gene shows that SB effectively blocks the Activin/Nodal pathway and disturbs the pluripotency. Consistent with previous studies, we showed that SB not only caused downregulation of *OCT-4* but also induced neural differentiation (Smith et al., 2008, Patani et al., 2009).

Previously, SB treatment alone has been shown to induce neural cells with dorsal identity of caudal neuraxis, identified by the expression of *PAX7* (Patani et al., 2009). It has been discussed earlier that auditory neurons are developed from the otic placodal derivative, next to hindbrain and *PAX7* is a marker for hindbrain during development. With this assumptions, we hypothesized that SB treatment could induce genes responsible for auditory like sensory neurons. Our results showed that SB treatment indeed upregulated key genes, *NGN1* (7.6 fold), *NEUROD1* (5.4 fold) and *BRN3A* (6.7 fold), important for development of auditory like sensory neurons.

Basic fibroblast growth factor (bFGF) has been shown to induce neuroectoderm proliferation as well as to act as a neurotropic factor for neuronal survival and differentiation. Some studies have also shown that fibroblast growth factor (FGF) signaling is important for otic placod induction (Martin and Groves, 2006, Freter et al., 2008) and ligand FGF3 and FGF10 are critical in inner ear development (Alvarez et al., 2003). FGF signaling can be activated by bFGF since it activates several FGF receptors including FGF3 and FGF10 (Groves and Bronner-Fraser, 2000). In the next step, we cultured EBs as adherent cultures in the presence of basic fibroblast growth factor (bFGF). This step further induced the expression of *NGN1* (25 fold), *NEUROD1* (45 fold) and *BRN3A* (10 fold). Interestingly, we have also observed a significant induction of *GATA3* (5 fold) in initial SB treated cultures. Further analysis by immunostaining showed that frequency of *BRN3A/TUJ1* and *ISL1/TUJ1* positive colonies were significantly increased in SB treated cultures in presence of bFGF compared to control. The results show that cultures have heterogeneous population with early progenitor and early mature population.

We then assessed the number of positive cells for *ISL1/TUJ1* and *BRN3A/TUJ1* by immunocytochemistry on cells cultured in the presence of BDNF and NT-3. *ISL1* expression can be detected in developing sensory neurons as well as in motor neurons and interneurons (Liang et al., 2011, Radde-Gallwitz et al., 2004).

We observed that all colonies were positive for *ISL1/TUJ1* in SB treated cultures compared to control. Moreover, *BRN3A/TUJ1* staining showed a significant increase in the number of colonies (90%) in SB treated cultures compared to control. This showed that the neurotrophins BDNF/NT-3 provided a survival effect as well as promoted further differentiation into auditory like sensory neurons.

In this study, we also used insulin like growth factor-1 (IGF-1) to indicate sensory neural induction. Previously, it has been shown that IGF-1 plays an important role in inner ear development in murine (Okano et al., 2011) and some studies have also shown the effect of IGF-1 in the induction of inner ear cell types, particularly hair cells, from stem cells (Li et al., 2003, Higashi et al., 2007, Oshima et al., 2010). This was, however, not investigated in thjs study. Our study, in line with previous studies, showed no significant impact on differentiation of auditory like sensory neurons from stem cells (Chen et al., 2012) and human fetal auditory stem cells (Chen et al., 2009).

Based on these results, we conclude that SB treatment has impact on early induction of sensory neural genes in EBs, which can be further enhanced by subsequent culture in the

presence of neurotrophic factors, bFGF and later BDNT/NT-3. This protocol showed the appropriate differentiation of markers for auditory like sensory neurons from pluripotent stem cells.

4.3 PAPER III

Induction of sensory neurons from neuroepithelial stem cells by the ISX9 small molecule

Efficient modulation of stem cell differentiation is very crucial to translate stem cell therapies from bench to clinics. There has been great interest in small molecules for modulating stem cells into particular cell lineages (Xu et al., 2013). In this study, we used three small molecules, SB431542 (SB), Isoxazole 9 (ISX9) and Metformin (MET), on long-term self-renewing neuroepithelial stem cells (Lt-NES) lines (C1GFP and AF22).

By selecting the ISX9 small molecule, we aimed to direct the cells to differentiate into neural cell types by inducing the *NEUROD1* gene (Schneider et al., 2008). It is known that *NEUROD1* expression guide early neural progenitor into auditory like sensory neurons during development. SB has been used widely to induce neural differentiation, either alone or in combination with other small molecules (Chambers et al., 2009, Chambers et al., 2012, Patani et al., 2009, Smith et al., 2008). MET (a first line drug for diabetics) has been shown to promote neural differentiation in mouse embryonic cortical radial precursors (Wang et al., 2012).

With the approach of using lt-NES cells we aimed to minimize the presence of cells from mesoderm and endoderm. Lt-NES cells lines are derived from manually selected neural rosettes and expanded as single cell long time culture systems maintaining neural progenitor identity with capacity to differentiate into different types of neurons like dopaminergic neurons, motor neurons and cholinergic neurons (Zhang et al., 2014, Fujimoto et al., 2012, Falk et al., 2012).

The ISX9 dose was titrated for our cell lines as it has been shown that the optimal doses vary between different cells lines (Schneider et al., 2008). Our initial experiments showed that proliferation decreased and apoptosis increased with increasing doses of ISX9. Isoxazole 9 has been shown to induce neural differentiation by directly activating the *NEUROD1* gene in mouse hippocampal neural stem cells (Schneider et al., 2008). Our dose response experiments showed significant reduction of *NEUROD1* expression but we observed dose dependent increased in *GATA3* expression, which is activated downstream of *NEUROD1*,

and showed a significant increased at the 20μM and 40μM doses. These results abstruse our initial idea of activating *NEUROD1* and suggested further kinetic analysis to explore *NEUROD1* expression in ISX9 treated cells. Based on the dose response we selected a ISX9 dose of 20μM. Optimal doses for SB (10μM) and MET (500μM) have been shown previously and the same doses were used in this study (Wang et al., 2012, Guo et al., 2013, Smith et al., 2008).

Pattern of gene expression revealed differentiation of sensory like neurons in the ISX9 treated group. ISX9 treatment resulted in a significant increase in the mRNA expression of *GATA3* (25 fold), *BRN3A* (5.5 fold) and *PERIPHERIN* (27 fold) compared to control in C1GFP cell line. In the AF22 cell line, the effects were even more prominent: *GATA3* (230 fold increase), *BRN3A* (7 fold increase) and *PERIPHERIN* (28 fold increase). In both C1GFP and AF22 cell lines immunocytochemistry revealed significantly higher numbers of *BRN3A/TUJ1* positive cells in the ISX9 treated cultures (16% vs 22%) compared to control (4% vs 8%), respectively.

Further, our results suggested that SB and MET were not effective to induced sensory neural differentiation in lt-NES cells lines. Interestingly, while this indicates that SB molecules are inadequate for sensory neural differentiation in the lt-NES cells, in Paper II we have shown that SB treatment can have a significant impact on early induction of sensory neural genes in EBs from the pluripotent hESC line HS181.

Next, we cultured cells treated with small molecules in BDNF/NT-3 containing medium to test further differentiation into sensory neurons. The neurotrophins BDNF and NT-3 are important for the survival of sensory neurons. Our data showed a further increase in *GATA3* expression in ISX9 (8 fold vs 60 fold) treated cultures in both the cells lines, CIGFP and AF22 respectively (figure 3.6 A). We also observed a further increase in *BRN3A/TUJ1* positive cells in ISX9 treated cultures (23% vs 28% in C1GFP and AF22, respectively). Protein and mRNA data suggest that more mature nature of sensory neurons in cultures since *GATA3* and *Brn3a* expression is present in late stages of auditory neuron during development.

In summary, we show that the ISX9 small molecule could differentiate lt-NES cells into cells with auditory-like sensory neuron phenotypes. This approach could further enhance the availability of neurons appropriate for a cell therapy approach for sensorineural hearing loss.

5 GENERAL DISCUSSION

This thesis investigated different strategies to induce sensory neurons from stem cells to be considered for future inner ear cell therapy. Chemical approaches using various types of small molecules are today central components of many differentiation protocols for stem cells (reviewed in (Li et al., 2013, Xu et al., 2013)). In Papers II and III we tested the small molecule SB431542 (inhibitor of Activin/Nodal signaling pathway by blocking phosphorylation of ALK4, 5, and 7 receptors), In Paper III we tested Isoxazole 9 (inducing *NEUROD1* gene) and Metformin (a first line diabetic drug) (in paper III). In line with previous studies, our results showed neural induction in SB431542 treated cells (Patani et al., 2009, Smith et al., 2008). In previous studies, SB431542 has been used in combination of other small molecules to differentiate hESC into peripheral sensory neurons (Kreitzer et al., 2013, Denham et al., 2015). Very recently, it has been shown that a combination of SB431542 and noggin applied at appropriate time points can enhance placodal derivatives from hESC (Dincer et al., 2013). In paper II, we have shown that SB431542 alone is sufficient to induce expression of early markers of sensory neural progenitor cells from hESC. However, no alteration in expression of these markers was observed in lt-NES cells when treated with SB431542 (paper III). Several previous studies on Lt-NES cells imply that lt-NES cells are already programmed to differentiate into CNS type neurons (Falk et al., 2012, Fujimoto et al., 2012, Koch et al., 2009, Zhang et al., 2014). On the other hand, we found that the small molecule ISX9 could induce expression of sensory neuron markers in lt-NES cells (Paper III). ISX9 has been reported to induce neural differentiation by activation of *NEUROD* gene (Schneider et al., 2008). Interestingly, we did not observe any change in *NEUROD1* mRNA expression level. However, an upregulation of *NEUROD1* downstream genes, *BRN3A* and *GATA3*, was observed after application of ISX9.

Our findings with expression of genes involved in sensory neuron development in ISX9 treated NES suggest that NES cells could in fact be appropriate for sensory neuron replacement. When we investigated the growth and integration of these cells in organotypic cultures with organ of Corti and spiral ganglion explants on plastic surface, our preliminary data showed that ISX9 treated cells not only differentiated into neurons, but also migrated and sent their projections towards spiral ganglion and hair cells in the organ of Corti (Figure 5; unpublished date). Further experiments are needed to investigate migration, possible synaptic connections, and differentiation into sensory neurons.

In paper II, we modified our approach and allowed hESC to differentiate into neuroectoderm by blocking mesodermal and endodermal differentiation using the small molecule SB431542. We showed that *NODAL* and *OCT-4* expression was reduced but our data did not confirm the absence of other germ layers. We could expect an unknown (likely small) percentage of cells to be present from mesoderm and endoderm lineages in our initial cultures and also the presence of different types of neurons in our final mixed cell populations. In future studies it is important to perform co-expression of lineage specific markers during all steps towards the identified pool of cells. The same genes are expressed in different cell populations during different stages of development or differentiation e.g., *NGN1* expression has been observed in sensory neurons from PNS and placodal derivatives. *NEUROD1* expression is present in early developing neurons in embryonic nervous system as well as otic placodal cells and some pancreatic progenitors. *ISL1* is expressed in sensory neurons as well by motor neurons during development.

For paper III, we used neuroepithelial stem cells to minimize the presence of cells from mesoderm and endoderm. Using the ISX9 small molecule, we obtained a pattern of gene expression indicating strong differentiation into sensory like neurons. However, since lt-NES cells used in this study are prone to differentiate into different types of neurons we could expect also the presence of different CNS types neurons in this population. Further studies are needed to confirm whether the obtained sensory like neurons are gluamatergic and electrophysiologically active. Future studies should also address survival and integration of these neural cells within cochlea and spiral ganglion using transplantation in animal models.

Based on previous studies from our group, we hypothesized that the appropriate neural progenitor cells are required for the successful replacement of degenerating sensory neurons in the cochlea in order to restore hearing and have beneficial effects, for example in combination with cochlear implants. Previously, three different types of cells or tissues were transplanted into the animal cochlea: adult neural stem cells, mouse ES cells and dorsal root ganglion (DRG) tissue (reviewed in (Ulfendahl et al., 2007, Hu and Ulfendahl, 2006)). Survival of DRG neurons were observed up to 10 weeks following transplantation, however the survival rate decreased with time (Hu et al., 2004b). The survival of adult neural stem cells and mouse ES were considerably lower as compared to DRG when implanted in the inner ear (Hu et al., 2005b, Hu et al., 2004a, Hu et al., 2005a). Interestingly, differentiation of transplanted stem cells into neurons was seen in the implanted cochlea but the occurrence ratio was very low.

Previously, Novozhilova et al. have reported that human neural progenitor cells, when co-cultured with brainstem tissue, differentiate and extend neurites towards the brainstem (Novozhilova et al., 2013). From the results obtained in Paper I we selected the NS7+1week protocol for further studies regarding growth and differentiation of this cell populations in conditioned medium derived from i) brainstem, ii) organ of Corti cultures, or iii) spiral ganglion cultures. Photos were taken from cultures at day 0, 3 and 7 and assessed by ocular inspection. These experiments showed that while all conditioned medium sources stimulated growth and migration, spiral ganglion medium exhibited the strongest stimulatory effect, compared to control medium (Figure 6; unpublished data). Most pronounced differentiation was obtained on day 7 with also longest neuritis growing in bundles (fascicles) (Figure 7; unpublished data).

We next tested organotypic cultures using membrane inserts and co-cultured NS7 cells with brainstem, organ of Corti, or spiral ganglion tissue. This resulted in extensive differentiation but the results were not conclusive due to technical challenges. No neurites were seen extended on the membrane inserts (Figure 8; unpublished data). Neurites did not extend towards the host tissue and instead mainly grew within the NS7 colonies, or if in close contact with host tissue within this tissue. Further experiments are needed to establish an appropriate protocol to test the growth and integration of sensory neural progenitor with the tissue from auditory system.

FIGURES; Unpublished data.

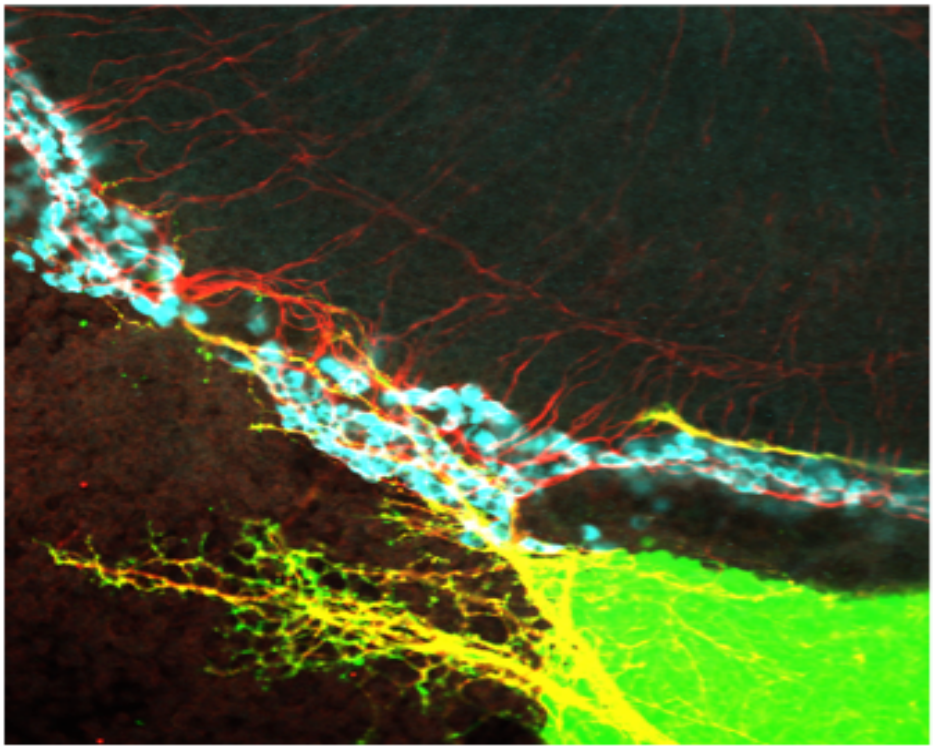

Figure 5: Innervation by neurite fibers from ISX9 treated NES sphere to the cochlear explant.

NES cell spheres were made from single cells using AggreWell plates one day before. The cochlear explants were prepared from 3-4 days old rat and cultured on membrane inserts coated with polyornathine and laminin. NES spheres were placed next to the explant on the spiral ganglion side and cultured in presence of NT3 and BDNF (10 ng/ml). After 1 week, the cultures were fixed and processed for immunocytochemistry for Neurofilament 200 (red), GFP (green) and myosin VII (blue) to identify neurite fibers, NES cells and hair cells respectively. The representative confocal image shows that the fibers generated from the NES cells innervate the explant and reach both hair cells and spiral ganglion neurons. (Ali et al., unpublished)

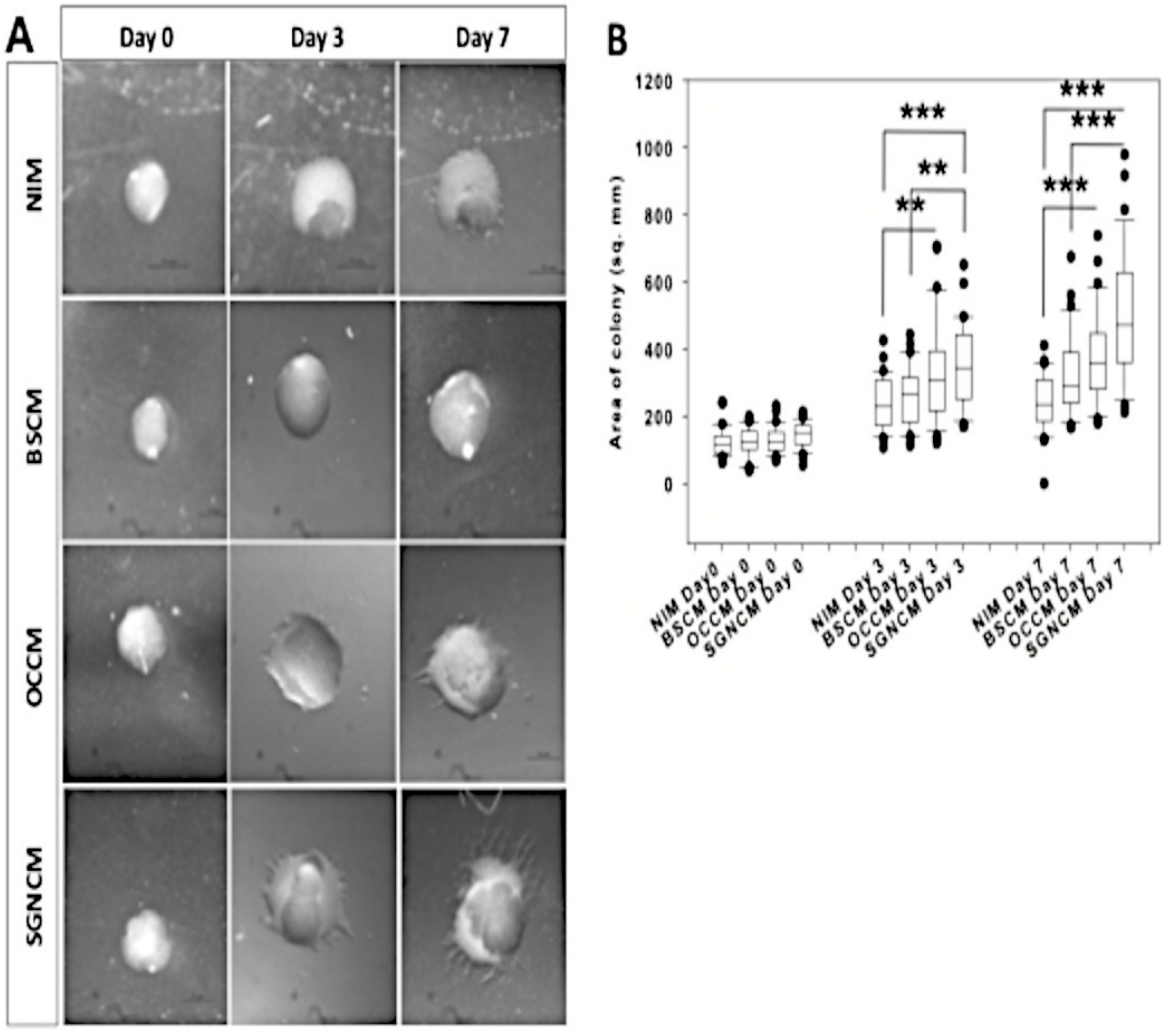

Figure 6: Migration of NS7 cells following culture in auditory tissue conditioned medium.

To study the effects of secreted molecules from different tissues of auditory system, the NS7 colonies were grown in the presence of conditioned medium from brainstem (BSCM), organ of Corti (OCCM) or spiral ganglion (SGNCM). The colonies from the different conditions were imaged on day 0, 3 and 7th day (A). Quantification of migration at days 0, 3 and 7 were performed by measuring the diameters of the spheres surrounded by the rim of migrating cells, illustrated here in a time course graph (B). The colonies showed similar size in all conditions on Day 0. Data represent 4 independent experiments with a total of >33 NS7 colonies examined in each time point. ** $P < 0.05$, *** $P < 0.01$. Scale bars: 10 mm. (Dash-Wagh et al., unpublished)

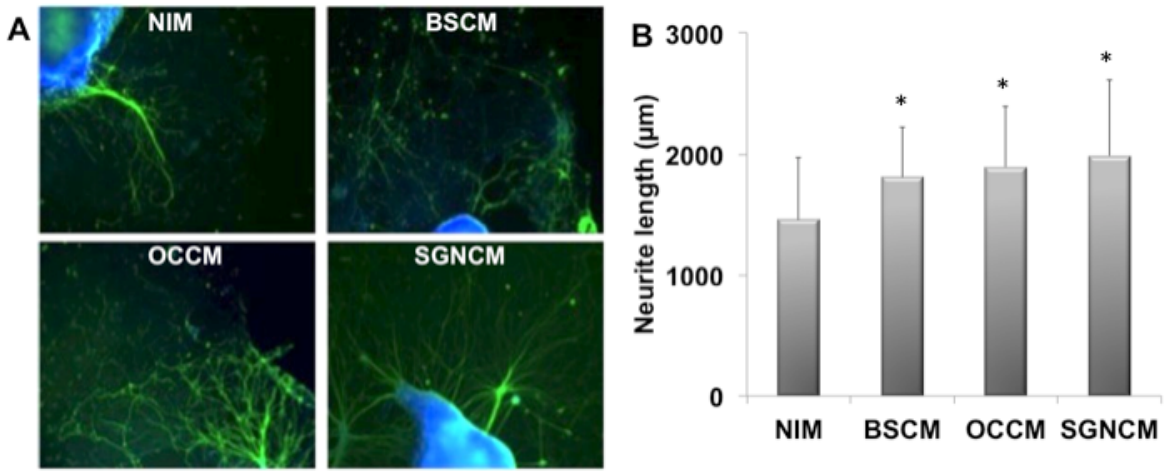

Figure 7: Enhanced neurite outgrowth from NS7 cells induced by conditioned medium.

(A) The NS7 colonies were grown in the presence of conditioned medium from brainstem (BSCM), organ of Corti (OCCM) or spiral ganglion (SGNCM) for 7 days. The colonies were then fixed, immunostained for the neuronal marker beta-tubulin III (green), and for DAPI (blue). **(B)** The colonies were imaged and neurite lengths measured by plotting a straight line from tip of neurite till the edge of the colony cell mass. Six to eight colonies were examined for each condition. * $P < 0.001$. (Dash-Wagh et al., unpublished)

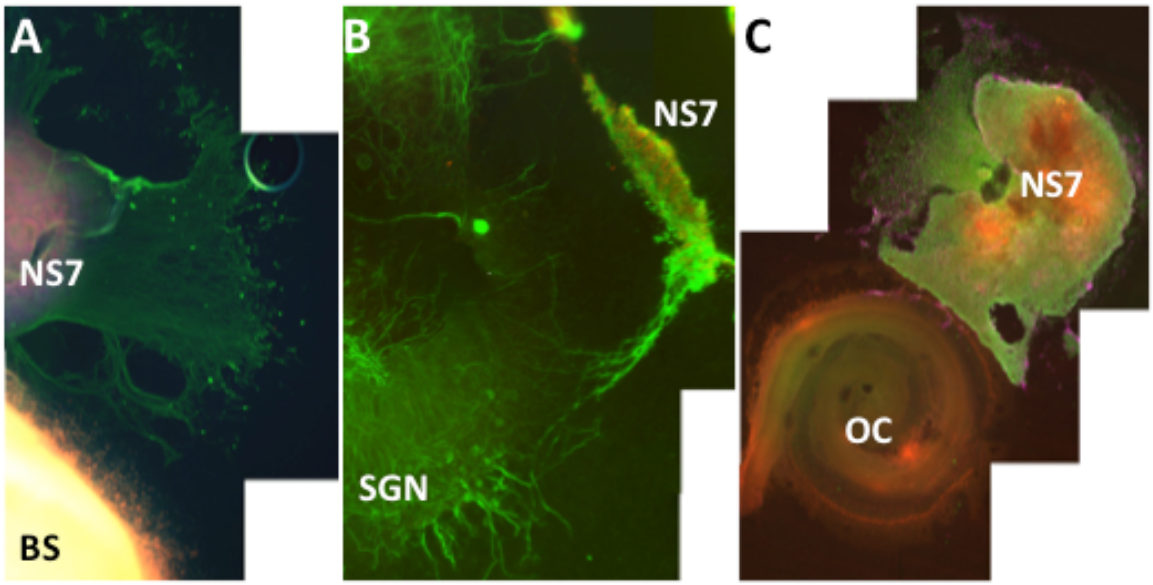

Figure 8: Differentiation of NS7 when co-cultured with auditory tissues.

The NS7 were co-cultured with brainstem (A), SGN explant (B) or organ of Corti (C) for 7 days. The cultures then stained for ß-tubulin III (green) or human nuclei (red). (Ali et al., unpublished)

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The results reported in this thesis emphasize hESC and lt-NES cells for modeling auditory like sensory neural differentiation. By using small molecules, hESC and neuroepithelial stem cells could be induced to candidate cell populations for future therapy studies for sensorineural hearing loss. Lessons learned include that small molecules are useful for initial induction of differentiation into neural and sensory neural progenitors as they can suppress hESC to differentiate into other cells lineages. Although our results suggest the induction of sensory neurons from hESC and lt-NES cells, it is important to further characterize the different cell population in the culture system. Appropriate time points are crucial and future studies must ensure that the cells are well differentiated into particular lineages and not mixed with pluripotent or other cell lineages.

Contrary to pluripotent stem cell, Lt-NES cells lack the potency to cause teratoma *in vivo* and are thus attractive for cell transplantations. Although, Lt-NES cells express mostly CNS types markers they can, as demonstrated here, be induced to express sensory neural linage markers by using ISX9 molecules. This could be a potential source for obtaining cells for inner ear cell therapy.

The differentiated neurons should next be characterized electrophysiologically to demonstrate potential for functional integration within the host tissue. Our preliminary studies with ISX9 treated cells showed signs of integration within organ of Corti but further experiments are needed with also other tissues to confirm these observations. These cells should also be transplanted into appropriate deafened animal models to explore the survival in the host environment, integration with host cells at the molecular level, and finally to demonstrate an improvement of auditory functions.

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