JARO 6: 341–354 (2005)
DOI: 10.1007/s10162-005-0012-9

JARO
Journal of the Association for Research in Otolaryngology

Survival of Partially Differentiated Mouse Embryonic Stem Cells in the Scala Media of the Guinea Pig Cochlea

MICHAEL S. HILDEBRAND,[1,2] HANS-HENRIK M. DAHL,[1,3] JENNIFER HARDMAN,[4] BRYONY COLEMAN,[4,5] ROBERT K. SHEPHERD,[4,5] AND MICHELLE G. DE SILVA[1]

[1]*Murdoch Childrens Research Institute, Royal Children's Hospital, Melbourne, Australia*

[2]*Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, Australia*

[3]*Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Melbourne, Australia*

[4]*The Bionic Ear Institute, Melbourne, Australia*

[5]*Department of Otolaryngology, University of Melbourne, Royal Victorian Eye and Ear Hospital, Melbourne, Australia*

Received: 10 February 2005; Accepted: 18 July 2005; Online publication: 6 October 2005

ABSTRACT

The low regenerative capacity of the hair cells of the mammalian inner ear is a major obstacle for functional recovery following sensorineural hearing loss. A potential treatment is to replace damaged tissue by transplantation of stem cells. To test this approach, undifferentiated and partially differentiated mouse embryonic stem (ES) cells were delivered into the scala media of the deafened guinea pig cochlea. Transplanted cells survived in the scala media for a postoperative period of at least nine weeks, evidenced by histochemical and direct fluorescent detection of enhanced green fluorescent protein (EGFP). Transplanted cells were discovered near the spiral ligament and stria vascularis in the endolymph fluid of the scala media. In some cases, cells were observed close to the damaged organ of Corti structure. There was no evidence of significant immunological rejection of the implanted ES cells despite the absence of immunosuppression. Our surgical approach allowed efficient delivery of ES cells to the scala media while preserving the delicate structures of the cochlea. This is the first report of the survival of partially differentiated ES cells in the scala media of the mammalian cochlea, and it provides support for the potential of cell-based therapies for sensorineural hearing impairment.

Keywords: cochlea, scala media, hearing loss, mouse embryonic stem cells, green fluorescent protein, xenotransplantation

Correspondence to: Michelle G. de Silva • Department of Gene Identification & Expression • Murdoch Children's Research Institute, Royal Children's Hospital • Flemington Road, Parkville, Melbourne, Victoria, Australia 3052. Telephone: 61-38-3416254; fax: 61-38-3416212; email: michelle.desilva@mcri.edu.au

INTRODUCTION

To achieve restoration of auditory function following sensorineural hearing loss, it will be necessary to regenerate or replace sensory hair cells. This is a complex task as the organ of Corti where the hair cells are located is difficult to access. The scala media is protected by a robust blood–endolymph barrier that maintains the stability of the endolymph bathing the apical surface of the organ of Corti (Juhn 1988). We therefore delivered embryonic stem (ES) cells directly into the scala media through the basilar membrane. Using this approach, we have achieved efficient delivery of ES cells into the scala media and have demonstrated their survival for at least nine weeks. Importantly, the structures of this highly regulated scalae were not adversely affected by the implantation of ES cells via this route.

Recent advances in ES cell technology have allowed us to culture and differentiate stem cells *in vitro* (Rathjen et al. 1999; Lake et al. 2000; Rathjen et al. 2002). It is possible that these cells could be

used to facilitate *in vivo* cell therapy of the inner ear, potentially replacing sensory hair cells lost or damaged by loud sound, exposure to ototoxic drugs (Palomar Garcia et al. 2001), aging, or hereditary gene defects (Kelsell et al. 1997; Tekin et al. 2001). Loss of mammalian hair cells is permanent and causes irreversible hearing defects in humans (Palomar Garcia et al. 2001). The discovery that hair cells of the avian basilar papilla (BP), the functional equivalent of the mammalian organ of Corti, are regenerated after preexisting hair cells have been destroyed (Corwin and Cotanche 1988; Ryals and Rubel 1988; Warchol and Corwin 1996) has stimulated much interest in the possibility of hair cell regeneration therapy in mammals (Staecker and Van De Water 1998). The continuous formation of cochlear sensory epithelial cells with and without insult to the auditory system has also been demonstrated in the lower vertebrate inner ear, but not in mammals (Cotanche and Lee 1994; Stone and Rubel 2000; Reng et al. 2001).

The delivery of ES cells to damaged tissues *in vivo* has been reported (Björklund et al. 2002; Rideout et al. 2002). The objective of cell therapy in the inner ear is to restore auditory function by regenerating or replacing damaged or lost sensory hair cells, auditory neurons, and supporting cells. One of the first reports of stem cell delivery to the inner ear was the study by Ito et al. (2001) that demonstrated survival and migration of adult rat neural stem cells implanted into the scala tympani of the rat cochlea. The beta-galactosidase (β-Gal)-expressing cells migrated to the organ of Corti, and some cells were shown to adopt hair cell-like morphology and to stain with phalloidin that binds to the F-actin in stereocilia and other structures (Ito et al. 2001). The authors speculated that if the stem cells could localize to the correct region of the cochlea, then they would take on hair cell characteristics (Ito et al. 2001). However, the correct targeting of stem cells to the organ of Corti alone is unlikely to be sufficient to promote hair cell development and differentiation as the appropriate developmental cues may not be present in the adult cochlea. The partial differentiation of ES cells *in vitro* prior to implantation may provide these cells with the developmental potential to form new hair cells.

Since then, other groups have reported on the transplantation of ES cells into the inner ear (Hu et al. 2004; Sakamoto et al. 2004). Sakamoto et al. (2004) reported the survival of ES cells predominantly in the vestibular region of the mouse inner ear and also some cells in the scala media of the cochlear duct after transplantation for four weeks. In comparison, the study by Hu et al. (2004) demonstrated the survival and migration of mouse ES cells along the auditory nerve after xenotransplantation into auditory nerve fibers (ANFs) of the rat cochlea. They showed that the ES cells could survive for up to nine weeks and that they migrated along the ANFs into the brainstem. Although these studies have demonstrated the survival of ES cells in the cochlea, the efficiency with which cells are delivered or migrate to the scala media has been low and neither have examined the survival of partially differentiated cell types.

The formation of new hair cells may require predifferentiation of progenitor ES cells prior to their transplantation *in vivo* as the damaged cochlear sensory epithelium may not be able to provide all the necessary developmental signals. Cochlear hair cells arise from neuroectodermal precursors and their development is dependent on regulation of many genes and growth factors (Malgrange et al. 2002; Pirvola et al. 2002). ES cells cultured in the presence of the medium conditioned by the human hepatocarcinoma cell line HepG2 (MEDII medium) have been shown to form morphologically distinct primitive ectoderm-like cellular aggregates (EBMs) (Rathjen et al. 1999; Rathjen et al. 2002). Treatment of the aggregates with basic fibroblast growth factor (bFGF) directs the cells down the neuroectodermal lineage (Torres and Giraldez 1998). In the current study, a differentiation strategy was established *in vitro* to generate cells along the neuroectoderm pathway. Cell types from this developmental model were selected for *in vivo* transplantation into the guinea pig cochlea to attempt cellular replacement or regeneration of auditory hair cells. The cell types included undifferentiated ES cells, ES cells partially differentiated in MEDII medium for three or seven days, and ES cells partially differentiated over nine days with MEDII medium and bFGF. The cells were delivered into 14 guinea pigs deafened by administration of aminoglycosides prior to implantation and into one normal hearing animal. It was demonstrated that mouse ES cells could survive for at least nine weeks in the guinea pig cochlea and that they could localize in the scala media. Some transplanted cells localized close to the organ of Corti. There was no evidence of significant immunological rejection despite the absence of immunosuppression, and the surgical approach for delivery of ES cells resulted in minimal extraneous trauma to the cochlea.

MATERIALS AND METHODS

All procedures involving animals had the prior approval of the Royal Victorian Eye and Ear Hospital Animal Ethics Committee and the Royal Children's Hospital Animal Experimentation Ethics

Committee. A total of 19 guinea pigs were used in this study. Fifteen guinea pigs were implanted with either undifferentiated or partially differentiated ES cell types. Fourteen of these animals were deafened using aminoglycoside antibiotic prior to implantation and one animal had normal hearing. In addition, four normal hearing control animals underwent mock surgeries with ES media alone delivered to the scala media. The guinea pigs were further divided into the experimental groups described in Table 1.

Cell culture

Mouse R1 B5-EGFP [Tg(GFPU)5 Nagy/J] cells were provided by Andras Nagy (Mount Sinai Hospital, Toronto, Canada) and cultured in ES media (DMEM; Trace Scientific, Australia) supplemented with 10% fetal bovine serum (Trace Scientific), 0.8 mM glutamine (Life Technologies, Scotland, UK), 0.2 mM β-mercaptoethanol (β-ME; Sigma, USA), and 1000 units of mouse leukemia inhibitory factor (mLIF; Chemicon, USA) under 5% CO_2 in a 37°C humidified incubator. Conditioned MEDII medium was prepared from the human hepatocarcinoma cell line HepG2 as described by Rathjen et al. (1999). The formation of embryoid bodies (EB and EBM) was conducted as previously reported (Lake et al. 2000). EBM cells were formed and maintained as embryoid bodies in suspension culture for seven days in media containing 50% MEDII conditioned medium and 50% ES media (excluding mLIF). EB and EBM embryoid bodies were then treated with 10 ng/ml basic fibroblast growth factor (bFGF; Invitrogen, USA) in 50% serum free DMEM (JRH Biosciences, USA) and 50% serum free Ham's F12 (Trace Scientific) media supplemented with 1× insulin–transferrin–sodium selenite media supplement (ITSS, Sigma) to day 9. EBMs were either mechanically disaggregated (guinea pigs 1–5, Table 2) or were disaggregated by incubation with 0.025% trypsin/EDTA for 1 min at 37°C (guinea pigs 6–15, Table 2) prior to implantation.

Isolation of total RNA from ES cells and generation of cDNA

Total RNA was isolated from B5-EGFP ES cells and EBM cell aggregates harvested at days 3, 7, and 9 using an RNeasy Midi Kit (Qiagen) according to the manufacturer's directions. RNA was combined with 1× hexonucleotide mix (Roche, Germany) and incubated at 65°C for 15 min. Then, 1× AMV buffer (Roche), 40 units RNase Inhibitor (Roche), 300 μM dNTPs (Promega, USA), and 40 units AMV reverse transcriptase (Roche) were added and the samples were incubated at 42°C for 1 h to generate cDNA.

Semiquantitative RT-PCR

The expression of the hair cell markers: myosin VI (*Myo6*); cholinergic receptor, nicotinic, alpha polypeptide 9 (*α9AchR*); and myosin VIIa (*Myo7a*) was determined in the differentiating ES cells by semiquantitative RT-PCR using 18S rRNA as the endogenous control. RT-PCR was performed with 1.5 mM $MgCl_2$, 100 μM dNTPs, 25 pmol of each primer, and 50 ng template cDNA. Primer sequences were as follows (forward, reverse, and cDNA product length): *Myo6*, 5-AGAATCAGGAGCTGGCAAAA-3′, 5′-ATTTCCAAGGTGCAGGACAC-3′, 598 bp; *α9AchR*, 5′-CCTGACCTTTGGTTCCTGG-3′, 5′-GCTTCATAGCGTGCACAGTAGC-3′, 762 bp; *Myo7a*, 5′-GCTGTATTATCAGCGGGGAG-3′, 5′-CAGGTGATGCAGTTACCCATG-3′, 409 bp; and 18S rRNA, 5′-AGAGCTAATACATGCCGACG-3′, 5′-ATAGGGCAGACGTTCGAATG-3′, 199 bp. The cycling parameters for each gene were optimized to generate products at

TABLE 1

Comparison of survival of cell types in the cochlea over different transplantation periods

	Cell type and transplantation period					
	Undifferentiated B5-EGFP		*EBM day 3*		*EBM day 7*	*EBM FGF day 9*
	2 weeks	*9 weeks*	*2 weeks*	*9 weeks*	*4 weeks*	*4 weeks*
Number of animals receiving transplanted cells	4	2	4	2	2	1
Number of animals containing surviving cells	4/4	2/2	4/4	2/2	2/2	1/1
In scala vestibuli	4/4	2/2	4/4	1/2	2/2	1/1
In scala media	4/4	2/2	4/4	2/2	2/2	0/1
In scala tympani	4/4	2/2	4/4	2/2	2/2	1/1

The survival of undifferentiated and partially differentiated mouse B5-EGFP cells in the three scala of the guinea pig cochlea. Surviving transplanted EBM FGF day 9 cells were not observed in the scala media cavity after four weeks. Transplanted EBM day 3 cells were only observed in the scala vestibuli cavity of one guinea pig after nine weeks.

TABLE 2

Analysis of auditory function of guinea pigs

Guinea pig	*B5-EGFP cell phenotype*	*Implantation duration*	*Kanamycin administered (mg/kg)*	*Initial threshold (dB)*	*Postdeafening threshold (dB)*	*Postsurgery threshold (dB)*
1	Undifferentiated	2 weeks	400	42–45	40	47–50
2	EBM day 3	2 weeks	400	42–45	95	95–97
3	EBM day 7	4 weeks	N/A #	47–50	N/A #	40–42
4	EBM day 7	4 weeks	400	45–47	100	90–92
5	EBM FGF day 9	4 weeks	400	47–50	75–77	67–70
6	Undifferentiated	9 weeks	520	40–42	≥100	≥100
7	Undifferentiated	9 weeks	520	42–45	≥100	≥100
8	EBM day 3	2 weeks	520	42–45	≥100	≥100
9	EBM day 3	9 weeks	520	48–50	≥100	≥100
10	Undifferentiated	2 weeks	520	40–42	≥100	≥100
11	Undifferentiated	2 weeks	520	45–48	≥100	≥100
12	EBM day 3	2 weeks	520	47–50	≥100	≥100
13	EBM day 3	2 weeks	520	35–37	≥100	≥100
14	Undifferentiated	2 weeks	520	37–40	≥100	≥100
15	EBM day 3	9 weeks	520	37–40	≥100	≥100
16	N/A*	2 weeks	N/A#	32–35	N/A#	45–47
17	N/A*	2 weeks	N/A#	38–40	N/A#	47–50
18	N/A*	2 weeks	N/A#	40–42	N/A#	45–47
19	N/A*	2 weeks	N/A#	38–40	N/A#	47–50

Auditory brainstem responses (ABRs) were recorded from all guinea pigs prior to deafening, after treatment with kanamycin, and postsurgery. A second ABR was not recorded from guinea pigs 3 and 16–19 as they were not deafened (N/A#). The normal-hearing control animals (guinea pigs 16–19; N/A*) were implanted with ES media that did not contain ES cells.

the linear portion of the amplification curve. The PCR cycle consisted of a single incubation at 95°C for 3 min, followed by *x* cycles of 94°C for 30 s, *y*°C for 30 s, and 72°C for 1 min. This was followed by a final extension at 72°C for 5 min on a PCR Express thermal cycler (Hybaid Limited, Middlesex, UK). The number of cycles, *x*, were 30 for *Myo6*, *α9AchR*, and *Myo7a* and 12 for 18S rRNA. Annealing temperatures, *y*, were 55°C for 18S rRNA and *α9AchR*, 56°C for *Myo6*, and 68°C for *Myo7a*.

Auditory brainstem responses

The hearing status of all guinea pigs was assessed by measurement of click-evoked auditory brainstem responses (ABRs) (Hardie and Shepherd 1999). Prior to deafening, all animals exhibited responses at thresholds of less than 50 dB peak equivalent sound pressure level (dB pe SPL), representing hearing in the normal range. The deafening process was considered to be successful if no ABR was detected using acoustic clicks at an intensity of 93 dB pe SPL. An ABR measurement was also recorded prior to sacrifice to assess any changes in hearing status after stem cell implantation or mock surgery.

Deafening procedure

All deafening procedures were conducted five days prior to surgery. Guinea pigs had their tympanic membranes otoscopically examined and normal auditory function confirmed by measurement of auditory brainstem responses (ABRs) to broad click stimuli. Animals were premedicated with 0.06 mg/kg atropine (Apex Laboratories, Australia) and anesthetized using 1–1.5% isoflurane (Bickford Inc, USA) and 1–1.5 l/min O_2. Procedures were conducted with animals on a heating pad maintained at 37°C and 2% lignocaine (Troy Laboratories, Australia) was delivered subcutaneously around the incision site. The jugular vein was surgically exposed using aseptic techniques and the loop diuretic, frusemide (100 mg/kg; Troy Laboratories), was delivered intravenously over a period of 60 s. The aminoglycoside kanamycin (400 or 520 mg/kg; Sigma) in 3 ml warmed Hartmann's solution was then administered subcutaneously to induce deafening. The loop diuretic is coadministered with the kanamycin as it increases the concentration of the aminoglycoside in the scala media (Russell et al. 1979). Deafening results from this process because uptake of high concentrations of the aminoglycoside by hair cells leads to a loss in the mitochondrial membrane potential (Dehne et al. 2002). This change in membrane potential induces the hair cells to undergo apoptosis (Dehne et al. 2002).

Surgical procedure

Guinea pigs were anesthetized by intramuscular injections of ketamine (40 mg/kg body weight;

Parnell Laboratories, Australia) and xylazine (4 mg/kg body weight; Troy Laboratories) five days after deafening. They were premedicated with 0.06 mg/kg atropine and placed on a heating pad to maintain body temperature at 37–38°C. Local anesthetic (2% lignocaine) was delivered subcutaneously to the site of incision. The condition of the animal was monitored throughout the procedure by assessment of respiration rate and pedal reflex. The head and neck were shaved and all transplantations were made into the left cochlea under sterile conditions. A postauricular approach was utilized to expose the tympanic bulla, which was then opened to a diameter of 3–5 mm with the tip of a scalpel (Figure 1A). A stereomicroscope (Carl Zeiss, Germany) was used to visualize the cochlea and a small hole was made in the round window membrane using a 30-gauge needle. A polyimide cannula (Cole-Parmer Instruments, USA) attached via PVC tubing (Critchley Tyco Electronics, Australia) to a Micro4 MicroSyringe Pump (World Precision Instruments, USA) was positioned through the basilar membrane (Figure 1B and C). A 0.5-μl volume of ES cells or disaggregated EBM cells, at a concentration of 5×10^5 cells/ml in ES medium or ES media alone, was delivered through the cannula into the scala media cavity over a period of 1 min. The cannula was then left in position for a further 1 min to ensure delivery of all cells. The round window was sealed with fascia and the wound sutured internally with 3.0 metric chromic gut (Ethicon, Mexico) and externally with 2.0 metric vicryl (Ethicon, USA). Postoperatively, 10 ml Hartmann's solution was administered subcutaneously, followed by intramuscular injection of antisedan (Novartis, Australia).

Perfusion and histology

Two, four, or nine weeks after surgery, guinea pigs had their hearing status assessed by click-evoked ABR. The animals were then given an overdose of pentobarbital (intraperitoneal; Virbac, Australia) and perfused intracardially with 0.9% saline at 37°C, followed by 10% neutral buffered formalin (NBF, Merck, Australia) at 4°C. The cochleae were carefully removed, trimmed, fixed in 10% NBF overnight, and decalcified for two weeks in 10% EDTA in NBF. This was followed by embedding in paraffin wax before slicing into 5-μm-thick serial midmodiolar sections. The sections were mounted on glass slides and the morphology was checked via hematoxylin and eosin (HE) staining. Representative sections were used for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry to detect GFP-expressing ES cell types in the cochleae was conducted using a

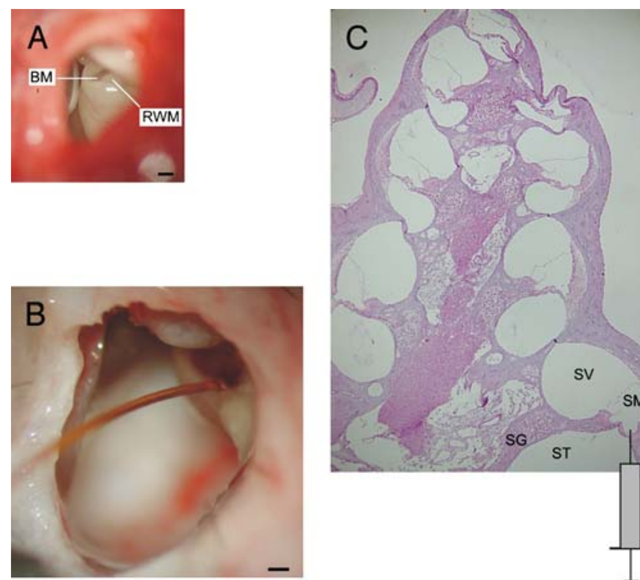

FIG. 1. Postauricular approach to expose the round window of the guinea pig cochlea and inject ES cell types. **A** View of the round window membrane (RWM) and underlying basilar membrane (BM) through a hole in the tympanic bulla. **B** Insertion of a polyimide cannula through the basilar membrane to facilitate implantation of cells into the scala media. **C** Schematic diagram illustrating delivery of ES cell types to the scala media of the guinea pig cochlea. SV, scala vestibuli; SM, scala media; ST, scala tympani; SG, spiral ganglion. Scale bars (A, B) = 1 mm.

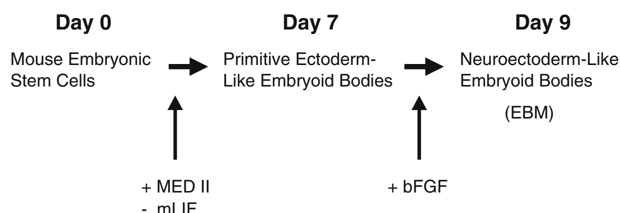

FIG. 2. Stepwise differentiation of mouse B5-EGFP cells. Mouse ES cells can be maintained in an undifferentiated state with mouse leukemia inhibitory factor (mLIF). Induction of ectoderm-like cells is achieved by removal of mLIF and addition of MEDII medium (Rathjen et al. 1999). Addition of the basic fibroblast growth factor (bFGF) directs commitment to the neuroectoderm lineage.

Vectastain Elite ABC Kit (Vector Laboratories, USA). Antigen retrieval was completed after dewaxing with xylene (BDH, Australia) and involved incubation of sections in 1 mM EDTA (Sigma), pH 8, at 95°C for 2.5 min. Sections were then blocked with 10% rabbit serum (Vector Laboratories) diluted in phosphate-buffered saline (PBS) for 20 min at room temperature (RT). This was followed by incubation with a goat anti-GFP primary antibody (Rockland Immunochemicals, USA) in PBS overnight at 4°C. A biotinylated secondary rabbit anti-goat IgG (Vector Laboratories) diluted in 10% rabbit serum in PBS was then applied to the sections for 30 min at RT. Sections were treated with Vectastain Elite ABC Reagent (Vector Laboratories) diluted in PBS for 30 min at RT. Dark brown precipitate was induced by incubation of the sections with 1× DAB/Metal Concentrate (Pierce Biotechnology, USA) in stable peroxide buffer (Pierce Biotechnology) for 10 min at RT. Sections were washed in PBS for 5 min and fixed in 4% formaldehyde in PBS for 15 min at RT.

Cell counting

Surviving implanted cells were detected in the guinea pig cochlea by direct visualization of GFP fluorescence. Sections through the whole cochlea of each transplanted animal were used to obtain cell counts. Approximately five sections were counted per animal, and examination of all scalae was conducted. Percentages were determined by averaging the results across all animals implanted with a particular ES cell type. Contralateral cochleae were also examined.

RESULTS

In vitro cell system

Ectodermal and neuroectodermal cell aggregates (EBM) were differentiated from B5-EGFP cells by using a previously reported stepwise approach (Rathjen et al. 1999; Rathjen et al. 2002). Mouse R1 B5-EGFP ES cells were maintained in an undifferentiated state by culturing in ES media supplemented with mouse LIF (mLIF). Primitive ectoderm-like bodies were formed by growing the cells in suspension culture in the absence of mLIF and in the presence of MEDII conditioned medium for six days. Directed differentiation of the cell aggregates to neuroectodermal cells was achieved by the addition of the growth factor bFGF to the culture for a further two days (Figure 2).

Undifferentiated ES cells and partially differentiated cell types collected at days 3, 7, and 9 were analyzed for a marker of early hair cell development: myosin VI (*Myo6*), and of differentiated cell types: cholinergic receptor, nicotinic, alpha polypeptide 9 (*α9AchR*); and myosin VIIa (*Myo7a*), by semiquantitative RT-PCR (Figure 3). Murine B5-GFP ES cells differentiated to early primitive ectoderm (EBM day 3) expressed all hair cell markers, albeit at lower levels when compared to undifferentiated ES cells in all cases. An increase in *Myo6* expression was observed in EBM day 7 cells compared to earlier developmental stages, whereas *α9AchR* expression was switched off in this cell type. Upon further differentiation to neuroectoderm, expression of all markers was decreased except for *α9AchR* whose expression was upregulated.

ABR and guinea pig deafening

All experimental animals exhibited normal hearing prior to deafening with click-evoked ABR thresh-

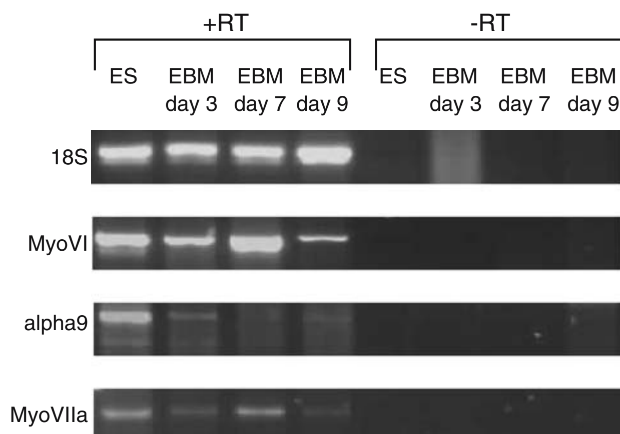

FIG. 3. Analysis of *in vitro* differentiation of ES cell types by semiquantitative RT-PCR. EBM embryoid bodies were tested for expression of early (*Myo6*) and more differentiated (*α9AchR*, *Myo7a*) hair cell marker genes. EBM aggregates were positive for both myosin motor genes.

olds of 50 dB pe SPL or lower (Table 2). Guinea pigs 3 and 16–19 did not undergo the deafening procedure. Although thresholds were generally higher in these control animals after surgery, in all cases thresholds remained in the normal hearing range (Table 2). Of the four animals that underwent the deafening procedure using 400 mg/kg kanamycin, guinea pigs 2 and 4 were considered to be severely–profoundly deaf as they displayed no response to 93 dB pe SPL clicks. Guinea pig 5 exhibited a moderate–severe hearing loss with no response below 75 dB pe SPL, whereas administration of kanamycin failed to deafen guinea pig 1, which responded to a click-evoked ABR of 40 dB pe SPL. When the concentration of kanamycin was increased to 520 mg/kg, all animals were profoundly deafened (guinea pigs 6–15; Table 2).

Assessment of surgical technique and immunological tolerance

There was no indication of significant trauma to the basilar membrane of the left cochleae of any of the guinea pigs. After surgical delivery there was no evidence of damage to the organ of Corti or Reissner's membrane attributable to the surgical procedure in any animal. However, it should be noted that some membranes of the cochlea, such as Reissner's membrane, can repair naturally following trauma (Lawrence 1983). The surgical approach did not significantly affect hearing function, with control guinea pigs 3 and 16–19 retaining normal hearing postsurgery (Table 2).

There was little evidence of immunological reactivity observed in any of the treated left cochleae. There was no indication of hyperacute rejection (HAR), which occurs almost immediately after transplantation, or acute rejection (AR), which usually occurs within the first six months following transplantation. No significant inflammation response was observed in any animal. In some cochleae, small amounts of fibrous tissue surrounding some transplanted cells were observed in the scala tympani, but this was not considered a significant response as viable engrafted cells were still observed in this cavity. Importantly, no signs of immunological reactivity were evident in the scala media of any cochlea.

Identification of implanted undifferentiated and partially differentiated ES cells

Mouse R1 B5-EGFP ES cells were transplanted into the guinea pig cochleae at four stages of differentiation. These included undifferentiated ES cells, cells partially differentiated in MEDII medium for three and seven days (EBM day 3 and EBM day 7 cells, respectively), and cells differentiated for seven days in MEDII medium and two days in medium containing FGF (EBM FGF day 9 cells; Table 1).

Undifferentiated ES cells ($n = 4$) and EBM day 3 cells ($n = 4$) were implanted into guinea pig cochleae for a period of two weeks. Undifferentiated ES cells were detected in the scala media, scala tympani, and scala vestibuli cavities by EGFP fluorescence at the end of the implantation period (Figure 4A). These cells were clearly distinct from the endogenous cells based on their expression of EGFP and localization in the cavity space. Most of the cells engrafted into the scala media were localized close to the stria vascularis and spiral ligament, particularly in turns 2 and 3 (Figure 4A and B). In the scala tympani, transplanted cells were most often seen close to the basilar membrane underneath the organ of Corti and spiral limbus (Figure 4A and B). Where transplanted cells were observed, they tended to be situated close together (Figure 4C, D, and E). Transplanted cells were also detected with an anti-GFP antibody (Figure 4F, G, and H). The surviving stem cells were comparable to the original cultured control B5-EGFP cells in terms of their EGFP expression (Figure 4I). However, examination of the morphology of transplanted cells and the control cells after HE staining (Figure 4B and J) indicated a change in the characteristics of the transplanted cells. The morphology of the transplanted cells after two weeks was similar to that of partially differentiated EBM day 3 cells (Figure 4J), in particular their propensity to exist as aggregations rather than as single cells.

Early partially differentiated EBM day 3 cells were detected in the scala media and scala tympani cavities after two weeks by EGFP fluorescence (Figure 5A and B). A small number of cells were also located in the scala vestibuli. Most of the cells in the scala media were localized near the stria vascularis, although some were identified close to the organ of Corti (Figure 5A and B). Aggregations of cells were identified in the scala tympani of the apical turn and in the scala media at turn 3 (data not shown). Analysis of the implanted cells by HE staining relative to control cultured ES cells indicated that they possessed a very similar morphology to EBM day 3 cells.

Two cell types, EBM day 7 ($n = 2$) and EBM FGF day 9 ($n = 1$), were implanted into the guinea pig cochlea for a period of four weeks. The majority of transplanted EBM day 7 cells were present in the scala tympani after four weeks (Figure 5C). A number of EBM day 7 cells were also identified in the scala media (Figure 5C and D). These cells were most often situated near the spiral ligament and stria

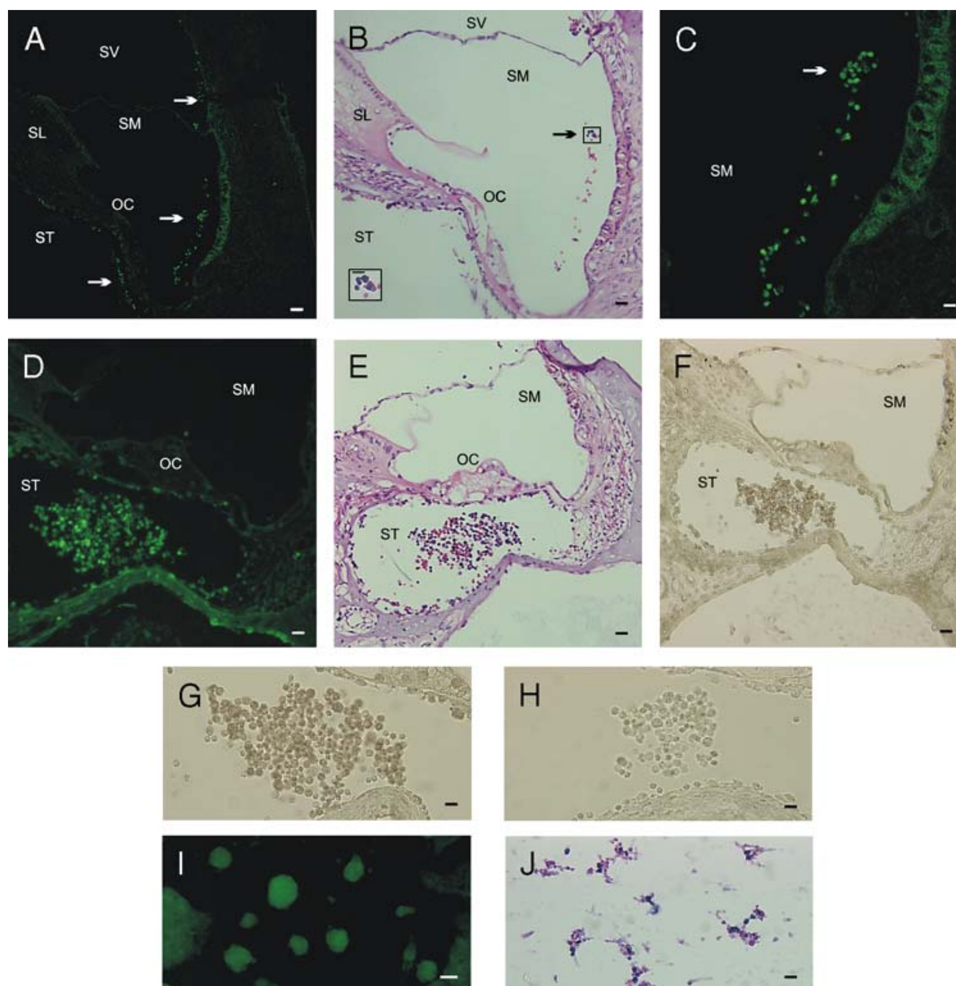

FIG. 4. Undifferentiated B5-EGFP cells after implantation into the cochlea for 2 weeks. **A** Confocal image of the guinea pig cochlea at turn 3 showing green fluorescent stem cells in the three scala. **B** Serial section of guinea pig cochlea (A) stained with hemotoxylin and eosin (HE) showing transplanted cells (arrow) and inset, transplanted cells at higher magnification. **C** Serial section of cochlea (A) showing B5-EGFP cells in the scala media under fluorescent light (arrow). **D** A large number of B5-EGFP cells in the scala tympani of the cochlea at turn 3 of another guinea pig. **E** Serial section of guinea pig cochlea (D) stained with hematoxylin and eosin (HE). **F** Serial section of cochlea (D) treated with anti-GFP antibody and DAB substrate. B5-EGFP cells expressing GFP are indicated by the dark brown DAB precipitate. **G** Higher magnification image of the GFP expressing cells in the scala tympani. **H** Serial section (D) not treated with primary antibody (negative control). No staining is apparent. **I** Undifferentiated B5-EGFP colonies under fluorescent light. **J** EBM day 3 embryoid bodies stained with hemotoxylin and eosin (HE). SV, scala vestibuli; SM, scala media; ST, scala tympani; OC, organ of Corti; SL, spiral limbus; SV, stria vascularis. Scale bars (A, B, D, E, F, I) = 10 μm; (C, G, H, J) = 20 μm.

vascularis. EBM day 7 cells were also observed in the scala vestibuli cavity (Figure 5C and D).

Undifferentiated ES cells ($n = 2$) and EBM day 3 cells ($n = 2$) were also transplanted into guinea pig cochleae for a period of nine weeks. Although transplanted cells were shown to survive in the cochlea for this period (Figure 5E and F), fewer cells were observed than after two or four weeks implantation. Most transplanted cells were found in the scala tympani and vestibuli, with only a small number located in the scala media cavity. None of the cells appeared to integrate into endogenous cochlear tissue, despite the longer transplantation duration.

In contrast to the other cell types, a relatively small number of EBM FGF day 9 neuroectoderm cells survived for four weeks in the scala tympani and scala vestibuli cavities of the cochlea. None of these cells were detected in the scala media cavity. The primary location of the stem cells in the scala vestibuli cavity was in turn 3 (data not shown). As had been observed with cells at other stages in the developmental pathway, the cells tended to aggregate close to each other.

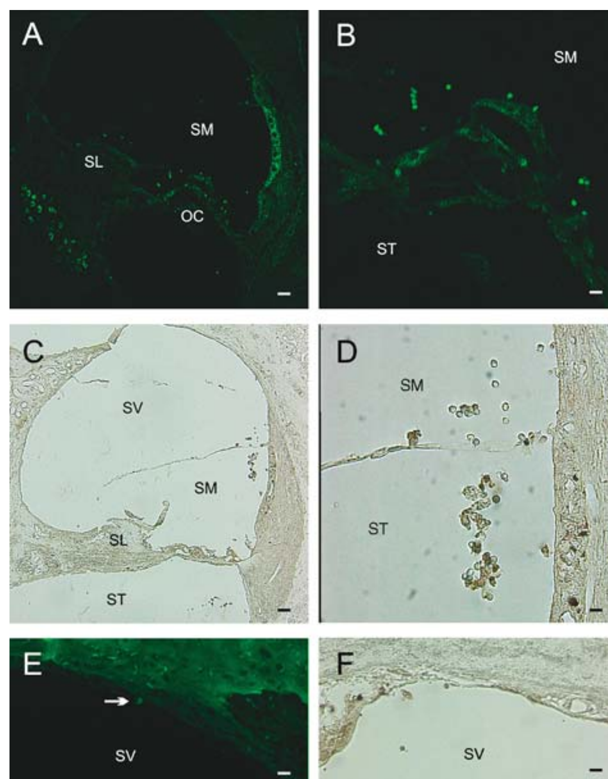

FIG. 5. Partially differentiated cell types after implantation into the cochlea. **A** EBM day 3 cells located close to the damaged organ of Corti in the scala media after 2 weeks implantation and viewed under fluorescent light. **B** Higher magnification image of the EBM day 3 cells. **C** Guinea pig cochlea at turn 2 implanted with EBM day 7 cells for 4 weeks. This section has been treated with an anti-GFP antibody and DAB substrate. Positive cells are labeled with the brown DAB precipitate. **D** Image showing the positive cells in the scala media and scala vestibuli. **E** A transplanted EBM day 3 cell in the scala vestibuli after nine weeks visualized under fluorescent light (arrow). **F** Serial section (E) treated with anti-GFP antibody. SM, scala media; OC, organ of Corti; SV, scala vestibuli; ST, scala tympani. Scale bars (A, C) = 10 μm; (B, D, E, F) = 20 μm.

Detection of implanted undifferentiated and partially differentiated ES cells by immunohistochemistry

To confirm the presence of B5-EGFP undifferentiated and partially differentiated cell types in the guinea pig cochlea, immunohistochemistry was performed using an anti-GFP antibody. The presence of transplanted EGFP expressing cell types in the cochleae was confirmed by the formation of brown precipitate upon addition of the DAB substrate. Data for the undifferentiated and EBM day 7 cell types transplanted into the cochlea and treated with anti-GFP antibody are shown (Figure 4F, G, and H; and Figure 5C, D, and E). It is clear in these figures that most, but not all, of the transplanted cells are expressing EGFP. Investigation of B5-EGFP cells in culture revealed that fluorescence is lost in some of the cells, particularly as the passage number of the cells increases (data not shown).

Survival rate

The number of surviving implanted cells was estimated by examining all three cavities of the cochleae. Five sections per cochlea were examined for EGFP fluorescence and morphology after HE staining. The survival rate for transplanted ES cells in the cochlea was relatively low (19.1%). The rate of survival of transplanted cells decreased as the level of differentiation and the duration of transplantation increased. Importantly, around 14% of the surviving cells were located in the scala media cavity.

Functional analysis of implanted guinea pigs

The ABR threshold of guinea pig 1 was higher after the implantation period, reflecting a deterioration in hearing ability (Table 2). Guinea pig 2 also displayed a marginally higher auditory threshold (Table 2). It is notable that both these guinea pigs

exhibited inflamed tympanic membranes prior to their final ABR measurements. The control animals (guinea pigs 3, 16–19) that were not deafened retained hearing ability in the normal range following surgical delivery (Table 2). Guinea pigs 4 and 5 both showed improvement in hearing ability after implantation, with decreases in threshold of approximately 10 dB. However, these changes were not significant and probably reflect recovery of hearing after the low concentration of kanamycin used in the deafening procedure. All guinea pigs treated with the higher concentration of kanamycin (guinea pigs 6–15; Table 2) were profoundly deaf (threshold: ≥100 dB) both postdeafening and postimplantation.

DISCUSSION

In this study, we have shown that undifferentiated and partially differentiated mouse ES cell types survive for at least nine weeks following transplantation into the guinea pig cochlea. Transplanted cells were found in the scala media and some had traveled from their implantation site in the basal turn to the apical turn. This movement could be attributable to active cell migration or fluid dynamics. In some cases, ES cell types were observed close to the organ of Corti, although integration of cells into endogenous tissue was not observed. Functional analysis of transplanted animals revealed no significant improvement in hearing ability, consistent with the lack of cell integration. This is the first report of transplanted differentiated cell types derived from ES cells surviving in the scala media.

Although studies have reported survival of ES cells in the cochlea (Hu et al. 2004; Sakamoto et al. 2004), none have demonstrated regeneration or replacement of auditory hair cells following a sensorineural trauma using stem cells. In previous reports of ES cell delivery to the scala media, transplantation efficiency and survival rates have been low (Hu et al. 2004; Sakamoto et al. 2004). In a promising recent study directed at replacing lost spiral ganglion neurons, the survival and differentiation of neural stem cells in the injured guinea pig inner ear was demonstrated (Hu et al. 2004). In the present study undifferentiated and partially differentiated ES cell types were shown to survive in the scala media for up to nine weeks following transplantation. These findings suggest that undifferentiated and partially differentiated ES cell types may be candidates for cell therapy approaches to regenerate or replace cochlear hair cells and possibly other cell types in the cochlea.

Cell transplantation studies directed at regenerating or replacing cochlear hair cells have traditionally involved accessing the cochlea via the scala tympani. The migration of neuronal stem and embryonic cell types into regions close to the organ of Corti after delivery into the scala tympani has been demonstrated (Hu et al. 2005a, b). However, this has been shown to occur at low efficiency (Hu et al. 2005a, b), and to date there has been no evidence of these migrated cell types developing hair cell characteristics *in vivo*. The successful replacement of hair and supporting cells is likely to require direct and efficient delivery of ES cells into the organ of Corti. Preliminary *in vitro* studies have shown that ES cells can survive in high potassium solutions such as endolymph. However, one of the major caveats to this approach is whether transplanted cells are able to access the basolateral regions of the hair cells from the scala media. Only the apical surface of the hair cells face into the scala media with their distinctive stereocilia projecting through the cuticular plate. The true division between the endolymphatic and perilymphatic compartments is the reticular lamina, which provides a tight junction barrier restricting the interchange of materials between these regions (Pickles 2001). The integrity of the reticular lamina has been shown to be maintained by the organ of Corti even after hair cell loss due to chronic gentamycin administration (McDowell et al. 1989). It is therefore unclear whether ES cells would be able to penetrate the reticular lamina to reach the normal location of the hair cell bodies. However, it is known that some cell types can migrate through tight junction barriers, such as binucleate cells that transverse the ionic barrier seal, the trophectodermal epithelium, throughout pregnancy in the ruminant placenta (Morgan and Wooding 1983; Wooding et al. 1994). An important clue that stem cell migration across the reticular lamina may be possible is that following noise trauma tight junctions are known to rearrange (Raphael and Altschuler 1991). It is thus conceivable that during the rearrangement of these tight junctions between the formation of phalangeal scars and the restoration of the reticular lamina, transplanted ES cell types would be able to cross the reticular lamina into the organ of Corti. The restoration of the reticular lamina is only transitory and over a prolonged period of time the organ of Corti of the deafened cochlea will collapse and disintegrate.

The postauricular approach that was utilized in this study to expose the tympanic bulla is a well-established technique (Goycoolea et al. 1990; Silverstein and White 1990). Accessing the scala media through the round window membrane minimizes trauma to the delicate structures of the cochlea as a

cochleostomy is not required. In a cochleostomy, the inner structures of the cochlea are accessed by making a hole in the bony labyrinth (Dimitrov et al. 1988). Our approach also has significant advantages over a lateral approach to the scala media, where it is possible to damage the stria vascularis and cochlear blood supply (Izumikawa et al. 2005). However, only the base of the cochlea can be directly accessed via the round window approach. To our knowledge, the direct delivery of cells into the scala media through the basilar membrane via this technique has not been reported. It has been demonstrated that this approach does not cause significant extraneous trauma to the cochlea. Analysis of the auditory function of the five control guinea pigs that were not deafened in this study revealed that they retained thresholds in the normal hearing range after delivery of cells or media alone. It must be kept in mind that the use of broad click stimuli to record ABR measurements does not rule out the possibility of damage in specific sites of the cochlea. Nonetheless, this data is consistent with our histological examination that found no significant trauma was sustained by the organ of Corti or Reissner's membrane during the surgical procedure.

Our surgical approach allowed efficient delivery of ES cells into the scala media. The survival rate for the transplanted ES cell types was relatively low, at approximately 19.1%; however, it was higher than has been reported previously (Hu et al. 2004). We speculate that this improvement may be attributable to the predifferentiation of the cells *in vitro* or the nature of the surgical approach to the scala media. Not all of the ES cells that were successfully transplanted into the cochlea were shown to express EGFP. Examination of B5-GFP cells in culture revealed that this phenomenon also occurs *in vitro*. We speculate that the ES cells turn off their expression of EGFP as a survival mechanism as GFP is known to be cytotoxic (Goto et al. 2003). It is also possible that the lack of GFP expression in some implanted cells could be attributable to the effects of the two-week decalcification period (Harms et al. 2002). The presence of transplanted cells in the scala tympani and scala vestibuli cavities was most likely attributable to inadvertent delivery to these regions during the surgical procedure. The transplanted cells could not have traveled from the scala media as this cavity is regulated by tight junctions that prevent cell movement (Juhn 1988). This inadvertent delivery is difficult to avoid given the small size of the scala media relative to the other cavities of the cochlea. The presence of significant numbers of transplanted cells in these cavities did not appear to have any adverse effects on the cochlea.

A major consideration for all xenotransplantation experiments is the potential for immunological rejection of the engrafted cells. There has been substantial investigation into this biological process and considerable debate over the efficacy of cross-species transplantation (Cascalho and Platt 2001; Cozzi and Ancona 2003). It was considered a distinct possibility that an immunological response would be initiated within the guinea pig cochlea after transplantation of the mouse ES cells. However, no significant immunological response was observed in any animal after implantation. There was no evidence of hyperacute or acute rejection in any of the guinea pigs. It is notable that none of the animals in this study were immunosuppressed prior to implantation. The lack of immunoreactivity to xenografted stem cells may be attributable to some degree of immunoprotection of the cochlea provided by the blood–labyrinth barrier (BLB) (Lin and Trune 1997; Trune 1997). The major evidence for the involvement of the BLB in immunoprotection of the inner ear comes from studies of the C3H/lpr autoimmune mouse. Animals with active disease were shown to have breakdown of their BLB leading to systemic autoimmune disease and other immune diseases, coincident with hearing loss (Lin and Trune 1997). It is also possible that the ES cells exhibit lower immunogenicity than terminally differentiated cells, although no evidence for this is available in the literature at present.

It has been established that stem cells exist in niches that provide a host of biological signals to direct their behavior (Fuchs et al. 2004). To artificially create these microenvironments *in vivo*, it may be necessary to provide exogenous factors. These factors will induce transplanted cells to migrate, differentiate, and integrate into host cochlear tissue (Hu et al. 2004, 2005a, b). In the present study, mouse ES cell types did not appear to integrate into the endogenous tissue of the guinea pig cochlea, despite the fact that they survived within the structure for up to nine weeks. The fact that some transplanted cells localized close to the damaged organ of Corti but did not appear to integrate suggests that the remaining cells in this structure either do not elicit the appropriate biological cues to induce integration or that these signals are produced but are unable to reach the implanted ES cells due to the tight junctions that protect the cells of the organ of Corti from the contents of the perilymphatic compartments. It is therefore likely that ES cell types will have to be cotransplanted with appropriate factors to encourage directed differentiation and integration. It is known that neurotrophic factors are important for hair cell development (Chabbert et al. 2003), and a recent report indicates that epidermal growth

factor (EGF) may be required to induce formation of new hair cells (Doetzlhofer et al. 2004). The formation of new hair cells may also require overexpression of Math1, a transcription factor downstream in the EGF pathway that is essential for hair cell development (Zheng and Gao 2000; Woods et al. 2004). The regeneration of mammalian hair cells *in vivo* and resultant recovery of auditory function by overexpression of Math1 (Izumikawa et al. 2005) supports this combined therapeutic approach. To achieve directed differentiation and integration of transplanted ES cell types into new sensory hair cells, it may be necessary to also deliver factors such as EGF and Math1.

The *in vitro* differentiation of ES cells prior to implantation was confirmed by analysis of markers of early hair cell development and markers expressed at later stages of hair cell differentiation. The undifferentiated ES cells expressed the three hair cell markers, *Myo6*, *α9AchR*, and *Myo7a*, at relatively high levels when compared to the partially differentiated cell types. The ES cells were differentiated along the ectodermal lineage (EBM day 3 and EBM day 7 cell types), the embryonic germ layer that forms the hair cells, and then to neuroectoderm (EBM FGF day 9 cells). Consistent with the cellular differentiation, the specific expression pattern of the hair cell markers changed over time with a trend toward decreased expression in all three markers tested. For future studies aimed at generating cell types with hair cell characteristics from stem cells, it may be necessary to use alternative differentiation conditions, such as pathways involving Math1. Of the cell types examined in this study, EBM day 3 partially differentiated cells appear to have the greatest potential for cell therapy of the damaged auditory system. EBM day 3 cells survived in the scala media for up to nine weeks, and some were localized close to the damaged organ of Corti. Importantly, EBM day 3 cells did not form teratomas and animals implanted for nine weeks with these cells appeared phenotypically normal apart from their profound hearing impairment. Although undifferentiated ES cells also survived in the scala media, the propensity for these cells to form teratomas (Hardy et al. 1990; Wakitani et al. 2003) means that they are likely to be unsuitable for use in clinical therapies.

In conclusion, we have demonstrated that mouse undifferentiated and partially differentiated cell types can survive in the adult guinea pig cochlea for at least nine weeks following xenotransplantation through the round window. This is the first report of partially differentiated cell types derived from ES cells surviving in the scala media. We have shown that the ES cell types can be delivered directly into the cochlea with minimal trauma and without significant immunological rejection. Some of the transplanted cells were shown to localize close to the damaged organ of Corti structure in the scala media; however, there was no evidence of integration. These results provide evidence that partially differentiated cells generated from ES cells may be useful in restoration of the damaged auditory system. However, we believe it will be necessary to induce specific interactions between the transplanted cells and the endogenous cochlear tissue to allow subsequent integration and differentiation of the engrafted cells into new sensory hair cells.

ACKNOWLEDGMENTS

The authors would like to thank Elisa Borg [Head of Biological Research Centre (BRC), Royal Victorian Eye and Ear Hospital], and Dr. Sue Pierce (Veterinarian, BRC) for their assistance with the welfare of the guinea pigs. Additionally, Anne Coco and Stephanie Epp (Department of Otolaryngology, University of Melbourne) are acknowledged for their assistance with the surgical aspects of guinea pig deafenings and perfusions. For excellent assistance and guidance with histology, Maria Clarke and Prudence Nielsen (The Bionic Ear Institute) are thanked. Professor Andras Nagy is thanked for generously providing R1 B5-EGFP cells. Dr. Chris Que Hee is thanked for sharing his expertise in surgery. We thank Bianca Bernardo (Cell and Matrix Biology, Murdoch Childrens Research Institute) for her assistance with RT-PCR analysis. Dr. Deon Venter (Cancer Functional Genomics Laboratory, Murdoch Childrens Research Institute) is acknowledged for his advice concerning histopathology. We acknowledge the financial support from the Murdoch Childrens Research Institute, the Garnett Passe and Rodney Williams Memorial Foundation, and J. & J. Calvert-Jones. H.-H. M. Dahl is a NHMRC Principal Research Fellow and R.K. Shepherd is a Wagstaff Fellow in Otology at the Royal Victorian Eye and Ear Hospital.

REFERENCES

BJÖRKLUND LM, SANCHEZ-PERNAUTE R, CHUNG S, ANDERSSON T, CHEN IY, MCNAUGHT KS, BROWNELL AL, JENKINS BG, WAHLESTEDT C, KIM KS, ISACSON O. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc. Natl. Acad. Sci. USA 99:2344–2349, 2002.

CASCALHO M, PLATT JL. Xenotransplantation and other means of organ replacement. Nat. Rev. Immunol. 1:154–160, 2001.

CHABBERT C, MECHALY I, SIESO V, GIRAUD P, BRUGEAUD A, LEHOUELLEUR J, COURAUD F, VALMIER J, SANS A. Voltage-gated Na^+ channel activation induces both action potentials in utricular hair cells and brain-derived neurotrophic factor release in the rat utricle during a restricted period of development. J. Physiol. 553:113–123, Epub 2003 Sep 08, 2003.

CORWIN JT, COTANCHE DA. Regeneration of sensory hair cells after acoustic trauma. Science 240:1772–1774,1988.

COTANCHE DA, LEE KH. Regeneration of hair cells in the vestibulocochlear system of birds and mammals. Curr. Opin. Neurobiol. 4:509–514, 1994.

COZZI E, ANCONA E. Xenotransplantation, where do we stand? J. Nephrol. 16:S16–S21, 2003.

DEHNE N, RAUEN U, DE GROOT H, LAUTERMANN J. Involvement of the mitochondrial permeability transition in gentamicin ototoxicity. Hear. Res. 169:47–55, 2002.

DIMITROV EA, DUCKERT LG, LONSBURY-MARTIN BL, MARTIN GK. The effect of cochleostomy on the development of endolymphatic hydrops—morphologic changes in the rabbit cochlea. Otolaryngol. Head Neck Surg. 98:283–290, 1988.

DOETZLHOFER A, WHITE PM, JOHNSON JE, SEGIL N, GROVES AK. *In vitro* growth and differentiation of mammalian sensory hair cell progenitors: a requirement for EGF and periotic mesenchyme. Dev. Biol. 272:432–447, 2004.

FUCHS E, TUMBAR T, GUASCH G. Socializing with the neighbors: stem cells and their niche. Cell 116:769–778, 2004.

GOTO H, YANG B, PETERSEN D, PEPPER KA, ALFARO PA, KOHN DB, REYNOLDS CP. Transduction of green fluorescent protein increased oxidative stress and enhanced sensitivity to cytotoxic drugs in neuroblastoma cell lines. Mol. Cancer Ther. 2:911–917, 2003.

GOYCOOLEA MV, MUCHOW DC, SCHIRBER CM, GOYCOOLEA HG, SCHELLHAS K. Anatomical perspective, approach, and experience with multichannel intracochlear implantation. Laryngoscope 100:1–18, 1990.

HARDIE NA, SHEPHERD RK. Sensorineural hearing loss during development: morphological and physiological response of the cochlea and auditory brainstem. Hear. Res. 128:147–165, 1999.

HARDY K, CARTHEW P, HANDYSIDE AH, HOOPER ML. Extragonadal teratocarcinoma derived from embryonal stem cells in chimaeric mice. J. Pathol. 160:71–76, 1990.

HARMS JF, BUDGEON LR, CHRISTENSEN ND, WELCH DR. Maintaining GFP tissue fluorescence through bone decalcification and long-term storage. BioTechniques 33:1197–1200, 2002.

HU Z, ULFENDAHL M, OLIVIUS NP. Central migration of neuronal tissue and embryonic stem cells following transplantation along the adult auditory nerve. Brain Res. 1026:68–73, 2004.

HU Z, ULFENDAHL M, OLIVIUS NP. NGF stimulates extensive neurite outgrowth from implanted dorsal root ganglion neurons following transplantation into the adult rat inner ear. Neurobiol. Dis. 18:184–192, 2005a.

HU Z, WEI D, JOHANSSON CB, HOLMSTROM N, DUAN M, FRISEN J, ULFENDAHL M. Survival and neural differentiation of adult neural stem cells transplanted into the mature inner ear. Exp. Cell Res. 302:40–47, 2005b.

ITO J, KOJIMA K, KAWAGUCHI S. Survival of neural stem cells in the cochlea. Acta Otolaryngol. 121:140–142, 2001.

IZUMIKAWA M, MINODA R, KAWAMOTO K, ABRASHKIN KA, SWIDERSKI DL, DOLAN DF, BROUGH DE, RAPHAEL Y. Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. Nat. Med. 13:13, 2005.

JUHN SK Barrier systems in the inner ear. Acta Otolaryngol. Suppl. 458:79–83. 1988.

KELSELL DP, DUNLOP J, STEVENS HP, LENCH NJ, LIANG JN, PARRY G, MUELLER RF, LEIGH IM. Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. Nature 387:80–83, 1997.

LAKE J, RATHJEN J, REMISZEWSKI J, RATHJEN PD. Reversible programming of pluripotent cell differentiation. J. Cell Sci. 113:555–566, 2000.

LAWRENCE M. Regeneration of Reissner's membrane. Acta Otolaryngol. 95:480–485, 1983.

LIN DW, TRUNE DR. Breakdown of stria vascularis blood–labyrinth barrier in C3H/lpr autoimmune disease mice. Otolaryngol. Head Neck Surg. 117:530–534, 1997.

MALGRANGE B, BELACHEW S, THIRY M, NGUYEN L, ROGISTER B, ALVAREZ ML, RIGO JM, VAN DE WATER TR, MOONEN G, LEFEBVRE PP. Proliferative generation of mammalian auditory hair cells in culture. Mech. Dev. 112:79–88, 2002.

MCDOWELL B, DAVIES S, FORGE A. The effect of gentamicin-induced hair cell loss on the tight junctions of the reticular lamina. Hear Res. 40:221–232, 1989.

MORGAN G, WOODING FB. Cell migration in the ruminant placenta: a freeze-fracture study. J. Ultrastruct. Res. 83:148–160, 1983.

PALOMAR GARCIA V, ABDULGHANI MARTINEZ F, BODET AGUSTI E, ANDREU MENCIA L, PALOMAR ASENJO V. Drug-induced otoxicity: current status. Acta Otolaryngol. 121:569–572, 2001.

PICKLES J. An Introduction to the Physiology of Hearing. Academic Press Ltd, London, 2001.

PIRVOLA U, YLIKOSKI J, TROKOVIC R, HEBERT JM, MCCONNELL SK, PARTANEN J. FGFR1 is required for the development of the auditory sensory epithelium. Neuron 35:671–680, 2002.

RAPHAEL Y, ALTSCHULER RA. Reorganization of cytoskeletal and junctional proteins during cochlear hair cell degeneration. Cell Motil. Cytoskelet. 18:215–227, 1991.

RATHJEN J, LAKE JA, BETTESS MD, WASHINGTON JM, CHAPMAN G, RATHJEN PD. Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. J. Cell Sci. 112:601–612, 1999.

RATHJEN J, HAINES BP, HUDSON KM, NESCI A, DUNN S, RATHJEN PD. Directed differentiation of pluripotent cells to neural lineages: homogeneous formation and differentiation of a neurectoderm population. Development 129:2649–2661, 2002.

RENG D, MÜLLER M, SMOLDERS JW. Functional recovery of hearing following AMPA-induced reversible disruption of hair cell afferent synapses in the avian inner ear. Audiol. Neurootol. 6:66–78, 2001.

RIDEOUT WM, HOCHEDLINGER K, KYBA M, DALEY GQ, JAENISCH R. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell 109:17–27, 2002.

RUSSELL NJ, FOX KE, BRUMMETT RE. Ototoxic effects of the interaction between kanamycin and ethacrynic acid. Cochlear ultrastructure correlated with cochlear potentials and kanamycin levels. Acta Otolaryngol. 88:369–381, 1979.

RYALS BM, RUBEL EW. Hair cell regeneration after acoustic trauma in adult Coturnix quail. Science 240:1774–1776, 1988.

SAKAMOTO T, NAKAGAWA T, ENDO T, KIM TS, IGUCHI F, NAITO Y, SASAI Y, ITO J. Fates of mouse embryonic stem cells transplanted into the inner ears of adult mice and embryonic chickens. Acta Otolaryngol. Suppl:48–52, 2004.

SILVERSTEIN H, WHITE DW. Wide surgical exposure for singular neurectomy in the treatment of benign positional vertigo. Laryngoscope 100:701–706, 1990.

STAECKER H, VAN DE WATER TR. Factors controlling hair-cell regeneration/repair in the inner ear. Curr. Opin. Neurobiol. 8:480–487, 1998.

STONE JS, RUBEL EW. Cellular studies of auditory hair cell regeneration in birds. Proc. Natl. Acad. Sci. USA 97:11714–11721, 2000.

TEKIN M, ARNOS KS, PANDYA A. Advances in hereditary deafness. Lancet 358:1082–1090, 2001.

TORRES M, GIRALDEZ F. The development of the vertebrate inner ear. Mech. Dev. 71:5–21, 1998.

TRUNE DR. Cochlear immunoglobulin in the C3H/lpr mouse model for autoimmune hearing loss. Otolaryngol. Head Neck Surg. 117:504–508, 1997.

WAKITANI S, TAKAOKA K, HATTORI T, MIYAZAWA N, IWANAGA T, TAKEDA S, WATANABE TK, TANIGAMI A. Embryonic stem cells injected into

the mouse knee joint form teratomas and subsequently destroy the joint. Rheumatology (Oxford) 42:162–165, 2003.

WARCHOL ME, CORWIN JT. Regenerative proliferation in organ cultures of the avian cochlea: identification of the initial progenitors and determination of the latency of the proliferative response. J. Neurosci. 16:5466–5477, 1996.

WOODING FB, MORGAN G, BRANDON MR, CAMOUS S. Membrane dynamics during migration of placental cells through trophectodermal tight junctions in sheep and goats. Cell Tissue Res. 276:387–397, 1994.

WOODS C, MONTCOUQUIOL M, KELLEY MW. Math1 regulates development of the sensory epithelium in the mammalian cochlea. Nat. Neurosci. 7:1310–1318, 2004.

ZHENG JL, GAO WQ. Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. Nat. Neurosci. 3:580–586, 2000.