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Stem Cell Replacement Therapy for the Mammalian Inner Ear: A Systematic Literature Review

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STEM CELL REPLACEMENT THERAPY FOR THE MAMMALIAN INNER EAR:

A SYSTEMATIC LITERATURE REVIEW

By

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A capstone project submitted to the Graduate Faculty in Audiology in partial fulfillment of the
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Abstract

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By

Robin Warwick

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Objective: The purpose of this investigation is to review current possible regeneration techniques for damaged hair cells of the inner ear in mammals. The avian has the ability to spontaneously regenerate damaged hair cells, and thus provides an animal model to simulate a similar response in the mammalian inner ear.

Methods: A systematic review of the literature was conducted using a PubMed database search to address the following question "What are the current investigations pertaining to regenerating hair cells using stem cell-based research?" The articles were analyzed and rated at Level Ia, Ib, IIa, IIb, or III level of evidence. All articles had to be related to the inner ear and stem cell, of English-language and be in peer-reviewed journals from 2005-2010.

Results: Study results indicate that hair cells generated from a stem cell show some morphological and electrophysical similarities to *in vivo* cells. Nonetheless, generation of hair cells *in vivo* has still a long way to go before being achieved. Stem cell investigations include not

only embryonic stem cells but also adult stem cells from the vestibular system, bone marrow and lateral ventricles. *In vivo* studies involving transplants of different types of stem cells have been conducted.

Conclusions: Since the finding that avians do spontaneously regenerate damaged hair cells, several researchers have attempted to replicate the factors indicated in the avian regeneration pathways in the human or mammalian model. Although successful replication has remained elusive, such attempts have shed light on the developmental process. The human inner ear is highly organized and extremely sensitive by nature of its function (being able to respond to movement micrometers in length); thus, any intervention—be it gene therapy based, or stem cell transplantation based—has proven to be extremely difficult.

Key Words:

Inner ear cell, stem cell, regeneration, avian

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I wish thank my wonderful family and close friends for their love, amazing support and full understanding.

Lastly, I would like to dedicate this to my parents, without whom, I would not have started with this endeavor.

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Explanation of Terms

Allograft A tissue or cell that has been transplanted from one organism to another of the same
species.

Blastocyst An embryonic sphere of cells produced by cell division following fertilization. It is
made up of various cell layers, the inner most called the inner cell mass.

Brachyury A marker of development of mesoderm.

BrdU A marker of cell proliferation.

Culture medium The liquid that covers cells in a culture dish and contains nutrients to nourish
and support the cells. Culture medium may also include growth factors that are added to produce
desired changes in the cells.

Differentiation The process whereby an unspecialized cell becomes a specialized cell such as a
liver, fat or muscle cell.

Embryonic stem cells The pluripotent cells derived from the inner cell mass of the blastocysts
of embryos.

Embryoid bodies Body of cells that arise when embryonic stem cells are cultured in medium.
Embryoid bodies contain cell types derived from all three germ layers.

Fibroblast An progenitor connective tissue cell that is fiber-producing.

Germ layers The inner cell mass of the blastocyst goes through a process called gastrulation
where the mass becomes organized into three distinct cell layers, called germ layers. The three
layers are the ectoderm, mesoderm, and endoderm.

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Growth factors Usually a protein or a steroid hormone which is capable of stimulating cellular growth via proliferation and differentiation.

Hematopoietic stem cell A stem cell that gives rise to all red and white blood cells and platelets.

Induced pluripotent stem cell (iPSC) A type of pluripotent stem cell, similar to an embryonic stem cell, formed by the introduction of certain embryonic genes into a somatic cell.

In vitro Latin for "in glass"; in a laboratory dish or test tube; an artificial environment.

Inner cell mass (ICM) The cluster of cells inside the blastocyst. These cells give rise to the embryo and ultimately the fetus. The ICM may be used to generate embryonic stem cells.

Mesenchymal stem cells Non-blood adult stem cells from a variety of tissues, though stem cells from different tissues may or may not be the same.

Mitosis Cell cycle process which produces daughter cells, thus is an important part of proliferation.

Murine Derived from the mouse.

Myosin VII-A: A marker for hair cell formation.

Nestin: A stem cell marker.

Neural stem cell A stem cell found in adult neural tissue that can give rise to neurons and glial cells.

p27kip1 A marker for supporting cell formation.

Pluripotent Having the ability to produce all of the various cell types of the body.

Proteomics Large scale study of structure and function of proteins (contraction of protein and genome).

Proliferation Expansion of the number of cells by the continuous division of single cells into two identical daughter cells.

Stromal cells Connective tissue cells found in virtually every organ. In bone marrow, stromal cells support blood formation.

Teratoma A multi-layered benign tumor that grows from pluripotent cells. These are usually formed from cells injected into mice with a dysfunctional immune system. A human embryonic stem cell line can be established by injecting stem cells into such mice and verifying that the resulting teratomas contain cells derived from all three embryonic germ layers.

Totipotent Having the ability to give rise to all the cell types of the body plus all of the cell types that make up the extraembryonic tissues such as the placenta. (See also Pluripotent and Multipotent).

Transcription factors: molecules that trigger cells into differentiating into their final cell type.

Transgenic A gene that can transfer from one organism to another.

Xenograft A transplanted cell from one species to another.

INTRODUCTION

It estimated that 36 million adult US citizens, approximately 17% of the US population, report suffering from some degree of hearing impairment. Approximately 8% of those between the age of 18 and 44, about 20% between 45 and 64, about 32% between 65 and 74, and about 50% of those 75 years of age and older have hearing impairment. Due to the rapidly aging population, that number is estimated to only increase (Pleis & Lethbridge-Cejku, 2007).

There are many causes of hearing loss (genetic disorders, noise exposure, ototoxic medicines, presbyacusis) as well as many sites of lesions (damage to the inner and outer hair cells, stria vascularis, spiral limbus, spiral ganglia). The primary cause of hearing impairment however is associated with aging. The many changes that occur throughout the auditory system due to the aging process include damage to the stria vascularis (Pauler, Schuknecht, & White, 1988), sensory cells and supporting cells (Scholtz et al., 2001), neurons of the eighth cranial nerve, and the entire auditory system. This paper will concentrate on repair to the inner ear hair cells, supporting cells and auditory neurons.

The loss of the sensory and supporting cells in the cochlea leads to permanent hearing loss as these cells are produced only during embryonic development in mammals and do not regenerate (Ruben, 1967). That is, mammalian hair cells stop differentiating during embryonic development and lose their ability to re-enter mitosis (Atar & Avraham, 2010; Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Zhong et al., 2010). Neither hair cells nor supporting cells of the inner ear divide under either normal conditions, or in response to damage (Laine, Sulg, Kirjavainen, & Pirvola, 2010) in the mammal after they have matured. Thus, if hearing

impairment is mainly due to cochlear hair cell and supporting cell loss, then replacing or repairing the damaged cells should ameliorate the hearing loss.

It was demonstrated in the late 1980s that birds could regenerate their cochlear hair cells following exposure to excessive noise levels or ototoxic aminoglycosides damage (Cotanche, 1987; Rubels, 1987). Recent experiments in the avian have demonstrated that damage from noise or ototoxic exposure leads to apoptosis (programmed cell death) of the hair cells (not the supporting cells) and to the eventual ejection of these cells from the sensory epithelium. In birds, apoptosis induces the adjacent supporting cells to revert to a more plastic state (easier to revert to a stem cell like state) to undergo regeneration to replace the dead hair cells (Matsui, Parker, Ryals, & Cotanche, 2005). Furthermore, new connections were found to be made to the efferent nerve terminals (Arnold et al., 2005) and recovery of auditory function was observed (McFadden & Saunders, 1989).

These recent studies on the mechanisms involved in cell death and regeneration of the avian cochlea has provided impetus for new research approaches on initiating hair-cell regeneration in the human cochlea. Success in extrinsically inducing regeneration in the mammalian cochlea *in vivo* has only been reported in one study however, as yet, no published experiments have replicated the results. The various methods thus far employed to investigate the possibility of regenerating inner ear cells include genetic manipulation, gene therapy, and stem cell transplantation (Cotanche, 2008; Vlastarakos, Nikolopoulos, Tavoulari, Kiprouli, & Ferekidis, 2008). Genetic manipulation involves turning off particular genes that would prevent hair cells from proliferating. Gene therapy involves inserting genes that signal supporting cells to divide and transform into hair cells. Stem cell transplantation involves replacement of hair cells.

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The stem cell can be derived from either embryonic or adult cells, and can be harvested from various sources including the inner ear epithelium, bone marrow, and ventricular zone of the brain.

Many review articles have been recently published. These reviews have discussed various aspects of current investigations. The purpose of this literature review is to fully describe the most recent developments in discovering and investigating new sources of stem cells, generating inner ear cells and the possibility of using these stem cells to replacing damaged inner ear cells *in vivo*. This literature review will be limited to issues regarding stem cell replacement therapies, an intensive review of using bone marrow transplants and other different types of sources of stem cells, how they can be differentiated into hair cells *in vitro*, and then the various studies that have been published on injecting these stem cells *in vivo* will be discussed. The main question to be answered is "What is the current state of stem cell transplantation to replace inner ear cells?"

Three areas will be covered in this literature review: (a) research conducted on the avian model since avian spontaneous regeneration was first documented; (b) the different sources of stem cells used *in vitro* studies; and (c) the different studies utilizing *in vivo* transplantation of stem cells.

METHODS

An initial search for the mammalian transplantation studies, using Pubmed via Endnote was performed using the search limits "inner ear" and "stem cell" from the years 2005 to 2010; the most recent search was accomplished on January 18, 2011. This yielded 297 articles in total over the 6 year span. Each title was manually read, and any paper that was not related to inner cell regeneration or repair was discarded. If the title was ambiguous, the abstract was then read. Inclusion criteria included cell regeneration in inner ear, or spleen. Articles not written in English also were discarded. Application of this process yielded 20 articles. Review articles were read in full, and a secondary list was made of relevant research papers cited by these review articles, which resulted in an additional 13 articles, bringing the total number of articles in this literature review to 30. These articles were further sorted into articles that dealt with 1) different sources of stem cells, 2) generation of a hair cell using stem cells, and 3) transplantation of the different types of stem cells into a recipient animal model.

RESULTS

Of the 30 articles reviewed, 16 related to different sources of stem cells, 3 related to generation of hair cells, and 14 related to transplantation studies. For the transplantation studies, various aspects examined included the donor cell type, recipient type, total number (n) per cell type, and type of experimental animal.

To illustrate both the explosion of interest with stem cell-based therapies and inner ear repair, an analysis was performed by running a search on published papers spanning an 11 year period from 2000 – 2010 comparing total numbers of papers involving the inner ear and stem cells, and comparing papers on stem cell implantation and gene therapy.

Various searches were run using PubMed via EndNote, with all searches confined to 2000 to 2010 and the English language. The searches were conducted as follows: for all searches, "stem cell" was searched in the abstract field, AND "inner ear" under all fields command, was used. The articles found using these field searches yielded the numbers for the "stem cell" articles. For *Transplant* articles: NOT "review" in all fields AND "transplant" in all fields, were used. For *Gene* studies, all fields contain "gene" with the NOT "review" command was used. For the *Review* articles the AND all fields contain "review" command, was used.
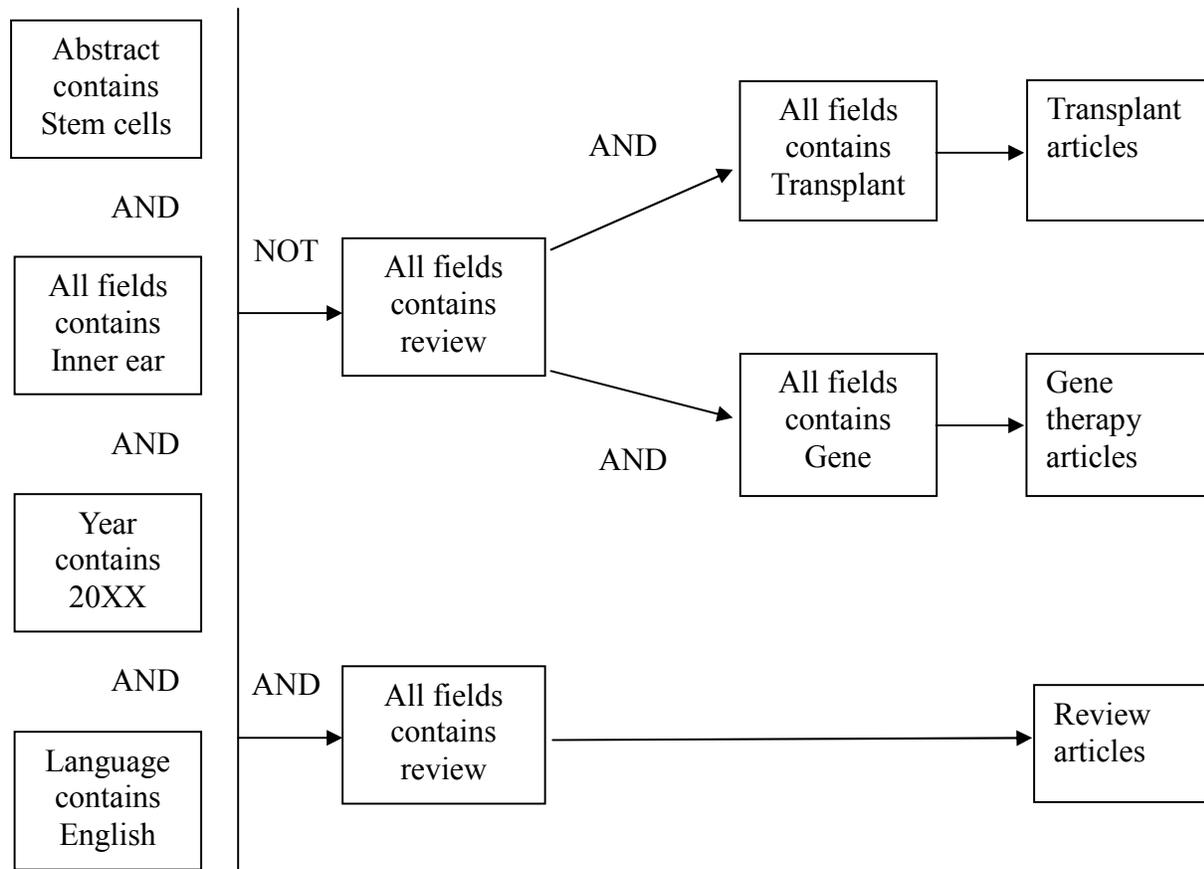
Figure 1. Flow chart paradigm for obtaining articles from January 1st, 2000 to December 31st, 2010
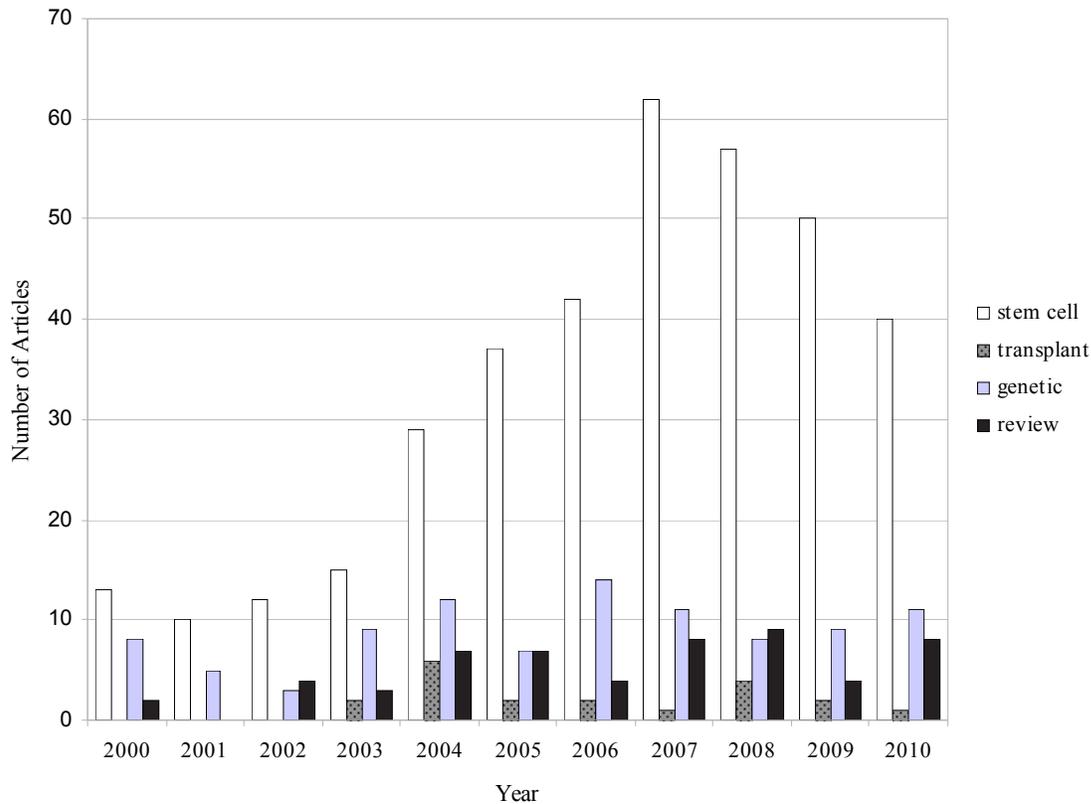
Figure 2. Inner ear regeneration articles published between January 1st 2000 and December 31st 2010, with keywords "stem cell", "transplant" "gene" and "review".

As can be seen from Figure 2 articles published concerning stem cells and inner ears increased from 13 in 2000, to over 60 in 2007, with a slight reduction over the next 4 years to 40 articles in 2010. This represents a 4.77-fold increase from 2000 to 2007, and a 3.08-fold increase from 2000 to 2010. Also apparent from this graph, from 2000 to 2002 there were few to no articles found concerning stem cell transplantation into the inner ear, but a jump in number of articles in 2004 and 2008. The four to eight year span may represent the length of time it takes to extract stem cells, transplant the cells, and then analyze the data (count the cells etc.). The number of articles concerning genes involved in the inner ear has been relatively consistent from

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2003 to 2010, with a peak at 2006. Interestingly, review articles outnumbered experimental articles on transplantation throughout the 11 year time period, with the exception of the year 2002. Review articles 50% of the time since 2002 were essentially at the same numbers as gene articles, the most notable difference in 2006 where the number of gene articles peaked and review articles experienced a decline. Undoubtedly, factors contributing to the number of articles include interest, funding, and time course of experiments.

The avian studies on hair cell regeneration were the impetus for studies on regeneration of the inner ear cells in the mammal. Although spontaneous recovery of hearing loss (except from temporary threshold shifts due to noise and some ototoxic medications) has not been observed in humans and other mammals, Corwin and Cotanche (1988), and Ryals and Rubel (1988) showed that in the bird, spontaneous recovery from noise exposure does occur.

Investigating the mechanisms behind avian auditory system recovery represents a starting point to search for methods to regenerate damaged hair cells in the mammalian system. First the initial investigations on avian regeneration will be described and next, current research into mammalian regeneration will be discussed.

Avian Research

Regeneration of Avian Inner Ear Cells

Both the Corwin & Cotanche (1988) and Ryal and Rubels (1988) experiments used thymidine-^{3}H as a radioactive tracer of mitotic activity to track cell division and regeneration of hair cells. Corwin and Cotanche used 9 to 13 day old chickens with a noise exposure of a 1.5kHz tone at 115-120dB sound pressure level for 48 hours, ($n = 1$ for controls, unstated for

experimental animals). From their findings they suggest that the labeled cells found post trauma originated from a different type of cell (a supporting cell) which replicates then differentiates into a hair cell. In the Ryal and Rubels (1988) experiment, the sexually mature quail with 10, 30 and 60 day survival times, ($n = 5$, 6 and 3) and a noise exposure time of 12 hours with a 115dB sound pressure was used. Ryal and Rubels (1988) were able to report the location of cell recovery on the papilla (the cochlea analog which houses mechanosensory cells with similar toxicities to noise and aminoglycosides as the mammalian sensory cells); and to show that age and extent of noise exposure is not a factor in recovery. Both research experiments were able to report thymidine-^{3}H labeled cells in the papilla post trauma which suggest mitotic activity in the adult avian. Mitotic activity is noted in an organ if replacement of cells is occurring (i.e., the cells have re-entered the cell cycle of duplication/replication and division). Ryal and Rubels (1988) found that the replacement of cells were progressive, with 30% of the middle of the papilla undamaged at 10 days, to essentially full recovery at 60 days. Thus, these papers were two of the first to document spontaneous hair cell recovery in the avian.

A subsequent paper by Lippe, Westbrook, & Ryals (1991) used gentamicin sulfate instead of noise exposure to induce hair cell damage in White Leghorn post natal day 3 (P3) chickens ($n = 6$) with control chickens ($n = 2$) which were reared with the experimental animals. They also evaluated mitosis activity using thymidine-^{3}H labeling. They found labeled cells only in damaged regions: hair cells and support cells, and very few in the auditory nerve, and their conclusion was that recovery of hair cells with aminoglycoside damage also resulted from the production of new cells by mitosis.

McFadden and Saunders (1989), using 1-day-old chicks and a noise exposure time of 48 hours, examined the effect of using a greater dosage of noise trauma, and investigated whether evidence of early recovery could be demonstrated. They sought evidence of recovery through evaluation of the cochlear nucleus auditory evoked potentials (ABR) rather than through examination of mitotic activity and structural recovery. For evoked potential evaluation, the electrode was inserted into the brainstem while 1.0kHz tone bursts at 115dB stimulation was used and the depth was adjusted until a maximum response was achieved (200-250µV). Latency, amplitude and waveform were compared to age matched control animals. They found complete recovery of the 0.9kHz tuning curves within 6 days post trauma, and of the 1.5-2.5kHz tuning curves at 9 days post trauma. By 15 days post treatment, essentially complete recovery of auditory function was observed.

Janas, Cotanche, and Rubel (1995) also used aminoglycoside toxicity to report on morphological changes. They used a single injection of a high dose of gentamicin to determine the extent of damage and the course of recovery from 3-5 days up to 5 weeks. They utilized three groups with different post-injection survival times: short term (3-5 days), intermediate (2 weeks) and long-term (5 weeks). They examined morphology using scanning electron microscopy (SEM) and quantified hair cell recovery on 4-7 chicks from each group. They found that immature stereocilia appeared by 3 days post injection; by two weeks post injection, the morphology of the hair cell appeared to return to normal.

Stone, Choi, Woolley, Yamashita, & Rubel (1999) investigated ongoing regeneration of cells in *undamaged utricles* as well as gentamicin damaged cochlea epithelia of post hatch day 7 (P7) White Leghorn chicks. For the undamaged utricle experiments, P7 chicks were sacrificed

from 2 or 6 hours, ($n = 5$-7) to 110 days ($n = 2$) post bromodeoxyuridine, (BrdU) injection. BrdU is a thymidine analog that is incorporated into nuclei during the DNA synthesis (S) phase of the cell cycle and is passed on to daughter cells during mitosis. BrdU was chosen rather than thymidine-^{3}H to study stem cell proliferation (replication and division) because it is rapid and non-radioactive. It is visualized by histology (mounted on slides and visualized via microscope). Stone et al., (1999) found that most labeled cells formed in pairs, one in the hair cell layer, the other in the supporting cell layer, suggesting that cells differentiate asymmetrically. They observed gradual decline in the proliferative cells during the first two post-natal weeks (during normal development). Some of the cells exited the cell cycle by gradual maturity and apoptosis. For the basilar papilla experiments they used various time courses between BrdU and thymidine-^{3}H injections after a single injection of gentamicin into P7 chicks. The main aim of this was to find double labeled cells which would indicate recycling of supporting cells to replicate. They reported only 1-3% of the total number of progenitor cells divide more that once.

Warchol & Corwin (1996) used laser microsurgery instead of ototoxic treatment or noise exposure to destroy individual hair cells in hatchling chick cochlea ($n = 60$) organ culture to have precise control over damaged hair cells to document onset of proliferation. Spatially patterned hair cell lesions were made in the sensory epithelia. Using laser microsurgery, they were able to destroy as few as 7 hair cells at one time. Using both thymidine-^{3}H and BrdU for labeling, separately and together, they found that the supporting cells began to proliferate at approximately 16 hours post laser injury. Proliferation of supporting cells was observed at about 200 μm from the lesions. These results suggest that the supporting cells are precursors of the regenerated hair

cells in the chick. They suggest that the proliferation of these cells are mitigated by signals that originate within the damaged epithelium.

Other species besides the avian have been found to regenerate hair cells spontaneously after gentamicin damage. Faucher, Aas-Hansen, Damsgard, Laukli, & Stenklev (2009) investigated the time course of recovery of fish inner ear (in this case, Atlantic cod) subsequent to local injection of gentamicin directly into the inner ear sacculus. The auditory evoked potentials (AEP) (ABR equivalent in Atlantic cod) were obtained for functional auditory assessment. SEM was used to investigate morphological changes. The AEP threshold was measured at 250 Hz (previous studies have shown that the lowest thresholds are obtained at this frequency). There were three groups (each with $n = 2$); control, sham (injection with saline solution) and treated fish. Over a three- week period, AEP response thresholds were measured weekly: once a week for sham and control fish, and three per week for the experimental fish. They found morphological improvement after 14 days, but AEP threshold improvement did not occur until a further 4 days had passed. The investigators did not report the obtained AEP thresholds and did not evaluate the mitotic state of the cells.

These series of investigations show that birds and fish can regenerate hair cells spontaneously, and with the case of the birds, supporting cells are kept in the mitotic state and can differentiate into hair cells.

Regeneration of the Avian Auditory Nervous System

Park, Girod, & Durham (2002) using adult chickens ($n = 6$) with gentamicin damage, labeled brainstem auditory neurons in the nucleus magnocellularis (the chicken homolog of the

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antero-ventral cochlea nucleus spherical bushy cells) using thymidine-^{3}H as a marker of mitotic activity, with gentamicin as the ototoxic treatment. The gentamicin causes a cochlear lesion which affects afferent input to the rostral high frequency half of the nucleus magnocellularis, according to frequency place maps of the chicken cochlea and the nucleus magnocellularis.

The gentamicin-treated, but not control cochleae, displayed hair cell damage and regeneration in the basal regions. No differences in glial cells between the experimental and control animals were noted; labeled glial cells were noted in both groups and were found throughout the rostral to caudal regions of the nucleus. Labeled glial cells were also noted throughout the surrounding brainstem and cerebellar regions of both groups.

Labeled neurons were found in both treated and untreated chickens, but more labeling was observed in the treated than control animals. Labeled neurons were found throughout the nucleus magnocellularis, but they were not found in the non-auditory abducens nucleus in either groups. (It is assumed that the investigators were reporting on labeled neurons found in other areas of close proximity to report on the extent to which the neurons reach in the central nervous system: regenerating neurons are desired to localize only to auditory neurons). Interestingly the cerebellum also displayed many labeled cells, but no differences in labeled cells occurred between groups. This may indicate that spontaneous regeneration is occurring throughout the CNS regardless of damage.

Park et al. (2002) concluded that the auditory central nervous system (CNS) retains some plasticity (ability for neurons to change in organization or function) in the avian, but further studies are needed to deduce the progenitors of the nucleus magnocellularis cells. They suggested three possibilities for the progenitors: the nucleus magnocellularis neurons, nucleus

magnocellularis glia, and stem cells in the ventricular zone. It is unlikely that the source is nucleus magnocellularis neurons, as no cell division was observed at that location. The nucleus magnocellularis glia is a possible candidate as glia have been shown to proliferate at low levels in control animals. Ventricular zone stem cells represent another possibility as they can migrate into the nucleus magnocellularis and differentiate into neurons. This was a well-documented study, particularly as the experimenter counting the labeled cells was blinded to group status (control vs. experimental).

Investigations into regeneration of the avian inner ear have shown that support cells are triggered into proliferation and then differentiate into hair cells. These studies on the avian auditory system have spear-headed research concentrating on repairing the inner ear cells of mammals. The remainder of this paper will focus on research conducted on mammalian models.

Mammalian Inner Ear Cell Regeneration

Various strategies for repair of damaged hair cells are under investigation, including gene based therapies and stem cell-based treatments. Researchers have demonstrated that stem cells can be grafted onto certain organ cells to replace damaged cells, and repair genes theoretically can be introduced to the inner ear via viral vectors. Many review articles have given a summary of these various approaches. This paper will give a detailed analysis of current research into stem cell approaches.

Stem Cells: Different Types

Stem cells can be characterized in a variety of ways. Before describing the different sources of stem cell, some definitions regarding different types of stem cells will be given.

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Undifferentiated stem cells are cells that have not yet specialized into a particular type of cell. Toti-potent stem cells such as an embryonic stem cells (ESCs) can differentiate into any type of specialized cell. A pluri-potent cell can specialize into any cell from the three germ layers (ectoderm, mesoderm and endoderm) but cannot generate all types of cells, as can an ESC. An inducible pluri-potent stem cell (iPSC) can be triggered by certain molecules (called triggering factors) into differentiating into a specialized cell. A multi-potent stem cell can divide into a multivariant of the same type of cell (for example, different types of blood cells), but cannot divide into a cell of a different organ.

Cell transplantation occurs when a cell or a group of cells extracted from a donor is injected into a target site of a recipient. An autograft is a transplant from one part of an organism into a new position of the same organism. An allograft is a transplant from one organism to another, where the donor is genetically different from the recipient but still remains a member of the same species. A xenograft is a transplant from an animal from one species to a recipient of another species. Since an autograft most closely resembles the organism's other tissues, it offers the least autoimmune rejection and, therefore, is often the most desirable of the three options. However autoimmunity may also occur.

As previously discussed, stem cells can be embryonic or adult. The next section will cover studies covering differing sources of embryonic cells from mice, rat and other mammalian models, as well as different sources for adult stem cells such as bone-marrow cells, cells from the lateral ventricular epithelium, as well as from the inner ear.

Most of the studies investigating difference sources for stem cells presented here followed similar protocols: they first isolated the cell from the tissue of interest, cultivated in cell culture in a medium, usually DMEM (Dulbecco's Modified Eagle's medium), to supply the cells with necessary nutrients for cell survival, and usually growth factors were added to enhance proliferation. Depending on the particular study further growth factors or triggering factors were added, to enhance differentiation or partial differentiation into the particular type of cell desired, such as ATOH1 for hair cell differentiation, and EGF and IGF-1, N2 and B27 for neuronal growth.

ESCs from mammalian vestibular and cochlea epithelia.

Li, Roblin, Liu, & Heller (2003) showed that sensory ESCs from the vestibular system are pluri-potent in the mouse as they have a high proliferative capacity and can differentiate into liver, heart, muscle and skin cells. A defining factor of these pluri-potent vestibular stem cells is their ability to form clonal, free-floating colonies, called spheres. Their study will be discussed in more detail under the heading of production of hair cells.

Progenitor and stem cells from the epithelia of postnatal mice: classification of 3 distinct sphere formations.

Diensthuber, Oshima, & Heller (2009) studied stem and progenitor (precursors to a particular differentiation path) cells from the sensory epithelia of newborn postnatal day 1, BALB/c mice and Math1/nuclear green fluorescent protein (nGFP) transgenic mice, (n = 3 to 4 for each experiment). After enzymatic and mechanical separation, and placing the single cell

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suspensions into non-adherent (free-floating) culture dishes they observed different types of morphologically distinct spheres. They were able to classify three separate sphere types: solid, hollow and transitional. Solid types were the smallest, hollow types were the largest. Transitional spheres were observed to have both solid and hollow regions and were generally larger than solid types. Hollow spheres were translucent, and displayed large and polygon-shaped cells. These cells were reported to appear more buoyant than the other two.

In a series of experiments the authors found that hollow spheres were formed from solid spheres, and that hollow spheres lost their ability for self-renewal. Thus their conclusions were that solid sphere types are more suitable for hair cell regeneration grafting experiments than the other two types of spheres.

Stem cells from the lesser epithelial ridge of neonatal rats.

Zhai et al. (2005) isolated cells from neonatal rat (P0-P1) cochleae, either from the lesser epithelial ridge or the outer spiral sulcus cells. These cells were cultured with epidermal growth factor (EGF) and were observed to proliferate and express selective lesser epithelial ridge cell markers. These lesser epithelial ridge cells formed spheres when separated from monolayer cultures and re-plated in aggregate cultures. Adeno-vectors were used to attach Math 1- EGFP or ad-EGFP with Myosin VIIA or EGFP/Myosin VI double immunocytochemistry markers to the cells to test for hair cell phenotype. Math 1 is a transcription factor known to be expressed when a cell is undergoing differentiation into a hair cell (or a hair cell-like cell). EGFP is an enhanced green fluorescent protein expressed when a cell proliferates (goes through the cell cycle process of cell division). The cells in the lesser epithelial ridge spheres were then co-cultured with

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dissociated mesenchymal cells prepared from postnatal rat utricular whole mounts. The lesser epithelial ridge spheres were tested for proliferation by using BrdU (previously described) and a large number of dividing cells were observed in the lesser epithelial ridge area as well as some in the inner sulcus cells. These cells were isolated and infected with Math 1-EGFP, and some became Myosin VIIA-positive, demonstrating that Math or Hath1 is sufficient for these cells to differentiate into a hair cell-like cell. Those infected with only Ad-eGFP did not convert to Myosin VIIA-positive cells, further supporting that Hath1 is required to trigger the cells into differentiation.

The authors showed that cells from the lesser epithelial ridge of neonatal mice show stem cell like properties and may be used in transplantation studies.

Multipotent stem cells from post-natal day 7 (P7) rat cochlea.

Lou, Zhang, & Yuan (2007) also used rats, but instead of neonatal rats, cells were taken from P7 Sprague-Dawley rat cochlea to study the ability of these cells to proliferate and differentiate into inner ear cells. Cells were extracted from rat cochlea ($n = 10$) and cultured with epidermal growth factor, (EGF) and basic fibroblast growth factor-2 (bFGF-2). These cells isolated from the organ of Corti formed 3±1 (number of labeled cells ± standard deviation, with *n* = 4 independent experiments) floating spherical shaped spheres after 8 days in culture. These numbers were blind-counted. Cells from these spheres were then cultured and passaged up to 10 times. These cells were labeled with markers for the following: Nestin (for stem cell), BrdU (for proliferation), Myosin VII A (for hair cells), p27kip (for supporting cells), map2 neurofilament (for neurons) and GFAP (for spiral ganglia growth). Nestin positive cells were counted using

counter staining with DAPI, and none were found on the adult rat cochlea sections. The following cells were reported as a percentage of total cells±standard error: 1±0.5% were Myosin VIIA positive and 3±1% were P27kip1-positive. All the differentiating cells were nestin-negative. The sphere-derived cells also expressed neuron-specific MAP-2 (7±1.7%) or neurofilament (7±0.7%) and glial fibrilary acidic protein (GFAP) (5±1.3%). It was noted that no nestin-positive cells were found in the adult cochlea uncultured cells. Stem cells were only found in the organ of Corti, including the basal membrane.

The authors concluded that stem cells can be isolated from young adult rat cochlea, up until a least a week old, and these cells can be cultured to produce cells that label for inner ear cell types.

Adult stem cells from the lateral ventricle

Wei et al. (2008) discussed the possibility of adult stem cells found in the lateral ventricle to be used to repair inner ear cells and spiral ganglia neurons as they have similar morphology and function. Using markers for proliferation and Myosin IIA (a hair cell marker), they showed that these adult stem cell could be used to produce hair cell-like cells. They reported that the ependymal cells assumed the hair cell-structural phenotype by performing immunostaining for Myosin VIIA *in vivo* (that is, using ependymal brain slices of cell layers), further labeling of ribeye (a hair cell synaptic protein) and Myosin VI markers (indicating a hair cell type of cell). Both cultured (*in vitro*) and *in vivo* cells expressed markers for hair cells.

Myosin VIIA positive ependymal cells showed functional characteristics of hair cells and were able to be incorporated into cochlear sensory epithelia: the cells were co-cultured with ESC

from Myosin VIIA -GFP transgenic mice with SGNs prepared from wild type (control) mice. They showed characteristic synaptic structures such as pre-synaptic vesicles, pre- and post-synaptic membranes, and a specialized synaptic cleft. Hair cells and ependymal cells expressed partially open large-conductance cation channels that were shown to be permeable to FM2-43, a dye with a small molecular structure used to indicate similarity to mechanical-sensitive channels in the hair cells. The hair cells were shown to have rapid uptake of FM2-43 that was inhibited by dihydrostreptomycin (an aminoglycoside). This is also indicative of hair cell-like qualities. In 3 of 7 synapses, there were responses between the Myosin VIIA-positive ependymal cells and SGNs. They isolated ependymal cells from the Myosin VIIA-GFP transgenic mice line and co-cultured these cells with cochlear sensory epithelia for 5 days. They qualitatively reported that the ependymal cells engrafted into the epithelial cells, without reporting a quantitative analysis. A question that must be asked is how feasible it is to obtain ependymal cells from the lateral ventricle in an adult, and then to transplant these cells into the inner ear.

Post-mortem stem cells from murine vestibular and cochlear stem cells.

Senn, Oshima, Teo, Grimm, & Heller (2007) isolated stem cells from the vestibular and cochlear epithelia from postmortem mice at 1,2,3,4,5,10 and 15 days post sacrifice. Animals were either newborn (P1 or P2) or 3- week old (P21), Math-1 nuclear green fluorescent protein (nGFP) mice. At the various times postmortem, cells were taken from the utricular maculae, organ of Corti, and the spiral ganglia and placed in culture for 7 days. They reported sphere numbers as spheres per 10^4 living cells as compared to 10^4 total cells used in the studies by Li et al. (2003) and Oshima et al. (2007).

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Propagation of isolated spheres was repeated at 5 day intervals, and fresh growth factors were added daily. The number of spheres was counted for each generation to ascertain the feasibility of longer term propagations. Half this number was used to produce the next generation.

The number of cells labeled with nGFP was counted in all three areas at 5, 10 and 15 days postmortem. They found that there was not a significant difference between 5 and 10 days, but there was significant reduction in cells between 10 and 15 days postmortem. The ratio of living versus dead cells in the total cell population of the organ of Corti was reported to decline gradually from 0.62-0.68, to 0.28-0.41.

At 15 days, none of the individual inner ear organs produced significant numbers of spheres under culture (1 single sphere was found in the utricle, $n = 3$). During the first 24 hours postmortem, the utricle had the fewest numbers of formed spheres per 10^4 living cells, with mean $= 168.5$ and SEM $= \pm5.4$ for organ of Corti spheres and 211.8 ± 18.2 for spiral ganglion cells ($n = 3$).

They found about 30 spheres per 10^4 cells were viable stem cells. The main conclusion that can be drawn from this study is that cells extracted from the organ of Corti from postmortem newborn and neonatal mice can produce solid spheres which can be renewed, that is, have stem cell qualities, for up to 10 days postmortem.

ESC-derived neuronal progenitors co-cultured with mouse auditory epithelia.

Matsumoto et al. (2008) studied the ability of murine ESC-derived neuronal progenitors to form synapses with auditory hair cells in epithelial explants from 3 day postnatal mice. The progenitor cells were co-cultured with the auditory epithelia for 7 days. They used

immunohistochemical analysis on cryostat (frozen) slices ($n = 15$, $n = 2$ controls) and transmission electron microscopy (TEM) for the whole mounts ($n = 5$ co-cultures and $n = 2$ controls) to identify and locate neurite and synaptic formation. For staining purposes they used Myosin VIIA to label hair cell location in the auditory epithelia and beta III tubilin for the ESC-derived neurons and their neurites. The study concentrated on neurite procession of ESC-derived neurons and the connection between hair cells and extended neurites. They concluded that murine ESCs co-cultured with postnatal auditory epithelia can differentiate and form synapses with the inner hair cells of the postnatal mouse, however, only three out of twenty co-cultured tissues contained ESC-derived neurites. These neurites thread through the auditory epithelium in a similar fashion as does cross-tunnel fibers.

The researchers also found that 7 days post implantation ESC-derived neurons formed colonies inside a single row of IHCs and projected their neurites toward IHCs of the auditory epithelia. ESC-derived neurons beneath the auditory epithelia were found to attach to the basal area of the IHCs. This is the location of type 1 SGN nerve fibers and nerve endings attachment *in vivo*. TEM analysis revealed contacts between ESC-derived vesiculated nerve endings and inner hair cell membranes, as well as synaptic densities in the membrane between IHCs and the nerve endings. Reportedly this is the first evidence of synapse formation between ESC-derived neurons and IHCs.

Stem Cells from the cochlea of 1 day to 4 month old mice.

Oshima et al. (2007) studied cells taken from the organ of Corti, the spiral ganglion and the stria vascularis of 1 day old to 4 month old Math 1/nGFP mice. They isolated cells from the

utricular maculae, saccular maculae, ampullary cristae, organs of Corti, spiral ganglia and striae vascularis from three to four Math-1/nGPF mice. They found that all tested areas can supply cells which, when cultured in EFG and bFGF, can form spheres. They differentiated cells from third generation spheres. Using RT-PCR, they found low or no expression of mature cell markers in the spheres. Cells from these spheres were able to be propagated for many generations which indicated that these cells were stem cells. Cells from the organ of Corti were found to lose their ability to propagate (approximately 100-fold) between postnatal weeks 2 and 3. Sphere-forming cells from the vestibular and cochlear sensory epithelia once differentiated, formed hair cell-like cells that had functional features similar to newly forming hair cells. Cells isolated from the spiral ganglia produced neurospheres that could be differentiated into neurons and glial cells.

Factors increasing the yield of generated cells: neurons from THY-1 or ATOH1-nGFP 1-3 day old mice utricular maculae.

Martinez-Monedero, Yi, Oshima, Glowatzki, & Edge (2008) obtained cells from 4-10 utricular maculae from 1-3 day old mice, that were either C57BL/6 mice, Thy1-CFP transgenic, or ATOH-1-nGFP, to investigate methods to increase the yield of cells that exhibit markers of inner ear cell progenitors (stem cells).

Cells were cultured to obtain spheres, for between 5-7 days. DMEM in conjunction with growth factors including EGF and IGF-1, N2 and B27 (for neuronal growth) were used as supplements. BrdU was used as a marker for proliferation. RNA was isolated and RT-PCR was used for qualitative analysis of *GATA3* (transcription factor expressed during development of spiral ganglion neuron marker), *Islet1, Pax 2,* (early markers of developing inner ear), *Pax 6*

(which has an opposing role with *Pax2* in development)*, Phox2b* (a marker of autonomic neurons in neural crest progenitors) and *GAPDH* (an enzyme that has also been implicated in transcription) expression.

In their study of murine utricular maculae stem cells, the investigators found that retinoic acid increased both the yield of neurons (based on marker expressions of *Pax 2*) and the percentage of differentiated sensory neurons obtained as compared to co-treatment with *noggin* and *sonic hedgehog* (two transcription factors known to be involved in early cell development). Addition of retinoic acid yielded 12.87 ±2.1% (*n* = 7) of neurons per total number of cells, *noggin* treatment yielded 6.42 ± 1.7% (*n* = 7) and *sonic hedgehog* 8.00± 2.1% (*n* = 7). The researchers also found that retinoic acid had a greater effect on promoting beta-III tubulin-positive cells, (p<0.05), Math-1 positive cells, and glial cells, compared to culture with *noggin* and *sonic hedgehog*.

The authors were also interested in tracking the differentiation of these stem cells into neuronal cells, to see if the process is the same as observed in the developing embryo. They found similar expression of early auditory and vestibular markers. After 3 days of differentiation of cells, embryonic auditory and other sensory neuronal markers *GATA3, Brn3a* and *islet1* were expressed, and after 10 days sensory phenotypical markers *peripherin, calretinin, TrkC* and *TrkB* were observed. Thus they concluded inner stem cells use a similar differentiation pathway taken by progenitors in embryonic development. This provides further evidence that inner ear stem cells have the potential to replace damaged afferent neurons.

Stem cells from the spleen.

Some approaches attempt to turn somatic cells into stem cells by introducing key transcription factors by retroviral vector or other vectors, a process called induced pluri-potent stem cells (iPSC). Splenic stem cells naturally express key transcription factors such as OCT4, SOX2, c-MYC and KLF4, which are used to induce somatic cells to produce pluri-potent stem cells.

Lonyai, Kodama, Burger, Davis, & Faustman (2008) found that stem cells taken from the spleen improve the degree of hearing loss associated with type 1 diabetes. Using stem cells from the spleen is a much safer method than other methods such as removing cells from the inner ear, and the lateral ventricle: that is, stem cells can be harvested from the spleen without serious risk of damage to the donor.

Dieguez-Acuna et al. (2010) characterized the set of unique proteins of CD45− (non-lymphoid) splenic stem cells, and compared these cells with CD45+ (lymphoid) cells. The CD45− stem cell-specific proteins were identical to those in iPSCs including OCT3/4, SOX2, KLF4, c-MYC and NANOG. They also expressed HOX11, GLI3, WNT2, and ADAM12, the benchmark transcription factors of embryonic stem cells. As they were attempting to identify stem cells that were not likely to cause tumors, they split the analysis into two: HOX11+CD45+ and HOX11+CD45- protein expressing stem cells.

They report that their discovery of HOX11+CD45- cells in the spleen that naturally express these key transcription factors for multi-lineage potential, avoids many of the pitfalls associated with using methods which may produce oncogenic transformations (tumor formation due to proliferating cells). Oncogenic transformation is more likely to occur when an attempt is

made to turn somatic cells into stem cells when introducing necessary transcription factors using retroviral vectors.

The experiment by Dieguez-Acuna et al. (2009) identified 809 proteins unique to the spleen's DC45- stem cell population relative to its CD45+ cells. Of the 809 proteins, they found 98 bore developmental functions. They identified the protein difference between splenic stem cells and cancer cells of closely related lineages. Although they did not discuss specific transcription factors known to induce hair cells, their protocol is a promising tool for further research into characterizing likely stem cell candidates.

Generating New Hairs Cells Using Stem Cells

In three experiments, to be discussed in detail presently, hair cell-like cells were produced from ESCs *in vitro*. Li, et al. (2003) compared three different ESCs from mouse lines and were able to produce progenitor cells. These cells were found to express hair cell markers, produce hair cell bundles, and be able to graft onto the epithelial layers that were damaged. Oshima et al., (2010) using both mouse ESCs and mouse iPSCs, were able to produce cells more efficiently than those produced in the experiment conducted by Li et al., (2003). The hair cell-like cells produced by Oshima were self-reported to have morphological and transduction-current similarities to hair cells *in vivo*. Chen et al. (2009) used human fetal ESCs to produce cells that expressed markers and had functional and electrophysical similarities to hair cells *in vivo*. These three experiments will now be more fully described to illustrate the method and complexity of generating hair cells (or hair cell-like cells) *in vitro*.

Li et al. (2003) used three murine ESC cell lines, (R1, YC5/EYFP, and ROSA26) to generate hair cells. The ESCs were maintained on mitotically inactivated primary mouse embryonic fibroblast feeders or on gelatin-coated culture plates with leukemia inhibitory factor in an ESC medium consisting of high glucose Dulbecco's modified Eagle's medium (DMEM) and other supplements. Embryoid bodies formed in 30 microlitre drops, each containing 300 ESCs Plating embryoid body-derived cells onto adhesive tissue culture surface initiated the step for progenitor cell enrichment. After an initial 16 hours of culture in ESC medium to promote adhesion, cells were incubated for 10 days in serum-free medium with supplements, EGF (epidermal growth factor) and IGF (insulin like growth factor). To further expand the cells, bFGF (basic Fibroblast growth factor) was used for 10 days. After selection of the progenitor cells, they removed the growth factors to initiate differentiation in serum-free medium with N2 supplements. The cells were analyzed by reverse transcription polymerase chain reaction or immunocytochemistry at 10-14 days of differentiation.

These progenitor cells were then transplanted into a chicken-mouse chimera (where zygotes from each animal are genetically engineered to develop together). The investigators reported that transplanted cells seemed to be able to engraft on damaged epithelial cells more easily that undamaged cells and these cells were able to adopt a phenotype similar to hair cells *in vivo*.

These cells expressed a battery of marker genes but the investigators did not examine any further similarities to hair cells (for example, did not examine morphology using SEM or transduction currents). In total, it took approximately 30-34 days to produce these hair cell-like cells.

Oshima et al. (2010) used transgenic mice (Math1/nGFP strain) which express an enhanced green fluorescent nuclear protein (nGFP) that is driven by an ATOH1 enhancer. ATOH1 is the single molecule required to differentiate pro-sensory cells into hair cells.

Oshima et al. (2010) generated a cell layer that was capable of differentiating into ectoderm that is, can be induced to become the otic layer Both ESCs and iPSCs (inducible pluripotent stem cells) were isolated from the same mouse model, and were compared to illustrate that they differentiated along similar otic descent. The ESCs and iPSCs both generated hair cell-like cells with stereociliary-like protrusions that were mechano-sensitive. Observation of voltage-dependent currents revealed no differences between the ESC and iPSC derived cells. Great differences were found, however, between the levels of currents with the major intracellular ion K+ ranging from 397pA to 4982pA (mean 2190 SD +/- 1595 ($n = 24$).

The authors stated that all sensory hair cells, unlike supporting cells, express nGFP when they begin to differentiate into nascent hair cells, and continuously express the protein until they reach adult status. Thus, isolated stem cells from this mouse can be identified at different stages of development.

Oshima et al. (2010) isolated four lines of ESCs that expressed typical ESC markers. These lines displayed ESC colony morphology when grown on mouse embryonic fibroblast feeders in the presence of leukemia inhibitory factor. All four Math1/nGFP ESC lines expressed the nGFP reporter.

To generate iPSC lines, Oshima et al. infected the Math1/nGFP transgenic mouse fibroblast using a retrovirus expressing OCT4, SOX2, KLF4 and cMYC transcription factors to induced the cells to become otic progenitors. Primary colonies were picked, sub-cloned and

expanded on mouse embryonic fibroblast feeder cells. These iPSC lines reportedly expressed both the typical ESC marker genes as well as the Math1/nGFP reporter.

Oshima et al. (2010) also reportedly randomly differentiated both the ESC and iPSC lines by generation of embryonic cell clusters, removal of Leukemia inhibitory factor (LIF) and culturing of the embryoid body cells prior to expression analysis. They analyzed expression of all three dermal layers by using markers for endo-, meso- and ectodermal layers such as *GATA6*, Brachyury, and microtubule associated protein 2 (MAP2), as confirmed by immunocytochemistry.

In differentiated cell populations, they reported that the nGFP reporter was either reduced or absent. Also, the cells that expressed the germline-specific markers consistently were nGFP negative. These observations reportedly indicate that the Math1/nGFP reporter is active in ESCs and iPSCs but is down-regulated in cells that have undergone differentiation.

Pluripotency of the ESC and iPSC lines was supported by the finding of typical teratoma tissue formation that contained all three germ layers when the lines were subcutaneously injected into immunodeficient mice.

Three gene transcription factors were used in isolation and in conjunction with each other to investigate growth efficacy: Dkk1, (a Wnt inhibitor), SIS, (selective inhibitor of Smad3, used to interfere with TGF-beta signaling), and IGF-1, a growth factor.

The researchers used different combinations and found that Wnt and TGF-signaling disruption using Dkk1 and SIS3 significantly reduced the number of Brachyury-positive cells from a mean of 65.0% (SE = ±14.9%) to a mean of 20.9% (SE = ± 6.9%) in ESC-derived cells and from a mean of 44.1% (SE = ± 9.6%) to 15.3% (SE = ± 6.5%) in iPSC-derived cells. For

Wnt and TGF-signaling disruption the combination of Dkk1, SIS3 and IGF-1 (D/S/I) was most effective, leading to a reduction of Brachyury-positive cells to a mean of 20.8% (SE = ± 13.1%) and of the GATA6-expressing cell population to a mean of 9.8% (SE= ± 5.0%) in ESC-derived cell populations. Similarly, the iPSC-derivative cells displayed reduction in the number of Brachyury-positive cells to a mean of 14.3% (SE = ± 5.8%) and in the number of GATA6-expressing cells to a mean of 12.1% (SE = ± 6.8%).

The Dkk1/SISI/IGF-1 treated cells were plated into gelatin-coated culture dishes and exposed to fibroblast growth factor to test for otic induction competence.

Oshima et al. (2010) used bFGF as a general otic inducer because they reported that it activates several different fibroblast growth factor receptor subtypes and has been previously used as a substitute for the proposed natural otic inducing FGF3 and FGF10 (Groves & Bronner-Fraser, 2000). They used PAX2 (as previously reported by Li et al, 2004) as a marker for otic induction.

At P3 day of treatment with basic fibroblast growth factor the number of Pax2-positive cells was greatest for Dkk1/SISI/IGF-1 treated cells. (They reported the number of particular cells marked, without reporting the overall number.)

They found that Dkk1 and SIS3 were mainly effective in suppressing endodermal and mesodermal lines.

The up-regulation of Pax2 in both the ESC and iPSC cultures were confirmed using reverse transcription polymerase chain reaction for DNA replication analysis. Oshima et al. (2010) concluded that PAX2 is not inner ear specific, but is co-expressed with the transcription factor ENGRAILED 1 when expressed in midbrain/hindbrain neural progenitors. Testing for

ENGRAILED 1, they found only 1.2% +/- 0.8% of ENGRAILED 1-positive cells, which all expressed PAX2, thus indicating the majority of PAX2-positive cells were otic progenitor cells. The strong up-regulation and co-expression of multiple early inner ear markers suggests that Dkk1/SISI/IGF-1 followed by basic fibroblast growth factor treatment sufficiently copies, in a culture dish, the process that induces cells to become the progenitors for the inner ear epithelia during normal embryonic development.

Following Li et al.'s (2003) protocol, Oshima et al. (2010) withdrew growth factors and serum from the culture on gelatin to initiate differentiation of ESC-generated otic progenitors. They observed up-regulation of hair cell markers, but the hair cell-like cells did not exhibit hair cell morphology.

NGFP-positive cells were detected using fibro-nectin, gelatin and mouse embryonic fibroblast feeders. Nonetheless, only a small sub-population was immunodeficient for the hair-cell marker Myosin VIIA (37 out of 1000). Hair cell bundles were not morphologically observed, nor were the marker for espin (hair cell bundle marker) expression. They subsequently plated the otic progenitors of mitotically inactivated chicken utricle stromal cells and then were able to observe patches of cells, although in small numbers (34 as compared with 0) that harbored nGFP-positive cells, espin and Myosin VIIA markers. In this group of inactivated chicken utricle stromal cells, hair bundle-like structures, with asymmetric tips and inter-stereociliary links were observed.

The authors then tested the cells response to mechanical stimulation. Using current displacement plots, they were able to obtain 24 positive responses from 42 ESC -derived and 10 iPSCs-derived cells.

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The researchers were able to show that the cell response of both types of derived hair cell-like hair cells were affected by an aminoglycoside, giving further evidence or corroboration that the elicited current was comparable to that evoked in native sensory hair cells. The voltage-dependent currents varied widely among individual cells, but no significant differences existed between the ESC and iPSC derived cells

Chen et al. (2009) defined a method to isolate human fetal auditory stem cells (hFASCs), reporting that their differentiating protocols can be used to study developing human cochlear neurons and hair cells, as well as serve as models for drug screening and toxicity experiments. Their method may also facilitate the development of cell-based therapies for hearing loss in humans.

So, whereas Oshima et al. (2010) used a murine model, Chen et al. (2009) identified and isolated a group of human auditory stem cells from 9-11 week old human fetal cochleae (hFASCs). They expanded these cells *in vitro* and these cells were able to retain their capacity to differentiate into sensory hair cells and neurons.

They also ran a separate set of experiments using one growth factor at a time to determine which growth factor was the most effective at producing progenitor cells.

Immuno-labeling methods were fully described, using antibodies for SOX2, NESTIN, p27kip1 and ATOH1, OCT4, and other markers for neuronal growth. Specific labeling was visualized with a secondary anti-mouse, anti-goat or anti-rabbit antibody or Rhodamine Red. Controls were performed by replacing the primary antibody with unspecific mouse or rabbit immuno-globulin G (antibody molecules).

The epithelial patches were placed in bFGF, EGF or IGF, or a combination of the three in a serum-free medium. After 72 hours, bFGF was found to support cell growth better than the other factors, with cells reaching a density of 98 ($\pm$ 13.1) cells per mm^2 against 49.8 ($\pm$ 8.0 cells per mm^2 (IGF) and 42.4 ($\pm$ 7.8) per mm^2 (EGF). The IGF only cells died after the first passage surviving for only a week. After a week post first passage, they observed a slight improvement in growth when using all three media (399.4 +/- 7.5 vs. 290.6 +/- 15.5 for bFGF alone, p < .001 (that is the effect was significant) or second passage (390.0 +/- 14.1 vs. 163.2 +/- 14.4 for bFGF alone, p < .001. Several independent stem cell lines from different donors (they report hFASC 1-5) were therefore derived and maintained in a culture media containing all three growth factors.

Interestingly this group expanded cells using a substrate that anchors the cells rather than using the free floating system. This anchoring tends to maintain monolayers of cells rather than producing floating spheres as described previously. These investigators argue that monolayers are less heterogeneous and culture conditions are easier to control.

Undergoing gene expression analysis, Chen et al. (2009) found that all cell lines expressed otic progenitor markers GATA3 and SOX2, as well as markers usually expressed by pluripotent embryonic stem cells (such as OCT4, NANOG, and REX1). The ABCG2 transporter marker was also observed, which according to the researchers has recently been reported in a population of supporting cells of the mouse cochlea with progenitory properties. Interestingly they found that the cells retained their proliferative ability for nearly 50 weeks, reaching 25-30 population doublings and were able to remain viable for several weeks after proliferation ceased. This was determined by using phospho-H3 staining.

Using immuno-fluorescent labeling at different passage intervals, they found that the cell lines remained positive for NESTIN, SOX2 and OCT4, and the epithelial origin of the cell lines was confirmed using cytokeratin staining.

They used different manipulations and culture conditions to explore the potential of these proliferated cells to differentiate into different inner ear lineages.

Trypsin dissociation produced cells with 82.7% of cells with neuronal characteristic bipolar morphology, whereas non-enzymatic dissociation produced only 4% of cells with neuronal characteristics. Thus, to maintain undifferentiated cells, non-enzymatic passaging was used for proliferation, whereas trypsin was used to trigger neuronal differentiation.

They found that the combination of Shh and bFGF was the most efficient in supporting neuronal differentiation as measured by bipolar morphology and BRN3A expression.

They also found that IGF-1 appeared to be important in preventing differentiation. They report that the best conditions to differentiate these hFESCs into neurons, are defined by splitting by trypsination, incubation with bFGF and Shh-*N* for 3 days, and then supplementing the culture media with BDNF and NT3.

Shh was removed from the media after 5 days and the cells were maintained in bFGF and neurotrophins. Neurons obtained in this manner expressed NEUROGENIN1, POU4F1, beta Tubilin III, NEUROFILAMENT 200 and PARVALABUMIN.

They also cultured the cells with retinoic acid and EGF, and found that about 90% retained their epithelial morphology forming small epithelial islands. About 85% of these epithelial like cells expressed ATOH1 (MATH1) and POU4F3 (BRN3C). They found that cells differentiated using EGF and retinoic acid also expressed the hair cell marker Myosin VII-A, and

some of these cells displayed a rearrangement of the actin cytoskeleton. They reported the formation of a circumferential ring and an actin-rich area reminiscent of the cuticular plate of the hair cell.

GFAP, which was also expressed in some cells, has been reported to be expressed by supporting cells along with with p27kip, a cell cycle inhibitor. GJB6 encoding CONNEXIN 30 and forming part of the gap junction between supporting cells *in vivo,* was also detected, using reverse transcription polymerase chain reaction. Thus these were defined as conditions to differentiate the stem cells into hair cell-like cells.

They measured membrane currents at 1 to 17 days post induction of differentiation and found that the potassium and calcium currents were characteristic of developing cochlear hair cells with cells differentiated under hair cell conditions (as opposed to neuronal cell conditions). Overall, about 56% of cells under hair cell conditions show hair cell phenotype, whereas 49% of cells produced under neuronal cell conditions show neuronal like bipolar morphology. They concluded that this is the first *in vitro* renewable stem cell system described obtained from the human cochlea.

Chen et al. (2009) were able to use human fetal auditory stem cells to produce hair cell-like cells and neuronal cells, with electrophysical similarities to *in vivo* nascent hair cells. However, in contrast with Oshima et al. (2010), Chen et al. (2009) were unable to produce cells with the formation of hair cell bundles. Chen et al. (2009) suggest that future experiments should aim to improve the conditions for hair bundle differentiation, as complex polarity cues are likely to be required. These cues are most likely to be absent in the culture conditions they used.

Chen et al. (2009) also discuss the interesting fact that differentiation was not triggered by just physically separating the cells. The notch signaling inhibitory pathways (i.e. inhibiting differentiation) has been thought to be controlled by ligands and receptors located in adjacent cells, and thus the signals to differentiate can be released by physically separating the cells. This is called lateral inhibition, and is mediated by delta/notch interactions. Thus it was unexpected that trypsin was required to induce differentiation in favor of non-enzymatic means. Future research, therefore, should investigate the role of this protease for induction to differentiate in human stem cells.

Some of the differences between the animal and human models were highlighted by Chen: IGF-1 although important for maintaining the cells in the undifferentiated state, failed to support growth and survival of the stem cells during isolation in the human model. The reverse was seen in rat, mouse and chick animal models. This emphasizes the importance of studying the human system as opposed to animal models.

Chen et al. (2009) suggest that further investigations should focus on optimizing the controlled differentiation of human ESCs into inner ear cells. The protocol employed by Chen et al. (2009) to isolate and expand hFACs, was designed using a chemically defined yet serum free medium without animal components. This, they report, should pave the way towards a method to derive clinical-grade cells which have potential therapeutic applications for humans.

Published studies involving the isolation of different type of stem cells and the subsequent transplantion into different hosts will now be discussed in detail.

Transplantation of Stem Cells into Adult Animal Models.

   Injection of embryonic stem cells (ESC)

   ESCs: Injection site: semicircular canal: male donor ubiquitously expressing the transgene EGFP and male recipient.

   Ahn et al. (2008) isolated ESC from blastocysts of inbred C57BL/6-green mouse ubiquitously expressing the transgene EGFP to investigate the survival of ESC after allotransplantation into the cochlea of other inbred C57BL/6 mice (*n* = 13*).* To maximize the number of proliferating stem cells found after transplantation, these investigators used male donors and male recipients, reducing the chance of rejection as has been observed when using female donors with male recipients. Alkaline phosphatase, stage-specific embryonic antigen 1, Oct-4 and Nanog were used to verify ESC pluripotency (ability to proliferate).

   Cisplatin was used in conjunction with kanamycin twice daily for 5 consecutive days, to deafen 15 four week old male mice. ABR was used to measure pre and post treatment thresholds to verify significant shifts for inclusion in the study and thus 13 of these mice were used for transplantation. The other two mice thresholds were not elevated sufficiently for inclusion (inclusion criteria were not explicitly given). Injection of cultivated ESCs was via the left posterior semicircular canal: the right inner ears were used for controls.

   Using histological methods (epifluorescence) grafted cells were found at the damaged organ of Corti in the scala media four weeks after implantation. ESC expressing EGFP were found incorporated into the area of the inner hair cells and the pillar cells underneath the tectorial membrane.

PCR amplification confirmed the incorporation of transplanted ESC in the cochlea. Hearing thresholds as measured by ABR were not found to be improved at 4 weeks post transplantation surgery. Incorporation of ESCs only occurred in two mice out of 13. Clusters of cells expressing EGFP were at the area of the stria vascularis and marginal cells of the scala media. Single cells were found inside the scala tympani. Some were also located in Rosenthal's canal close to the spiral ganglion neurons and auditory nerve fibers reaching the organ of Corti.

As it was found that some cells did indeed survive, this shows that survival of ESCs injected into the semicircular in the adult mouse is possible.

ESCs: Injection site: vestibular utriculus.

Praetorius, Vicario, & Schimmang (2008) transplanted murine ESCs into the vestibular utriculus via the bulla of adult C57BL/6 mice (*n* = 5, data not explicitly supplied). They suggested that the vestibular site of injection carried less risk of damage to the cochlear tissues than utilizing a cochleastomic procedure. The ESCs carried a beta-galactosidase gene integrated at the *ROSA26* locus (gene location) to enable immunostaining labeling for detection. Animals were sacrificed at only 4 days post injection. They found ESCs in the scala vestibuli and the scala tympani underneath the organ of Corti and attached to the spiral limbus, but not in the scala media, (quantitative data was not supplied). The researchers concluded that although damage to the cochlea was limited, cells did not reach the scala media. The short survival time of the animals post injection should also be noted, as other researchers have used longer periods to assess location and counts of ESCs in the cochleae.

ESCs: Injection site: vestibulocochlear nerve.

Regala, Duan, Zou, Salminen, & Olivius (2005) investigated how well ESCs extracted from E13-16 fetal mice survived in the vestibulocochlear nerve of both adult guinea pigs ($n = 3$) and Sprague-Dawley rats ($n = 46$) 2 and 4 weeks post transplantation. Adult neural stem cells (ANSC) were dissected from the lateral wall of the lateral brain ventricles of *ROSA26* transgenic mic and cultured to produce sufficient quantities for transplantation. Secondary neurospheres passaged from primary neurospheres were used for transplantation. 10 embryonic dorsal root ganglions were randomly transplanted into five hosts, giving two DRGs per host animal. There were five groups in the study: EGFP mouse DRG into rat vestibulocochlear nerve for 2 and 4 weeks ($n = 4$, $n = 8$ respectively) postoperative survival; LacZ mouse DRG in rat, 2 and 4 weeks, ($n = 6$, $n = 6$ respectively); EGFP mouse ESC into rat vestibulocochlear nerve for 2 and 4 weeks ($n = 4$, $n = 8$ respectively); LacZ mouse ESC in rat, 2 and 4 weeks, ($n = 6$, $n = 4$ respectively), and lastly, LacZ mouse marker for ANSC transplanted into the rat vestibulocochlear nerve for two ($n = 6$) and 4 weeks ($n = 6$). LacZ is used for neuronal stem cell identification.

Injection of ESCs was through the basal turn of the cochlea below the round window, through the osseous spiral lamina into the modiolus. The vestibulocochlear nerve was severed and four DRG halves were placed within the severed nerve in the modiolus. Cyclosporine was used for prevention and treatment of organ transplantation rejection reactions and doxycycline was used as an antibiotic to prevent infections in the perioperative period. They used histological methods to investigate location of ESC and DRG cells.

The investigators found ESC-derived cells at the site of injection and in the brainstem.

EGFP-positive DRG neurons survived in 75% of animals at 2 weeks, but only in 12% of animals at 4 weeks. The surviving cells appeared healthy, with axonal profiles seen growing within the proximal vestibulocochlear nerve, usually growing towards the brain stem. LacZ expressing mouse DRG in vestibulocochlear nerve were found in 83% of animals, at 2 weeks post transplantation, but at 4 weeks, none were observed out of 4 animals.

For the ESC mouse injected into rat animals: EGFP-positive cells were found at 2 weeks in 62% of animals, at 4 weeks: 25% of animals. In vestibulocochlear nerve, at 4 weeks some were seen at the brain stem. In the transplanted damaged guinea pig: 67% were observed to have EGFP-positive cells at 4 weeks. LacZ expressing cells were found in 33% of guinea pigs at 2 weeks, 83% of animals at 4 weeks post transplantation. LacZ profiles were seen within brain stem in 50% of animals at 4 weeks postoperative survival.

This study showed that ESC, DRG and adult neural stem cells have the ability to survive in the vestibulocochlear nerve and the brainstem of both rat and guinea pigs, albeit in small numbers, and not in all animals injected with the cells. They propose that a possible factor of reduction of survival over time may be due to immuno-rejection, and future research could concentrate on using neuronal growth factors to facilitate survival.

ESCs: differentiated and undifferentiated: Injection site: the scala media.

Hildebrand et al. (2005) the same year also used adult guinea pigs but used both undifferentiated and partially differentiated murine ESCs as transplants injected into the scala media via the round window. Mouse embryonic stem cells can remain in an undifferentiated state when maintained in a culture with mouse leukemia inhibitory factor (mLIF). ESCs become

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partially differentiated when mLIF is removed, and MEDII medium is added. This produces

primitive ectoderm-like embryoid bodies. To produce neuroectoderm-like embryoid bodies, basic

fibroblast growth factor (bFGF) can be added to direct commitment to the neuroectoderm

lineage. Hildebrand et al., (2005) used this technique to partially differential stem cells towards

neurite differentiation.

The authors used 19 animals in total, and had three different groups: implanted with

undifferentiated or partially differentiated ESCs and deafened using kanamycin ($n = 14$),

implanted with undifferentiated or partially differentiated ESCs and normal hearing ($n = 1$),

sham implantation and normal hearing ($n = 4$).

Both types of cell types were collected at 3,7 and 9 days post placing in culture (called

EBM for partially differentiated cells), for hair cell development analysis. Partially developed

ESCs expressed fewer markers than did undifferentiated ESCs by EBM day 3. By EBM day 7

Myo6 expression was observed to increase compared to earlier developmental stages, and alpha

9AchR expression was switched off. At EBM day 9, expression of all markers were decreased

except for an upregulated expression of alpha 9AchR.

Survival times were 2 weeks ($n = 4$) and 9 weeks ($n = 2$) post implantation (ESC cells, n

$= 4$ and EBM day 3 cells ($n = 4$). At these times the number of undifferentiated and partially

differentiated cells were counted. They found surviving cells in all animals in the scala vestibuli,

scala media and scala tympani, except only one animal out of two had surviving cells in the scala

vestibuli in the 9 week period. They reported a survival rate of 19.1%. It is noted that they

suggest that this is a small number, however, this is a much higher value than reported by other

studies discussed.

Injection of Neural Stem Cells (NSCs) ESC AND DRG.

Hu et al. (2005) investigated the survival, differentiation and integration of transplanted mouse ESC into the scala tympani of adult guinea pig cochlea, via the round window. Both ESC alone and ESCs co-grafted with dorsal root ganglion (DRG) cells were used. The researchers hypothesis was that DRG cells could enhance the survival of the ESCs.

Four groups were used: normal hearing animals transplanted with ES cells only; normal hearing with both ESC and DRG cells; deafened with neomycin, ESCs only; and deafened with ESC and DRG cells. Animals were sacrificed at 2 and 4 weeks post transplantation ($n = 6$ for each survival group). Histological and immunohistochemical methods were used to assess location, survival and integration of the ESC and DRG cells. ESCs identified by GFP fluorescence, were found in all inner ears of all groups at both week 2 and week 4, however, there was a significant reduction (by at least factor of 7) in cell count between the two time periods in the non co-graft groups. In the co-graft groups, there was a reduction by a factor of 3. There was no difference in the number of ESCs between normal hearing animals and deafened animals. ESC labeled with TUJ1 antibodies indicated those cells that had differentiated into neuronal cells. It was found that although there was a reduction of differentiated cells found between 2 and 4 weeks post transplantation, a greater number of cells were found when co-grafted with DRG in both normal hearing and deafened animals. After 2 weeks post transplantation more than 80% of surviving ESCs were labeled with the neuronal marker when co-grafted with DRG, whereas only 12% of ESC with co-grafting differentiated into neurons.

There was no significant differences between normal hearing and deafened animals.

Transplanted ESCs with cografted DRG were found to produce projections into the osseous modiolus, with TUJ1 positive staining suggesting neuronal differentiation. In 11 out of 24 animals, it was noted that these cells were in close proximity to cochlear afferent nerve fibers. These two results were not noted in the non-DRG co-graft groups. The main conclusions drawn by the authors were that ESC survival was enhanced by being engrafted with DRG and that this is most likely due to one or more extrinsic factors supplied by the DRG but not present in the adult inner ear.

ESC partially differentiated; Injection site: scala tympani

Coleman et al. (2006) also used mouse ESCs partially differentiated into neuroectoderm as transplants into kanamycin damaged adult guinea pigs, and used the scala tympani as the injection site. The target site was Rosenthal's canal. Recovery time before implantation was 2 weeks, and guinea pigs were injected with day 9 differentiated ESCs ($n = 15$) or a sham media ($n = 5$). Injection was through the round window of the left cochlea of each animal. Animals were sacrificed at 1, 2 or 4 weeks post transplantation, ($n = 5$ each group) and 2 week post sham transplantation ($n = 5$). Both left and right cochleae were removed for histochemical analysis to detect neuronal ESC in the scala tympani. ESCs were detected using direct fluorescent microscopy for GFP and DAPI (a marker for nuclei). A small number of ESCs were observed near the injection site. Quantitative analysis showed that there was a significant decline in the number of surviving ESCs in the lower basal turn of the scala tympani between 2 and 4 weeks. ESCs were detected in Rosenthal's canal in the left cochleae. Quantitative analysis did not show

any differences between numbers detected at 1, 2 and 4 weeks, however, the mean number of stem cells observed was less than 1 cell per section analyzed. The researchers concluded that using the scala tympani as the delivery site for ESC-derived cells lacked efficiency. They proposed that the implanted cells may have migrated to the cochlear aqueduct and/or into the CSF, and that further studies should investigate these possibilities. This study confirmed the results of Hu et al. (2005) study, but did not seem to add any new evidence. The animal model was identical, , as was the injection site and transplant.

NSCs from the hippocampus of mice: Injection site: scala tympani

Fu et al. (2009) using neural stem cells infected with Ad-GFP from the hippocampus of mice transplanted cells into the scala tympani of mature undamaged Sprague-Dawley rats. 45 animals were assigned to 1 of 3 groups ($n = 15$ each) randomly: NSC implantation; sham implantation; and non-implantation. ABR was used to record pre treatment (baseline) and 14 days post surgery hearing thresholds. No significant differences were found between pre and post transplantation hearing thresholds. 5 animals from each group were used to observe the structure of the cochlea and track the expression of GFP to count surviving NSCs; the whole organs of Corti were used for surface preparation from another 5 animals from each group (for histology), and the last 5 from each group were processed for scanning electronic microscopy (SEM) for morphological assessment. Animals were sacrificed for assessment after 14 days post NSC transduction.

The researchers found grafted NSCs (using the expression of GFP as a marker) in each turn of the cochlea, in all the animals transplanted with NSCs. Most of the grafted NSCs were

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found as a cluster of cells, especially in the perilymphatic spaces. Some cells attached to the walls of the scala vestibuli, others to the scala tympani. The vestibular membrane also was found to be a site for attachment, as well as the basilar membrane in the scala tympani. Some grafted NSCs were found in the spiral ganglions, and more NSCs were observed in the endolymphatic spaces attached to the tectorial membrane. No grafted cells were found in the organ of Corti or the spiral ligament of the cochlea. No cells expressing GFP were found in the control group. The structures of the cochlea including the organ of Corti, spiral ligament and spiral ganglions were found to be intact and undamaged by surgery or transplantation. However, the researchers found that there were some losses of outer hair cells in all basal turns of cochlea of every group, total losses being less that 1% and no significant differences between groups. This study used non-treated animals. In summary, Fu et al. (2009) obtained the following results:

1. The NSCs survived in the cochlea in each grafted group.

2. No statistically significant difference existed between the pre- and post surgical eABR thresholds.

3. No adverse side-effects of the surgery occurred such as blood effusion, wound infection, lost weight and head tilts;

4. and morphological damage did not occur in the treated animals.

Their main conclusion was that the NSCs survived transplantation via the round window and expressed GFP efficiently.

They reported that further studies should focus on the following:

1. longer periods of observation,

2. final fate of grafted cells,

3. increasing the differentiation towards neurons from the grafted cells,

4. promoting migration of the cells into the sensory epithelia,

5. and differentiating these cell into hair cells,

They omitted to suggest that further investigations should included using host animals that have been treated with ototoxic agents, to study the effects of engrafted cells onto damaged cochlea.

ESCs partially differentiated into neurospheres: Injection site: scala media

Han et al. (2010) used ESCs from between E12.5 and E14th day (embryonic days) of rats. Neuroepithelium was used to produce neurospheres that had been cultured for 6 days. Markers such as nestin (for immature neural cells), SOX2 (transcription factor expressed by stem cells), GFAP (for astrocytes) and TUJ1 (neurons) were used to identify secondary neurospheres at this time. Tertiary neurospheres were harvested for transfer and transplantation. Ad5-Atoh1-enhanced green fluorescent protein (EGFP) and Ad5-EGFP as control were placed with the neurosphere culture for 2-3 hours, for gene transfer, for *in vivo* experiment. For the *in vitro* transfer, a 10 hour culture was used. Cells were harvested 7 days after culture for both groups.

Twelve young adult guinea pigs were used as recipients. NSCs were transplanted into the endolymph of the scala media of the cochlea, via a small hole in the bone wall in the pigmented area of the stria vascularis of the 2nd turn of the left cochlea. 6 animals were injected with NSC infected by Ad5-Atoh1-EGFP and 6 with Ad5-EGFP and fast green dye.

Two animals from the experimental group and 2 from the control group were sacrificed 14 days post surgery. The cochleae were removed for whole mount immunohistological staining. The other 8 animals had their cochleas decalcified and prepared for cryostat for sectioning. *In*

vivo testing for NSCs were determined using the reporter gene EGFP whereas hair differentiation of NSCs by Atoh1 gene transfer was determined by immunohistochemistry for Myosin VII A.

In vitro it was found that the ESC could produce neurons and glial cells by day 14. They found that using Ad5-Atoh1-EGFP about 0.5-1% of all the surviving cells were Myosin VIIA positive. In the control group, none were found to be Myosin VIIA positive.

In vivo, 14 days post injection, grafted NSCs were found in the endolymphatic space as clusters attached to the basilar membrane in scala media, close to the organ of Corti, and some were found suspended in the endolymph or attached to the endolymphatic surface of Reissner's membrane.

Myosin VIIA-positive cells were found in the endolymph of the cochlea suggesting differentiation into hair cells utilizing Atoh1 gene transfer. This study used normal hearing rats as recipients of the transplanted ESCs. Further studies should include a similar injection site, i.e. the scala media, but using damaged cochleas instead.

DRG from mice: injection into the scala tympani of guinea pigs

Hu, et al. (2009) transplanted embryonic DRGs into the scala tympani of 31 adult guinea pigs. They used kanamycin to deafen the guinea pigs and ABR to document thresholds at 0,7,11,17,24, and 31 days. The purpose of this experiment was to determine the effect of using chronic electrical stimulation (CES) and neuronal growth factor (NGF) on the cells, to determine if neuronal growth would be enhanced. They split 46 animals into 7 groups: sham($n = 7$), DRG ($n = 7$) DRG+CES ($n = 8$), NGF ($n = 8$), DRG+NGF($n = 8$), DRG+CES+NGF ($n = 8$).

Although they found transplant survival in 58% of the animals they found no statistically

significant threshold differences between the 7 groups.

Injection of Bone Marrow Derived Stem Cells (BMSCs)

There are different types of cells that can be derived from the bone marrow: stoma cells and blood derived cells. To use bone-marrow derived cells as stem-cell transplants, first the cells must be isolated, then culured (or passaged) to increase the number of cells for efficacy, as stem cells are relatively small in number. The standard procedure to isolate and grow stem cells seems to be first extract cells from the bone-marrow (often from the epiphyses) of the femur and tibia of the donor animal. The isolated bone marrow, composed of both hematopoietic and stromal cells (blood and regular "body" cells) are maintained in a medium called Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine (cow) serum (blood). This prevents the cells from drying out and dying. Often an antibiotic is used to reduce infection. The medium is replinished, sometimes as often as twice a week. Non-adherant cells are removed while the medium is changed. The stem cells are "passaged" (doubled via the cell cycle) usually between 3 to 5 times to increase the numbers to a sufficient amount for transplantation. At this stage the cells are still undifferentiated.

This process of extracting stem cells is similar for all sources. For induction to different cell types, a growth factor must be added to the cells. In the case of neural induction, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) is added. Often the growth factor is added every 3 days, and then fixed after 7 days in culture. Immunohistochemistry is often used to detect nestin and Musashi-1 which are marked for cells that have differentiated into neuronal cells .

Naito et al. (2004) used autologous bone marrow cells from the femur of the individual experimental animal labeled with DiI (a fluorescent marker for cell transplantation studies). These cells were implanted into the modiolus via the round window of 5 chinchillas treated with gentamicin and ethacrynic acid (GM-EA).

At two weeks post bone marrow harvest, a single dose of GM-EA was injected into the chinchillas. At four weeks post damage, the cultured autologous marrow cells labeled with DiI were injected into the damaged cochlea.

Histological examination was performed at 3 weeks post BMSC injection. Naito et al. (2004) found DiI positive BMSCs transplanted into multiple regions of the cochlea. At the basal turn of the cochlea they found the greatest number of BMSCs to be in the scala vestibuli and scala tympani, and the smallest number of BMSCs to be in the lateral wall and scala media. The mean number of surviving transplanted cells in the basal turn was 130.7 cells/section. Also, NF200-positive (a marker for neuronal proliferation) transplant derived cells were found in the osseous spiral lamina and in the spiral ganglia, suggesting that some BMSCs had differentiated into neuronal cells. Bone marrow stem cells were scarce in the scala media, which suggests that high potassium concentration may interfere with survival of transplanted cells. Naito et al. (2004) suggest that micro-environment adjustment may be required to increase the survival rate. Since the scala media houses the hair cells, BMSC transplantation may be more applicable for nerve regeneration.

BMSCs from mouse donors; recipients: normal hearing gerbil; injection sites: scala

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tympani and modiolus.

Matsuoka, Kondo, Miyamoto, & Hashino (2006) used normal hearing gerbils as experimental animals and injected bone-marrow derived stem cells (BMSCs) isolated from the *TgN (ACTbEGFP)* mouse line. Stem cells from this mouse line expresse green fluorescence under microscope. The BMSCs were isolated from the femurs and tibias of the mice at 6 weeks of age. These stem cells were tested for ability to differentiate into neurites in vitro by growing in neuronal induction medium. Morphological changes were noted: cells changed from a flat mesenchymal cell shape into a round cell body with neurite-like processes. These neuron-like cells stained positive for GluR4, a marker for neurons, thus confirming that these BMSCs can differentiate into neurites.

Transplant recipients were 3 to 4 month old Mongolian gerbils. There were four groups and two injection sites: BMSCs and sham solution injected into the scala tympani, and BMSCs and sham solution injected into the modiolus via the bony wall of the basal turn of the cochlea (n = 4 each group). Cyclosporine for immunosuppression was administered via drinking water from 2 days before surgery up until they were sacrificed, 7 days post injection. GFP-positive cell counts were made at four anatomic subdivisions of the cochlea: scala vestibule, scala media, scala tympani and modiolus. They report that the overall survival rate defined by number of GFP-positive BMSCs divided by the total number of injected cells (e.g. 10^5) was 0.54% with scala tympani injection, and 0.59% with modiolar injection which was not a significant difference. Issues with this study that need to be addressed: early sacrificial time (only 7 days for recovery), used gerbils instead of mice, rats or guinea pigs as recipients, and used BMSC from

mice instead of using autologous cells. The authors discuss both survival times and not using partially differentiated cells and suggest that future studies could address these issues.

BMSCs from mouse; recipients: irradiated mice; injection site: intravenous (IV).

Tan, Lee, & Ruan (2008) in their study used lethally irradiated 7 week old mice (to destroy their own bone marrow cells) as recipient animals and GFP mice as bone marrow donors. About 3x 10^7 stem cells derived from the bone-marrow of the femurs and tibias of the 16 week old GFP transgenic mice were injected into the irradiated mice (*n* = 20). 3 months post-transplantation, the mice were deafened by 120dB SPL for two hours, and then sacrificed at day 0, 1 and 3, and weeks 1, 2, 4 and 8 post deafening.

The following markers were used: Calretinin, (for supporting cells); MyosinVII-A and S100A1 (for Deiter's, Hensen's and pillar cells) and NF200 (for afferent and efferent fibers of the cochlea innervation). Na, K-ATPase type II and IV were used for fibrocytes of the spiral ligament and marginal cells in the strias vascularis.

Various components of the cochlea were examined for survival of BMSC: the spiral ligament, perilymphatic compartment walls, limbus spiral ganglion, and modiolus. Cell count in these areas were found to be the greatest during the first week, with the maximum count at 3 days in the spiral ligament and perilymphatic wall. Most of the GFP cells were identified as leukocytes, specifically macrophages. Although the researchers showed that intrinsic host bone marrow stromal cells can migrate to the damaged cochlea, using ABR they found no functional improvement in the time period tested. The main conclusion from this study is that there may be a brief time span (3 days) in which cells can be most successfully and therapeutically transplanted.

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BMSC injected into normal and damaged guinea pig cochlea: target: SGNs.

Ogita, Nakagawa, Sakamoto, Inaoka, & Ito (2010) transplanted bone marrow derived neurospheres (spherical clusters of precursor neuronal cells, produced in vitro) from adult guinea pigs into the modioli of damaged and normal guinea pig cochlea. They report that this model is better suited for cochlea transplantation studies as the cochlea is larger than the murine model, and functional analysis using ABR is easier to conduct.

They harvested BMSCs from the tibias and femurs of 6-8 week old guinea pigs ($n = 4$) and first cultured these cells into neurospheres. The neurospheres were then transplanted into both normal hearing and ouabain (an aminoglycoside) damaged cochleae of 300-330 g guinea pigs. 4 control guinea pigs (intact cochleas, with transplantation) and 10 damaged cochlea with transplantation (only 8 were used, as 2 animals did not sustain enough hearing loss, criteria not reported). The damaged cochlea were further group into transplanted with BMSCs and sham medium ($n = 4$ each group).

In vitro, the BMSCs taken from the young adult guinea pig began to form spheres after 2-3 days. By day 7, over 10^4 spheres were identified in each dish, with $89.2 \pm 2.8\%$ of the cells labeled with beta-III tubilin, a marker for neuronal differentiation.

DAPI, a DNA stain, becoming fluorescent when bound to DNA, was used to label BMSC-derived neuronal cells in vivo. Cells that were stained with both DAPI and DiI (a nuclei stain) were counted as survived transplants.

DiI positive transplants were identified in all tranplanted cochleae (74.1 ± 44.4 were counted in undamaged cochlea and 72.1 ± 53.1 in damaged cochlea), thus there was no significant difference in the number of surviving transplants between the two. However, the

majority of cells in the intact cochlea were found in the scala tympani, with none in the internal auditory meatus, whereas in the damaged cochleae, the location of the majority of transplants was observed in both the modiolus and the internal auditory meatus, however, very few cells were found in the scala tympani. Expression for beta-III tubulin, a marker for neuronal differentiation, was found in $18.6 \pm 6.4\%$ of transplants into intact cochleas, and $24.1 \pm 5.3\%$ of damaged cochlea transplants.

Ogita et al. (2010), using electrical auditory brainstem response (eABR) testing to monitor spiral ganglion neuron (SGN) function, found no difference in SGN function between the sham and transplanted group. Thus, the transplantation of BMSC derived spheres into the cochlear modiolus failed to induce significant functional recovery. They concluded that future investigatations involving growth factors or neurotrophins injected locally may improved the likelihood of functional recovery.

Human Cord Blood Transplanted into Deafened Mice.

Revoltella et al. (2008) used stem cells derived from human cord blood to assess survival of these cells after IV injection into 39 deafened adult mice. Mice were either deafened by noise or ototoxic agents, and either injected with human cord blood or non-transplanted as controls (n = 10). Mice were sacrificed as late as 2 months post injection.

Revoltella et al. (2008) transplanted stem cells derived from 9 donors of human cord blood into nod-scid mice. Nod-scid (non-obese diabetes, severely immunodeficient) mice are used extensively for transplantation studies as they are unable to have an immune response to exogenous tissues or cells.

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In this study 70 mice were used. A preliminary study was made with three different groups to evaluate the damage to the cochlear structure with kanamycin only, noise only and kanamycin with noise. The evaluation of the damage was made at days 12, 49 and 60 post treatment, with at least 2 animals per time and treatment group. From their results they concluded that noise with kanamycin produced the most amount of damage in the shortest time. However, with the second part of the study, when they transplanted the cord blood into the mice, they still used the three damage paradigms, as well as different time courses between implantation and sacrifice (0, 12, 30, 49, 60, 61 and 62 days). They also varied the number of cells of cord blood injected into the mice (0.7, 1.5, 1.7, 2.0, and 2.5 x 10^5). They used controls for the different damage treatment paradigms, as well as saline solution as a dummy treatment. They also controlled for treatment and no injection, at various times from transplant.

PCR analysis were used to analyze the presence of *HLA.DQalpah1*. This is a marker for the presence of human stem cells. Qualitative analysis was reported as either a weak, weakly positive or a stong positive signal. They found positive *HLA.DQalpah* in the cochlea of most deafened animals that had at least 1.7 x 10^5 injected cells, at least 49 days from transplantation time. Interestingly, they reported that only one out of 6 control animals (saline solution instead of kanamycin, and no noise, and 2.0 x 10^5 injection cells) tested positive for *HLA.DQalpah*, and it was a weakly positive signal.

As hair cell and supporting cell markers they used Math1, MyosinVII-A, beta-tubilin, and protein gene product 9.5 (the latter is expressed in neuronal tissues by both mature and immature neurons). Histological methods were used to identify and locate cells containing these markers.

Of extreme interest they found that 60 days post transplantation all mice treated with a deafening protocol, and transplanted with human stem cells, showed morphologically intact cells throughout the cochlea. Whereas the controls lacked hair cells, supporting cells, neurons and severely damaged tissue, the experimental animals showed well maintained cells. Cells lining the modiolus, the organ of Corti, vestibular membrane of Reissner, the lateral wall, the basilar membrane, hair and supporting cells, spiral ganglia and neurons were all intact.

The main conclusions that Revoltella et al. (2008) report are the following: 1) that HSC IV transplantation in ototoxically damaged mice not only favored but improved recovery of the cochlear tissue; 2) the higher the number of injected cells, the greater the therapeutic effect, 3) the longer the time difference between IV injection and morhological analysis, the greater the observed recovery of cells.

This report appears to be the only study: 1) using human fetal cord blood as a source for stem cells, and 2) showing actual repair of hair cells in particular, and cochlea epithelia in general. This study was based in Italy but reported in *Cell Transplantation.* A Google Scholar search using "human cord blood and inner ear" did not result in any further articles, or any articles referencing this paper.   Suggestions for further research would be to study the effects on mice that have an immune system, and to assess functional recovery as well as morphology. A better design may have been to use three donors instead of nine, as this dilutes the analysis by introducing a greater number of variables.

In all, 14 experiments in cell transplantation into the inner ear were reviewed. In these 14, 5 different types of implanted cells were used: ESC, NSC, BM, DRG and HSC. The total number of different types of experimental animals used was 5: guinea pig, rat, mouse, gerbil and chinchilla.

Table 1. Recipient animal models versus donor animals and stem cell types.

Donor	Guinea Pig	Rat	Mouse	Gerbil	Chinchilla
Mouse	5	1	3	1	
Rat	1	1			
Guinea Pig	1				
Human			1		
Chinchilla					1
Stem Cell					
ESC	3	1	2		
NSC	2	2			
DRG	2	1			
BMSC	1		1	1	1
HCB			1		

There were 5 studies that used guinea pigs as the recipient and mouse stem cells as transplants. There were 2 studies which used autologous cells, where the donor and the recipient were the same animal model. Only 1 study used human cord blood as a source of stem cells, and only 1 study used chinchilla cells as a source. Only 1 study used the gerbil as a recipient animal model.

A total of 6 studies used ESCs as a source of stem cells, 4 studies used NSCs, 3 DRG, and 1 each for BMSC and HCB. Experiments utilizing guinea pig used ESCs the most often, as did the mice studies. Mice were implanted with ESCs and HCB whereas the gerbil and chinchilla animal models were only implanted with BMSCs. DRGs were only implanted into guinea pig and rat.

Table 2. Levels of evidence

Category of Evidence	Origin of evidence
I a	Evidence from meta-analysis of randomized controlled trials
I b	Evidence from at least 1 randomized controlled trial
II a	Evidence from at least 1 controlled study without randomization
II b	Evidence from at least 1 other type of quasiexperimental study
III	Evidence from nonexperimental descriptive studies such as comparative studies, correlation studies, and case-controlled studies
IV	Evidence from expert committee reports or opinions, or clinical experience of respected authorities, or both

Each article was examined for evidence, and categorized in terms of levels of evidence. No randomized controlled trials were conducted, so there were no level I experiments. The highest level of evidence achieved was IIa, with nine papers achieving that rank. The rest of the articles were ranked at IIb, with none providing evidence from non-experimental descriptive studies.

Table 3. Article, *n*, number of controls, type of injected stem cell and level of evidence.

Article	Type of injected stem cell										LoE
	ESC		NSC		DRG		BMSC		HCB		
	n	C	n	C	n	C	n	C	n	C	
Naito et al. (2003)							5^a	--			II b
Matsuoka et al. (2007)							8^b	8			II a
Table 3. (cont.)											
Ogita, et al. (2010)							4^f	8			II a
Tan et al. (2008)							20^d	5			II b
Hu et al. (2005)	24^d	--			24^d	--					II b
Hildebrand et al. (2005)	14^c	5									II a
Ahn et al. (2008)	13^b	13									II a
Praetorius et al. (2008)	$5^{d,e}$	2									II b
Regala et al. (2005)	15^d	--	12^d	--	22^d	--					II b
Hu et al. (2009)					31^f	15					II a
Coleman et al. (2006)			15^b	5^b							II a
Fu et al. (2009)			15^f	30							II a
Han et al. (2010)			6^f	6							II a
Revoltella et al. (2008)									39^d	10	II a
total per type of stem cell	71	20	48	41	77	15	37	21	39	10	

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Table 3. (cont.)
[a]Ear and number of controls not specified. [b]Left ear used for experimental animals, right ear used as controls. [c]Left ear only, control not specified. [d]Ear not specified. [e]n not explicitly reported. [f]Left ear used for experimental animals, different animals used for control.

Notes. Control animals for the purpose of this evaluation are animals that have either received no injection of stem cells, or have received a sham injection. Controls in experiments that use damaged vs undamaged cochleae as controls were not included in this analysis. C = number of control animals. LoE = level of evidence.

Table 4. Number of experimental animals (n) per type of stem cell and recipient.

	RECIPIENT									
	GP		RAT		MOUSE		GERBIL		CHINCHILLA	
CELL	*n*	C	*n*	C	*n*	C	*n*	C	*n*	C
ESC	41	5	12	0	18	15				
NSC	21	11	27	30						
DRG	55	15	22	0						
BMSC	4	8			20	5	8	8	5	
HCB					39	10				
EXP T	121		61		77		8		5	
CTL T		39		30		30		8		
Total *n*	272									
Total C	107	0.4								
No. of expmts	8	5	4	1	4	3	1	1	1	0

Notes. C = controls, GP = guinea pig, EXP T = total *n* per recipient type, CTL T = total controls per recipient type

Guinea pig animal models were used most often, with a total of 8 experiments out of 18 (some articles had more than one experiment). Experiments using gerbils and chinchilla's were the fewest with one each. Only 12 out of 18 experiments had negative controls, that is, used animals with sham injections. Some experiments used different types of damage as controls, thus were not included in these results as controls, as I have restricted the definition of controls as animals either not injected or injected with saline solution instead of the transplanted stem cell. A total of 272 animals were recipients of stem cell transplants (n), and 107 were used as controls (C) resulting in a ratio of 0.39 C:n.
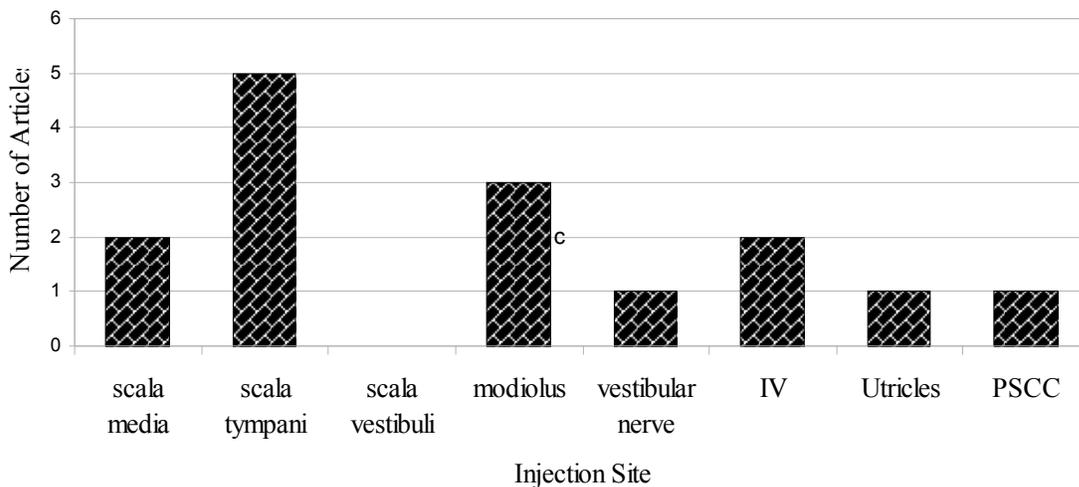
Figure 3 Number of articles per injection site.

The most common injection site was the scala tympani, with 6 studies, and no studies used the scala vestibuli. 3 studies used the modiolus and 2 used IV injection. One study compared both the scala tympani and the modiolus.
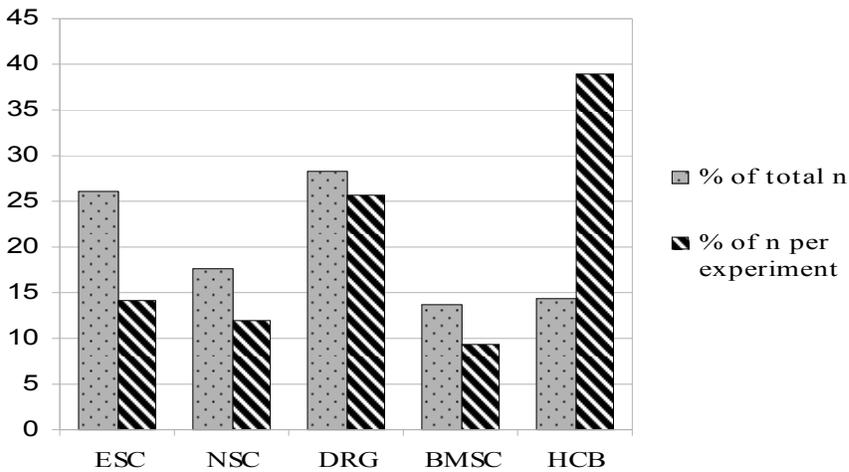
Figure 4. Percentage of type of stem cell per total *n* and percentage of *n* per experiment.

As a percentage of number of experimental animals implanted with a particular cell type vs total number of experimental animals used in the 14 articles, DRG type cells were the most used transplant with 28.31% of all experimental animals (total n), with ESCs the next with 26.10%. Although there was only one paper involving human cord blood, the number of experimental animals (*n*) was high, thus per paper, HSCs had the greatest count, with DRGs being the next most used implants per paper, and BMSC the least.

Sacrificial Time from Implant
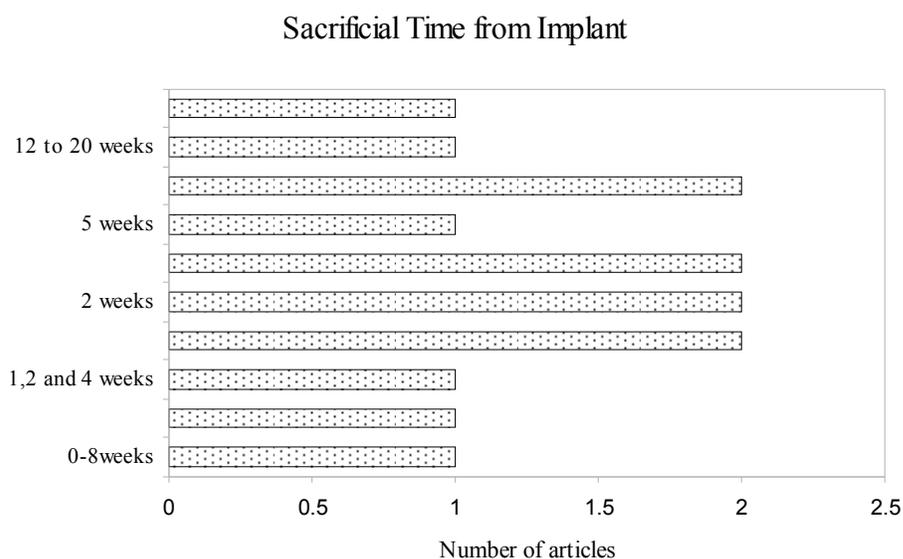
Figure 5. Sacrificial time from implantation of stem cell.

There was a wide spread of different sacrificial times post implantation of stem cells. The shortest was at day 0 from implantation, used for a control animal, but the same article used other time points through to 8 weeks. The next shortest was 4 days, and the longest time period was 20 weeks.

Table 5. Type of cell tracked with corresponding cell marker, and number of articles.

Function or cell type	Marker	No. of articles
Hearing thresholds	ABR (Auditory Brainstem Response)	5
Hair cells	Alpha9AchR	1
Neuronal cells (differentiated)	Beta-III tubilin	1
HC troponin-C intracellular calcium binding protein	Calretinin	1
BM mast cells	CD117	1
Leukocytes	CD45	1
Macrophoages	CD68	1

Table 5. (cont.)

Function or cell type	Marker	No. of articles
DNA stain (fluorescent when bound to DNA)	DAPI	3
Nuclei stain	DiI	2
Cochlea (histological staining)	Eosin	2
Astrocytes	GFAP	8
Cochlea (histological staining)	Hematoxylin	2
Human blood cell	HLA-DQalpha1	1
Neural stem cells (Adult)	LacZ	2
Hair cell progenitor	Math 1	1
Neuronal cells (differentiated)	Musashi-1	1
Hair cell	Myosin VI	1
Hair cell	Myosin VIIa	4
Fibrocytes type II and IV(spiral ligament) and marginal cells in the strias vascularis	Na K-ATPase	1
ESC	Nanog	1
Neuronal cells (immature)	Nestin	2
Neuronal development	Neurofilament	1
Fibers (afferent and efferent)	NF200	2
ESC	Oct-4	1
Hair cell	Phalloidin	1
DNA analysis	RT-PCR	1
Supporting cells, including Deiter's Hensen's and pillar cells	S100A1	1
Mouse stromal-derived factor	SDF-1	1
Supporting cell transcription factor	Sox2	1
ESC	SSEA-1	1
Neural stem cell (Adult)	Thy 1.2	1
Neuronal cells	TUJ1	2

There were a total of 30 different types of markers used for structural and loci analyses, and one method used for functional analysis (ABR). The most popular marker used was the glial fibrillary acidic protein (GFAP) (for astrocyte location, in eight experiments). ABR was used to test hearing thresholds after deafening in 5 articles, and used to test functional hearing in one article. MyosinVII-A was used in 4 articles to detect hair cells.

Table 6. Location of Transplanted Cells

Article	Inject Site	Cell Type	Damage	ST	SM/OC/BM/RM	SV/LatWall/SpLgmt	SGN/RC	SL	OSL	Mod	IAM	BS/VC
						SITE OF FOUND TRANSPLANTED CELLS						
Naito et al. (2003)	Mod	BMSC	GENT	++	+/NR/NR/NR	+++/+/NR	+++		+	++		
Matsuoka et al. (2006)	ST	BMSC	OUB	++	+/NR/NR/NR	++				0		
	Mod	BMSC	OUB	++	+/NR/NR/NR	++				+++		
Ogita, et al. (2010)	Mod	BMSC	NONE	+++	+/NR/NR/NR	NR/+/NR	NR		NR	++	0	
			OUB	+	0/NR/NR/NR	+	NR	0	NR	++	+++	

Table 6. (cont.)

Article	Inject Site	Cell Type	Damage	SITE OF FOUND TRANSPLANTED CELLS								
				ST	SM/OC/BM/RM	SV/LatWall/SpLgmt	SGN/RC	SL	OSL	Mod	IAM	BS/VC
Tan et al. (2008)	IV (blood)	BMSC	NOISE	+++	NR/-/-/++	NR/NR/+++	++	++		++		
Hu et al. (2005)	ST	ESC only	NEO	+	-/+/NR/NR	NR/+/NR	+	+		+		
		ESC + DRG	NEO	+	-/+/NR/NR		+			+		
		ESC	NONE	+	-/+/NR/NR		+			+		
		ESC + DRG	NEO	+	-/+/NR/NR		+			+		
Hildebrand et al. (2005)	SM	ESC	NONE	+	+/-/NR/NR	+/NR/NR						
Ahn et al. (2008)	PSCC	ESC	CIS	+	NR/+++ [a]/+ [b]/+	+++	++					

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Table 6. (cont.)

Article	Injection site	Cell type	Damage	SITE OF FOUND TRANSPLANTED CELLS								
				ST	SM/OC/BM/RM	SV/LatWall/SpLgmt	SGN/RC	SL	OSL	Mod	IAM	BS/VC
Praetorius et al. (2008)	Utricle	ESC	NONE	+++	0/NR/NR/NR	+++						
Regala et al. (2005)	VC	ESC	NONE									+
		NSC	NONE									+
		DRG	NONE									+
Hu et al. (2009)	ST	DRG	KAN	+	NR/+^{c}/NR/NR	+ SV^c	+			+		
Coleman et al. (2006)	ST	NSC	KAN	NS	NS	NS	+					
Fu et al. (2009)	ST	NSC	NONE	+ BM	+ TM/0/NR/NR	+/NR/0	+	NR				
Han et al. (2010)	SM	NSC	NONE	-	NR/NR/+ +/ +	-						

Table 6. (cont.)

Article	Injection site	Type of cell	Damage	ST	SM/OC/BM/RM	SV/LatWall/SpLgmt	SGN/RC	SL	OSL	MOD	IAM	BS/VC
					SITE OF FOUND TRANSPLANTED CELLS							
Revoltella et al. (2008)	IV (blood)	HSC	KAN & NOISE	+	++ (OHC plus 1IHC)/+/+	+/+++/+	+++		0	0	0	0

[a]marginal cells. [b]IHC and pillar cells. [c]Close to.

Notes. As most articles did not give actual numbers of cells, estimates have been given here for comparisons. +++ = most of the survived cells found, ++ = 30-60% of the cells found, + = 5-29% of the cells found,- = 1 or 2 cells found, 0 = no cells found, BM = basilar membrane, BS = brain stem, CIS = cisplatin, GEN = gentamicin, IAM = internal auditory meatus, IHC = inner hair cell, KAN = kanamycin, Mod = modiolus, NEO = neomycin, NR = not reported, NS = Reported as not significant, OC = organ of Corti, OHC = outer hair cell, OSL = Osseous Spiral Ligament, PSCC = posterior semicircular canal. RM = Reissner's membrane, RC = Rosenthal's canal, SGN = spiral ganglion neurons, SL = spiral limbus, SM = scala media, ST = scala tympani, SV = scala vestibula, TM = tectorial membrane, VC = vestibulocochlear nerve.

BMSCs were injected into the modiolus in 4 experiments, and 3 out of 4 found cells migrating into the scala media. In the paper by Ogita et al. (2010) it was found that there was a difference between migration ability to the internal auditory meatus between oubain damaged cells and non-damaged cells. All BMSC experiments found the injected cells in the scala tympani and scala vestibuli or lateral wall of the scala vestibuli.

ESCs were injected into various sites: scalae tympani and media, posterior semicircular canals, utricles, and vestibulocochlear nerve. ESCs when injected into the scala tympani were

found to migrate to the scala media although in extremely small numbers; some in the lateral wall of the scala vestibuli, Rosenthal's canal, spiral limbus and modiolus. When injected into the non-damaged scala media, some cells were found in the scala tympani, and scala vestibuli. Ahn et al. (2008) found that when injected into the cisplatin damaged posterior semicircular canals, ESCs mostly migrated to the marginal cells of the scala media, scala vestibuli, and Rosenthal's canal. However, Praetorius et al. (2008) found that when ESCs were injected into the non-damaged utricle, these cells migrated to the scala tympani and scala vestibuli, but not the scala media.

Regala et al. (2005) injected ESCs into the vestibulocochlear nerve and mainly found that although cells survived after two weeks in the nerve, there was a substantial reduction in survival at 4 weeks post implantation.

NSCs were injected into scala tympani, and scala media. Fu et al. (2009) injected into undamaged scala tympani, and found cells in the scala tympani, tectorial membrane and the walls of the scala vestibuli, as well as in Rosenthal's canal. However, Coleman et al. (2006) found when injection into damage scala tympani, a significant number in Rosenthal's canal only.

Han et al. (2010) injected NSCs into undamaged scala media and found cells in the basilar membrane and Reissner's membrane, but very few in the scala tympani and scala vestibuli.

Revoltella et al. (2008) using human stem cells and IV injection, with noise and kanamycin damaged mice, found the cells in most parts of the cochlea, but not the spiral ligament, modiolus, IAM or vestibulocochlear nerve.

From this summary, it seems that future experiments could concentrate on using the damaged scala media as an injection site, instead of being undamaged. As most of the experiments do not have substantial survival rates of transplanted cells, further studies could incorporate utilizing transcription factors to trigger the ESCs into transdifferention while in vivo.

CONCLUSIONS

Studies in avian automatic repair of damaged inner ear cells have sparked an interest and pushed forward the search for a method for repairing the inner ear of humans. Various pathways have been studied: using viral vectors to inject genes into the cochlear to trigger the supporting cells to differentiate into new hair cells, or to transplant stem cells directly into the cochlea. This review of the literature published between 2005 and 2010 has concentrated on stem cell-based research into transplantation of stem cells into the inner ear.

In the first section, articles covering spontaneous recovery of hair cells in the avian were discussed, laying the ground work for the more recent studies on mammalian systems. The second section discussed the variety of different sources for stem cells so far discovered and studied. Embryonic stem cells were thought to have the greatest possibility for therapeutic use, however, issues of immuno-reactivity and ethics have supported the search for other more suitable stem cells. It was found that cells from young adult tissues from the vestibular and cochlear systems have stem cell like qualities. Other tissues, such as the spleen, bone-marrow, and the epithelial layers of the lateral ventricles can also supply cells which can proliferate and differentiate into inner ear cells. Most studies have used stem cells from mice, rats, or guinea pigs, whereas a few have used chinchillas or the gerbil as a mammalian model. The mouse seems to be favored as a model as more work on the mouse genome has been done than on the other animal models. However, other animals have auditory systems anatomically and functionally more similar to the human system, so a future research direction could lie in obtaining the complete genome for these animals.

The third section of this review presented the studies on stem cell transplantation. Some studies used stem cells from mice or rats, and transplanted them into different parts of the cochlea of different recipient animals. Different injection sites included the scala vestibular, scala tympani, scala media and the modiolus. Different recipient animals included mice, guinea pigs and Sprague-Dawley rats. Most studies reported very few stem cells or differentiated cells surviving transplantation. None bar one reported functional gain post transplantation.Most of the studies using adult stem cells showed neuronal growth success rather than repair of the hair cell. Although damage to the hair cell is the most common cause of hearing loss, with cochlear implants, the hair cell is "bypassed" and stimulation of nerve cells becomes more important. If stem cells that are ear marked to differentiate into auditory neurons can be successfully transplantated into humans, this would greatly enhance the efficacy of cochlear implants.

One of the major hurdles with using adult stem cells is that as there are usually so few, they need amplification, and with amplification comes the possibility of tumor formation. Amplification requires many rounds of replication, thus more research into the possibility of tumor formation *in vivo* must be carried out before this method is tested on humans, (Carricondo, Iglesias, Rodriguez, Poch-Broto, & Gil-Loyzaga, 2010).

The study by Revoltella is an exciting advancement to stem cell research, but there are still many hurdles to overcome and questions to be answered. Some of the hurdles include immuno-reactivity, some of the questions include how can replication be achieved without tumor formation, will functional repair be observed, will there be long term effects. This is a very exciting field, however, it is very complex, and many more questions need to be answered before direct benefits will be seen in the clinical setting. Already there has been an exponential growth

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in knowledge of the development of the inner ear and we are seeing advancement towards the goal of repairing damaged inner hair cells. However, whether of not it will be safely achieved in the human cochlear in my lifetime, remains to be seen.

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