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Hydrogel limits stem cell dispersal in the deaf cochlea: implications for cochlear implants

Bryony A. Nayagam[1,2,*], **Steven S. Backhouse**[1,2], **Cengiz Cimenkaya**[1], and **Robert K. Shepherd**[1,2]

[1]Department of Otolaryngology, University of Melbourne, Australia, 3002

[2]The Bionics Institute, East Melbourne, Australia, 3002

Abstract

Auditory neurons provide the critical link between a cochlear implant and the brain in deaf individuals, therefore their preservation and/or regeneration is important for optimal performance of this neural prosthesis. In cases where auditory neurons are significantly depleted, stem cells (SCs) may be used to replace the lost population of neurons, thereby re-establishing the critical link between the periphery (implant) and the brain. For such a therapy to be therapeutically viable, SCs must be differentiated into neurons, retained at their delivery site and damage caused to the residual auditory neurons minimized. Here we describe the transplantation of SC-derived neurons into the deaf cochlea, using a peptide hydrogel to limit their dispersal. The described approach illustrates that SCs can be delivered to and are retained within the basal turn of the cochlea, without a significant loss of endogenous auditory neurons. In addition, the tissue response elicited from this surgical approach was restricted to the surgical site and did not extend beyond the cochlear basal turn. Overall, this approach illustrates the feasibility of targeted cell delivery into the mammalian cochlea using hydrogel, which may be useful for future cell-based transplantation strategies, for combined treatment with a cochlear implant to restore function.

Keywords

cochlear implant; hydrogel; stem cell; auditory neuron; deafness

Introduction

Cochlear implants electrically stimulate auditory neurons (ANs) in patients with severe-to-profound hearing loss. However, there is often a lengthy delay between the loss of hearing and cochlear implantation, resulting in ongoing degeneration of ANs which comprise the auditory nerve. The health and integrity of the auditory nerve is considered to be one of the factors affecting cochlear implant performance [Shepherd and Javel, 1997], and thus, preserving a population of robust neurons is an important factor in improving outcomes with this neural prosthesis. Many laboratories have now published studies describing the delivery and survival of murine stem cells (SCs) into the mammalian cochlea for the replacement of degenerating ANs, using a variety of delivery techniques [Hu et al., 2004a, Hu et al., 2005,

*Correspondence: Dr Bryony Nayagam, Department of Otolaryngology, Royal Victorian, Eye and Ear Hospital, Level 2, 32 Gisborne Street, East Melbourne, 3002, Australia., Phone: +61 3 9929 8385; Fax: +61 3 9663 1958; b.nayagam@unimelb.edu.au.

Note that S.S. Backhouse is presently working at: Department of Ear, Nose and Throat Surgery, Princess of Wales Hospital, Coity Road, Bridgend, South Wales, CF31 1RQ, UK

Author Disclosure Statement

The authors declare no competing commercial associations or potential conflicts of interest in connection with the submitted manuscript.

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Regala et al., 2005, Coleman et al., 2006, Corrales et al., 2006, Sekiya et al., 2006, Ahn et al., 2008, Altschuler et al., 2008, Lang et al., 2008, Reyes et al., 2008].

Despite differences in SC differentiation protocols and transplantation technique adopted, the results from these studies support four main conclusions: 1) SCs can survive in the deaf mammalian cochlea, 2) SCs are capable of extensive migration/dispersal following their delivery into the mammalian cochlea, and 3) a proportion of transplanted SCs express neuronal and glial proteins *in vivo*. Initial attempts to transplant exogenous cells into the cochlea adopted a conservative surgical approach, that is, cells were delivered directly into the scala tympani in an attempt to minimize trauma to the delicate cochlear structures. While minimizing trauma, the delivery of cells into the scala tympani frequently resulted in the extensive dispersal of cells within the cochlea, and/or low detection of transplanted cells in the target site [Olivius et al., 2003, Hu et al., 2004b, Coleman et al., 2006].

Recent attempts at delivering SCs to the cochlea have investigated a more invasive approach into the modiolus or auditory nerve [Hu et al., 2004a, Okano et al., 2005, Regala et al., 2005, Corrales et al., 2006]. While successful in terms of improving the numbers of exogenous cells detected within the target site, these studies also report the extensive dispersal of cells following transplantation. Although some dispersal of transplanted cells is likely to be necessary for effective replacement of ANs, extensive dispersal has the potential disadvantage of transporting the cells to regions distal to their target site. In addition, the effect of the surgical procedure on the endogenous AN population and the inflammatory tissue response in the cochlea are also important to evaluate when considering these potential therapies. Whilst a variable proportion of ANs will likely have degenerated after deafness, it will be important to preserve those ANs that remain. An effective cell replacement strategy is therefore likely to incorporate both the targeted delivery strategy which minimizes damage to the existing cochlear structures, in combination with the retention of transplanted cells in a matrix that restricts their widespread dispersal until they can become integrated.

Cell encapsulation is one solution which may minimize cell dispersal following transplantation. Effective cell encapsulation for *in vivo* studies would need to incorporate biocompatible materials and encourage cell growth. A product which meets these specifications is PuraMatrix™ peptide hydrogel. This hydrogel forms synthetic three dimensional matrices at a physiological pH, and can be customized to create specific microenvironments for cell growth and differentiation *in vitro* and *in vivo*. Hydrogel is biocompatible comprising >99% water and mimicking a bare extracellular matrix, thereby enabling three dimensional growth analogous to the *in vivo* scenario http://www.puramatrix.com/index.html. Several reports have described improved growth and differentiation of neurons in hydrogel scaffolds [Holmes et al., 2000, Semino et al., 2004], including neurite outgrowth and formation of functional synapses [Holmes et al., 2000, Ellis-Behnke et al., 2006]. However, the application of hydrogels in conjunction with SC delivery into the inner ear has not yet been explored.

The current study explored the delivery of SCs into the deafened mammalian cochlea, using hydrogel to limit their dispersal (Fig. 1). The efficacy of this delivery procedure was examined based upon the location of transplanted SCs, the density of residual ANs, and the inflammatory tissue response elicited by the procedure. These data were then considered in terms of future cell therapy for combined cochlear implant/SC transplantation.

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Materials and Methods

Experimental animals

Ten adult guinea pigs (400–600g) from a mixed gender and gene pool were used for the current study. Half the cohort of animals were ototoxically deafened, allowed to recover for two weeks and then transplanted with 11-day differentiated mouse embryonic SCs (n=5). Untreated contralateral cochleae served as untreated, deafened controls (n=5). Age-matched, untreated, normal hearing animals served as additional controls (n=5). All procedures were conducted in accordance with the guidelines set by the Royal Victorian Eye and Ear Hospital Animal Research and Ethics Committee (approval numbers: 02/090A and 05/122A).

Deafening procedure

The hearing status of all guinea pigs was evaluated using standard click-evoked auditory brainstem responses [Hardie and Shepherd, 1999]. Five normal hearing guinea pigs were bilaterally deafened by co-administration of the ototoxic aminoglycoside kanamycin monophosphate and the loop diuretic frusemide, as previouslydescribed [Coleman et al., 2006]. These animals were confirmed profoundly deaf via auditory brainstem responses, one week post-deafening (click-evoked auditory brainstem response thresholds 98dB peak equivalent sound pressure level; [Hardie and Shepherd, 1999]) and also immediately prior to termination.

Differentiation of stem cells

The mouse embryonic SC line used in this study [R1 B5-EGFP (Tg(GFPU)5 Nagy/J)], was obtained from Dr A. Nagy (Mt Sinai Hospital, Toronto) and expressed enhanced green fluorescent protein (GFP). Stem cells were maintained in standard medium and differentiated *in vitro* using retinoic acid and conditioned media (collected from organ of Corti explants cultures [Coleman et al., 2007]). Briefly, following differentiation in retinoic acid, SC neurospheres were collected and washed twice for 5 minutes in 10% sterile sucrose in phosphate buffered saline (PBS; Invitrogen). After the final wash, all of the solution was removed and 5 μL of the neurospheres were carefully mixed with 5 μL of liquid *PuraMatrix*™ Peptide Hydrogel (BD Biosciences) in 4 well chamber slides (Nunc; one pellet per well), to give a neurosphere/hydrogel pellet of 10 μL total volume. The neurosphere/ hydrogel pellet underwent several gentle washes by emersion in 0.5 mL of sterile PBS for 2 minutes, followed by 0.5 mL of sterile DMEM/F12 media (Invitrogen) for 5 minutes, before being immersed in conditioned media collected from post-natal day 5 organ of Corti cultures. Each day, the media was removed and replaced with conditioned media from the organ of Corti cultures. Following 3 days growth in conditioned media, neurosphere/ hydrogel matrices were ready for transplantation (Fig. 2). Additional neurosphere/hydrogel matrices were fixed in 4% paraformaldehyde (PFA; BDH Laboratories), and embedded in OCT freezing medium on the day of transplantation. These were later cryosectioned and stained for the neural marker βIII tubulin (Millipore AB9354, 1:1000).

Mechanical properties of PuraMatrix™ peptide hydrogel

Hydrogels have tissue-like mechanical properties, providing a hydrated environment which is both biocompatible and biodegradable and making them ideally suited as tissue regeneration scaffolds [Mawad et al., 2012b]. Hydrogels possess a degree of flexibility very similar to natural tissue, due to their significant water content. Adjusting the water content can therefore change the mechanical properties of the gels slightly, as can the wide range of polymers from which they can be fabricated. The *PuraMatrix*™ hydrogel consists of standard amino acids (1% w/v) and 99% water, which is synthesized in small (16 amino

acids long, 5 nanometers) oligopeptide fragments that self-assemble into nanofibers on a scale similar to the *in vivo* extracellular matrix (a fibrous structure with an average pore size of 50–200 nm). The material comprises amphiphilic peptides that have alternating repeating units of positively-charged lysine or arginine and negatively-charged aspartate and glutamate residues. These peptides contain 50% charged residues and are characterised by their periodic repeats of alternating ionic hydrophilic and hydrophobic amino acids; thus, the interaction between the distinct polar and non-polar surfaces facilitates self-assembly of the material into a nanofiber hydrogel scaffold which can coat surfaces or encapsulate cells as a 3-D weak gel (http://www.puramatrix.com/technology/tec1.html).

Transplantation of stem cells

Two weeks post-deafening, guinea pigs were anaesthetized with ketamine (60 mg/mL, Parnell Laboratories) and xylazil (4 mg/mL; Troy Laboratories), and 2% Lignocaine (Troy Laboratories) was administered subcutaneously to the incision site. The left tympanic bulla was exposed under aseptic surgical conditions via a post-auricular incision. The bulla was drilled open and the basal turn of the cochlea visualized under a microscope. A small cochleostomy was made into the scala tympani at the level of the basal turn and a fine probe used to puncture the osseous spiral lamina wall overlying Rosenthal's canal. The prepared SC neurospheres embedded in hydrogel were then delivered to Rosenthal's canal using the fine tip of a micro-electrode. Following cell delivery, the cochleostomy was sealed with a muscle plug and the wound sutured in two layers. All transplantation surgeries were performed by a trained otologist using aseptic technique.

Processing of tissues for histology and immunohistology

All guinea pigs were transcardially perfused one week post-transplantation with 4% PFA. The cochleae were quickly removed, the apex gently punctured with a 30 gauge needle, and the cochleae post-fixed for a further 90 minutes in separate vials containing 4% PFA, and maintained on gentle rotation at room temperature. The cochleae were then decalcified in 10% EDTA for approximately two weeks (confirmed via radiography), and were trimmed and embedded for immunohistology as previously described [Coleman et al., 2009]. All cochleae were cryosectioned in the modiolar plane at 12 μm increments.

To examine AN density and the extent of any tissue response every second slide was stained with Hematoxylin and Eosin (H&E). Stem cell identification and survival *in vitro* and *in vivo* was detected by immunochemical labeling using antibodies to GFP and neurofilament 68 kDa (NFL) or βIII tubulin, in combination with the nuclear counterstain DAPI, in order to determine the location and differentiation status of transplanted cells. Briefly, cryosections were rinsed in PBS and post-fixed for 5 minutes in a weak solution of PFA (1.5%). After 2 rinses in PBS, sections were placed in blocking solution (comprising 3% bovine serum albumin, 4% goat serum, diluted in 0.1% Triton-X in PBS) for 2 hours. The primary antibodies (mouse anti-GFP, 1:200, Millipore; rabbit anti-neurofilament 200 kDa (NFH), 1:800, Millipore, Chicken anti-βIII tubulin, 1:1000, Millipore) were diluted in the blocking solution and tissues immersed overnight in an humidified chamber at 4°C. Any unbound primary antibody was removed by thorough rinsing in 0.1% Tween 20 (Promega) in PBS and the fluorescent secondary antibodies applied (Alexa Fluor 488 goat anti-mouse; Alexa Fluor 594 goat anti-rabbit; Alexa Fluor goat anti-chicken 647) at a 1:500 dilution in 3% BSA, 4% goat serum in 0.1% Tween 20 in PBS, for 4 hours at room temperature. Unbound secondary antibody was removed by thorough rinsing in PBS and then sections mounted in DAPI ProLong Gold fluorescent mounting medium (Molecular Probes). Sections were evaluated under a Zeiss Axioplan Microscope (Zeiss, Victoria, Australia) using a fluorescent lamp with appropriate filters (Zeiss filter set 00; 488000-0000, Zeiss filter set 02; 488002-0000 and Zeiss filter set 13; 488013-0000). Photomicrographs were

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taken using a Zeiss, AxioCam 12V monochrome digital camera and the computer software AxioVision 4.7.

Quantitative analysis

Detection of transplanted stem cells—Immunolabeled sections were examined for the presence of GFP-positive SCs. Surviving SCs were identified based upon their co-expression of GFP and DAPI. Results were expressed qualitatively to show whether cells were present in the basal, middle or apical cochlear turns.

Auditory neuron density measurements—Sections stained with H&E were examined using light microscopy. To minimize the potential for bias, twenty random sections were selected and counted blindly. Neural density measurements (cells/mm^2) were calculated using NIH Image software (http://rsb.info.nih.gov/nih-image/). The number of ANs with a visible nucleus was divided by the cross-sectional area of Rosenthal's canal, in both the lower basal turn and upper basal turn of the deaf treated, deaf untreated, and normal hearing cochleae. A non-parametric Kruskal-Wallis one-way analysis of variance was used to detect significant differences between these groups.

Inflammatory tissue response—The same cohort of H&E stained sections described above was then used to quantify the tissue response in the basal, middle and apical turns in the cochlea, in each group. The inflammatory tissue response was expressed as a percentage of the total area of the perilymphatic compartment that it occupied, and mean values expressed ± standard error of the mean (SEM). Sections in which a clear cochleostomy or perforation in the osseous spiral lamina wall was visible were deemed "surgical sites" and compared against adjacent sections. A Mann-Whitney Rank Sum Test was used to compare the tissue response measured between treated and untreated groups.

Results

Transplanted stem cells retain a neural phenotype during transplantation

Differentiated SC neurospheres retained their neural phenotype for the duration of the implant period, illustrated by expression of βIII tubulin both before (Fig. 3B) and after (Fig. 3D) transplantation into the deaf cochlea. Furthermore, weak expression of the neuronal marker NFH was detected within transplanted SC neurospheres at the end of the implant period (Fig. 4). Note, the variable luminescence of GFP expression in the R1 B5-EGFP SC line both *in vitro* (Fig 3. A,B) and *in vivo* (Fig 3. C,D).

Hydrogel retains transplanted stem cells in the basal turn of the cochlea

Stem cells encapsulated in hydrogel were retained in the basal turn of implanted cochleae (arrowheads, Fig. 4A,B; see also Fig. 6B), effectively limiting their dispersal throughout the perilymphatic compartments for the implantation period. Transplanted cells were identified via co-expression of GFP and DAPI (Fig. 4C–F). GFP-positive SCs were delivered to and detected within Rosenthal's canal and also in the scala tympani, in the lower basal turn of treated cochleae (arrowheads, Fig. 4C). No GFP-positive SCs were observed in the contralateral control cochleae (Fig. 4D). In one instance, a small number of SCs were observed in the upper basal turn scala tympani, proximal to the surgical site, however no SCs were observed in the middle or apical turns of the SC treated cochleae. Weak expression of the neuron-specific NFH protein was detected within transplanted GFP-positive SC neurospheres (red, Fig. 4F). The location of transplanted SCs within the treated versus untreated cochleae is summarized in Fig. 4G.

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Auditory neuron density is not significantly decreased following stem cell transplantation proceudres

To quantify the extent of AN loss following stem cell delivery, we calculated the density of neurons in the basal turn (Rosenthal's canal) for each experimental group. The basal turn was chosen due to the localization of the surgery and cochleostomy site to this region. Photomicrographs of Rosenthal's canal (Fig. 5A–F) illustrate the degree of AN loss in the lower (Fig. 5A–C) and upper (Fig. 5D–F) basal turns in deaf treated, deaf untreated and normal hearing animals. The box plot shows quantified data and statistically different groups (Fig. 5G). Statistical evaluation of data revealed that there was no significant difference in AN density in the lower basal turn of deaf treated (5A; 601 ± 37 cells/mm^2) versus deaf untreated (5B; 681 ± 27 cells/mm^2) animals. Both groups did however, have statistically lower AN densities in the lower basal turn than those calculated for normal hearing controls (5C; 912 ± 27 cells/mm^2; P 0.001). The same trend was observed in the upper basal turn, which showed no statistical difference in AN density measurements between deaf treated (5D; 737 ± 32 cells/mm^2) and deaf untreated (5E; 821 ± 32 cells/mm^2) animals. Again, both upper basal turn AN densities in the treated and untreated groups were significantly lower than those measured in normal hearing controls (5F; 1115 ± 33 cells/mm^2; P 0.001).

Transplantation of stem cells causes a localized tissue response, which is restricted to the surgical site

The tissue reaction at the surgical site was dominated by a lose areolar fibrous tissue (arrows; Fig. 6B, C). This tissue response reduced significantly away from the surgical site but did extend into other regions of the basal turn scala tympani (Fig. 6C). The extent of the tissue response was expressed as a percentage of the perilymphatic compartment occupied. As expected, no tissue response was observed in the lower, middle, or upper turns of any of the normal hearing (Fig. 6A) or deaf untreated cochleae (not illustrated). Conversely, animals that received SC transplants showed evidence of an extensive tissue reaction (44.7% ± 4.5%), that was confined to the surgical site in the lower basal turn (Fig. 6B, D). A small tissue response was measured in sections adjacent to the surgical site in the lower (3.9% ± 1.5%; arrow Fig. 6C) and upper (6.9% ± 1.8%; arrow Fig. 6B) basal turns of transplanted animals (illustrated graphically in Fig. 6D). These minor responses were not statistically different to one another (P = 0.817), but were significantly smaller than the tissue response measured at the surgical site (P 0.001; Fig. 6D). The tissue reaction measured at the surgical site was variable between treated cochleae, with some showing a minimal response (7.3% ± 1.4%) and others a much greater reaction (76.5% ± 9.8%; illustrated in Fig. 6B). Moreover, when quantified in mm^2 and compared to surgery only controls from a previous study [Backhouse et al., 2008], no significant difference was detected (0.5691 mm^2 ± 0.0658 mm^2 versus 0.5231 mm^2 ± 0.0478 mm^2, respectively).

Discussion

Some of the challenges associated with transplantation of SCs into the deaf mammalian cochlea include choice of delivery site and dispersal of transplanted cells after injection. The purpose of this study was primarily to evaluate the effectiveness of hydrogel at retaining SCs at their site of implantation. The study illustrated that hydrogel was effective at retaining cells at the implant site for the duration of the study, and that the density of ANs was not significantly altered following the delivery of SCs into Rosenthal's canal. Furthermore, a localized inflammatory tissue reaction was observed in treated cochleae, however this did not prevent the survival of SCs in the lower basal turn, and the localized nature of the tissue response supports our previous findings describing these surgical procedures in guinea pigs [Backhouse et al., 2008].

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The present study demonstrated that SCs survive *in vivo* delivery into the deaf mammalian cochlea within a hydrogel matrix, and retain expression of the neural protein βIII tubulin. In addition, the data illustrate that hydrogel was effective at retaining SCs in the lower basal turn for the duration of the study. Transplanted cells encapsulated in hydrogel were observed both within Rosenthal's canal and the scala tympani of the basal turn of the cochlea (Fig. 4A; Fig. 5A), an observation supported by others describing this surgical approach [Lang et al., 2008]. This is likely to be a consequence of cell delivery through the fluid-filled scala tympani, which makes precise cell delivery (even using micro-pipettes) challenging. The promising combination of using hydrogel with the Rosenthal's canal approach, is that even if some cells are delivered into the scala tympani during transplantation, they are retained at the site of implantation. Prevention of cell dispersal has been previously reported *in vitro* [Semino et al., 2004], and *in vivo* [Davis et al., 2005], however, this is the first report using hydrogel to minimize dispersal of SCs following delivery of SCs into the deaf cochlea.

In future, hydrogels may confer the benefit of providing a three-dimensional scaffold for new neurite outgrowth, before naturally biodegrading after 6–8 weeks *in vivo*. Because hydrogels comprise >99% water they are readily biodegradable and biocompatible (http://www.puramatrix.com/index.html). In this way, peptide hydrogels could provide an environment whereby newly transplanted SCs could more readily establish connections with endogenous neurons [Holmes et al., 2000, Ellis-Behnke et al., 2006], providing a scaffold until such time as connections could be made. Moreover, they can be fabricated from natural polysaccharides such as chitosan (cationic), alginate (anionic) or dextran (neutral), natural proteins such as collagen or gelatin, hyaluronan and fibrin, or, from a range of synthetic polymers [Mawad et al., 2012a, Pakulska et al., 2012], making them suitable to a variety of *in vivo* niches. For cell delivery or encapsulation for example, it is important that the hydrogel matrix be capable of solute transportation, to ensure allow effective exchange of nutrients and waste between exogenous cells and the surrounding environment. Moreover, the natural polymers agarose and chitosan have functional groups available for chemical modification [Pakulska et al., 2012], facilitating the design of tailor-made hydrogels for specific *in vivo* niches. The incorporation of specific neurotrophins and growth promoting molecules for example, may in future allow for sustained growth and specific differentiation of SCs *in vivo,* including the possibility of directed neurite outgrowth toward central and peripheral targets in the cochlea.

A fundamental consideration in transplanting SCs into Rosenthal's canal is to preserve the existing population of ANs, particularly given the persistence of a proportion of these neurons in deaf human subjects [Fayad and Linthicum, 2006]. An effective cell-based therapy would ideally avoid depleting the cells that it intended to replace, therefore the density of ANs was an important indicator of the efficacy of this approach. We observed that the density of ANs in deaf treated animals was not significantly different to the density of ANs in deaf untreated animals, in both the lower and upper basal turns of the cochlea. These findings illustrate the feasibility of this approach for future cell-based therapies given that the trauma from such invasive surgery does not result in a significant loss of endogenous neurons and is localized to the surgical site. With many studies now aimed at regenerating/replacing the ANs following deafness, it will be important to quantify the survival of endogenous ANs in order to compare the efficacy of various cell replacement strategies in the deafened cochlea in future.

The delivery of SCs into the deaf cochlea in the present study involved a cochleostomy into the lower basal turn scala tympani, followed by the perforation of the osseous spiral lamina wall overlying Rosenthal's canal. The inflammatory tissue response observed was moderate considering the invasive nature of this approach, and most importantly, was localized to the surgical site. The tissue response was identified histologically and comprised primarily

areolar fibrous tissue which was limited to the surgical site. New bone was also evident in the vicinity of the surgical site (arrowheads; Fig. 6B) and was likely associated with the presence of bone chips that entered the scala tympani during the drilling to create the cochleostomy [Clark et al., 1995]. In the treated cochleae, the mean cross-sectional area that inflammatory cells occupied was less than 50% of the perilymphatic space in the lower basal turn scala tympani. This was reduced to <10% in sections adjacent to the surgical site and in the upper basal turn, demonstrating the localized nature of this response. This is consistent with previous experimentation describing localized trauma to the osseous spiral lamina wall using the same approach [Backhouse et al., 2008], and during the insertion of a cochlear implant electrode array [Shepherd et al., 1983, Shepherd et al., 1995, Shepherd et al., 2005]. Such a response is undesirable clinically, due to the fibrous tissue which can surround the cochlear implant electrode array and contribute to increased impedance and, as a result, increased power consumption, of this electrical device [Xu et al., 1997, Newbold et al., 2004]. The tissue response observed in the current investigation is likely to resolve into mature fibrous tissue over more extended periods, and it may assist to retain transplanted cells within the perilymphatic space after the eventual degradation of the hydrogel. Using the described approach, SCs could be delivered at the same time as a cochlear implant electrode array. This may prove important, given the likelihood of a localized tissue response to SC implantation, and the effect this might have on electrode insertion at a later time.

Future studies will investigate the long-term survival and viability of SCs delivered in hydrogel, and these are currently underway in our laboratory. We envisage that the hydrogel will biodegrade over 6–8 weeks *in vivo*, leaving stem cells at the implantation site. However, we do not expect any subsequent migration of these cells, but rather that they will be contained within the fibrous tissue matrix that develops around the hydrogel. In addition, future studies will need to incorporate the development of delivery techniques that allow SCs transplanted into Rosenthal's canal to spread along the length of the modiolus. Previous studies have illustrated that widespread migration of SCs within the modiolus is indeed possible and that exogenous SCs survived in the denervated cochlea for the maximum implant period of 14 weeks [Corrales et al., 2006]. Yet to be elucidated is whether these transplanted cells are capable of forming functional and tonotopic connections, a vital step to the long-term success of SC replacement therapy in the cochlea. Combining cell transplantation with electrical stimulation from a cochlear implant, may confer the advantage of maintaining the residual population of auditory neurons whilst depolarizing the newly transplanted cell population. Previous studies have illustrated that chronic depolarization provides important trophic cues for auditory neurons *in vitro* [Hansen et al., 2001], and *in vivo* [Leake et al., 1999] and this may improve survivability and connectivity of newly transplanted SC-derived neurons.

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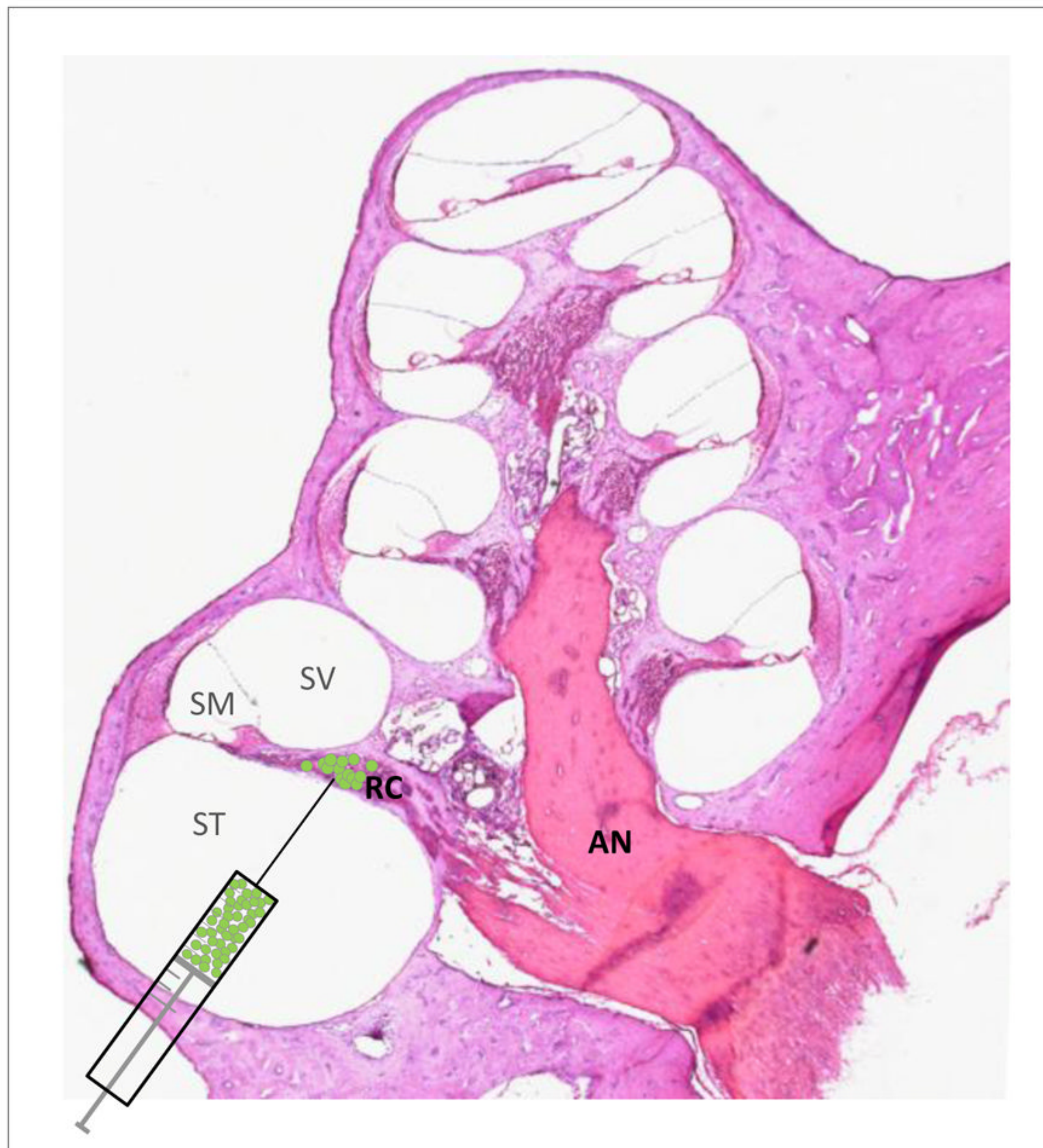

Figure 1. Transplantation approach for stem cell delivery into Rosenthal's canal

A post-auricular approach was used to transplant green fluorescent protein-positive SCs directly into Rosenthal's canal in the deafened guinea pig cochlea. ST: scala tympani; SM: scala media; SV: scala vestibuli; AN: auditory nerve; drawing not to scale.

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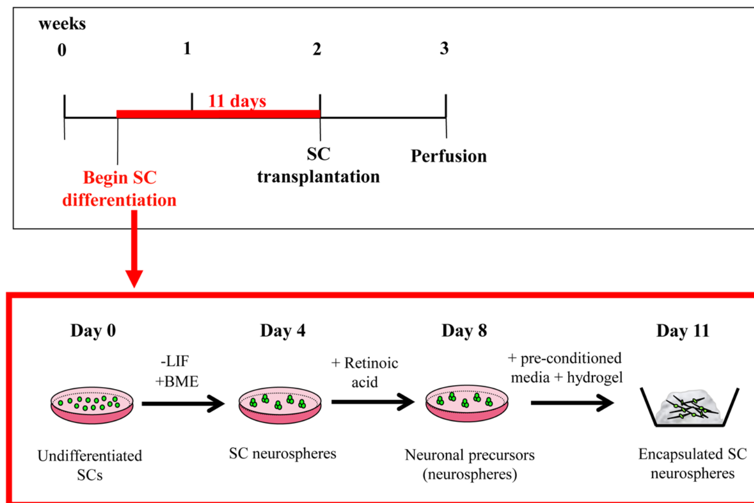

Figure 2. Timeline of stem cell differentiation and encapsulation in hydrogel

Detailed timeline showing differentiation of stem cells (SCs) into neuronal precursors *in vitro*. LIF: leukemia inhibitory factor; BME: β-mercaptoethanol.

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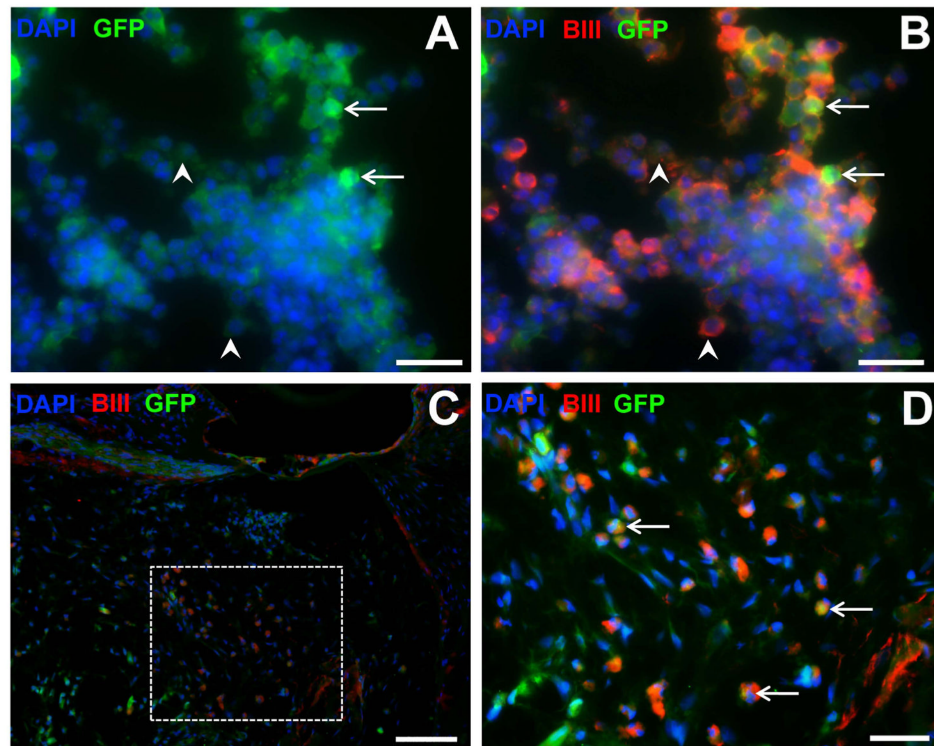

Figure 3. βIII tubulin labeling of stem cells *in vitro* and *in vivo*

Immunofluorescence photomicrographs of differentiated stem cells embedded in hydrogel *in vitro* (A,B) and *in vivo* (C, D), expressing green fluorescence protein (GFP, green), βIII tubulin (BIII, red) and counterstained with the nuclear marker DAPI (blue). *In vitro* preparations (A,B) illustrate the variable expression of GFP within this cell line (low expression, arrowheads; high expression, arrows). Low magnification image in (C) showing location of stem cells in the cochlea, and higher magnification of inset in (D) showing immunopositive labeling. Scale bars = 20 μm (A,B,D); 50 μm (C).

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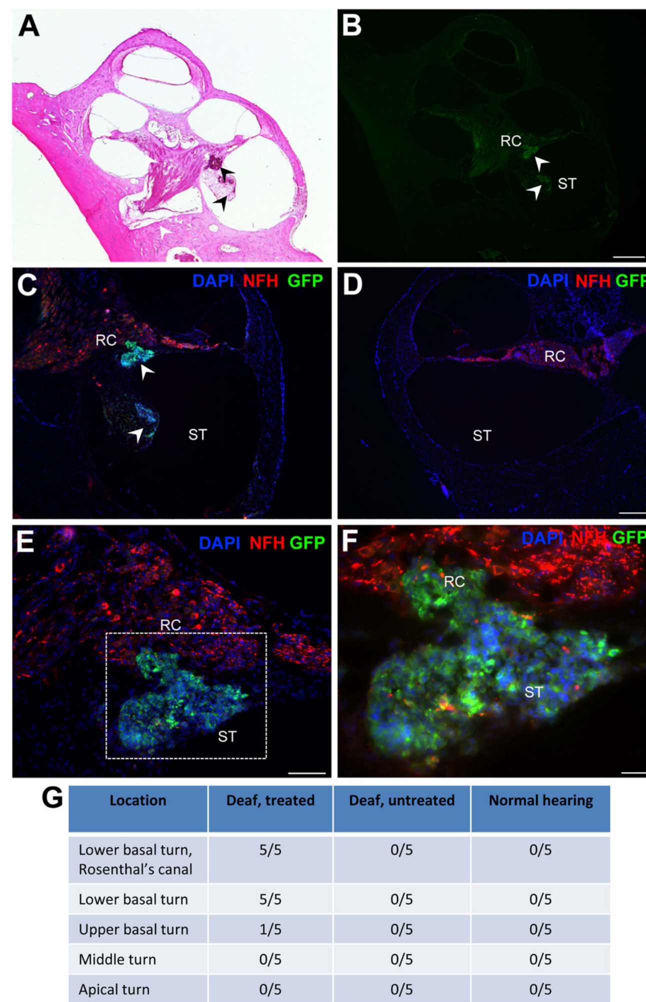

Location	Deaf, treated	Deaf, untreated	Normal hearing
Lower basal turn, Rosenthal's canal	5/5	0/5	0/5
Lower basal turn	5/5	0/5	0/5
Upper basal turn	1/5	0/5	0/5
Middle turn	0/5	0/5	0/5
Apical turn	0/5	0/5	0/5

Figure 4. Detection of GFP-positive stem cells in the deaf cochlea

Low magnification images show implanted stem cells in the lower basal turn of the cochlea (arrowheads in A: H&E stained, and B: GFP-stained). Sequentially higher magnification images of the lower basal turn are shown (C–F) illustrating implanted stem cells (GFP, green, arrowheads C) and endogenous auditory neurons and processes (NFH, red) in the lower basal turn Rosenthal's canal (RC) and scala tympani (ST). The same region in the deaf untreated cochlea is shown in (D), with no stem cells observed. High magnification image (F) of inset region in (E), illustrating stem cells in RC. Note implanted stem cell sphere is weakly positive for NFH. All images are counterstained with the nuclear marker DAPI (blue; C–F). Table in (G) gives summary of location of stem cells detected in each cochlea examined. Scale bars = 500 μm (B, relative to A); 200 μm (D, relative to C); 100 μm (E), 50 μm (F).

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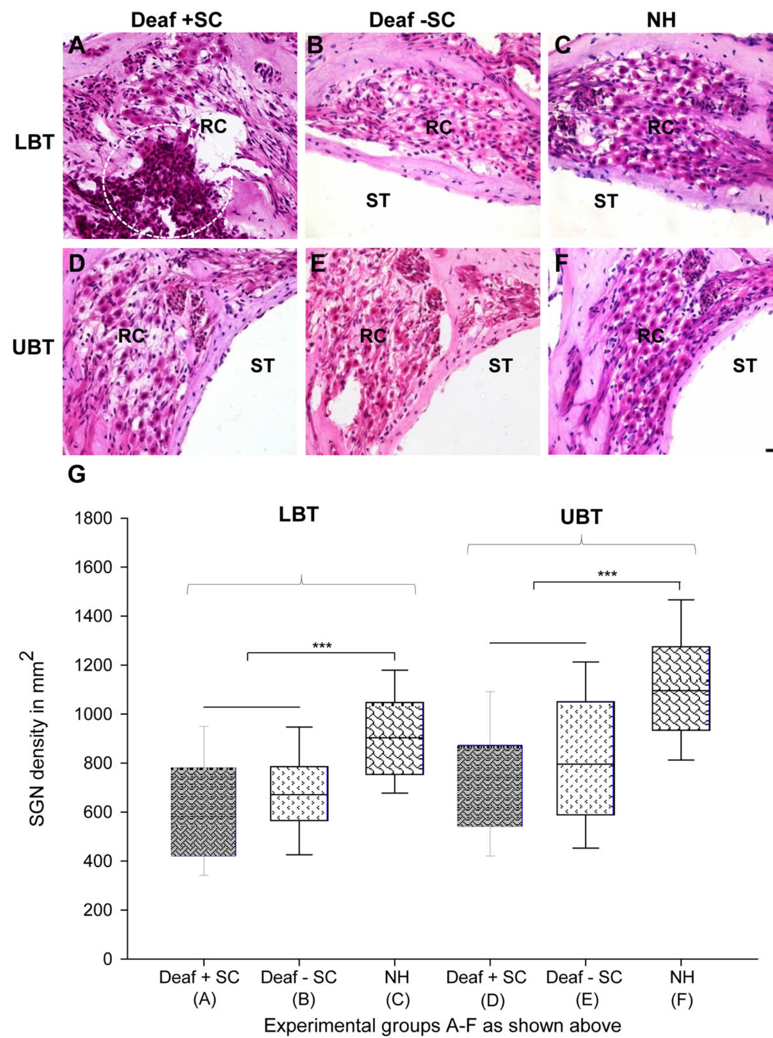

Figure 5. Auditory neuron density measurements and histology

Representative histological sections (A–F) from the upper and lower basal turns (UBT and LBT, respectively) from each treatment (Deaf + SC; Deaf – SC; NH), illustrating surviving auditoryneurons (ANs) and transplanted stem cells (circled, A). Auditory neuron densities from each treatment group (A–F above) were measured and graphed (G), and no significant difference was detected between deaf treated and deaf untreated animals in either the upper basal or lower basal turns. There were significantly fewer ANs in deaf cochleae (A,B, D,E) in comparison to normal hearing cochleae (P 0.001; ***). SC: stem cells; NH: normal hearing; ST: scala tympani; RC: Rosenthal's canal. Scale bar in (F) 20 μm (relative to A–F).

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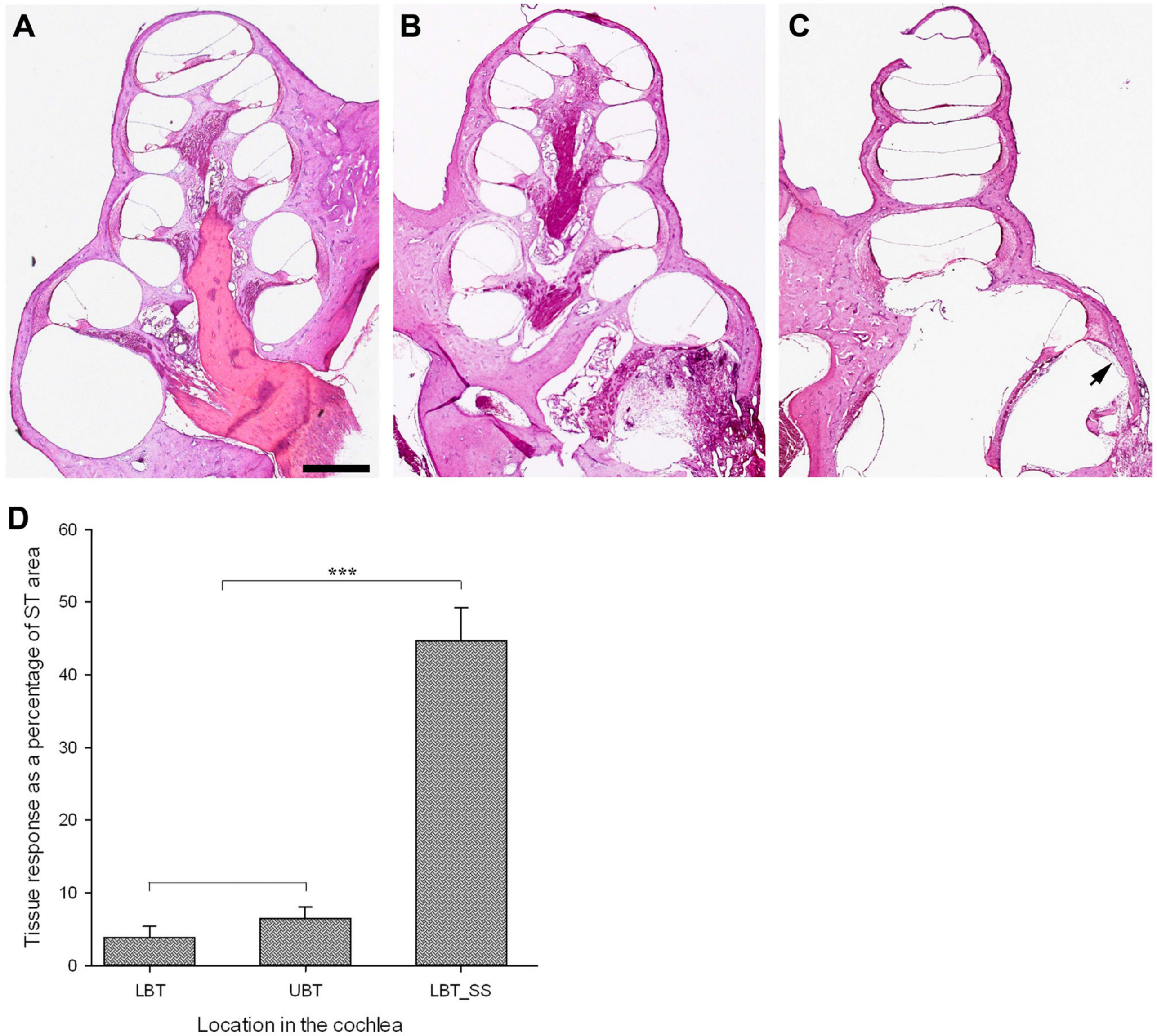

Figure 6. Tissue response measurements and histology

No tissue response was observed in untreated, normal hearing cochleae (A), however a localized areolar tissue response was observed at the surgical site in deafened, SC-treated animals (arrows B,C) along with a small amount of new bone growth (arrowheads, B). The most aggressive response observed is shown in (B); mean response measured across all five treated animals was $0.5691\ mm^2 \pm 0.0658\ mm^2$. Importantly, only a tiny response was observed in sections adjacent to the surgical site (arrow, C). Measurements and statistical analyses of the tissue response in the treated cochleae showed a small and non-significant reaction in the lower and upper basal turns (LBT and UBT, respectively), but a significant response which was localized to the surgical site (LBT_SS; D). Scale bar in (A) 1000 μm (relative to A–C).