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A PROTOCOL FOR CRYOEMBEDDING THE ADULT GUINEA PIG COCHLEA FOR FLUORESCENCE IMMUNOHISTOLOGY

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Abstract

Green fluorescent protein (GFP) has been used extensively to label cells *in vitro* and to track them following their transplantation *in vivo*. During our studies using the mouse embryonic stem cell line R1 B5-EGFP, we observed variable levels of fluorescence intensity of the GFP within these transfected cells. The variable fluorescence of this protein coupled with the innately autofluorescent nature of several structures within the cochlea collectively made the *in vivo* identification of these transplanted stem cells difficult. We have modified previously published protocols to enable the discrimination of an authentic GFP signal from autofluorescence in the adult guinea pig cochlea using fluorescence-based immunohistochemistry. The protocol described can also be used to label tissues of the cochlea using a chromogen, such as 3,3'-diaminobenzidine tetrahydrochloride (DAB). Moreover, the described method gives excellent preservation of structural morphology making the tissues useful for both morphological and quantitative studies in combination with robust immunohistochemistry in the adult guinea pig cochlea.

Keywords

Cochlea; stem cells; GFP; cryosectioning; cell transplantation; deafness

2. Introduction

The structural heterogeneity of tissues in the mammalian cochlea, its small size, the large volumes of fluid spaces within it and the fact that it is embedded in the temporal bone make the collection of well preserved morphological sections challenging. This is particularly true for the adult guinea pig (GP) cochlea, which contains four fluid-filled turns (the most of any mammalian species), is spherical in shape, and is approximately 4 mm high × 2 mm wide. Previously, the processing and collection of cochlear tissues for immunohistochemistry has been described as a compromise between the maintenance of morphology and preservation of antigenicity (Hurley et al., 2003). Although paraffin sections are generally considered superior to frozen sections in terms of their preservation of cochlear morphology, this technique involves high temperatures and treatment with strong chemicals. This often results in decreased immunogenicity of tissues and increased autofluorescence, making authentic signals difficult to detect. Conversely, frozen sections generally result in excellent antigenic preservation but

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freezing artifacts and crystal formation cause alterations to morphology (Whitlon et al., 2001). Although gelatin embedding has been used to achieve good morphology and immunochemical staining of the adult rat cochlea, the thinnest sections produced using this method were reportedly 20 μm (Hurley et al., 2003). More recently, 5 μm frozen sections were obtained from both the mouse and the rat cochlea embedded in OCT compound (Donadieu et al., 2007). To date, there are no detailed reports describing methods to preserve good morphology for immuno-analysis in the adult guinea pig cochlea.

For our initial attempts to transplant GFP expressing stem cells into the cochlea we chose paraffin wax for embedding, both to preserve the cochlear architecture and because we had experience using this technique. We thought it imperative in these early experiments to gain an appreciation of cell location and dispersal following transplantation, and needed to preserve morphology in order to achieve this. Although we were able to detect transplanted stem cells using direct fluorescence microscopy for GFP, the transplanted cells were not easily distinguished from the high background fluorescence observed after paraffin embedding the cochlea (Fig 1) (Hildebrand et al., 2005; Coleman et al., 2006). Indeed, GFP can be difficult to detect in biological materials which contain metabolites and structural components that can autofluoresce at similar wavelengths to GFP (Billinton and Knight, 2001). In an attempt to more accurately identify the transplanted cells, we then used an antibody directed against GFP combined with the avidin-biotin complex and 3,3'-diaminobenzidine tetrahydrochloride (DAB). Although we observed improved cell detection using DAB, we had to use antigen retrieval techniques to stain the tissues. The high temperatures required to perform this technique regularly caused damage to the 5 μm sections by altering their morphology, probably due to the diversity of tissues reacting differently to the heating procedure. Furthermore, the chromogen labelling was limited to one colour and we needed to detect multiple proteins in the same section.

Collectively, these observations and experiments led us to explore alternative ways in which to embed and detect antigens in the adult GP cochlea. The present protocol was therefore developed in order to detect exogenous green fluorescent protein (GFP) expressing stem cells following their transplantation into the deafened adult GP cochlea. The method gives clear discrimination of GFP from endogenous autofluorescence, whilst maintaining the superior tissue morphology often only observed using plastic embedding techniques. Notably, Parker and colleagues (2007) have since illustrated the potential to fluorescently label transplanted neural stem cells in paraffin embedded cochlear sections. Based on the method of Whitlon et al., (2001) for embedding, cryosectioning and immunolabelling early post-natal to 21 day old mouse cochleae, this manuscript describes an embedding protocol for use in the adult guinea pig cochlea. To the best of our knowledge, this is the first detailed report describing the collection of quality cryosectioned cochlear tissue from the adult guinea pig, that can be used for both histological and immunohistochemical analyses. The protocol described maintains excellent morphology in all four turns of the cochlea, and is suitable for multiple labelling with fluorescent antibodies and for chromogen-based detection.

3. Experimental procedure

All procedures were compliant with the Royal Victorian Eye and Ear Hospital Animal Research and Ethics Committee Guidelines (project approval numbers 02/090A, 03/099A).

Maintenance, differentiation and transplantation of stem cells

Genetically modified EGFP-expressing mouse embryonic stem cells [R1 B5-EGFP (Tg (GFPU)5 Nagy/J)] were obtained from Dr A. Nagy, (Mt Sinai Hospital, Toronto) (Hadjantonakis et al., 1998). They were maintained in standard media comprising DMEM (Invitrogen), 1000 units/mL leukaemia inhibitory factor (LIF; Chemicon) and 1 mL/L (1000X)

β -mercaptoethanol (BME; Chemicon), and passaged every 2-3 days using 0.025% trypsin in 1 mM EDTA (Applichem). When required for transplantation, stem cells underwent 11 days of differentiation using retinoic acid (Sigma) and pre-conditioned media from organ of Corti explants (Coleman et al., 2007b). Briefly, undifferentiated stem cells were induced to form embryoid bodies using retinoic acid treatment (Bain et al., 1995). After 8 days *in vitro* embryoid bodies were embedded in PuraMatrix™ hydrogel (BD Biosciences) and then exposed to pre-conditioned media taken from organ of Corti explants for a further 3 days prior to transplantation (Coleman et al., 2007a).

Pigmented adult GPs aged 8-12 weeks (400-600g) and taken from a mixed gender and gene pool were used in this study. All GPs were systemically deafened using a combination of the aminoglycoside antibiotic kanamycin monophosphate and the loop diuretic frusemide, as previously described (Coleman et al., 2006). Animals were deafened so as to maintain clinical relevance for future studies, however the deafening procedure was not essential to the development of this protocol. One week post deafening the left cochlea was exposed via a dorsal approach and a cochleostomy made directly below the round window into the lower basal turn scala tympani. A small opening was made in the osseous spiral lamina wall adjacent to the cochleostomy site and stem cells (embedded in hydrogel) were delivered directly into Rosenthal's canal and the lower basal turn scala tympani using the fine end of a titanium microelectrode (Fig 2). The goal of this technique was to deliver stem cells directly into their target site, while minimising their dispersal by delivering them in a three dimensional, biocompatible matrix (hydrogel). The cochleostomy was then sealed with a muscle plug and the wound sutured in two layers. All right cochleae remained untreated and served as contralateral controls. Animals were transcardially perfused one week after transplantation with 4% paraformaldehyde (PFA; BDH Laboratories) made in 0.1 M phosphate buffer (PB), pH 7.4 and pre-warmed to 37°C. The quality of the perfusion is critical for the collection of good quality tissue for immunocytochemical analysis.

Fixation and decalcification of cochleae

Cochleae were quickly removed from the temporal bone and a small hole made in the apex with a 30G needle to assist in solution infiltration. The cochleae were then placed into 10 mL of fresh 4% PFA (pre-warmed to 37°C) and maintained in this solution at room temperature for 90 min on rotation. After fixation, the cochleae were washed three times for 5 min in PBS (0.1 M, pH 7.6) and then each transferred into 20 mL of 10% EDTA (0.1 M PBS, pH 7.4) with rotation at 4°C for decalcification. The EDTA was changed daily until decalcification is complete (approximately 2 weeks). We confirmed complete decalcification via radiography.

Tissue infiltration and embedding of cochleae

The cochleae were gently removed from the EDTA and washed three times in PBS (0.1 M, pH 7.6) for 5 min. The cochleae must remain moist throughout the described procedure. Excess bone was trimmed from the decalcified cochleae using a scalpel and plastic forceps. Care was taken so as not to dislodge the cells delivered into the scalae. The cochleae were transferred into small glass vials (Wheaton 'snap cap' specimen vials; ProSciTech) and washed for 30 min in 10% sucrose (Univar; Ajax Finechem) on rotation at room temperature. The 10% sucrose solution was removed and replaced with a 15% sucrose solution and kept on rotation for a further 30 min at room temperature. The vials were then moved directly to 4°C and maintained on rotation overnight. The following morning, the solution of 15% sucrose was replaced with 1:1 solution of 15% sucrose and OCT. The vials were then moved back into the refrigerator and kept on rotation at 4°C overnight.

Approximately 2 mL of OCT was transferred into the remaining glass vials and they were placed into a vacuum desiccator under gentle vacuum overnight to ensure the removal of all

air bubbles (Whitlon et al., 2001). The cochleae were transferred into the degassed OCT, taking care not to introduce any air bubbles. Cochleae were incubated at 4°C overnight with rotation. The tissues were then placed under gentle vacuum for 30 min to remove any air trapped within the cochleae (Whitlon et al., 2001).

Tissue orientation and cryosectioning of cochleae

OCT was aliquoted into plastic cryomoulds to a depth of approximately 3mm and was degassed under vacuum for at least 30 minutes prior to use (Whitlon et al., 2001). Each cochlea was transferred into a cryomould with the round window facing downwards. A stereomicroscope was used to orient the cochleae such that the round window lay flat on the bottom of the cryomould and the modiolus was parallel to the base. Note that the guinea pig modiolus is visible once the otic capsule is decalcified.

As described by (Whitlon et al., 2001) once the cochleae are in the correct orientation, cryomoulds are transferred into a freezing slurry of solid CO₂ and 100% ethanol for 7-10 sec until tissue was immobilised. More OCT was then added to completely cover the tissue, and the sample was left in the freezing slurry until solidified. Each mould was then dried, wrapped in aluminium foil and stored in an air tight container at -80°C until required for sectioning (Whitlon et al., 2001).

The chamber and chuck temperature of the cryostat were set to -20°C and the cryomoulds were allowed to equilibrate in the cryostat for at least 60 minutes prior to sectioning. The OCT blocks were removed from the cryomoulds and cut to the shape of an isosceles trapezium. A small amount of OCT was placed onto a cold chuck and the cut OCT block pressed flat to the surface with the bottom surface (i.e. the round window) facing up. The angle of the chuck was adjusted until the blade was parallel with the flat surface (and therefore the modiolus). Sections were cut at 10 µm and mounted onto Fisherbrand SuperFrost Plus slides (3-4 sections per slide). As previously described by Whitlon and colleagues (2001) the slides were then air dried for 2 hr before they were transferred into plastic slide boxes, sealed inside snap-lock bags, and stored at -80°C until required for staining or immunochemistry. A quick protocol for the cryoembedding of the GP cochlea is provided in supplementary data 1.

Haematoxylin and eosin staining

Slides were air dried for 2 hr and then washed twice for 1 min in H₂O to rinse away the OCT. Sections were post-fixed onto the slides for a further 5 min in 1.5% PFA (in 0.1M PBS, pH 7.4) and then washed three times for 5 min in PBS (0.1 M, pH 7.6) (Whitlon et al., 2001). Sections were then stained with Haematoxylin and Eosin as follows: sections were emersed in Haematoxylin for 3 min, rinsed three times for 2 min in H₂O, placed into acid alcohol for 6-8 seconds (the staining intensity checked under the microscope before proceeding), rinsed twice for 2 min in H₂O, placed into Scott's solution for 30 seconds, placed directly into Eosin (6 seconds), and then rinsed briefly in tap H₂O. Sections were then dehydrated in 100% ethanol three times for 1 min. The dehydration step should be adjusted for each batch because the staining intensity can vary slightly depending on the number of slides stained at once. An acceptable stain will give a good contrast of colour in the spiral ganglion neurons, so that the nucleus can easily be distinguished from the soma. When a satisfactory contrast has been achieved, rinse the sections three times for 1 min in Histoclear. Mount the slides using DPX and leave to cure overnight before examining.

Fluorescence immunohistochemistry

Slides were air-dried for 2 hr prior to starting the procedures. Sections were post-fixed for 5 min in 1.5% PFA (in 0.1M PBS, pH 7.4) and then rinsed in H₂O (three times for 5 min) (Whitlon et al., 2001). The brief treatment in a weak solution of paraformaldehyde improved the

adherence of the tissue sections to the slide during lengthy immunohistochemical staining procedures. Standard techniques were used for fluorescence immunohistochemistry and have been described by others' (Whitlon et al., 2001; Alam et al., 2007; Donadieu et al., 2007). A PAP pen was applied around the sections and each section was blocked in 5% serum (relative to secondary antibody, in this case goat serum, Vector Laboratories) containing 0.1% Triton X-100 (Sigma; diluted using 0.1M PBS, pH 7.4) for 2 hr at room temperature. The first primary antibody mouse anti-GFP (Chemicon MAB3580) was applied diluted at 1:200 in blocking serum above and incubated overnight at 4°C in sealed chamber to prevent dehydration. The sections were subsequently rinsed five times for 5 min in 0.1% Tween 20 (Promega) diluted using 0.1M PBS, pH 7.4. The relevant secondary antibody Alexa Fluor goat anti-mouse 488 (Molecular Probes) was then applied, diluted at 1:200 in 0.1% Tween 20 for 4 hr at room temperature. The sections were then washed five times for 5 min in 0.1% Tween 20. The next primary antibody rabbit anti-neurofilament 68 kDa (Chemicon AB1983) was then applied diluted at 1:400 in 5% blocking serum and incubated overnight at 4°C in a sealed chamber. The sections were then washed five times for 5 min in 0.1% Tween 20 and the relevant secondary antibody Alexa Fluor goat anti-rabbit 594 (Molecular Probes) applied diluted at 1:200 in 0.1% Tween 20 for 4 hr at room temperature. The sections were washed five times for 5 min in PBS (0.1M PBS, pH 7.4) and then mounted directly in DAPI fluorescent mounting medium (Vector Laboratories). The coverslip edges were sealed with nail varnish and left to cure overnight at room temperature in the dark.

DAB immunohistochemistry

Standard chromogen-based immunolabelling techniques were used to detect GFP in cells that had been transplanted into the cochlea. The sections were air dried for 2 hr at room temperature then rinsed in PBS three times for 5 min before blocking for endogenous peroxidase in a solution containing 125 mL PBS, 90 mL H₂O, 10 mL 30% H₂O₂ and 25 mL MeOH for 10 min. Sections were then rinsed three times for 5 min in PBS (0.1M, pH 7.4). Each section was blocked in 5% serum (relative to secondary antibody, in this case donkey) containing 0.1% Triton X-100 (diluted in 0.1M PBS, pH 7.4) for 2 hr at room temperature. The primary antibody mouse anti-GFP (diluted 1:100 in 5% serum) was added to the sections and incubated overnight at 4°C in a sealed chamber to prevent dehydration. The primary antibody was removed the following day by rinsing five times for five minutes in 0.1% Tween 20 in PBS. The secondary antibody biotinylated donkey anti-mouse (Vectastain Elite ABC Kit PK-6102) was then applied for 4 hr at room temperature and was diluted 1:100 in 0.1% Tween 20 in PBS. The secondary antibody was rinsed from the sections three times for 5 min using 0.1% Tween 20 in PBS. Reagents A + B (Vectastain Elite ABC Kit PK-6102) were combined as per the manufacturer's directions (20µL A + 20µL B in 2mL of dH₂O) and incubated with the sections for 30 min at room temperature and then washed three times for 5 min in PBS. A nickel solution of DAB (Vector Laboratories) was prepared and applied to the sections for no more than 30 seconds. The DAB was then thoroughly rinsed from the sections using H₂O. The sections were then dehydrated three times for 1 min in 100% ethanol, then cleared in HistoClear three times for 1 min. Sections were mount directly in DPX and allowed to set overnight at room temperature.

4. Results and discussion

The increase in the number of investigations using cell- and molecular-based therapies for the treatment of hearing loss means that there is a requirement for robust detection techniques for exogenous cells and genes delivered into the mammalian cochlea. This is essential for the thorough analysis and comparison of various experimental treatments.

The protocol described herein, enabled the routine collection of 10 μm sections from the adult guinea pig cochlea for histological analysis for both H & E staining and for immunohistochemistry. Figure 3 shows a representative mid-modiolar section from a deafened adult GP following stem cell transplantation (3A). Note the maintenance of the outer cochlear wall and intact fluid-filled scalae. The thin Reissner's membrane is well-preserved and the described infiltration protocol does not dislodge cells that have been transplanted into the scalae of the cochlea. The described embedding protocol has also been used for morphological analyses in the normal hearing guinea pig cochlea (Backhouse et al., 2008). Figure 4 shows representative examples from the cochleae of normal hearing animals stained with H & E. Both the inner and outer hair cells of the organ of Corti are readily identifiable (4C) and the spiral ganglion neurons can be quantified based upon their morphology (their nucleoli can be visualized under high magnification, 4D) (Backhouse et al., 2008). These features make the described protocol useful for both the identification of transplanted cells in the cochlea and for morphological analyses in the guinea pig cochlea.

The stepwise infusion of 10-15% sucrose is an important step in the maintenance of good morphology for cryosectioning of the adult GP cochlea. The slow infiltration of increased concentrations of sucrose was applied by Barthel and Raymond (1990) and first applied to the cochlea by Whitlon et al (2001). The sucrose acts as a partial dehydrant which protects the tissue against freezing artefacts caused by ice crystal formation when the tissue is frozen (Tokuyasu, 1973). A concentration that is approximately isotonic with the tissue is ideal because it does not cause either swelling or shrinking of the tissues. The final concentration of sucrose should therefore be adjusted to suit the type of tissue being processed, so that the tissues are both protected without excessive dehydration and so as to give the desired hardness for sectioning. (Note that lower concentrations of sucrose will produce harder blocks). In addition, the overnight incubation steps with gentle rotation ensure that the solutions are consistently distributed throughout the scalae of all turns in the GP cochlea. When the whole cochlea is subsequently frozen, there are minimal variations in embedding medium between scalae, which contributes to maintaining tissue structure integrity after freezing.

Initial attempts to transplant stem cells into the cochlea, described the delivery of cell suspensions directly into the fluid-filled compartments (Iguchi et al., 2003; Hildebrand et al., 2005; Coleman et al., 2006). While this technique may have facilitated cell migration to sites of interest, it also allowed for cell dispersal throughout the cochlea, and potentially into the cerebrospinal fluid via the cochlear aqueduct (Coleman et al., 2006). In the present study we therefore delivered stem cells into the cochlea in hydrogel - a biocompatible, three dimensional matrix - which limited the dispersal of transplanted cells to the delivery site, the lower basal turn scala tympani. The hydrogel may also have assisted in keeping transplanted cells from dislodging during the trimming and embedding process, because once decalcified, the GP cochlea becomes quite malleable. If exposed to external pressure during this process, fluid (and potentially transplanted cells) could be forced out of the cochlea. It is therefore imperative to trim the cochleae under a dissecting microscope to ensure that no pressure is put on the decalcified cochlear wall. The cochleae should also be handled carefully throughout the entire embedding process so as to avoid any force that might cause internal movement of fluids.

Using an antibody to GFP, transplanted stem cells could be identified at their delivery site - the lower basal turn scala tympani and Rosenthal's canal - with very little background fluorescence observed (Fig 5). A second antibody to neurofilament (NFL) was used to identify endogenous spiral ganglion neurons and labelling was restricted to the soma and processes of these neural elements. All cells in the tissue sample were counterstained using the nuclear marker DAPI. Figure 5 shows both low and high power photomicrographs of all three labels, GFP (green), NFL (red) and DAPI (blue). The overlay (F) illustrates co-localisation of these

proteins in particular DAPI/NFL in the spiral ganglion neurons and DAPI/GFP in the transplanted stem cells.

Green fluorescent protein expression in transplanted stem cells was also examined using DAB-based immunohistochemistry (Fig 6). Green fluorescent protein expression was localised to transplanted cells (6B) but was not detected in negative control sections which were treated identically but without the addition of the primary anti-GFP antibody (6C). A weak background signal was observed in both the experimental and control sections, which is common for DAB-based immunohistochemistry. High magnification photomicrographs further illustrate the positive signal in transplanted stem cells and their *in vivo* morphology (6D).

A complicating factor in this line of research has been the variability in the intensity of the GFP observed in the R1 B5-EGFP mouse embryonic stem cell line. Although rarely published, this observation is consistent with the observations of other researchers in the field. It is not known why fluorescence levels are variable, although it is likely that this is related to the efficiency in which the GFP gene was initially incorporated into the stem cells and the number of cell divisions the cell has undergone. However, using immunohistochemistry against GFP we were able to reliably detect transplanted stem cells because the protein is still present in the cell (even if the fluorescence has faded to background levels). This finding reiterates the requirement for good morphological sections from which immunohistochemical labelling can also be performed, so that transplanted cells can be labelled with multiple probes which confirm their identity. It also confers the advantage of giving a better overall understanding of the interaction between exogenous and endogenous tissues.

This present study describes the application of a method originally described by Whitlon and colleagues (2001), for microscopic analysis of the adult guinea pig cochlea. Although previous studies have reported rapid processing of mouse and rat cochleae for immunohistochemical analyses (Whitlon et al., 2001; Hurley et al., 2003; Donadieu et al., 2007) this is the first report combining a detailed procedure for cryoembedding, cryosectioning and double-immunofluorescence labeling of the adult GP cochlea. The adult GP cochlea is a challenging mammalian organ to successfully embed, cryosection and immunolabel for several reasons. Firstly, it contains four fluid-filled turns, each containing three compartments which are separated by thin membranes. The number of turns in the GP cochlea makes the sequential infiltration of solutions time consuming, and increases the likelihood of introducing air into the tissue freezing medium throughout the embedding process. Air trapped within the cochlea compartments will result in the collection of poor tissue sections (Whitlon et al., 2001). In addition, tissue fixation via transcardial perfusion is critical in order to avoid apoptosis-induced auto fluorescence in adult GP cochlear tissue. A poor or incomplete perfusion can initiate apoptotic cell signalling cascades and the subsequent release of cytochrome c (a small autofluorescent heme protein) into the cytoplasm. Moreover, the adult GP cochlea must be adequately decalcified for two weeks prior to embedding as any residual calcified tissue will cause differences in tissue rigidity, resulting in the collection of distorted sections. We used radiography to confirm complete decalcification of the cochleae. Collectively, these procedures are integral to obtaining high quality cryosectioned cochlear tissue for histological and immunohistochemical analyses from adult GPs. Similar procedures are being optimized for use on cat cochleae by other members of our laboratory (Fallon et al., 2008).

This manuscript describes a protocol for the cryoembedding of the GP cochlea and detection of transplanted cells using either fluorescence-based immunohistochemistry (for two or more labels, Fig 5) or chromogen-based immunohistochemistry (for a single label, Fig 6). The described protocol has also been used successfully for immunofluorescent confocal microscopy on adult cochlear tissues from normal hearing GPs (see supplementary data 2), and this can be customized to suit different experimental aims by adjusting the thickness of

the tissue sections collected. Moreover, the same protocol gives excellent preservation of cochlear morphology making it suitable for morphological quantification studies using haematoxylin and eosin staining (Figs 3 and 4), and in the quantification of inflammatory tissue response and/or biocompatibility resulting from different experimental treatments. We envisage these procedures will assist in the identification of exogenous cells and/or genes delivered into the adult guinea pig cochlea, thereby improving the quality of analysis of such studies in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1. Autofluorescence in the paraffin-embedded guinea pig cochleae

Fluorescence microscopy images illustrate the autofluorescent tissues in the adult ototoxin-treated guinea pig cochlea, following embedding in paraffin wax. These transverse mid-modiolar sections are 5 μm thick and are taken from a control cochlea (A; no stem cell transplant) and a treated cochlea (B; 4 weeks following stem cell transplant into the lower basal turn scala tympani, ST). Photomicrograph (B) shows transplanted stem cells located along the wall of the ST (arrows). Transplanted cells were difficult to detect because they had a similar level of fluorescence to background autofluorescence observed using paraffin embedding. RC = Rosenthal's canal. Scale bar in B = 50 μm and relative to A.

Fig 2. Surgical approach for stem cell transplantation into the lower basal turns

(A) The cochlea (C) was exposed via a dorsal approach and a cochleostomy (CO) made directly below the round window (RW) to expose the osseous spiral lamina wall in the lower basal turn. A tiny opening was made in the medial osseous spiral lamina wall using the sharp tip of a fine probe (*). (B) Stem cells (SC) were delivered directly into the lower basal turn and Rosenthal's canal (*) on the tip of a microelectrode and were held together in hydrogel to minimise their dispersal. (C) The transplanted stem cells (SC) in hydrogel were observed in the lower basal turn scala tympani (circled). Scale bar = 1 mm (A), (relative to B and C).

Fig 3. Preservation of structure and morphology of the deafened adult guinea pig cochlea embedded in OCT and stained with haematoxylin and eosin (H & E)

A) Low power photomicrograph of a mid-modiolar transverse section through the adult mammalian cochlea. The cochlea has been ototoxically damaged via systemic delivery of kanamycin monophosphate. Note the excellent preservation of tissues, demonstrated by the intact Reissner's membrane in every turn. Stem cells were delivered into the lower basal turn scala tympani (ST).

B) Photomicrograph of a single cochlear turn (boxed region in A) illustrating more clearly the intact Reissner's membrane (arrow) and well preserved cochlear morphology. The spiral ganglion neurons are situated in Rosenthal's canal (RC).

C) and D) High magnification images of the organ of Corti and Rosenthal's canal (RC), respectively. Note the collapsed organ of Corti (arrow, C) as a result of ototoxic deafening and subsequent reduction of spiral ganglion neurons, located in RC. Scale bars = 500 μ m (A), 100 μ m (B), 50 μ m (C,D).

Fig 4. Preservation of structure and morphology in the normal hearing adult guinea pig cochlea embedded in OCT and stained with haematoxylin and eosin (H & E)

A) Low power photomicrograph of a mid-modiolar transverse section through the normal hearing guinea pig cochlea. Note the preservation of tissues throughout all four turns of the guinea pig cochlea.

B) Photomicrograph of a single turn in the normal hearing cochlea (boxed region in A) illustrating the well preserved morphology. The organ of Corti is intact in the normal hearing cochlea (arrow). Scala tympani (ST), Rosenthal's canal (RC).

C) and D) High magnification images of the organ of Corti and RC, respectively. Note the structure of the organ of Corti is maintained and the four rows of hair cells can be distinguished (one inner hair cell and three outer hair cells, arrows, C). The spiral ganglion neurons located in RC can be quantified based upon their visible nucleoli (arrows, D).

Fig 5. Detection of transplanted GFP expressing stem cells in the adult mammalian cochlea using fluorescence immunohistochemistry

A) Haematoxylin and eosin stained adjacent cochlear section illustrating site of interest (boxed).

B) Double-labelled section illustrating transplanted stem cells (green) and endogenous neurons and processes (NFL, red). All cells can be identified by the nuclear stain DAPI (blue). The delineation between Rosenthal's canal (RC) and the scala tympani (ST) is shown by the perforated white line.

C)-F) High magnification images of the boxed region in B) are shown in images C-F; DAPI (blue) GFP (green) and NFL (red) are shown separately in C), D) and E) respectively. Co-localisation of these markers is shown in the overlay in F).

F) Transplanted GFP expressing stem cells were detected both in the ST and RC (arrows). The approximate separation between the ST and RC is illustrated by the white perforated line. Note the specificity of NFL labelling to endogenous spiral ganglion neuron soma and processes and GFP labelling to transplanted stem cells, with very low background. Scale bars = 500 µm (A), 100 µm (B), 50 µm (C-F).

Fig 6. Green fluorescent protein immunohistochemistry in the adult GP cochlea using a DAB chromogen label

A) Cochlear schematic illustrating orientation of sections shown in B and C.

B) Low magnification image illustrating GFP labelling in exogenous stem cells transplanted into the scala tympani (ST) of a deafened guinea pig (arrows).

C) Low magnification image of negative control section (no primary antibody), demonstrating the absence of a positive signal in transplanted stem cells (arrows). The delineation between Rosenthal's canal (RC) and the ST is depicted by the perforated line in both B) and C).

D) High magnification image of boxed region in B, further illustrating the positive signal detected in transplanted stem cells and the *in vivo* morphology of these transplanted cells. Scale bars = 500 μ m (A), 100 μ m (B,C), 20 μ m (D).