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INJECTION OF ADULT MOUSE MESENCHYMAL STEM CELLS INTO THE DEVELOPING CHICK INNER EAR

by

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Abstract: This series of experiments attempted to characterize the abilities of stem cells derived from bone marrow and adipose tissue to integrate into the sensory epithelium of the inner ear and to differentiate into hair cells or neural cell types.
Abstract

Interest in the therapeutic possibilities of stem cells has led to preliminary studies involving stem cell use in the treatment of inner ear disorders. Deafness brought on by hair cell or spiral ganglion loss is permanent in mammals. The regenerative properties of stem cells suggest that these cells could be introduced to the damaged inner ear to possibly restore function. This study used mesenchymal stem cells from the bone marrow and omentum of mice. In some cases cells were transfected with a plasmid containing GFP and Math-1 as well as with hair cell intercellular proteins. Donor cells were injected into chicken embryos at embryonic day 3 (E3) Hamburger and Hamilton’s (1951) stages 14 - 18. Inner ear portions of the embryo were dissected out and fixed at 1 - 4 day intervals. Once frozen, these blocks were sectioned and processed for hair cell, neuronal, and mesenchymal cell markers with immunohistochemical staining. Results show that injected cells were present in the developing otocyst and survived until day E7. Some cells integrated into the periotic mesenchyme, but none expressed any hair cell markers. It can be concluded that this injection protocol is effective in delivering enriched stem cells to the inner ear, but the fate of these cells remains undetermined. Further evaluation of the molecular properties of these injected cells and more accurate phenotyping may help to better characterize the possible transdifferentiation, integration, and therapeutic use of stem cells in the inner ear.

Introduction

 Permanent sensorineural deafness occurs as a result of loss of hair cells in the cochlea or of their primary afferents the spiral ganglia neurons, or due to damage along the auditory nerve. The mechanisms of damage include: genetic mutation, autoimmune or infectious diseases, chemical
ototoxic agents, mechanical acoustic trauma, or metabolic oxidative stress. Therapies involving the use of hearing aids or cochlear implants provide benefit to most people with hearing loss, but full restoration of “normal hearing” is limited by the anatomy and physiology of the impaired system.

Stem cell therapies aim to restore “normal” cellular function by inducing undifferentiated cells to a specific fate needed by the recipient. In the case of hearing loss, stem cells have the potential to differentiate into new hair cells, neurons, and supporting cells. This technology is still in its early stages. Several recent studies have explored the ability of stem cells to adopt a neural fate in vitro. Fewer studies have demonstrated in vivo or inner ear applications; however, there are still many studies showing that stem cells from a variety of sources can differentiate into mature cells expressing certain genes, labeling for target cell markers, or even functioning in the inner ear. Variables in these studies have yet to indicate an ideal animal model, cell source, or protocol based on survival rate and differentiation ability of the stem cells; therefore, a review of the literature can serve only to discover new combinations of variables yet to be attempted to further illustrate the role of stem cells as a therapy for inner ear cell loss.

**Sources of Stem Cells**

“A stem cell is a cell that gives rise to progeny with more than one differentiated phenotype and that may be greatly expanded in an undifferentiated form” (Pittenger and Marshak 2001).

*Embryonic Stem Cells* (ESCs) are cells derived from the inner cell mass of the blastocyst (Smith 2001). Some articles claim that these cells are ideal for stem cell therapies because they are “totipotent,” able to differentiate into any cell type. Most authors suggest that “pluripotent” is a more appropriate term. ESCs to give rise to cells of all three germ layers and to germ cells, but they cannot generate a blastocyst (Smith 2001). They are useful for somatic cell nuclear transfer
cloning and for somatic cell regeneration. As pluripotent cells, ESCs are an attractive source for research. In theory, ESCs could be expanded and partially differentiated into any desired cell type \textit{in vitro} and then injected or grafted \textit{in vivo}. Even undifferentiated ESCs can differentiate \textit{in vivo} by responding to developing embryonic environmental cues or damage-induced trophic factors in the adult. Drawbacks to ESC use include the teratogenic (tumor forming) ability of these cells and the current restricted use of human embryonic stem cells due to legal and ethical concerns (Parker and Cotanche 2004).

\textit{In vitro} studies using ESCs attempt to characterize the ability and efficacy of certain factors to induce these cells into a neuronal fate. For example, Matsumoto et al (2005) demonstrated the ability of ESCs enriched with SDIA (PA6 stromal cells) to differentiate into TuJ1-expressing neuron-like cells after seven days of co-culture with the auditory epithelia of neonatal mice. \textit{In vitro} these cells had neurites that elongated toward the basolateral surface of the hair cells in the cochlear explants. In addition, the ESCs expressed synaptophysin near the hair cells indicating the presence of synaptic vesicles at the neurite terminals and the ability for these new neurons to be functional.

To show ESC interactions in the vestibular epithelium, Kim et al (2005) used the same protocol with neonatal mouse utricles instead of auditory epithelia. Results indicated that ESCs had invaded the vestibular epithelium and labeled for TuJ1. To further define the characteristics of these cells, the authors also labeled for NR1 (a Scarpa’s ganglion post-synaptic marker). Expression of certain neurotransmitter markers (like NR1, GABA, glutamate, cholinergic and catecholaminergic markers) indicated that these ESCs fully differentiated into a variety of desired inner ear neuronal subclasses. It is particularly interesting that these ESCs mostly expressed markers for glutamate—the afferent neuron sensory transduction neurotransmitter. In
addition, these cells had neurites that projected to the basolateral edge of the vestibular hair cells and expressed synaptophysin, demonstrating that they are functional glutaminergic neurons.  

*In vivo* studies demonstrate the ability of ESCs to use host cues for differentiation, integration, and function. ESCs injected into the modiolus and auditory nerve can integrate/engraft and differentiate into functional neurons. Okano et al (2005) reported that ESCs can preserve function both in normal hearing and in kanamycin-deafened guinea pigs when injected into the modiolus through the scala tympani. These mouse ESCs enriched with retinoic acid (RA) and SDIA were able to survive for three weeks. These cells exhibited neuronal marker TuJ1 (labeling for β III Tubulin) and projected neurites all the way to the brain stem. Auditory brainstem responses (ABR) indicate that the thresholds of the injected group—although not as good as normals—still showed improvements in ABR thresholds compared to damaged ears without stem cell treatment.

Similarly, Hu et al (2005a) injected ESCs directly into the auditory nerve fibers through the modiolus. These cells expressed GFAP (for glial cells) and neurofilament (for neural cells). By three weeks post-injection the cells could be found in the basal turn of the cochlea and in the internal auditory meatus. By six weeks, the ESCs had begun to migrate centrally; so that by nine weeks ESCs were located in the brain stem near the ventral cochlear nucleus.

Sakamoto et al (2004) explored the use of ESCs in neomycin-damaged posterior semicircular canals of adult mice. Donor cells were either undifferentiated or partially-differentiated into a neuronal cell fate (by co-culture with SDIA PA6 stromal cells). At four weeks post-injection, few injected cells were present in the scala media of the cochlea and none of these cells expressed the hair cell marker myosin VIIA. Most of the injected cells were in the vestibular areas expressing β III tubulin. There was no expression of a mesodermal marker in
any of the injected cells, indicating that these ESCs had undergone differentiation into ectodermal neurons.

Using both *in vitro* and *in vivo* methods, Li et al (2003b) demonstrated that ESCs could differentiate into hair cells, in addition to neurons and glial cells. *In vitro*, hair cells were generated via stepwise differentiation of ESCs. Using RT-PCR (to characterize gene expression) and immunohistochemical markers at different stages of development, these authors showed that ESCs respond to an enriched medium of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) and develop into clones expressing neural progenitor cell markers like *nestin*, *pax2* and *BMP7*. When the growth factors were removed from the media, the cells no longer expressed the progenitor genes. They had differentiated even further into cells colabeling with Math-1 and either *myosin VI A* or *Brn3.1* in ways similar to developing hair cells. Finally, these differentiated cells were injected into the developing chick inner ear at stage 16 - 17. At 16 hours post-injection, some of the ESCs had already integrated into the host epithelium at the site of damage from the injection. Others integrated elsewhere into the supporting cell layer. By three days post-injection, ESCs that had integrated into the epithelium had morphology similar to the mature hair cells of the surrounding chick host. In the vestibular epithelium of the utricle, injected ESCs labeled with espin for F-actin rich stereocilia. These data suggest that embryonic stem cells injected into the developing inner ear of the chick are able to integrate into the epithelium and differentiate into hair cells; however it was not determined whether these hair cells were functional.

In summary, ESCs can integrate into embryonic or damaged adult sensory epithelia in the ear. Also, ESCs can differentiate into neural cell types such as hair cells, supporting cells, glial cells, and neurons of several subclasses.
Neural Stem Cells (NSCs) are neural cells from the dorsal telencephalon, the dorsal root ganglia (DRG), the lateral wall of the lateral ventricle, or the hippocampus that have stem cell properties. NSCs are multipotent and expand in culture without undergoing further differentiation. Unlike ESCs, NSCs can be derived from adult, neonatal, or embryonic cells. Also, NSCs have a more limited self-renewing ability and are more restricted in their ability to differentiate into other cell types (Panicker and Rao 2001). Despite this, NSCs seem to be a more-natural choice when considering stem cell inner ear therapy because both inner ear cells and NSCs are derived from the ectoderm and neural progenitors. Also, cells of the otocyst are influenced by neural tube factors during development (Parker and Cotanche 2004), making it likely that inner ear cells and injected NSCs may continue to be receptive to neural cell signals at later stages too. Several in vivo studies pertaining to the inner ear can be found in the published literature on NSCs.

Tamura et al (2004) used NSCs from the dorsal telencephalon of fetal mice. These cells still expressed the neural progenitor nestin, but had yet to express TuJ1 or GFAP. Two weeks after injection into the modiolus of cisplatin-damaged adult mice, NSCs had integrated throughout the inner ear. Donor cells robustly survived in the basal and apical scala tympani and in the modiolus. Among the spiral ganglia, injected NSCs predominantly expressed GFAP, indicating a glial cell fate. Out of all the integrated NSCs in the osseous spiral lamina, only ~10% expressed TuJ1. This suggests a limited potential for NSCs to differentiate into spiral ganglion neurons. To verify any functional benefit of these cells, the authors admit that these NSCs would have to show more extensive neural outgrowth to be therapeutically useful.

In a study by Tateya et al (2003), fetal mouse dorsal telencephalon NSCs expressing nestin, but not MAP2 or GFAP were injected into the second cochlear turn of adult mice.
damaged with neomycin. After 25 days, NSCs had integrated into the utricle and the ampullar cristae and had labeled with myosin VIIA. In cochlear structures, NSCs in the perilymph spaces of the scala tympani expressed GFAP and MAP 2. These cells did not integrate into the epithelium, but they did adopt neuronal and glial cell fates. Data from these two comparable studies demonstrate that fetal dorsal telencephalon NSCs can differentiate into vestibular hair cells, spiral ganglia, or glial cells by 4 weeks post-injection. These multipotent dorsal telencephalon cells are useful for therapy in the inner ear by restoring an array of cell types when damaged with cisplatin or neomycin.

Regala et al (2005) injected fetal DRGs or adult lateral wall of lateral ventricle rat NSCs into adult rats damaged only mechanically by the injection. At four weeks post injection NSCs were located along the proximal vestibulocochlear nerve extending into the brainstem as labeled with Thy-1.2, a neuronal marker. The fetal DRGs had fewer cells surviving at four weeks than at two weeks; however, the adult lateral ventricle NSCs were greater in number at four weeks than at two weeks. The authors conclude that since more spiral ganglia would be more useable for therapy, the proliferating adult ventricle NSCs are preferred over the fetal DRGs that diminish over time.

Hu et al (2005a) grafted fetal mouse DRGs into transected auditory ganglia damaged by neomycin. These NSCs expressed neurofilament and survived until nine weeks post-injection. Even though NSCs were injected at the basal turn of the cochlea, neurites from these cells migrated from the modiolus toward the brainstem during a 3 – 9 week period. However, most NSCs remained near the injection/grafting site. Compared to the pluripotent ESCs also used in this study, NSCs demonstrated less differentiation into fewer cell types and less migration toward the brainstem. These results are to be expected from the less multipotent NSCs.
Hu et al (2005b) attempted another study using NSCs from the lateral wall of the lateral ventricles of adult mice. After enrichment with EGF and bFGF and transfection with \textit{GFP} and \textit{neurogenin}, these cells were injected into the scala tympani at the basal turn of undamaged cochleae or cochleae damaged with neomycin. By two weeks post-injection, NSCs attached to the osseous Rosenthal canal integrating among the auditory nerve fibers. These NSCs were close to the spiral ganglion neurons and expressed GFAP, suggesting a glial cell fate. NSCs were also in the lumen of the scalae tympani and vestibuli from base to apex. This confirms the migration ability of NSCs noted in other studies. Only in deafened animals did these NSCs express \textit{β III tubulin and GFAP}; however some TuJ1 expression was noted in normal hearing animals injected with \textit{neurogenin}-transfected NSCs. None of the injected cells in any of the animals express myosin VIIA. These results show that lateral ventricle NSCs are multipotent—able to differentiate into several cell types throughout the inner ear, but not into hair cells. These data suggest that compared to normal cochleae, inner ears damaged with neomycin had more NSC integration and differentiation; but these data cannot determine if the injected cells were in fact functional.

Finally, Ito et al (2001) used yet another NSC source: adult rat hippocampal cells enriched with N2 and FGF. They injected these cells into the cochleae of presumably normally developing neonatal rats with no predisposition to hearing loss. At two weeks post-injection, NSC spheres merely lined the wall of the cochlear cavity. By four weeks, surviving NSCs had integrated into the organ of Corti among the inner and outer hair cells. These cells expressed phalloidin indicating presence of hair cell morphology. According to the authors, NSCs also differentiated into astrocytes and oligodendrocytes in addition to hair cells, illustrating the multipotent ability of these hippocampal NSCs.
No studies were found that used NSCs applied to the developing chick inner ear. However, Fontaine-Pérus et al (1995, 1997) grafted embryonic mouse NSCs into embryonic chicks’ somites at stages similar to the time period of the developing otocyst (day E2 – E8). NSCs managed to integrate into all three germal layers of the chick and to develop similarly to the neighboring host cells by responding to environmental cues.

In summary, NSCs have a somewhat diminished capacity (compared to ESCs) to differentiate \textit{in vivo} into hair cells, neurons, and glial cells somewhere between 3 - 4 weeks post-injection. Results indicate that NSCs can proliferate and integrate into both normal and damaged inner ears of both adult and post-natal mammals, making them a useful stem cell source for inner ear therapy.

\textit{Mesenchymal Stem Cells} are derived from bone marrow, umbilical cord blood, adipose tissue, and muscle (Krabbe et al 2005). Subclasses of mesenchymal stem cells like Hematopoietic Stem Cells (HSCs), Marrow Stromal Cells (MSCs), and Adipose-derived Stem Cells (ASCs) may serve as therapeutic cell sources for use in the inner ear. HSCs are rare, multipotent, self-renewing cells derived from mesoderm. Everyday, HSCs reconstitute blood cells in the bone marrow by differentiating into progenitors for erythrocytes, lymphocytes, macrophages, platelets, natural killer cells, osteoclasts etc. in the process of hematopoiesis. Bone marrow transplant (BMT), a therapy for blood disorders, utilizes the ability of HSCs to reconstitute all blood cells for the host (Orkin 2001). MSCs are derived from the bone marrow stroma that supports HSC hematopoiesis. Marrow stromal cells constitute a complex mixture of uncharacterized cells. The stem cells in the stroma regenerate tissues such as bone, tendons, skeletal and cardiac muscle (Pittenger and Marshak 2001). Both HSCs and MSCs can be extracted from the iliac crest of the pelvis, the tibia and femur, and the thoracic and lumbar spine.
Finally, ASCs are stem cells found in fat tissue. These self-renewing, multipotent cells are as accessible and replenishable as HSCs and MSCs, making mesenchymal stem cells excellent sources for stem cell therapy.

In order for these cells to be useful source for inner ear cell therapy, mesenchymal cells must demonstrate the ability to transdifferentiate from their mesoderm cellular fate into a neural cell fate. According to Krabbe et al (2005), transdifferentiation describes the “plastic ability of an adult stem cell to differentiate into cell lineages of the tissues different from the lineage in which the somatic cell resides—even into cells originating from other germ layers.” Mesoderm-derived stem cells would need to be as pluripotent as ESCs to undergo transdifferentiation into ectoderm cell types like hair cells or spiral ganglia. To test the ability of mesenchymal stem cells to transdifferentiate into inner ear ectoderm-derived cells, both MSCs and ASCs were used in our experiment.

There are several studies that explore the neural differentiation of all types of mesenchymal stem cells into neural structures of the eye, spinal cord, and hippocampus. For thorough reviews including both in vitro and in vivo studies see Ortiz-Gonzalez at al (2004) and Lu & Tuszynski (2005). The few published studies that pertain to the inner ear, to the chick, and to omental cells are described below.

One way of using mesenchymal cells in the ear is through BMT: replacement of all of the host blood cells with donor stem cells after complete irradiation. Studies of animal models disposed to autoimmune inner ear disease and presbycusis, illustrate that HSC and MSC transplants can restore functional hearing. Iwai et al (2005) and Lee et al (2000) used the auditory brainstem response (ABR) at 24 weeks post-transplantation to verify that experimental animals had thresholds similar to normals and much better than control deafened animals that did
not receive BMT. Iwai et al (2001) also showed that BMT can stop the loss of hair cells and spiral ganglia that occurs with presbycusis. These authors attribute the therapeutic ability of BMT in the inner ear to the lack of IgG deposits in the stria vascularis in the injected mice. This, in combination with macrophage release of neuron growth factor, helps to preserve hearing in these BMT models. However, the destruction of all host blood cells is not a useful strategy for patients with hearing loss. In addition, other types of hearing loss not caused by a faulty immune response may not be responsive to this therapy. In our study, a more-conventional injection protocol was used to localize the mesenchymal stem cell therapy to only the inner ear.

Naito et al (2004) described the autologous injection of enriched MSCs into the inner ears of adult chinchillas damaged with gentamycin. At three weeks post-injection, the MSCs were located in the scalae vestibuli and tympani, the spiral ligament, and the stria vascularis of all cochlear turns. Most notably, some cells were found in the modiolus. The lack of injected cells in the scala media was attributed to the high potassium ion concentration of the surrounding endolymph. The authors suggest that the microenvironment of the scala media may not be a suitable for injected MSCs. The MSCs that did integrate expressed neural markers NF200 and GFAP. The authors state that these cells could have some use clinically; but compared to other studies in their lab with NSCs, the MSCs have less differentiation potential.

The only other use of MSCs in inner ear comes from an in vitro study by Kondo et al (2005). First these authors isolated MSCs and cultured them in an enriched media containing retinoic acid and sonic hedgehog. Only when combined did these two agents cause the MSCs to become neural progenitors expressing nestin. Next, the media was supplemented with FGF causing the flat MSCs to change morphology into more round cell bodies with neurite-like processes. These cells were post-mitotic and had differentiated into neural cells. Then, the
authors grafted these partially-differentiated MSCs into an embryonic mouse organ of Corti. After one week of co-culture, the MSCs expressed sensory cell markers in both the vestibular and cochlear ganglia, in the trigeminal ganglia, and in the DRG. The cells in the vestibulo-cochlear ganglia labeled positive for TuJ1, calretinin, NeuN, Gata-3, NF and MAP 2. In most cells, the expression profile was similar to that of glutamatergic neurons, the type of afferent neurons in the ear.

Bone marrow hematopoietic and stromal stem cells have been used in experiments describing neural development of the embryonic chick, but not specifically in the ear. In one study by Pochampally et al (2004), MSCs were grafted into stage 12 – 13 (~ day E2) chicks at a damage site along the somite region of the embryo. Four days later, engrafted cells had migrated to and integrated into the heart, liver, brain, and spinal cord. The MSCs only comprised a small portion of the total number of cells indicating that even though MSCs can expand and develop into all three germal layers; they do so much more slowly and less effectively than the host chick somites. These authors did not detect any multinuclear cells, suggesting a lack of cell fusion (mouse/chick hybridization at the cellular level). Sigurjonsson et al (2005) injected CD34 positive human HSCs into the lumbar spinal cord of stage 15 – 16 embryonic chicks. Nine days later, most of the embryos showed some integration of HSCs into neural tissue. Particularly in the DRG, HSCs expressed neural markers MAP 2, NeuN, GABA and synaptotagmin and stopped expressing the hematopoietic cell marker CD34. Also, voltage sensitive action potentials could be measured from these HSC-derived neurons using whole-cell recordings. These findings suggest that a mouse/chick chimera can illustrate the transdifferentiation ability of mesenchymal stem cells into functional neural cells in vivo.
Stem cells have also been found to exist in the adipose tissue. Like all other stem cells described above, these ASCs can transdifferentiate in vitro into neural cells. Safford et al (2002) isolated ASCs from human fat readily-available from easily-accessible, discarded liposuction waste. The ASCs were induced along a neural cell fate with the help of butylated hydroxyanisole (BHA). After one day of culture, ASCs expressed the neural progenitor, nestin. With further enrichment by bFGF and EGF, the neural progenitors adopted a more mature neuron-like morphology. ASCs derived-neural cells did not survive longer than 2 weeks in culture. Despite their ability to transdifferentiate, the culturing of ASCs must be improved to prolong survival if they will prove to be a useful source for inner ear stem cell treatment. An unpublished series of experiments from Shin et al utilize ASCs derived from the stromal mesenchymal cells of the adipose tissue in the adult mouse omentum. Once expanded in culture, these cells expressed CD34, CD29, GAPDH, vimentin, and FSP1 similarly to ESCs. Studies are underway to determine the further neural differentiation abilities of these ASCs.

In summary, with the functional recovery of hearing in animals transplanted with stem cells via BMT already established, further research is needed to fully understand the transdifferentiation abilities of all types of mesenchymal stem cells and their possible uses to repopulate lost neural cells in the inner ear.

*Inner Ear Stem Cells* (IESCs) are inner ear supporting cells that maintain the ability to self-renew in culture. To be classified as a stem cell, these IESCs must also be multipotent—able to differentiate into cell types other than those inherent to the corresponding somatic cells. Li et al (2003a) set out to find and characterize these IESCs. First, the utricular maculae and the underlying stroma were removed from adult mice. The cells were isolated and eventually self-renewed by the formation of spheres—all without evidence of cell fusion. *In vitro*, these IESCs
could be induced along a mesodermal cell fate by co-culture with myogenic cells. The IESCs then further differentiated into myotube-forming cells that expressed muscle cell markers myosin heavy chain and MyoD. *In vivo*, IESCs were grafted into the amnionic cavity of stage 4 chick embryos. Early implantation allowed the IESCs to distribute during gastrulation into all three germ layers. Two days later, IESCs had integrated into developing mesodermal cells of the kidney and heart, endodermal liver cells, and ectodermal cells in the skin. Just like the host cells, these multipotent IESCs were able to respond to the local environmental cues and differentiate into a variety of cells.

Once Li et al defined these IESCs as true stem cells, they then determined the potential of these cells to differentiate into inner ear cell types *in vitro* and *in vivo*. Isolated IESC cultures were enriched in a medium to induce *nestin* expression. Most of the cells advanced out of the self-renewing cell cycle and began to differentiate into neural progenitors. These partially-differentiated cells also expressed inner ear development markers *Pax-2*, *BMP-4* and *BMP-7*. The authors then allowed more time for further differentiation to occur. Two weeks later, some IESCs had differentiated into hair cells indicated by *myosin VIIA* and *Brn3.1* expression. Some cells co-labeled for myosin VIIA and espin markers, indicating the presence of F-actin stereocilia. Since this expression profile of markers is exclusive to inner ear hair cells, these experiments can conclude that the IESCs are able to differentiate into mature hair cells. Differential staining showed that supporting cells expressing pan-cytokeratin could also be derived from these IESCs. *In vivo*, *nestin*-expressing IESCs were injected into the developing chick otocyst at stages 16 - 17. Five days post-injection, these cells expressed myosin VIIA at the same time and in the same locations as the surrounding chick hair cells. These data show
that IESCs are capable of integrating into the sensory epithelium and of receiving environmental
cues from the chick to differentiate into hair cells.

Kojima et al (2004) injected cells from the developing otocyst of rats at embryonic day
12 into postnatal rats that had been exposed to damaging levels of noise. One month after
injection into the middle turn of the cochlea, IESCs had integrated along the injection tract to the
cochlea, the lateral walls, the modiolus, and the scalae tympani and vestibuli. IESCs either lined
up along the inner lumen of the perilymph-filled spaces or accumulated into a mass of cells in
these areas. A few cells had migrated to the basilar membrane areas where hair cells and
Claudius cells had died from acoustic over-stimulation. These IESCs only integrated into the
supporting cell layers, adopting supporting cell morphology. No IESCs integrated into the organ
of Corti to become hair cells.

IESCs may be involved in hair cell regeneration. In non-mammals, some supporting
cells receive signals from the environment after damage. These cells then re-enter the cell cycle
to asymmetrically divide into a new hair cell and another supporting cell. In about two weeks,
new, mature hair cells re-populate the epithelium by this mechanism. These re-differentiated
cells innervate with existing neurons to restore function. Mammals have demonstrated limited
hair cell regeneration in the vestibular epithelium, but are unable to regenerate hair cells in the
cochlea by this mechanism (Parker and Cotanche 2004). Whether these supporting cells are
multipotent and stem-cell-like has yet to be determined. Nevertheless, the existence of IESCs
may prove to be the best therapeutic option for inner ear stem cell therapy. What is lost in
pluripotency may be gained by fewer intermediary steps needed for differentiation. These cells
may only need one master switch to be enabled—like Math-1, and possibly new hair cells could
be generated.
**Stem Cell Enrichment**

Stem cells are pluripotent; but with hearing loss, only a few—not many—cell types are needed for therapy. In order to coax stem cells to differentiate along the desired neural fate toward hair cells and spiral ganglia, cultures of stem cells can be supplemented or enriched with a variety of factors.

Stem cells of all types can use an array of environmental factors present in the host or in the culture medium to adopt inner ear cell fates. Some studies use growth factors like insulin-like growth factor (IGF-1), glial-cell derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and most successfully, epidermal growth factor (EGF). EGF either alone or in combination with other agents begins the partial differentiation of ESCs into neural progenitors expressing *nestin* (Li et al 2003b). EGF also induces sphere forming and proliferation in NSCs (Tamura et al 2004). EGF together with IGF is most effective in IESC neural induction (Li et al 2003a). Even partially differentiated MSCs respond to bFGF in culture and go on to adopt more mature neural cell types. Aside from growth factors, other agents that normally take part in inner ear development, like retinoic acid and sonic hedgehog, can be introduced to stem cells in culture media to aid in neural induction prior to injection (Kondo et al 2005).

Other enrichment protocols involve co-culture with either stromal cells or neuronal cells. A process called stromal cell-derived inducing activity (SDIA) describes the ability of PA6 stromal cells to induce ESCs toward a peripheral or sensory neuronal fate by differentiation into neural crest cells. Many of the ESC studies discussed earlier used SDIA to induce *nestin* expression prior to stem cell injection (Okano et al 2005, Kim et al 2005, Matsumoto et al 2005, Sakamoto et al 2005). ESCs can also be co-cultured with embryonic DRGs for neural induction.
Hu et al (2004, 2005a) compared outcomes from an enriched group of ESCs to the outcomes of ESCs without enrichment. Whether or not ESCs were enriched with the co-graft, both groups of cells integrated into the host animal and expressed TuJ1 as early as 2 weeks post-injection. Enriched ESCs survived longer, expressed more TuJ1, and had neurite projections reaching further towards the modiolus than the ESCs without co-culture.

In addition to environmental factors and co-culture, donor stem cells can be transfected with certain genes to influence cell fates. Plasmids coding for a promoter and the desired genes can enter the cells via viral vector infection or electroporation. In studies of the ear, a hair cell gene *Math-1* and a neural gene, *neurogenin* have been transfected into cells of the inner ear with the hopes of creating these neural cell types.

Studies in *Drosophila* have indicated that the basic helix-loop-helix transcription factor, *atonal* is necessary for hair cell development. Homologs of *atonal*, *Math-1* (mouse) and *Hath-1* (human), influence *nestin*-expressing neural progenitor cells to differentiate into hair cells. By acting as a “master switch” *Math-1*-expressing cells develop into hair cells in the appropriate culture medium. For example, dissociated embryonic otocyst cells expressing *Math-1* mature into hair cells with a combination of EGF and peri-otic mesenchymal cells that act as a supportive layer (Doetzlhofer et al 2004). These otocyst cells eventually label for myosin VIIA, calretinin, and phalloidin, indicating that *Math-1*-expressing cells develop into mature hair cells with calcium channels and stereocilia. However, without *Math-1* neural progenitors do not become hair cells. *In vitro* experiments by Chen et al (2002) illustrated that embryonic mouse cochlear explants first transfected with a *Math-1* enhancer could express *Math-1* upon exiting the cell cycle and could differentiate into cells labeling for myosin VIIA. But in the same
conditions, transgenic Math-1-null mice did not express any Math-1 or label for myosin VIIA indicating a lack of hair cell formation due to the lack of the Math-1 gene.

Zheng and Gao (2000) reported that postnatal rat cochlear and utricular explants infected with a Math-1 plasmid generated extra hair cells. Six days after infection, numerous new hair cells were located in the greater epithelial ridge and expressed myosin VIIA. Results with the utricular explants suggest that Math-1 facilitates hair cell regeneration by supporting cell re-differentiation. These authors conclude that Math-1 is necessary for hair cell differentiation in both cochlear and vestibular structures. Shou et al (2003) published similar findings with Hath-1 expression in postnatal rat cochlear explants. Although, none of the existing spiral ganglia or hair cells labeled for Hath-1, supporting cells in the greater epithelial ridge and in the lateral organ of Corti (lesser epithelial ridge) were infected with Hath-1 via electroporation and six days later these cells labeled for myosin VIIA. These authors attempted another similar experiment with mature utricular explants damaged with aminoglycosides. In these mature utricles, none of the Hath-1-infected cells labeled for myosin VIIA at 3 days post-infection; but by 7 – 18 days following infection, nearly all of the Hath-1-expressing cells also labeled for myosin VIIA. These studies confirm the need for expression of an atonal homolog for hair cell differentiation in the developing cochlea and utricle.

Kawamoto et al (2003) demonstrated that in vivo, Math-1 transfects supporting, Hensen cells and greater epithelial ridge inner sulcus cells when introduced to the mature inner ear of the guinea pig. One to three months following injection, these supporting cells had changed their morphology to resemble a pear-shaped hair cell with stereocilia visible with scanning electron microscopy. The authors point out that the number of transfected cells was few and that these new, immature hair cells were ectopically placed in unpredictable locations. Despite these
possible detriments to a clinical use for *Math-1* gene therapy, the authors also report (by labeling with neurofilament) that axons extended from the organ of Corti all the way over to the ectopic cells. These results are exciting because if new hair cells are to be made via gene or stem cell therapies, they still must prove functional *in vivo*. The possibility of synapses with spiral ganglia is a good first step.

Li et al (2003b) performed both *in vitro* and *in vivo* experiments showing that *Math-1* is also required for stem cells to differentiate into hair cells. *Math-1* was expressed only after the IESC spheres exited the cell cycle. These cells then became neural progenitors expressing *nestin*. After 14 days in culture, all IESCs labeling for *Math-1* also expressed myosin VIIA, suggesting a hair cell fate. When undifferentiated IESCs were injected into embryonic chick utricle sensory epithelium *in vivo*, *Math-1* was expressed only by cells that eventually became hair cells, and was not expressed by any injected cells that became supporting cells.

Donor cells should also be labeled in order to discriminate between donor and host cells in both *in vitro* and *in vivo* studies. Oftentimes the stem cells are transfected with genes like *lacZ* that labels with β-gal staining or with green fluorescent protein (GFP) for easy identification of injected cells.

In our experiments with omental cells, we injected hair cell proteins of unknown composition into the isolated ASCs. The effect of these proteins was also unknown, but perhaps they served as a hair cell induction mechanism or as enrichment proteins. To direct these ASCs toward more-desirable cell fates, the cells were transfected with *neurogenin* (for neurons) or *Math-1* (for hair cells). To more clearly identify the injected cells from the surrounding host cells, we also transfected the ASCs with a GFP marker.
Host Animals

In choosing an experimental model, it seems logical to use a clinically analogous mammalian paradigm: adult hosts subjected to damage simulating an acquired deafness or postnatal hosts with a genetic mutation approximating a congenital hearing loss. In the studies already mentioned, adult guinea pigs, mice, and chinchillas were damaged with noise, cisplatin, loop diuretics, aminoglycosides, or mechanical damage. Sometimes host animals included embryonic chicks and postnatal rats, but rarely have researchers used a congenital deafness model (i.e. “knocking out” normal inner ear development genes or coding for one of the known deafness gene mutations).

Sometimes, a clinically applicable design may not yield a strong enough result and no firm conclusions can be drawn from the data. For example, Sakamoto et al (2004) used two different protocols with two different hosts. As already discussed, undifferentiated rat ESCs were injected into the inner ears of adult mice. Injected ESCs were primarily located in the vestibular area four weeks after implantation. These cells labeled for neural markers (NCAM and β III tubulin), but none labeled for myosin VIIA. As hair cell differentiation was the goal of this experiment, the authors tried to produce a stronger result by using a chick model instead. Rat ESCs enriched with SDIA and BMP-4 were injected into the chick otocyst at stage 10 – 17. By two days post-injection (approximately day E5), cells were located in the lumen of the otocyst and in the mesenchyme near the dorsal edge of the otocyst and in the delaminating vestibulo-acoustic ganglia, but none of these cells labeled for myosin VIIA either. Even though neural induction mechanisms were assumed to be stronger in the developing chick than in the adult mouse, integration and differentiation were as limited in the chick as in the mouse for this experiment.
Embryonic chicks have been useful for the study of vertebrate developmental physiology. Hamburger and Hamilton (1951) staged the chick embryo by appearance of morphological features. Knowlton (1967) described inner ear development of sensory and neural cochlear and vestibular structures. Additional resources for inner ear development include Bissonnette and Fekete (1996) and Lang and Fekete (2001). Also, chicks can effectively serve as host animals in implantation and grafting experiments involving stem cells and the inner ear. As previously reviewed Li et al (2003a, 2003b) used chick inner ear explants in their ESC and IESC experiments. Furthermore, neural induction studies by Fontaine-Pérus (1995, 1997), Pochampally et al (2004), and Sigurjónsson et al (2005) all used the embryonic chick host model with success, although not within the inner ear. Opposed to a mammalian model, we chose to use embryonic chicks as host animals for our mesenchymal stem cell injection experiments.

**Injury to Host**

As reviewed previously, adult host animals are often damaged with noise, cisplatin, loop diuretics, or aminoglycosides (neomycin or kanamycin). Sometimes, no damage is caused—except by the actual injection of the stem cells into the inner ear structures. In a comparison of deafened and normal ears, Okano et al (2005) found no significant differences in the number of surviving injected ESCs between the two groups. Also, this study did not report any additional integration or expression with the deafened group over the normal hearing group.

Similarly, Hu et al (2005a) showed that for animals exposed to kanamycin damage, there was no difference when compared to normals for longevity of ESC survival or for amount of TuJ1 expression. But in another experiment (Hu et al 2005b), treatment of the cochlea with neomycin did affect survival and expression of injected NSCs at two weeks post injection. The number of animals showing NSC survival was about 40% for normal hearing animals (regardless
of whether they were transfected with neurogenin or not), but 58% for the animals deafened with neomycin. TuJ1 expression was only observed in 30% of the neomycin-deafened animals. These injected cells had been transfected with neurogenin and all expressed TuJ1. None of the NSCs (even those transfected with neurogenin) in normal hearing hosts co-labeled for TuJ1. For all experiments the actual number of surviving NSCs was typically low (at 2 weeks ~ 0.5%) with no significant differences between groups.

Kojima et al (2004) injected cells from the developing rat embryonic otocyst (day E12) into postnatal rats that had been exposed to damaging levels of noise (1 kHz – 20 kHz white noise at 120 dB SPL for 1 hour). This damage caused more grafting of the IESCs into the cochlea as integration occurred in 3/8 of damaged hosts vs. 1/8 of undamaged hosts. These data show that damaging the host was not required for cell integration, but it did help. However, it is possible that all damage was due to the mechanical damage from the injection—not from the noise exposure. No ABRs were performed to confirm the extent of hearing loss caused by the acoustic over-stimulation. Even if the noise resulted in merely a temporary threshold shift, some trophic factors in the local environment may still assist stem cells integration and differentiation. By this same reasoning, a more-damaging, permanent loss could yield more-successful grafting.

In our experiments, the otocyst was only minimally damaged by the disruption of the developing sensory epithelium with the micropipette used for injections.

In vivo injection

There are many ways to deliver therapeutic agents to the inner ear including: installation of an osmotic pump (useful for delivery of pharmaceuticals), creation of a niche at the round window (for drug delivery only to the basal end), and transection of the modiolus (to engraft neural cell tissue). Sekiya et al (2006) even tried grafting ESCs into the internal auditory meatus.
of damaged rat inner ears. The ESCs migrated along the vestibulo-cochlear nerve to integrate into Rosenthal’s canal near the scala media and spiral ganglia. They expressed β III tubulin and changed morphology, suggesting neural differentiation. In addition, this injection caused minimal trauma and maintained the endolymph and perilymph compartments in the inner ear.

Most *in vivo* manipulations involve injections that disrupt the membranes of the inner ear in order to deliver stem cells or viral vectors for gene therapy. Generally these methods result in donor cell aggregation around the injection site with only a few cells migrating or extending processes away from the injection site. The differentiation of these cells may be limited by location because injected cells are not subjected to an array of environmental factors—only to what is nearby. In our experiment, we injected cells into the developing chick otocyst. In this model, cells are injected into the unstructured common lumen of the otocyst and have the ability to diffuse/migrate throughout the entire developing inner ear. Unlike the adult model, injected cell survival and differentiation are likely to be affected by numerous factors in the surrounding embryonic environment.

**Methods**

All procedures were performed in accordance with institutional board ethical guidelines for use of experimental animals.

**Egg Preparation**

Fertilized White Leghorn chicken eggs were maintained at 4°C for no more than 7 days before being placed in an automatic, rotating incubator at 37°C at approximately 60% humidity (Day E0). At day E3, eggs were removed from the incubator carefully so as to maintain embryo orientation. Using an 18 gauge needle, 3 mL albumen was withdrawn from the egg. A larger
window was slowly cut out of the shell using scissors. Care was taken to allow time for the embryo to drop away from the shell if it had adhered. Any embryos that did attach to the shell were discarded.

At this point, the developmental stage of the embryo was estimated by visualization of morphological features under a Leica MZ6 dissecting microscope. Development of the otocyst, amnion, and overall embryonic features were compared to Hamburger and Hamilton’s atlas (1951) for staging of the embryo. Using a 23 gauge needle the outer, chorion membrane that surrounds the amniotic sac and yolk sac was punctured so that a few drops of PBS could be injected for clearer visualization and manipulation of the embryo. The entire chorion was then pulled away. Using a tungsten needle, the amnion was removed exposing the otocyst.

**Stem Cell Cultures**

Whole mouse bone marrow was collected from the tibias and femurs of an adult mouse by flushing the shaft with filter sterilized PBS. The sample was placed in a cold centrifuge for five minutes and re-suspended in 20 μL DMEM until injection. Cultured mouse bone marrow stem cells were obtained in the same manner but were plated out on media until confluent for three passages. Remaining cells were assumed to be marrow stromal stem cells. Adipose stem cells were obtained from the omentum of an adult mouse. Cells were purified in culture and then were transfected with a neuronal specific Thy-1 promoter. The promoter controlled the expression of green fluorescent protein (GFP) and *Math-1* or *neurogenin* genes. These ASCs cells were also enriched with undefined, intracellular hair cell proteins. After 5 passages, cells were expanded *in vitro*. At the time of injection, cultured cells were trypsinized, centrifuged, and finally re-suspended in 20 μL DMEM.
**Stem Cell Injection**

Fast green dye was added to the suspension fluid to aid in visualization under the dissecting microscope. Cells were loaded into a Nanoliter 2000 micropipette (World Precision Instruments), and approximately, 500 - 5,000 cells were injected into each exposed otocyst. The eggs were then covered with tape and placed in a non-rotating incubator at 37˚C for 1 - 4 days.

**Dissection**

At days E4 - E7 embryos were removed from the incubator and survival was verified by visualization of the heart beat. The vitelline was punctured in two places and the embryo was removed from the egg using a forceps and spatula. Embryos were placed in a dish of M199 solution, and any remaining amnion membrane was removed with forceps. Both otocysts were visualized. For the three omental cell experiments, any injected cells expressing GFP were visualized at this time with inverted phase contrast and epifluorescent microscopy (Nikon TE2000-S). To dissect out the otic region in all embryos, two cuts were made: one just rostral to the superior edge of the otocyst to free the head and another caudal to the inferior edge of the otocyst to free the tail.

Dissected otic regions were fixed in 4% Para formaldehyde to fix for 30 minutes to 1 hour—depending on embryo size and age. Following fixation, dissections were washed with PBS and placed in 30% sucrose (a cryoprotectant) overnight at 4˚C.

The following day, dissections were positioned in 10mm x 10mm x 5mm tissue tek wells filled with OCT. To insure consistent orientation during cryostat sectioning, the left side of embryo faced up, the dorsal side was on the left, and the caudal side was on top. The well was frozen into a block of OCT using a bath of dry ice and 2-methylbutane. Specimens were maintained in -80˚C freezer until sectioning.
Sectioning

Tissues were sectioned in the coronal plane from caudal end to rostral end in 10μm slices using a cryostat (Microm hm505N or 505E) kept at -20°C. Sections were mounted on slides that had been coated with chromium aluminum and were placed in a -80°C freezer until immunohistochemical processing.

Immunohistochemistry

First, non-specific binding of secondary antibodies was blocked with 1.0% BSA in Normal Goat and Normal Horse sera in PBS with Triton X-100 for two hours. Tissues were reacted with primary antibody at 4°C for 1 - 3 days, rinsed with PBS, and then placed in secondary antibody for one hour. After rinsing with PBS, sections were double stained using streptavidin (Alexa 488) for biotinylated IgG diluted 1:500 in PBS for one hour. This step was deleted for the three experiments using GFP labeled cells. Next, all specimens were rinsed with PBS again and were stained with nuclear marker bis-benzimide diluted 1:1000 in PBS for 30 minutes. After a final PBS rinsing, sections were coverslipped in 90% glycerol in PBS. All mounted sections were stored in 4°C until viewing.

Primary antibodies included a rabbit polyclonal anti-calretinin antibody diluted 1:1000 in PBS; a rabbit polyclonal anti-β III Tubulin antibody diluted 1:1000 in PBS; a rabbit polyclonal anti-myosin VI antibody diluted 1:200 in PBS; a mouse polyclonal anti-β III tubulin (TuJ1) antibody diluted 1:500 in PBS; a mouse polyclonal anti-CD29 antibody diluted 1:500 in PBS; a rat polyclonal anti-CD34 antibody diluted 1:200 in PBS. Secondary antibodies were CY3 anti-rabbit diluted 1:500 in PBS and Biotinylated IgG anti-mouse or anti-rat diluted 1:130 in PBS.

Specimens were examined using a Nikon TE2000-S inverted epifluorescence microscope using 20, 40, and 100X objectives and were recorded using a side-mounted Q-Imaging Retiga
1300 camera. Colorized processing of images was performed using Image Pro imaging software.

**Results and Discussion**

**Donor Stem Cells and Pre-treatment**

Five experiments were performed using the protocol stated above. First, whole mouse bone marrow (directly from the donor without purification) was injected into 8 embryos (Einj 9.14). This cell source has an unknown composition of many cell types. Many of these cells (i.e. erythrocytes) are known to die off, but remaining hematopoietic stem cells (HSCs) and marrow stromal stem cells (MSCs) may continue to live and possibly integrate (Ortiz-Gonzalez 2004).

Cultured mouse bone marrow was utilized in the second experiment (MBM 10.26). By culturing the whole bone marrow, only adherent cells were used for injection into 8 embryos. These cells were assumed to be MSCs by their morphology and self-renewing abilities.

Finally, a series of three experiments were performed using ASCs from the adult mouse omentum. First, all purified ASCs were transfected with intracellular proteins from a line of hair cells. Then, the cells were transfected with *neurogenin* and *GFP* (~80% transfection rate, Ngn 2.20) or with *Math-1* and *GFP* (2 experiments: ~ 10% transfection, Math-1 2.20; ~ 50% transfection Math-1 3.6). In comparing two day E4 specimens—one from each Math-1/GFP experiment, it is evident that the injection with a reportedly higher rate of transfection resulted in embryos with more GFP-labeled cells than the injection with Math-1/GFP cells with a reportedly lower rate of transfection (Figure 1). Seven embryos were injected with *neurogenin/GFP*-
transfected cells, and a total of 15 embryos were injected with *Math-1/GFP* transfected cells (6 - low transfection rate, 9 - high transfection rate).

**Host Animals and Pre-treatment**

Despite the clinically relevant and successful use of mammalian host animals, we chose to use embryonic chicks for the following reasons. First of all, chick development is well categorized and of short duration. An extensive atlas is available from Hamburger and Hamilton (1951), and developmental stages of the chick inner ear are described by Knowlton (1967). Also, the developing chick inner ear is easily accessible for experimentation. Eggs can be opened and re-sealed for experimentation—exposing the otocyst region without compromising the embryo. In addition, the inductive interactions leading to cell differentiation in the chick are similar to those in mammals, allowing mammalian, injected cells to respond to avian environmental cues for development (Goldstein et al 2002). Finally, existing protocols for use of chicks were readily available in our lab.

As already mentioned, many studies have shown that stem cells integration and differentiation can be made more effective with pre-injection ototoxic, noise induced, or mechanical damage. Since the egg is a protected environment for the developing chick, pre-treatment of the embryo with intentional damage may not have long-lasting effects on the local environment. If damage is too severe, the embryo may not survive or the structures may heal on their own without incorporating the stem cells at all.

Our injection protocol caused only minimal mechanical damage at the site of the micropipette injection into the otocyst where the pars superior epithelium was punctured. Also, more damage may have occurred by the injection of the additional volume of cells and suspension fluid into the confined otocyst. With embryo #1 in the Math-1 3.6 experiment, an
intentional attempt was made to induce more mechanical damage with the pipette during the injection by repeatedly poking the otocyst epithelia. Figure 2 compares this embryo to another embryo also at day E6 (#3 Math-1 3.6) with a more typical injection-induced damage. There were no differences in the appearance of the host structures or in the properties of the injected cells regardless of degree of damage.

In all experiments, injected cells either remained near the injection site along the lateral wall of the otocyst, or they migrated to three locations: dorso-laterally to the developing superior sinus, ventro-medially to the junction of the developing endolymphatic and cochlear ducts, or dorsally up into the endolymphatic duct. Since cells were found in other places besides the injection area—regardless of stage of development or extent of damage, it can be concluded from this study that any environmental factors released during damage do not play a role in stem cell migration by preferentially recruiting injected cells to the site of lesion. However, with only one embryo as an example of more extensive damage and without any damaged control animals, this conclusion may be faulty. Also, due to poor sectioning and processing, rarely was a complete otocyst fully sectioned, and never were all sections able to be photographed for any embryo. It is possible that injected cells may have migrated to several other locations not shown in these limited data.

**In vivo injection**

All injections took place on day E3 (approximately 72 - 78 hours after beginning incubation). After opening the shell and peeling away the chorion and amnion membranes, the embryo’s stage of development was determined. According to Hamburger and Hamilton (1951) and Knowlton (1967), embryos at day 3 should be at stages 19 - 23. These authors admit that classifying embryos by chronological timing is flawed due to variability; hence they proposed a
morphologically based staging system. Bissonnette and Fekete (1996) also observed variability in development as embryos were staged one half day behind normal development. This delay can be attributed to prolonged onset of incubation of already fertilized eggs. In our experiments, embryos ranged from stage 14 (presence of otic cup) through stage 18+ (heavy vascularization around otocyst). Younger embryos where the otic cup was still open (such as embryo #4 Einj 9.14) were injected with cells into the neural tube and into the medial mesenchyme in addition to into the developing otocyst (Figure 3).

Despite any developmental delays, all embryos had to at least progress to Hamburger and Hamilton’s (1951) stage 12 (approximately day E2) to undergo the injections. Embryos at this stage have begun to turn to the right side, exposing the right otocyst for injections. The undisturbed left side served as a control. In our experiments, two of the 38 embryos had turned to the left. In these cases, the left otocyst was injected and the right side served as a control.

Survival of embryos was typical of previous chick manipulations in this lab and similar to other studies (Sakamoto et al 2004). In each round of injections, approximately 2 - 4 embryos adhered to the shell and could not be injected. Sometimes, the injection resulted in minimal embryo trauma, such as small extra-embryonic bleeds in the vitelline (N=7), larger extra-embryonic bleeds (N=2), and carotid bleeds into the otocyst (N=2). These injuries were not always detrimental to survival as only 3 of the 11 deaths (post-injection) were attributable to bleeds. Of the total 38 embryos that were injected, 11 (28.9%) died before the otocyst could be dissected out for processing.

In each experiment, cells rested in the micropipette without agitation from the injection of the first embryo to the injection of the last embryo, totaling approximately 4 – 6 hours. Over this time period cells began to coagulate—especially during the Math-1 3.6 study. In this case, cells
were re-trypsinized before injection for the last two embryos in this experiment. These embryos died before dissection and could not be examined further. Cell clumping had two effects. First, cells were injected as a large ball or mass of cells. This could prevent interactions of these cells with environmental factors or integration into the epithelium. Another disadvantage to clumping was that fewer cells were actually injected among the majority of suspension media. Because of this, some embryos had few injected cells—if any—in the otocyst region. However, a lack of cells in the lumen may also be attributable to an unlikely, complete integration of injected cells without detection, or more likely to faulty injections blocked by any remaining amnion covering the otocyst.

**Dissection of otic region**

Prior to otocyst dissection, whole embryos could be viewed with inverted phase contrast and epifluorescent microscopy to visualize the efficacy of injections for the three ASC studies (Ngn 2.20, Math-1 2.20, Math-1 3.6). In both Math-1 experiments, GFP-expressing cells were seen in the otocyst—sometimes only as a green blur (Figure 4). Visualization became more difficult as density of the embryo increased with age. At this stage, the neurogenin/GFP experiment did not show any cells clearly expressing GFP; only a minimal green blur was visible in one embryo. After sectioning and before immuno-processing, these sections were checked again for GFP expression. No sections from the neurogenin experiment expressed GFP; therefore, no further processing was completed. Neurogenin is not normally found in epithelial cells. Perhaps, the transfected cells had difficulty existing in the lumen of the inner ear and died early.

In all experiments, at least one embryo was dissected at days E4 and E5. Embryos were dissected at day E6 in the cultured MSC study (MBM 10.26) and in all of the ASC studies—with
the exception of the Math-1 2.20 experiment. A total of two embryos were incubated up to day E7 and survived—both from the Math-1 3.6 experiment. In the whole mouse bone marrow experiment (Einj 9.14), embryo #8 survived until E8. No further processing was completed due to time constraints.

All embryos were sectioned caudal to rostral except for embryo #5 from the Einj 9.14 whole bone marrow study. It had been positioned incorrectly in the OCT and all images should be reversed.

**Immunohistochemistry**

The *Whole Mouse Bone Marrow* injection experiment (Einj 9.14) had minimal results. As this was the first experiment, injection and immuno-processing errors left few intact sections to be examined. The sections that could be visualized show that injected cells do survive at least until day E5 (2 days post-injection). Injected cells were present in the otocyst lumen at days E4 and E5; however differential staining of chick versus mouse cells with bis-benzimide was difficult to determine. All injected cells were CD34 and calretinin negative and some were positive for TuJ1.

At day E4, all injected cells were located near the injection site and some lateralized along the epithelium (Figure 5A). It is unclear from the bis-benzimide staining as to whether any of the injected cells actually integrated into the chick otocyst epithelium. Furthermore, if any injected cells did integrate, it cannot be determined whether they are part of the group of cells expressing calretinin. These cells may have transdifferentiated into calretinin-positive cells by receiving environmental cues from the neighboring chick epithelial cells. Whether the injected cells had integrated or not, it is possible that the intense calretinin expression in the epithelium could be due to the presence and activity of the injected mouse cells. For reasons not fully
understood, the calretinin antibody used in our lab consistently labels this area of the common crus in this banding pattern at day E4. As no sensory cells ever develop in this region (Knowlton 1967) and since a complete comparison to the left control side is not possible, no clear conclusions can be made with these data.

At day E5, injected cells were located in the otocyst lumen and near calretinin-positive sensory patches (Figure 5B). Again, if these cells integrated and whether they express calretinin cannot be determined from these data. The influence of the injected cells on the surrounding epithelium is also unknown because chick cells normally express calretinin in this area of the developing lateral semicircular canal. It is clear that no local environmental cues from the chick caused any of the injected cells in the lumen to express calretinin.

The difficulty in distinguishing between donor and host cells using bis-benzimide has also been cited in the literature. Studies by Fontaine-Pérus (1995, 1997) also used differential bis-benzimide staining to distinguish between the chick and mouse cells. According to these authors, chromatin of chick nuclei is more dispersed than the nuclear chromatin of the mouse. Because of the more condensed chromatin network, mouse cells will fluoresce brighter than chick cells. These authors state that the difference in nuclear staining with bis-benzimide is less between mouse and chick than it is between quail and chick; and therefore, it can be difficult to assess which cells belong to which animal.

_Cultured Mouse Bone Marrow_ experiments (MBM 10.26) involved culturing of whole bone marrow to isolate the MSCs for injection. No injected cells were identified in day E6 specimens. In a day E5 embryo (#6), injected cells were identified with bis-benzimide staining in the medial end of the developing cochlear duct. All of these cells were CD34 and calretinin negative (Figure 6A and 6B). The absence of CD34 labeling indicates that no HSCs were part of this
stem cell group. By culturing the bone marrow, only CD34 negative MSCs that adhered to the culture plate were injected. None of the cells labeled for calretinin; perhaps other markers like TuJ1 or GFAP would have labeled the MSCs. This is one of only two examples from all experiments where injected cells were found in the region of the cochlear duct. The other example was another day E5 embryo from the Math-1 2.20 experiment, embryo #6 (Figure 6C). In these cases, as before, it is difficult to determine injected cell integration into the epithelium due to poor visualization of differential bis-benzimide staining. Also, it is possible that more embryos had injected cells in the area of the developing cochlear duct; but due to poor processing, few slides were intact enough to be viewed.

In general, the injections of the MSCs were not successful due to a small volume of cells available for injection and experimental errors injection and processing. Other authors have demonstrated neural-differentiation in vivo with this type of cells (ear-Naito et al (2004), brain-Zhao et al (2002), eye-Kicic et al (2003), and spinal cord-Pochampally et al (2004)). These experiments used damaged adult hosts injected with enriched MSCs. Results indicated that injected cells migrated to sites of neural damage and expressed appropriate neural markers; therefore another attempt of this model is worthwhile. Modifications would include: obtain more cells through more cultures, use a more reliable GFP marker for easier injected cell identification, and prolong the amount of incubation time post-injection prior to dissection.

Three experiments using cells from the Omentum (Math-1 2.20, NGN 2.20, and Math-1 3.6) used transfected, purified ASCs for injection. With both of the bone marrow experiments, it was difficult to distinguish between mouse and chick cells in the epithelial region. To aid in determining whether the injected omental cells did integrate into the host epithelium, the next three experiments used green fluorescence to mark the injected cells. Results of the neurogenin

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experiment were discussed earlier (*Results: Dissection of otic region*) and are not discussed further in this section.

Two experiments were performed with ASCs transfected with *Math-1/GFP*. They varied in success of gene transfection into the donor cells (Figure 1). The experiment performed on 2.20 not only had a low rate of *Math-1/GFP* transfection, but also a low experimental yield as only three embryos survived to dissection. When viewed prior to dissection, only two of these embryos had any GFP expression. Once fully processed, all three embryos did show successful cell injections. Due to the low *Math-1/GFP* transfection rate in this example, screening embryos for GFP post-injection prior to processing did not reflect failure or success of the injection. It was only indicative of the presence or the absence of GFP-expressing cells. Screening embryos prior to the extensive sectioning and immuno-processing was a useful tool to identify the *Math-1/GFP* cells that are of the most interest.

All three embryos were sectioned and processed. The two day E4 embryos had bis-benzimide positive cells in the lumen of the developing otocyst. Embryo #1 did show some GFP-expressing cells that were negative for myosin VI (Figure 1A). Embryo #3 also had some GFP-expressing cells prior to dissection; however, due to poor processing resulting in few intact sections, none of the available data show any GFP-expressing cells after immuno-processing. None of the injected cells expressed myosin VI (Figure 7A & 7B). At day E5, only one embryo had survived to dissection (embryo #6), but this embryo did not have any GFP-expressing cells when viewed prior to dissection. Once completely processed, this embryo did have injected cells located in the lumen of the otocyst. The available sections are from the most caudal end of the otocyst, and bis-benzimide staining in the otocyst lumen may in fact be the wall of the otocyst (Figure 8). In injected cells in this area all express CD29, a mesenchymal cell marker, and some
of these cells label for GFP/Math-1 too (Figure 9). The morphology of these cells cannot be determined from these data; but expression of both markers indicates that the ASCs have not fully-differentiated yet. As these cells continue to express CD29, they may become mesenchymal cells. A few examples of injected mesenchymal stem cells maintaining their mesoderm fate can be found in the published literature: Castro et al (2002) and Massengale et al (2005).

Another section of this embryo did show a GFP-like green haze in the cochlear duct that was not present on the opposite (Left control) side (Figure 10B & 10C). There was no co-labeling with bis-benzimide for this section in this area indicating an absence of cellular nuclei in the lumen. However in a preceding section (Figure 10A), non-GFP-expressing nuclei are located in the ventro-medial cochlear duct. Perhaps, the green haze is a remnant left behind by GFP-expressing, injected cells that died by this stage, leaving only non-GFP-expressing cells in the lumen.

From the second Math-1/GFP experiment performed on 3.6, many more sections are available for data as 7 out of 9 injected embryos survived until dissection. Also, by this time experimental error decreased due to acquired skills in sectioning and processing. One embryo was dissected at day E4. In this specimen, injected cells were labeled with GFP and bis-benzimide, but none were labeled with calretinin. The red staining visible in the mass of cells does not co-label with bis-benzimide. Therefore, there was no true calretinin expression by any injected cells—only dead cell remnants without nuclei that auto-fluoresce (Figure 1B). In more rostral sections, the mass of cells invaded the developing endolymphatic duct. In the most caudal sections, the neighboring chick epithelium expressed calretinin in a similar way to what was seen in the whole bone marrow injections (Figure 5A). The calretinin labeling was on the
dorsal edge of the otocyst (Figure 11A). Additional imaging of the left side of this embryo (Figure 11B), illustrates that calretinin expression in this region is normal at this stage—but it is not as intense as on the affected side. These findings suggest that the mass of GFP/Math-1-expressing cells can have some effect on the surrounding chick epithelium, resulting in an increase in calretinin expression. Unlike with the previous two experiments, here it is clear that the injected cells are not in the group of calretinin-expressing cells in the host membrane because all of these cells are GFP negative. However, as green fluorescence only indicates the transcription of the plasmid containing GFP and Math-1, it does not indicate the location of the Math-1 protein once made by the injected cells. Therefore, it is still possible that Math-1 (or another agent) was secreted by the injected cells to increase calretinin expression in the host cells.

Two embryos were dissected at day E5. In both cases, there were fewer GFP-expressing cells present than in day E4 embryos. In fact, there were fewer injected cells overall. Results show migration of the GFP-expressing cells up into the endolymphatic duct (Figure 12). There was little evidence of injected cells anywhere else in the developing inner ear. Most likely, many of the injected cells have died off at this point post-injection, or perhaps fewer cells were injected into this embryo due to the clumping discussed earlier.

As before, all injected cells were calretinin negative; but in contrast, all cells of the neighboring epithelium of the endolymphatic duct were also calretinin negative. These results indicate that the injected cells had no detectable effect on the host cells. Since the host cells in this area would normally never express calretinin at any point in their development, perhaps they are not responsive to Math-1. GFP expression is very strong in these cells. It can be assumed that Math-1 is strongly expressed as well. Since the injected cells themselves do not express
calretinin at this point in development, perhaps more time is needed for these cells to transdifferentiate and label for the mature sensory cell calcium channel protein, calretinin. Even though sensory patches were staining calretinin positive in other appropriate areas of the chick, it is possible that the transdifferentiating ASCs may have a delay in their development as compared to the typically developing host cells. Staining with an earlier neural progenitor marker, like nestin, might be helpful at this stage.

At day E6, two embryos were dissected. In both specimens, GFP was expressed in some of the injected cells located in the lumen of the otocyst. None of the injected cells expressed calretinin. These two embryos have been discussed previously in the section on damage to the host epithelium (Host Animals and Pre-treatment). Figure 2 shows the location of the injected cells in these embryos. The large cell mass in embryo #1 (A) was typical throughout this experiment; however, embryo #3 (B) warrants further discussion. In the most dorsal edge of the superior sinus, some GFP-expressing cells appeared to be arranged in line with other cells in the host epithelium. Upon further examination of each filter color channel, it is clear that all of the injected cells have not integrated into the epithelium, but are merely part of the mass of cells (Figure 13). Previous and subsequent sections of this area confirm that no injected cells integrated into the epithelium.

At day E7, two embryos were dissected and processed; however embryo #4 contained no GFP-expressing cells. The efficacy of the injection into this embryo was questionable at the time of injection, but processing was completed for verification. Embryo #7 had a limited number of cells expressing GFP when checked prior to dissection and also once fully processed. Unlike all other experiments, the GFP-expressing injected cells crossed the epithelial barrier and were integrated into the host mesenchyme along the ventro-medial edge of the endolymphatic duct at
day E7 (Figure 14). As there were no bis-benzimide labeled cells in the lumen, all non-integrated cells had died by this point. In other embryos at day E5, cells were found in the lumen of the endolymphatic duct. Embryo #7 must have also had cells tucked into that space on previous days too. By day E7, injected cells had integrated into the mesenchyme adjacent to where the injected cells had been in day E5 specimens.

The morphology of the GFP-expressing cells had also changed (Figure 15A). The cell bodies were globular, and they had processes extending in a bipolar fashion—indicating differentiation into a neural cell type. This was confirmed by calretinin labeling; all of the GFP/Math-1-expressing cells co-labeled for calretinin (Figure 15B). Some additional calretinin staining in the region did not co-label for bis-benzimide, and therefore it was probably a result of the autofluorescence of dead, injected cell remnants (Figure 15C). None of the host cells labeled with calretinin; and therefore, they must not have received any signals from the injected cells. In this area of the inner ear, no cells would express calretinin at any point in normal development. Despite the common lineage of the injected cells and neighboring cells of the mesenchyme, the injected ASCs transdifferentiated into neural cells and did not continue along a mesenchymal cell lineage. This could be attributed to Math-1 expression encouraging these cells to adopt a neural cell fate. However, Math-1 expression was not enough to encourage these cells to adopt a hair cell fate. In development, Math-1 usually acts once cells leave the cell cycle and after they have become neural progenitors. The time window for Math-1 to influence the injected cells may have passed in our specimens. Expression of Math-1 may decrease over time as these cells have not adopted epithelial cell morphology and will not become hair cells. Other studies confirm these findings. Mesenchymal stem cells have differentiated into neurons, but not hair cells (see Stem cell sources: Mesenchymal stem cells).
Summary

As discussed earlier, several researchers have illustrated the capacity of mesenchymal stem cells to transdifferentiate along a neural path, but perhaps with only limited ability: few become neurons, whereas a majority of cells become glial supporting cells.

From all of our experiments with all types of mesenchymal stem cells, only a few examples of differentiation and integration were obtained. Therefore, these injected mesenchymal stem cells cannot be completely characterized. Most of the mass of cells, labeled only for the nuclear stain, bis-benzimide; other dead cell remnants merely autofluoresced. In the first experiment with whole bone marrow, some cells labeled for TuJ1; but none of the ASCs labeled for $\beta$ III tubulin. In the final ASC experiments, many cells labeled for Math-1 expression, but only a few co-labeled with calretinin and none labeled for myosin VI. In addition, some injected cells continued to label with mesenchymal cell marker CD29. As our data in these experiments was very limited, one cannot conclude the true phenotype of these injected mesenchymal stem cells.

That being said, our data suggest that mesenchymal stem cells have a very limited ability to transdifferentiate into neural cells \textit{in vivo}. These cells only expressed calretinin in the one case of integration. With calretinin expression and the change in morphology, it is likely that these cells are beginning to differentiate into neural cells. It can be concluded that—even with Math-1 expression—stem cells derived from the mesenchyme cannot transdifferentiate into hair cells when injected into the developing otocyst of the chick; but they may begin to integrate and differentiate into neurons by day E7.

In order for injected cells to become useful for functional recovery, they must either integrate into the sensory epithelium as a hair cell or into the area of the developing vestibulo-
cochlear ganglia. Our results did not identify any integrated stem cells in either of these areas. If injected cells survived long enough to integrate, they did so into the mesenchyme surrounding non-sensory areas of the inner ear instead.

**Future directions** would include modifications of the stem cell sources, the host animals, and the immuno-processing. First, to ease injection, stem cell clumping should be minimalized. Also, the properties of the omentum-derived enriched cells need to be further defined in vitro. The transplanted hair cell proteins and their effects on the stem cells require further characterization too. In addition, other stem cell sources could be tried. Preliminary injections of IESCs from the supporting cell layer of the quail utricle have been attempted. Perhaps these cells will be easily induced into a hair cell fate as they have been known to do in non-mammalian hair cell regeneration.

Modifications could also be made to the host. As opposed to the highly inductive and nurturing environment of the normally developing chick inner ear, better stem cell integration and differentiation may be demonstrated in a more clinically analogous paradigm such as adult mammals with acquired hearing loss or postnatal mammals with congenital hearing loss. If chick embryos are used, more injections should be done to control for experimental variables. In our study, the left side served as a control for each animal. The addition of normally developing controls could help to determine the normal expression of different cellular markers for comparison. Embryos injected with a mock solution could show the effects of the injection in damaging the epithelium. And finally, more embryos should be dissected at older stages. As integration was finally seen at day E7 with these experiments, further study utilizing longer post-injection incubation periods (E8 or E9) is warranted. By waiting until the chick has reached
near-adulthood, more integration and further differentiation of injected stem cells could be characterized. Furthermore, a more well-defined damage protocol using an aminoglycoside could be used.

Other improvements could be introduced to better characterize the phenotypes of the injected stem cells. As few of our embryos survived 4 days post-injection, it would be worthwhile to label with a neural progenitor cell marker that can detect the intermediate stages of stem cell differentiation at the earlier embryonic stages. As normal hair cell differentiation (visible with calretinin and myosin VI labeling) is limited to day E6 - E12, it may be difficult to determine hair cell phenotype in early embryos. To better define more-mature cells, supporting cell markers, glial cell markers, or other mesenchymal cell markers could be implemented too.

Additional research is needed to determine what environmental cues play roles in the normal development of inner ear cell types and in the differentiation of injected stem cells. Also, an ideal mechanism of cell delivery that preserves the intact structures of the host has yet to be determined. Stem cells must preferentially migrate from the injection/engraftment site to the damaged areas within the inner ear. And finally, these stem cells must prove effective in restoring hearing function in order to be implemented clinically.

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References:


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Figure 1  GFP Transfection rate reported from culture was verified *in vivo*.
A: Section from a day E4 embryo with a low transfection rate had few cells expressing GFP.
B: Section from a day E4 embryo with a high transfection rate had more GFP-expressing cells for a comparably-sized mass of injected cells.
Figure 2 Excessive damage during injection had no effect on the migration of injected cells. 
A: Section from a day E6 embryo with a mass of injected cells in a common location, near the junction of the developing cochlear and endolymphatic ducts—not near the site of damage along the dorso-lateral edge of the otocyst. 
B: Section from another day E6 embryo with more-typical damage. Injected cells had migrated to the dorso-lateral edge of the developing superior sinus.
Figure 3 Whole mouse bone marrow injection results at day E4. Embryo was injected at stage 14+.
A: Injected cells are in the lumen of both the otocyst and the neural tube. Some express TuJ1, but only cells of the lateral epithelium of the otocyst express calretinin.
B: Image of same embryo. Cells are not present in the control otocyst, but are in the neural tube. Some injected cells express TuJ1, but none express calretinin. It is not possible to visualize the entire control otocyst to verify calretinin expression normally occurring in the lateral otocyst region.
Figure 4 Views of GFP expression in day E4, E5, and E6 embryos prior to dissection. Visualization at this point served as a helpful intermediate step to identify unsuccessful injections prior to processing.
Figure 5 Injected whole mouse bone marrow cells were present near calretinin-positive areas of the epithelium.  
A: Section from a day E4 embryo with injected cells lined up along the lateral wall of the otocyst.  This section was not photographed for TuJ1 expression, but previous and subsequent slides do not show any TuJ1 expression in this area (40x).  
B: Section from a day E5 embryo with injected cells near the chick sensory patches of the developing lateral semicircular canal (20x).
Figure 6 A (caudal) & B (rostral): Two sections from the same embryo illustrating that injected cells migrated down ventrally into the developing cochlear duct at day E5. These cells label only with bis-benzimide for nuclei.
Figure 7 Results of two embryos at day E4 from the low transfection rate Math-1 experiment.
A: Injected cells survive in the otocyst one day post-injection, but do not express GFP or myosin VI.
B: Very few cells labeled with GFP at E4; none co-labeled with myosin VI.
**Figure 8** Injected cells continue to express mesenchymal cell marker, CD29 at day E5.

A: GFP/Math-1 is expressed by most of the injected cells.

B: CD29 labels all injected cells in otocyst region. Some CD29 cells co-label for GFP.

C: CD29 cells have nuclei and label with bis-benzimide.

D: Merge of all 3 color channels.
Figure 9 Injected cells continue to express mesenchymal cell marker, CD29 at day E5.
A: GFP/Math-1 is expressed by most of the injected cells.
B: CD29 labels all injected cells in otocyst region.
C: Some CD29 cells co-label for GFP.
D: Merge of GFP/Math-1, CD29, and bis-benzimide labeling.
Figure 10 Results from Math-1/GFP 2.20 day E5 continued.
A. Injected cells migrated to the ventro-medial end of the cochlear duct, but do not express CD29 or GFP.
B. GFP expression in the developing cochlear duct did not co-label with bis-benzimide for the presence of nuclei.
C. Left otocyst (control) confirmed that affected side results were not artifact due to sectioning and processing error.
Figure 11 Injected cells were present near calretinin positive chick epithelial cells.  
A: GFP expression of injected cells near developing endolymphatic duct induced more intense calretinin expression in neighboring host cells.  
B: Left otocyst (control) confirmed that results from the affected side are not caused by sectioning and processing errors.
Figure 12 GFP-expressing injected cells congregated in the lumen of the developing endolymphatic duct at day E5.
Figure 13 Separate color channel images of day E6 embryo showed no integration of GFP-expressing injected cells into the epithelium of the superior sinus.
**Figure 14** GFP-expressing injected cells integrated into the host by Day E7.  
A: Section with injected cells located in the mesenchyme, ventromedial to the endolymphatic duct—not in the lumen.  
B: Successive section 20x  
C: Same section as 12B at 40x

Calretinin
Figure 15 Injected cells begin to differentiate by day E7.
A: GFP/Math-1 expressing cells adopt neural cell morphology
B: Some GFP cells co-label for calretinin.
C: Some calretinin staining does not co-label with bis-benzimide.
D: Merge of all 3 color channels