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Cochlear Repair by Transplantation of Human Cord Blood CD133$^+$ Cells to *Nod-Scid* Mice Made Deaf With Kanamycin and Noise

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We investigated the fate of human cord blood CD133$^+$ hematopoietic stem cells (HSC) transplanted intravenously (IV) into irradiated *nod-scid* mice previously made deaf by ototoxic treatment with kanamycin and/or intense noise, to verify whether HSC engraft the cochlea and contribute to inner ear restoration, in vivo. We tested the presence of HLA.DQα1 by PCR, used for traceability of engrafted cells, finding evidence that HSC migrated to various host tissues, including the organ of Corti (OC). By histology, antibody and lectin-staining analysis, we confirmed that HSC IV transplantation in mice previously damaged by ototoxic agents correlated with the repair process and stimulation ex novo of morphological recovery in the inner ear, while the cochlea of control oto-injured, nontransplanted mice remained seriously damaged. Dual color FISH analysis also provided evidence of positive engraftment in the inner ear and in various mouse tissues, also revealing small numbers of heterokaryons, probably derived from fusion of donor with endogenous cells, for up to 2 months following transplantation. These observations offer the first evidence that transplanted human HSC migrating to the inner ear of oto-injured mice may provide conditions for the resumption of deafened cochlea, emerging as a potential strategy for inner ear rehabilitation.

Key words: Cochlea repair; Organ of Corti; Stem cell; Transplantation; Deafness

INTRODUCTION

Deafness is one of the most widespread disabilities in the world, particularly affecting the elderly population. People suffering from noise-induced hearing loss also tend to develop increased vulnerability to ototoxic chemical insults (44). Hearing loss in mammals achieved by ototoxic drugs, intense noise, or age is considered permanent because cochlear neurons and hair cells do not regenerate (15,16,33,34,37,46). The most sensitive cells are hair cells of the organ of Corti (OC), vestibular and auditory ganglion neurons, cells of the stria vascularis, mesenchymal cells, and fibrocytes present in the spiral ligament and basilar membrane (10,23,27,34,40,43). In contrast, mesenchymal cells are fairly able to regenerate themselves for a short time from the moment the damage occurs (14,25,37). Also cochlear fibrocytes of the spiral ligament thus damaged can regenerate progenies that differentiate, although their capacity to proliferate diminishes with age (14,25,37). These cells and their role in regulating ionic concentration and the replacement of fluids in the inner ear have recently attracted increasing attention (9,30,42). However, the conditions required for stimulating mesenchymal cells, fibrocytes, and sensory neuron regeneration are still unknown, as are the origin of these cells and the role played by the different functional support cell categories for the resumption of the neural function in a damaged OC. Re-

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cently, Lang et al. (24) transplanted murine bone marrow (BM), GFP-expressing hematopoietic stem cells (HSC) into irradiated syngeneic mice, demonstrating that inner ear cells can derive from circulating HSC. The results of these studies provided evidence for the origin of mesenchymal inner ear cells from BM-derived HSC, suggesting the possibility that mesenchymal cells, including fibrocytes, in the adult inner ear derive continuously from HSC in vivo.

On the basis of these findings, the present studies aimed to further investigate the contribution of hematopoietic stem/progenitor cells to adult inner ear resumption in mice deafened by treatment with the ototoxic aminoglycoside kanamycin and/or acoustic noise. For this purpose, we used the human/mouse xenotransplant model and injected human cord blood (CB) CD133$^+$ HSC intravenously (IV) into adult *nod-scid* mice, by themselves unable to reject foreign transplanted cells. We then evaluated the capacity of donor HSC or their progeny to engraft in these mice. Highly sensitive tracing methods for transplanted human HSC were used to examine whether these cells were capable of migrating into the cochlea, particularly into the OC, in vivo, and to evaluate whether they or their progeny could contribute to regenerating neurons and sensory epithelium in the cochlea, piloting the recovery of the original phenotype.

MATERIALS AND METHODS

Human CB-HSC

CB was obtained from informed consenting mothers (Bone Marrow Transplantation Unit, Careggi Hospital, Florence, Italy) and processed between 24 and 56 h after delivery. Mononuclear cell fractions were isolated from individual CB by density gradient centrifugation onto a Ficoll-Hypaque layer (Ficoll-Hypaque, Amersham Biosciences Europe GmbH, Freiburg, Germany), and subjected to red cells lysis using 0.8% ammonium chloride solution. CD133$^+$ HSC were then obtained by magnetic isolation with MACS® MicroBeads conjugated to a monoclonal mouse anti-human CD133 antibody (clone AC133, which recognizes epitope CD133/1), using the CD133 Cell Isolation Kit following the manufacturers's procedure (all from Miltenyi Biotec GmbH Gladbach, Germany). The efficiency of purification was verified from flow cytometry counterstaining with CD133/2 (293C3)-PE (Miltenyi Biotec GmbH). In the nine different CB samples used in these experiments, the mean purity of the injected CB-CD133$^+$ samples was >86.7%.

Mice

Seventy 2-month-old inbred *nod-scid* mice (Charles Rivers, Laboratories Clinical, Tranent, UK) were employed. All animal experiments were performed in compliance with institutional guidelines, according to protocols approved by the Institutional Review Board and the Ethics Committee of the Institute for Research against Tumors (IST) of Genua and the National Research Council of Italy (C.N.R.), Italy.

Animal Treatments

We used the xenotransplant human–murine model (7) on *nod-scid* mice, made deaf by treatment with kanamycin, noise, or a combination of these oto-damaging agents.

Kanamycin. Kanamycin sulphate (Azienda Farmacologica Italiana. S.r.l., Genua, Italy) was administered subcutaneously (SC) at 800 mg/kg body weight twice a day for 12 days to induce progressive damage to inner ear cells. At high doses, kanamycin frequently induces side effects including severe degeneration of sensitive ear cells (3).

Acoustic Noise. Animals were exposed for a total of 5 h, with no pauses, to 120 dB SPL, 8 kHz O.B. (octave band) noise. A noise-generating box (Audiopack, Medea, Italy), connected by a serial cable to a personal computer, produced noise, and specific software (Audiopack-panel, Medea, Italy) controlled each experimental parameter. Noise was amplified by a Yamaha AX 396 stereo amplifier. The exposure to noise was boosted by four tweeter speakers (20 Watt, 68 Ohm, Motorola, USA) placed on the top of the cages. A Bruel and Kjaer impulse precision sound level meter type 2209, connected to a 1-inch condenser microphone type 4145 for free field measurements, was used to ensure that the noise level remained the same throughout the 5-h exposure.

Combination of Kanamycin and Acoustic Noise. Another group of animals was exposed to acoustic noise (120 dB SPL) for 4 h before the 2-week treatment with kanamycin that was administered at 400 mg/kg total body weight SC, twice a day, on alternate days, for 12 days. This lower dose of kanamycin has been shown to be equally ototoxic, but has a reduced likelihood of side effects compared with the higher dose used in the single study (3).

As listed in Table 1, in a preliminary experiment the cochlear tissue phenotype was assessed histologically in mice made deaf, after 12, 30, 49, or 60 days from the three above ototoxic treatments, confirming that, in comparison to untreated normal controls, the inner ear structure was seriously damaged in all these mice, revealing a highly disorganized profile in the OC, with complete loss of inner/outer hair and support cells, replaced by empty vacuoles, or detected only sporadically in severely damaged tissue, and the absence of normal neurons and fibrocytes (see Fig. 2).

Table 1. Histologic Evaluation of the Effects on the Cochlear Structure After Exposure to Ototoxic Treatments

Mouse	Ototoxic Treatment	Time From Treatment	Cochlear Structure (Organ of Corti)*
K1	kanamycin	12 days	++
K2	kanamycin	12 days	++
K3	kanamycin	49 days	+++
K4	kanamycin	49 days	++
K5	kanamycin	60 days	+++
K6	kanamycin	60 days	+++
N1	noise	12 days	++
N2	noise	12 days	+++
N3	noise	30 days	+++
N4	noise	30 days	+++
N5	noise	62 days	+++
N6	noise	62 days	+++
KN1	kanamycin + noise	12 days	+++
KN2	kanamycin + noise	12 days	+++
KN3	kanamycin + noise	49 days	+++
KN4	kanamycin + noise	49 days	+++
KN5	kanamycin + noise	60 days	+++
KN6	kanamycin + noise	60 days	+++

See Materials and Methods for details on treatment.
*Histological assessment of cochlear damages induced in deafened mice sacrificed at different days from the ototoxic treatment, compared to the profile of three untreated normal mice of the same age. Score: –, no difference; + to +++, from moderate to severe and highly disorganized profile, with complete hair and neuron cells loss, replaced by empty vacuoles.

Xenotransplantation. As listed in Table 2, group A mice were injected with kanamycin; group B mice were exposed once to high-intensity noise; group C mice were exposed once to noise followed by kanamycin. Controls included two additional groups of mice: group D (6 mice) received IV saline solution followed by CD133$^+$ HSC transplantation, and group E (5 mice) were untreated and not transplanted. After the ototoxic treatment, all mice were total body irradiated (350 rads, Caesium source), 24 h before IV injection of human HSC in saline solution or only saline without HSC. Each mouse transplanted with human CD133$^+$ HSC received cells that had been isolated from an individual umbilical cord. Three batches of mice at the same age were tested in these experiments, indicated as groups I, II, or III.

To control the effects of variation in HSC concentration for transplantation, some mice deafened by kanamycin (group AI, 7 mice) or noise (group BI, 4 mice; group BII, 7 mice) were irradiated and after 24 h transplanted with a different number of CD133$^+$ HSC (from 0.7×10^5 to 2.5×10^5 cells injected IV per mouse). As listed in Table 2, mice were sacrificed at different time intervals from IV HSC transplantation (12, 30, 49, or 60–62 days), and the cochlear tissue phenotype was assessed histologically, by polymerase chain reaction (PCR) for HLA.DQα1 detection and by antibody and lectin staining for the detection and distribution of neuronal cell markers. Experiments with a larger number of mice were then performed to evaluate a significant cochlear tissue phenotypic recovery using a constant high dose of transplanted HSC ($\geq 2 \times 10^5$ HSC IV per mouse) (group AIII, group BIII, groups CI and CII): these mice were allowed to survive for 60–62 days from HSC IV transplantation.

The mice were euthanized by CO_2 inhalation and decapitated. Prostate, skin, bone marrow (BM), liver, spleen, heart, cochlea (from both ears), eye, and kidney were collected. One half of each sample was frozen at −80°C and then analyzed by PCR. The second half of each sample was fixed overnight in Glyofixx (Italscientifica, Genua, Italy), paraffin embedded, and then further processed for histology, antibody and lectin staining, and fluorescence in situ hybridization (FISH) analysis (see below).

Cochlea Preparation, Histology, and Antibody and Lectin Staining

Fixed cochleas were washed in 0.1 M phosphate saline solution, pH 7.4, and then decalcified with EDTA solution for 15 days at 37°C. After removal of the temporal bones the bullas were opened and the bony otic capsule was exposed under a stereomicroscope. The cochlea was opened at the apex and 5 ml Glyofixx was poured through the perforated round window. The final fixation step of the cochlea was carried out overnight in the dark at 4°C in Glyofixx, followed by rinsing in PBS, for 1 h. The decalcification of the whole cochlea was performed in 10% EDTA, pH 7.4, at 37°C for 12 days, changing the EDTA solution daily. Samples then were paraffin embedded and processed for hematoxylin and eosin staining (H&E) and immunofluorescence. Sections (5 μm) were cut and permeabilized with Triton 0.1% in PBS, for 30 min at room temperature, blocked with 5% rabbit, mouse, and goat serum for 40 min and then incubated separately with four primary antibodies: i) rabbit polyclonal IgG anti-Math1 (a transcription factor, expressed in postmitotic differentiating neurosensory outer hair cells and support cells) (4,48) (Abcam, Cambrigde, UK, cat. 13483) and ii) rabbit polyclonal IgG anti-Myosin VIIa (a marker of fully differentiated hair cells) (13,39) (Abcam, cat. No. 3481): both antibodies identify two markers highly expressed by hair cells in the OC; iii) mouse monoclonal antibodies against β-tubulin isotype III (32) (Sigma Chemical, St. Louis, MO, cat. No. T 8660), a protein involved in neuronal differentiation at an extremely early stage of commitment prior to the extension of neuritis; iv) rabbit polyclonal antibodies against protein gene product 9.5 (PGP 9.5) (8,47) (DAKO

Table 2. Experimental Procedure and Evaluation of the Effects of the Engraftment

Group/ Mouse	Treatment	CB $CD133^+$ HSC		Time From Transplant	PCR: HLA-DQα1*								
		Donor	No. of Injected Cells IV		Pr.	Sk.	B.M.	Li.	Sp.	He.	Co.	Eye	Ki.
AI													
1A	kanamycin	679	1.5×10^5	12 days	+/–	–	–	+/–	+/–	–	–	–	n.d.
2A	kanamycin	679	1.5×10^5	12 days	+/–	–	–	+/–	+/–	–	–	–	n.d.
3A	kanamycin	679	1.5×10^5	49 days	+	+	–	+	+	+/–	+	–	n.d.
4A	kanamycin	129	1.7×10^5	49 days	–	+/–	–	+	+/–	+	+/–	–	+
5A	kanamycin	129	1.7×10^5	49 days	–	+/–	–	+/–	+/–	+/–	+	–	n.d.
6A	kanamycin	268	0.7×10^5	49 days	–	–	–	+	–	–	–	–	n.d.
7A	kanamycin	—	—	49 days	–	–	–	–	–	–	–	–	–
AII													
A2	kanamycin	430	1.5×10^5	60 days	n.d.	+	n.d.	+	–	+/–	+	n.d.	–
A3	kanamycin	430	1.5×10^5										
A4	kanamycin	430	2.5×10^5										
A5	kanamycin	430	2.5×10^5	60 days	n.d.	+	n.d.	+	+	+	+	n.d.	+/–
AIII													
A11	kanamycin	176	2.5×10^5	60 days	n.d.	–	+	+	–	–	+	n.d.	–
A22	kanamycin	176	2.5×10^5	60 days	n.d.	–	+	+	–	–	+	n.d.	–
A33	kanamycin	176	2.5×10^5	60 days	n.d.	–	–	+	–	–	+	n.d.	–
A44	kanamycin	176	2.5×10^5	60 days	n.d.	+	–	–	–	–	+	n.d.	–
BI													
1B1	noise	716	0.7×10^5	30 days	–	–	–	+/–	–	–	–	–	n.d.
2B1	noise	—	—	0 days	–	–	–	–	–	–	–	–	–
3B1	noise	716	1.7×10^5	62 days	+/–	–	–	+/–	+	–	+	–	n.d.
4B1	noise	716	1.7×10^5	62 days	–	–	–	+/–	+	–	+	–	n.d.
BII													
8B2	noise	716	1.2×10^5	30 days	–	+/–	–	–	–	–	–	–	n.d.
9B2	noise	716	1.7×10^5	30 days	–	+/–	–	–	–	+	+/–	–	n.d.
10B2	noise	716	1.7×10^5	30 days	+/–	+/–	–	+/–	+	+/–	+	–	n.d.
11B2	noise	716	1.7×10^5	30 days	+/–	–	–	+	+	+/–	–	–	n.d.
12B2	noise	716	1.7×10^5	30 days	+/–	+	–	–	+/–	+	–	–	n.d.
13B2	noise	—	—	0 days	–	–	–	–	–	–	–	–	–
14B2	noise	—	—	62 days	–	–	–	–	–	–	–	–	–
BIII													
2B11	noise	214	2.5×10^5	61 days	–	–	–	+	+	–	–	–	–
2B22	noise	214	2.5×10^5	61 days	–	–	–	+	+	–	+	n.d.	–
2B33	noise	214	2.5×10^5	61 days	–	–	+	+	–	–	+	n.d.	–
2B44	noise	214	2.5×10^5	61 days	+	–	–	+	–	–	–	n.d.	–
CI													
B1	kanamycin + noise	430	2.0×10^5	60 days	n.d.	+	n.d.	–	+	+	–	n.d.	+
B2	kanamycin + noise	430	2.0×10^5	60 days	n.d.	+	n.d.	–	+	+	+	n.d.	+
B3	kanamycin + noise	—	—	60 days	–	–	–	–	–	–	–	–	–
B4	kanamycin + noise	181	2.0×10^5	60 days	n.d.	–	n.d.	+/–	+	+	+	n.d.	–
CII													
B11	kanamycin + noise	260	2.5×10^5	61 days	–	+	–	+	+	+	+	n.d.	–
B22	kanamycin + noise	260	2.5×10^5	61 days	n.d.	+	–	–	+	+	+	n.d.	–
B33	kanamycin + noise	260	2.5×10^5	61 days	n.d.	+	–	–	–	–	–	n.d.	–
B44	kanamycin + noise	260	2.5×10^5	61 days	n.d.	+	n.d	–	+	+	+	n.d.	–
DI													
C1	saline	181	2.0×10^5	60 days	n.d.	–	n.d.	+/–	+	–	+/–	n.d.	+
C2	saline	155	2.0×10^5	60 days	n.d.	–	n.d.	+	+	–	–	n.d.	–
C3	saline	806	2.0×10^5	60 days	n.d.	–	n.d.	+	+/–	+/–	–	n.d.	–

Table 2. Continued

Group/ Mouse	Treatment	CB CD133⁺ HSC Donor	No. of Injected Cells IV	Time From Transplant	PCR: HLA-DQα1* Pr.	Sk.	B.M.	Li.	Sp.	He.	Co.	Eye	Ki.
DII													
C11	saline	274	2.5×10^5	61 days	n.d.	–	n.d.	–	+	–	–	n.d.	–
C22	saline	274	2.5×10^5	61 days	n.d.	–	n.d.	+	–	–	–	n.d.	–
C33	saline	274	2.5×10^5	61 days	n.d.	–	n.d.	+	–	–	–	n.d.	–
E													
D1	—	—	—	60 days	–	–	–	–	–	–	–	–	–
D2	—	—	—	60 days	–	–	–	–	–	–	–	–	–
8A	—	—	—	60 days	–	–	–	–	–	–	–	–	–
DG1	—	—	—	0 days	–	–	–	–	–	–	–	–	–
DG2	—	—	—	0 days	–	–	–	–	–	–	–	–	n.d.

Pr.: prostate; Sk.: skin; B.M.: bone marrow; Li.: liver; Sp.: spleen; He.: heart; Co.: cochlea; Eye.: eye; Ki.: kidney.
*PCR amplification limit: 1 ng human DNA/1 mg mouse DNA (see Materials and Methods). Score: (–) no signal, (+/–) weak positive signal, (+) strong positive signal, n.d.: evaluation not done.

Cytomation, Glostrup, DK, cat. No. Z 5116), a member of the ubiquitin C-terminal hydrolase (UCH) family of proteins that is expressed in neuronal tissues by mature and immature neurons. Secondary FITC-conjugated anti-IgG antibodies were employed for visualization of anti-Math1 and anti-Myosin VIIa. Peroxidase-labeled second anti-rabbit IgG antibodies were employed to visualize anti-β-tubulin and anti-PGP 9.5. One biotinylated lectin, *Lycopericum esculentum* agglutinin (LEA) (10 μg/ml, Vector Laboratories, Burlingame, CA), carbohydrate specificity: *N*-acetyl-D-glucosamine, and a horseradish-peroxidase-conjugated lectin, *Triticum vulgatis* (wheat germ) agglutinin (WGA) (10 μg/ml, Sigma), carbohydrate specificity: D-acetyl-D-glucosamine oligomers, and neuroaminic acid (syalic acid), were used to identify the sensory and nonsensory regions of the cochlea (12,22,38).

PCR Analysis

Genomic DNA was extracted from frozen tissues according to the manufacturer's protocol (QIAamp DNA Mini Kit, Qiagen GmbH, Hilden, Germany) and analyzed by PCR for the presence of human HLA.DQα1 as marker for human DNA sequence, using the forward (5′-GTG CTG CAG GTG TAA ACT TGT ACC AGT TGT-3′) and reverse (5′-CAC GGA TCC GGT AGC AGC GGT AGA AGT TG-3′) primers, as described (1). Taq Platinum (Invitrogen, Merelbeke, Belgium) was used in all PCR reactions, as recommended by the manufacturer. Single positive 242-bp PCR bands were sequenced to confirm the human HLA.DQα1 DNA sequence.Standard curves were run to establish the sensitivity of our PCR assays on murine samples. Dilutions were prepared containing decreasing amounts of human DNA (from 1000 to 1 ng) in normal mouse DNA to 1.0 μg of total DNA sample in 10.0 μl. The method of amplification with Taq Platinum detects 1 ng (for HLA.DQα1) of human DNA in 1 μg of mouse DNA after 40 cycles (Fig. 1A).

Dual Color (DC)-FISH and Confocal Scan Analysis

DC-FISH analysis was performed only on tissue positive for HLA.DQα1 by PCR. Pan-centromeric probes for either human (labeled with FITC) or mouse (labeled with Cy3) (both from Cambio, Cambridge, UK) were employed according to the manufacturer's protocol (www.cambio.org) as previously reported (21). Slides were then observed in multiple fluorescence channels using an Olympus Bx61 epifluorescent microscope system and Smartcapture X software (Digited Screnhitz, Cambridge, UK). In addition, some specimens were studied using a Zeiss L5M 510 confocal microscope system to establish that human centromere signals were within cell.

RESULTS

Engraftment in Different Body Tissues of Mice After Human CD133⁺ HSC Transplantation, Detected by PCR Analysis

DNA isolated from all transplanted mice revealed the presence of the human HLA.DQα1 sequence in various tissues varying in presence or absence among the tissues of the same mouse (Table 2 and Fig. 1B). Engraftment (Fig. 1B and C) appeared more frequently in the spleen and liver of all transplanted animals (including group D mice) than in other tissues; in addition, oto-injured, transplanted animals (groups A, B, and C) also showed human engraftment (presence of the HLA.DQα1 sequence) in the cochlea (Table 2). In particular mice treated with kanamycin (group A) or kanamycin and

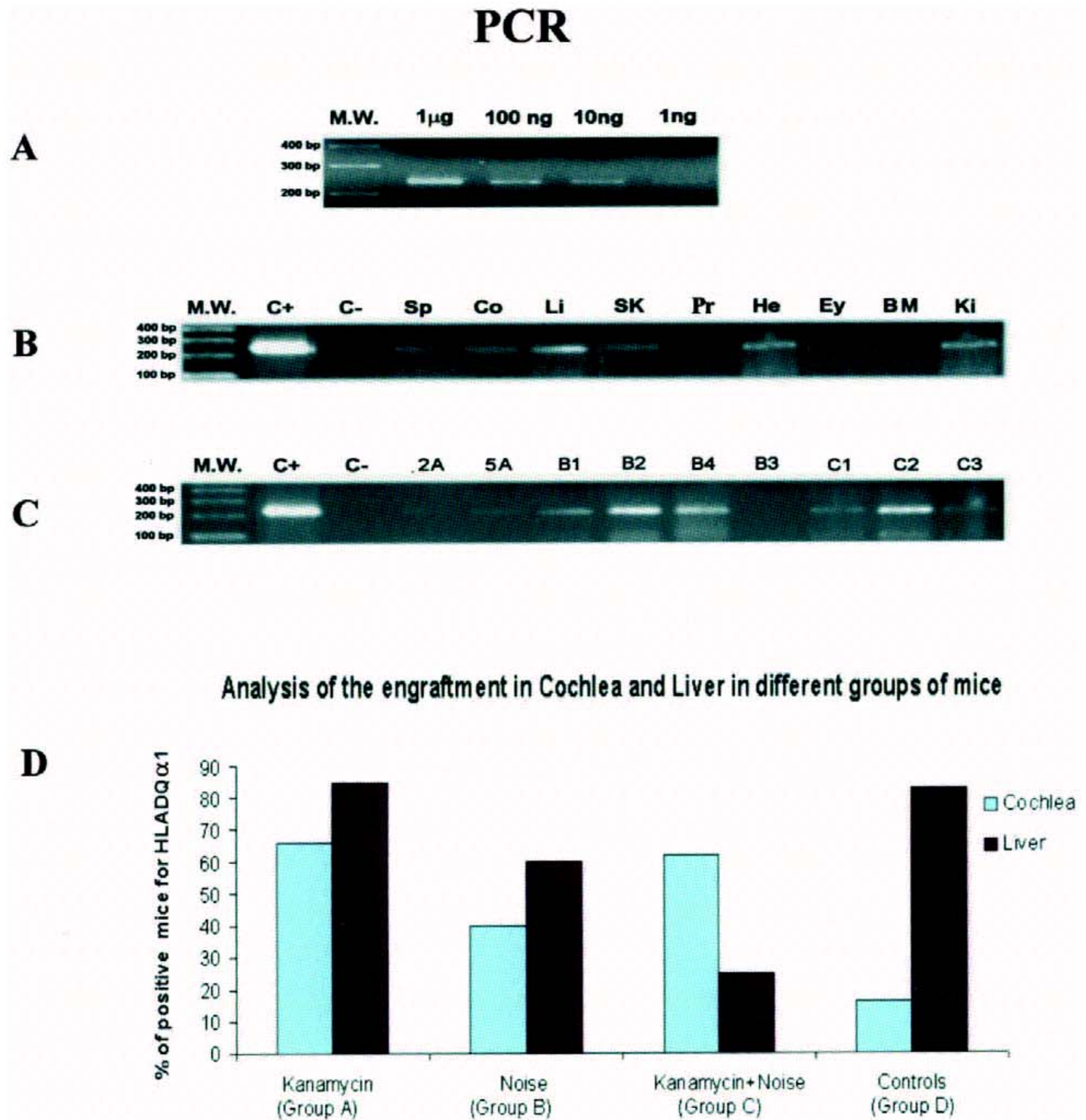

Figure 1. PCR for HLA-DQα1 on mouse DNA. (A) Sensitivity of PCR assay with human DNA . (B) Mouse 4A. (C) Spleen of a pool of different untreated mice. C+: positive control, human cord blood; C−: negative control, mouse 8A; Li: liver; Sp: spleen; Co: cochlea; Li: liver; SK: skin; Pr: prostate; He: heart; Ey: eye; BM: bone marrow; Ki: kidney. M.W.: standard molecular weight.

noise (group C) showed engraftment more frequently in the cochlea (Fig. 1D) than mice treated with noise only (group B), probably reflecting the consequence of greater damage induced by kanamycin or the combination of the two oto-toxic agents in the cochlea. We also found that positive detection correlated with high dose of HSC transplantation ($\geq 2 \times 10^5$ cells IV per mouse) particularly in mice that were sacrificed after 49 or 60 days from transplantation (Table 2 compares results of groups A, B, and C).

Morphology of the Cochlea and the OC in the Different Groups of Mice

Figure 2 shows the normal morphology and cytoarchitecture of the cochlea, and particularly the OC with well-maintained hair cells and inner/outer support cells and the presence of normal neuron and ganglion phenotype, found after 60 days from the beginning of the experiment in a normal untreated mouse of Group E (mouse 8A). In contrast, in mice not injected with HSC cells, both kanamycin (Fig. 2B and C; mouse 7A) and noise (Fig. 2F and G; mouse 14B2) or the combination of both oto-toxic agents (not shown) severely damaged or destroyed the architecture of the OC examined, with

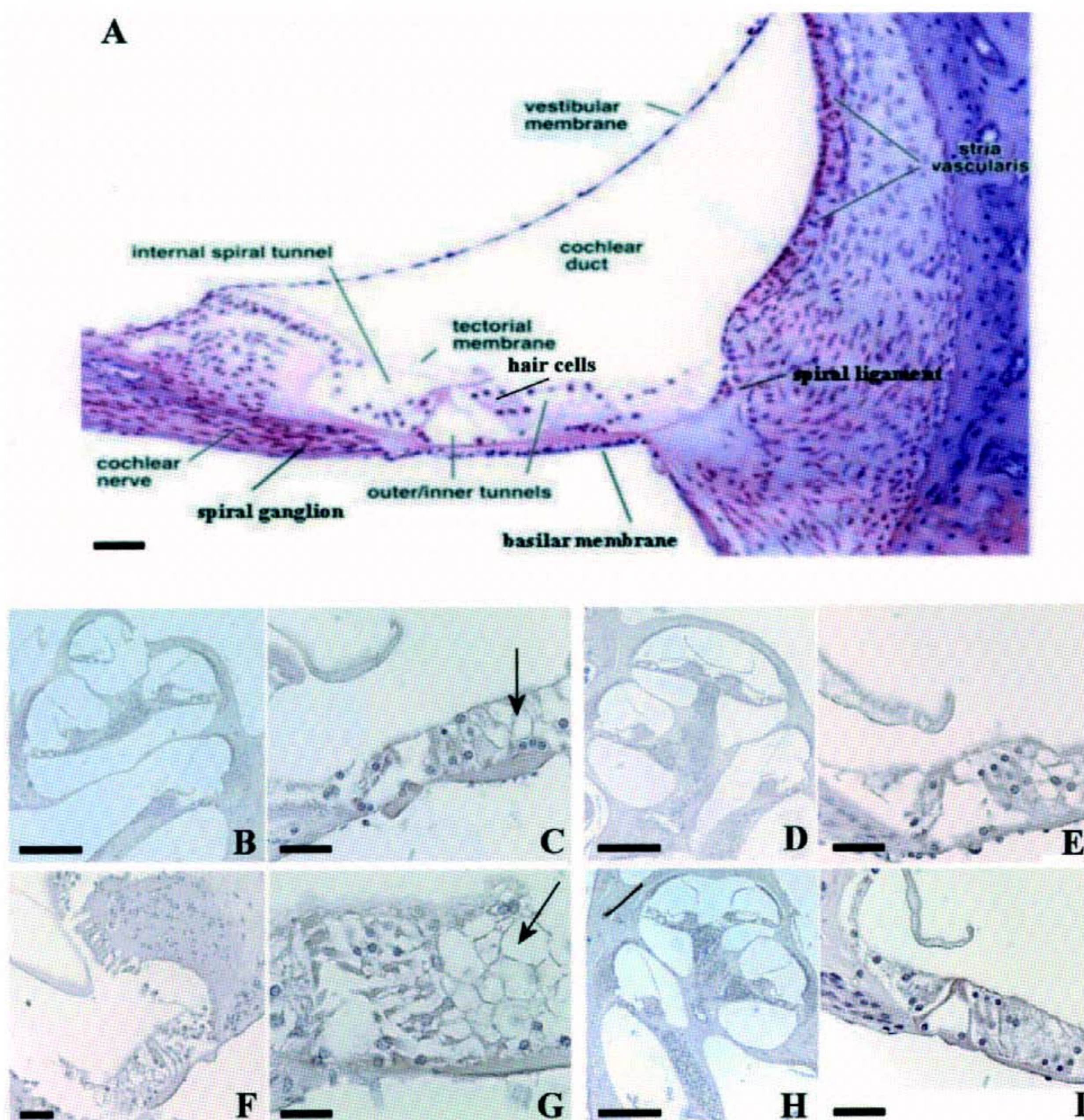

Figure 2. Hematoxylin and eosin staining of the cochlea in mice. Control mouse 8A (A). Cochlea (B) and OC (C) of kanamycin-treated nontransplanted mouse 7A. Cochlea (D) and OC (E) of kanamycin-treated and transplanted mouse 5A. Cochlea (F) and OC (G, different section) of noise-treated, nontransplanted mouse 14B2. Cochlea (H) and OC (I) of noise-treated transplanted mouse 10B2. (C, G: arrow shows vacuoles). Scale bar: 20 μm for (A, C, E, I); 100 μm for (B, D, F, H); 10 μm for (G).

marked loss of both hair and support cells, clearly showing empty vacuoles (arrows in Fig. 2C and G). After 60 days from the transplant, all mice transplanted with HSC following treatment with kanamycin and noise alone or kanamycin plus noise (not shown) showed well-repaired cochlea (Fig. 2D, E, H, and I). Particularly, abundant cells lining modiols and the OC vestibular membrane of Reissner, the lateral wall (including stria vascularis and the spiral ligament), the basilar membrane, sensory and support cells, and tectorial membrane surrounding the limbus were clearly evident, with spiral ganglion and nerve cells well maintained, showing abundant mesenchymal cells and fibrocytes, the latter surrounding neural tissue. Conversely, tissues of nontransplanted mice lacked hair and neural cells, replaced by empty vacuoles or detected only sporadically in severely damaged tissues (Fig. 2B, C, F, and G). Cochlear regeneration in noise-treated mice (group B) was generally lower than other transplanted mice, implying that damage was more severe when induced by noise than by kanamycin.

Sixty days after transplantation, the tectorial membrane and the Corti organ showed a strong reaction after WGA (Fig. 3A) and LEA (Fig. 3B) binding.

Morphological evaluation of these mice thus provided important information: 1) HSC IV transplantation in mice previously damaged by ototoxic agents favored and improved cochlear tissue phenotypic recovery; 2) regenerative effects detected in the inner ear were enhanced in mice injected with a higher number of donor HSC cells ($\geq 2.0 \times 10^5$ cells per mouse); 3) effects of cochlear tissues resumption were more evident in mice sacrificed after 49 and especially after 60–62 days from transplant, compared to shorter intervals (12 or 30 days).

Antibody Staining on the OC of the Different Groups of Mice

In the OC, phenotypic recovery and hair cell regeneration was further confirmed in transplanted mice by positive antibody staining for two hair cell and support cell markers (i.e., Math1 and myosin VIIa). Figure 4 shows loss of both antigen expressions induced by ototoxic treatment (Fig. 4C, D, I, and J) and partial recovery of both antigen expressions after human HSC transplantation (Fig. 4E, F, K, and L). As a representative positive example, Figure 5 shows that after 60–62 days from the CD133$^+$ HSC transplantation the outer hair cells and one inner cell expressed a moderate β-III-tubulin staining while the tectorial membrane was strongly labeled (Fig. 5A). The hair cells were also positive after the immunohistochemical detection for PGP 9.5 (Fig. 5B). PGP 9.5 was also expressed by the spiral ganglion; the tectorial membrane was negative.

Chimerism Revealed by DC-FISH and Confocal Scan Analysis

To demonstrate the presence of human chimerism within transplanted mice, we applied the DC-FISH method, revealing both mouse and human chromosome centromeres in cell nuclei. Cochlea and liver of transplanted animals positive for HLA.DQα1 and from negative control mice were subjected to DC-FISH analysis. We detected a small fraction of chimeric human cells, mostly small mononuclear cells, in the liver of the engrafted mice (<2.0%) (see, for example, Fig. 6A, arrows). Subsequent sections of the same liver also revealed small numbers of mouse/human cell hybrids (heterokaryons) with large nuclei typical of parenchymal/mesenchymal-like cells (Fig. 6A, arrowhead). On examining the cochlea of the same mouse, small numbers of heterokaryons were detected (Fig. 6B–D) together with human chimeric mononuclear cells. These heterokaryons contained very little human genome and might derive from fusion between endogenous cells and human HSC, losing most of the human genomic material with subsequent cell divisions. Table 3 compares the level of the

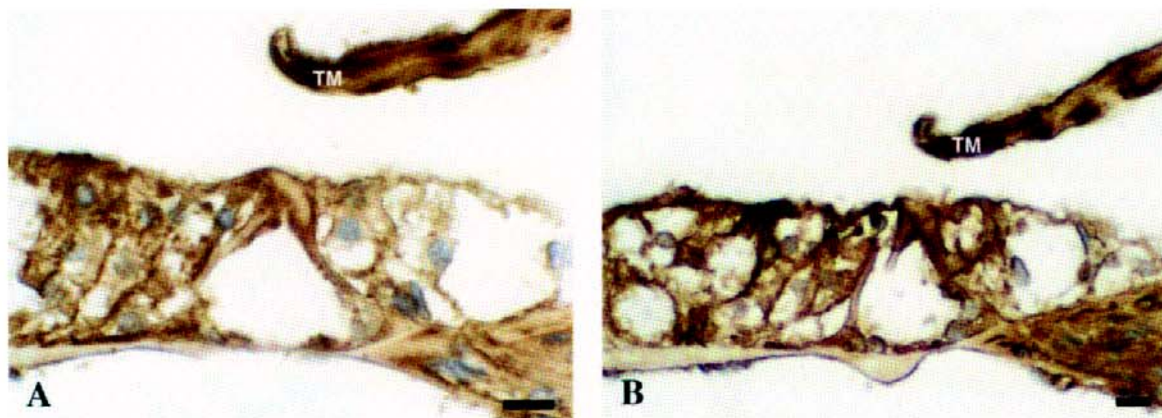

Figure 3. Lectin histochemistry in the OC of mice. WGA (A) detection in CD133$^+$ HSC transplanted, noise-treated mouse 10B.2 and LEA (B) detection in a CD133$^+$ HSC transplanted, kanamycin-treated mouse 5A. TM: tectorial membrane. Scale bar: 10 μm.

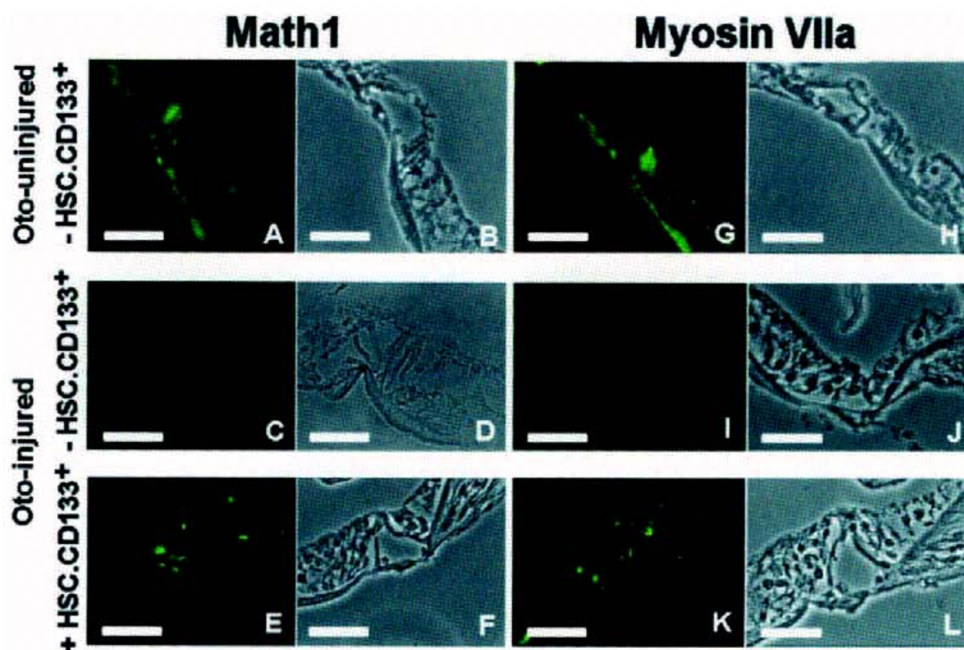

Figure 4. Immunofluorescence staining for Math1 and Myosin VIIa proteins in the OC of mice. Math1 expression in control mouse 8A (A), noise-treated mouse 14B2 (C), and CD133+ HSC transplanted, noise-treated mouse 10B2 (E). Myosin VIIa expression in control mouse 8A (G), noise-treated mouse 14B2 (I), and CD133+ HSC transplanted, noise-treated mouse 10B2 (K). Phase contrast shown in (B, D, F, H, K, M). Scale bar: 20 μm.

engraftment detected in the OC of the mice of all five groups, reporting the number of positive cells for human centromeres counted in tissue sections in several ears evaluated in each group. Group A mice showed a higher number of chimeric cell nuclei compared to groups B and C, and the number was the lowest in group D mice. These positive cells were seen in several areas of the inner ear lining, very frequently on the bone surface of scala vestibuli, in scala tympani, and modiols (Fig. 6B). In the OC, small numbers of mononuclear cells positive for only human centromeres were seen in the lateral wall (stria vascularis and spiral ligament) (Fig. 6C) and, to a lesser degree, in Reissner's membrane and beneath the basilar membrane, within or around capillaries that were clearly evident. Positive staining for human DNA was never seen in hair cells, saccule, ganglion, and vestibular

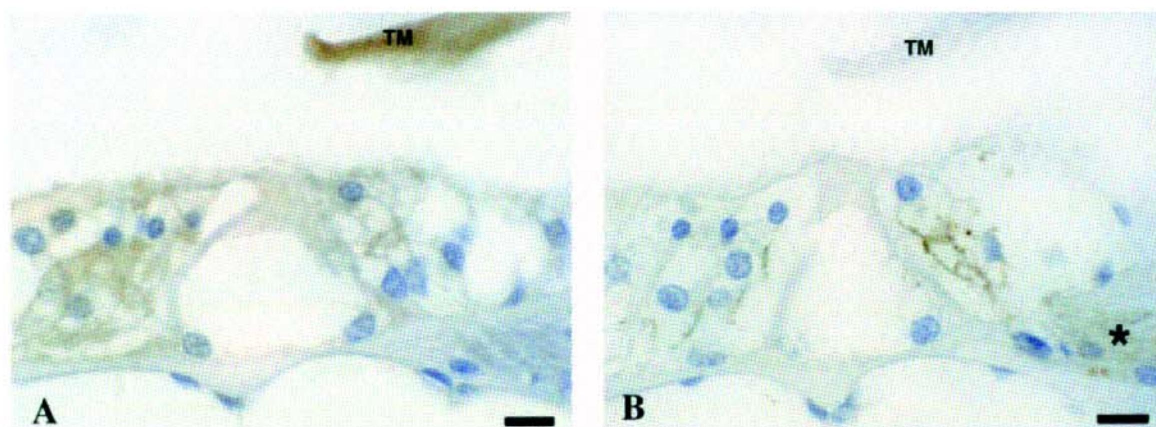

Figure 5. Immunostaining for β-III-tubulin and PGP 9.5 proteins in the OC of mice. β-III-tubulin (A) and PGP 9.5 (B) expression in CD133+ HSC transplanted, noise-treated mouse 10B2. TM: tectorial membrane. Spiral ganglion shown with an asterisk. Scale bar: 10 μm.

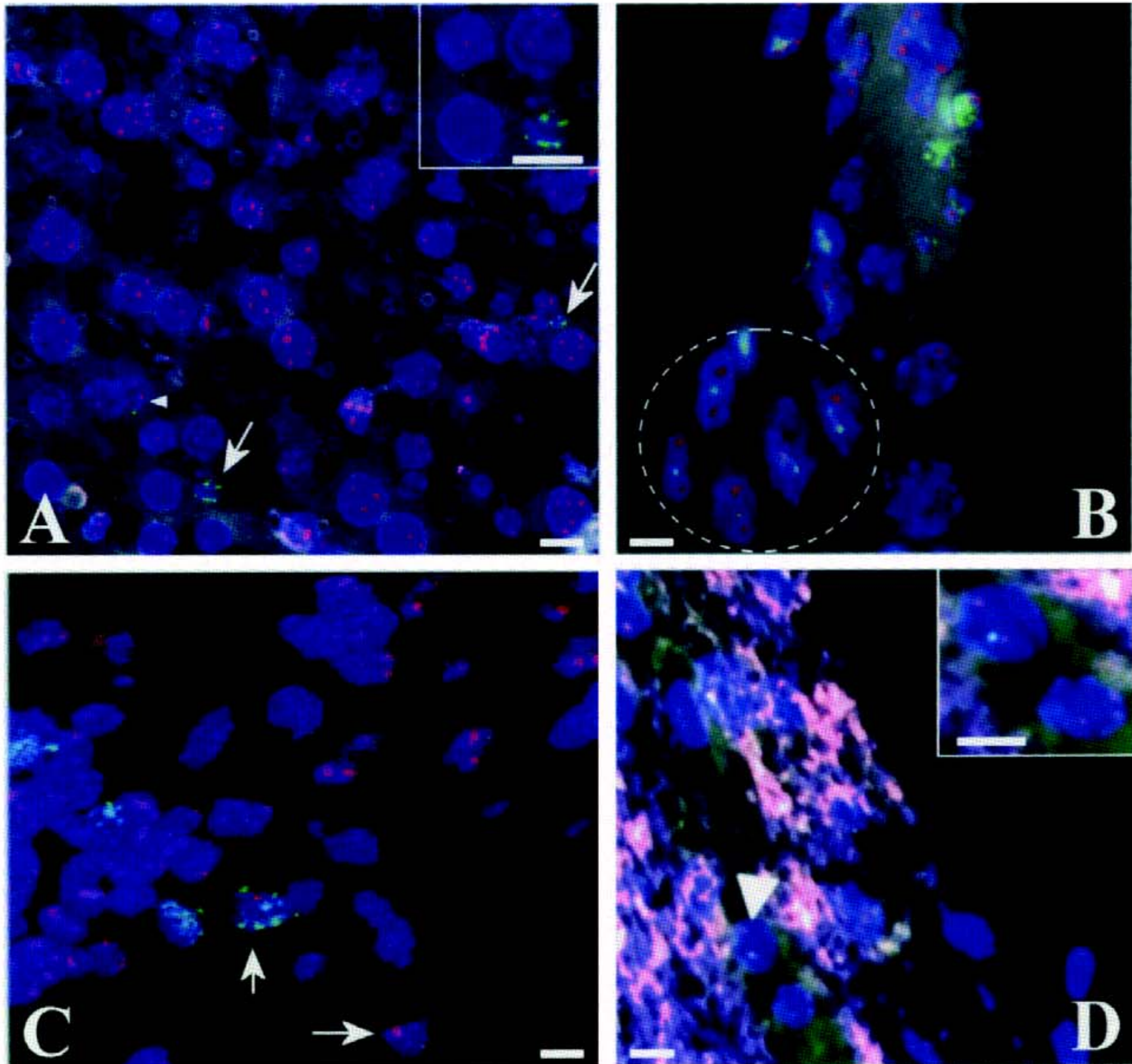

Figure 6. DC-FISH assay in different tissues of mice. Red: mouse centromeres. Green: human centromeres. (A) mouse 3A: liver. Arrows: human cells. Arrowhead: heterokaryon. Inset: enlargement of a human cell among mouse cells. (B) mouse 3A: cochlea. Dashed circle: heterokaryons. (C) mouse 10B2: cochlea . Arrows: heterokaryons. (D) mouse 10B2: cochlea. Arrows: heterokaryons. Inset: heterokaryon. Scale bar: 10 μm.

neurons nor among the cochlear nerve fibers. Additionally, in sections of the modiols (Fig. 6D), the lateral cochlear duct and in the lateral wall and spiral ligament in the OC, in zones normally abundant in stromal, mesenchymal, and endothelial cells forming capillaries, we also detected a small number of heterokaryons similar to the liver of the same and other engrafted mice. Confocal scan analysis (not shown) confirmed that the positive human centromeric signals were in the nucleus of the positive cells.

DISCUSSION

In these studies, using the human–mouse xenotransplant model, we demonstrated that in *nod-scid* mice, previously exposed to kanamycin and/or intense noise to provoke permanent damage to their inner ear apparatus and deafness, injecting human HSC IV favored tissue regeneration and repair with dramatic resumption of hair cells and neurons loss in the OC of these mice. Our findings raise several important points.

Table 3. Engraftment in the Inner Ear (Organ of Corti)

Mice	No. of Ears	Total Cells Counted	Human Centromeres Positive Cells (%)	Heterokaryons Positive Cells (%)
Group A	4	3440	142 (4.12%)	38 (1.10%)
Group B	3	3256	96 (2.94%)	28 (0.86%)
Group C	3	2860	63 (2.20%)	22 (0.77%)
Group D	2	3218	26 (0.81%)	15 (0.47%)
Group E	2	2125	0	0

Semiquantitative analysis of human cells and heterokaryons in the inner ear of mice at 49 or 60 days after human HSC IV transplantation. Cell counts were made on 4–5 midmodiolar sections, at about 20 μm distance, for each cochlea. Total cells were counted based on nuclear counts.

The resulting effects of cochlear recovery were enhanced in mice pretreated with kanamycin, compared with those exposed to acoustic noise, probably reflecting dissimilar damage intensity provoked by the ototoxic agents. In contrast, in mice injured but subsequently not transplanted with HSC, the inner ear compartments remained severely damaged. Our present findings of regeneration of ganglion cells in this human–mouse xenotransplantation model correlate with a similar finding reported after embryonic stem cell-derived neural progenitor cell transplantation in a mouse model of neuronal degeneration, with donor cells constitutively expressing the EYFP marker (6).

The human DNA marker HLA.DQα1 was detected mainly in liver and spleen of HSC transplanted mice and was more often present in tissues of oto-injured mice (i.e., liver, spleen, and cochlea) than in others. The cochlea was principally damaged by the ototoxic pretreatment, confirming that “homing” and engrafting of transplanted HSC cells were probably enhanced by the intensity of tissue damage and target tropism induced by the chemical or physical insults. These results agree with previous observations by Jereczek-Fossa et al. (18), who, observing enhanced stem cell migration derived from bone marrow in a mouse transplantation model, suggested that better engraftment is associated with tissue damage compared with only irradiation treatment.

By means of DC-FISH and confocal scan analysis, on liver and cochlea of transplanted animals, we demonstrated a small but constant presence of mononuclear chimeric cells after up to 60–62 days from transplant, positive for only the centromeric human chromosomal marker, showing that there are intact human cells, confirming the human chimerism assessed by PCR. In the cochlea these positive cells were seen in several areas of the inner ear, particularly lining the bone surface of the modiols, the scala vestibuli and the scala tympani. In the OC, mononuclear cells positive for only human centromeres were seen in the lateral wall, spiral ligament, and beneath the basilar membrane, zones containing capillaries and abundant mesenchymal cells and fibrocytes; but never among the sensory hair epithelium of the OC, in the saccula, or in neurons of the auditory and vestibular ganglion. These findings clearly demonstrate that, in vivo, transplanted human HSC renewed themselves in the mouse while they were circulating and were able to migrate to the inner ear.

Histologic and immunofluorescent analysis confirmed that sensory hair cell loss resumption was better in mice where host cells were more frequently detected, in mice pretreated with the ototoxic agents and subsequently transplanted. We suggest that among the circulating host HSC, some divided in vivo, generating progenies along different lineages able to contribute to the renewal of damaged tissue. This would not be surprising because precursor cells along the blood myeloid or endothelial lineages are normally present in $CD133^{+}$ HSC isolated from umbilical CB. The results of lectin binding analyses confirm this hypothesis. In fact, the intense WGA- and LEA-positive reaction we detected 60–62 days after the HSC transplantation in transplanted *nod-scid* mice is comparable to that detected from the last days of gestation until 8–10 days after birth in the cochlea of mice by Rueda et al. (38), in a study on the distribution of glycoconjugates during development. The cell surface glycoconjugates have been shown to mediate specific cell adhesion events that take place during development and regeneration [for references, see (49)]. Therefore, the presence of the *N*-acetyl-D-glucosamine residues, detected by LEA, and D-acetyl-D-glucosamine oligomers and syalic acid, detected by WGA, may reflect specific cell–cell interactions needed during the restoration of the auditory organ.

We provide evidence of partial resumption of expression in OC of oto-injured transplanted mice of host hair cell markers, Math1 and Myosin VIIa. Moreover, the positive immunostaining detected for β-III-tubulin involved in neuronal differentiation at an extremely early

stage of commitment (32), and for PGP 9.5, expressed by mature and immature neurons (8,47), provides evidence of the neuronal nature of these cells.In contrast, the phenotypic rescue of hair cells in nontransplanted oto-injured mice was never observed.

Further regeneration of cochlear sensory hair cells and neuronal cell loss by transplanted HSC probably required "homing" to appropriate local niches, where they were then stimulated to contribute to generation of cells along the appropriate phenotype. Li et al. (26) and Rask-Andersen et al. (36) have shown that, in adult mice, stem and early progenitor cells are present in the inner ear and in spiral ganglion tissue, because they are capable of giving rise to new hair cells and neurons ex vivo. In contrast, regeneration of new neuronal cells is difficult to prove in vivo, particularly because the stem cell population is difficult to identify and markedly decreases after birth (35). Several examples of HSC-derived stem cells able to generate progenies differentiating along different lineages in vivo were reported; for example, the derivation of glomerular mesangial cells in the kidney, and microglial cells in the brain from transplanted BM-derived HSC (5,28). The origin of myofibroblasts in the kidney and brain from engrafted HSC strengthens the possibility that inner ear fibrocytes and their precursors may derive from HSC. In a mouse transplantation model, cells derived from a single HSC isolated from BM were able to home, divide, and differentiate towards mesenchymal phenotypes in the adult inner ear of mice (24). Because the stromal cells of the inner ear derive from mesoderm, it was speculated that hematopoietic stem cells may be involved in inner ear repair processes (5,24,36). Multipotent mesenchymal stem cells, isolated from bone marrow and from the umbilical cord, in culture in vitro, can in fact differentiate, giving rise to cells of different lineages, consequently leading to hope that they might become a powerful source of cells for reconstructive therapy in many tissue types (5,19,20,36). However, it appears that in vivo the postulated plasticity of these cells, even using powerful traceability methods, is questionable because positive proof has been scarce so far and is absent in many tissues (17,45).

By DC-FISH and confocal scan analysis, in addition to small numbers of human chimeric mononuclear cells we also found heterokaryons both in the liver and cochlea of our mice, which showed the presence of mostly mouse centromeres and a little human genomic material in the nuclei. This suggests that a limited fraction of circulating human HSC migrated into the liver or the inner ear after transplantation to these mice and fused with resident mouse cells, generating hybrids that retained only a small part of the human genomic material, probably gradually lost after repeated cell divisions. Cell fusion may result in multinuclear cells, but here we found integrated cells that had only a single nucleus. Many cases of somatic fusion of stem cells with differentiated adult parenchymal cells or adherent cells of other lineages has been confirmed by others (2,11,21,41, 50), opening up the possibility that the resulting cells can acquire a multipotential condition from which they start to reorganize their genome and differentiate, controlled by the damaged adult tissue (epimorphic regeneration). Cell fusion reflects a phenomenon that probably requires a high number of HSC in situ. In the cochlea of our mice, both engrafting and fusion of HSC were apparently facilitated and intensified by the injuries that enhanced interaction between host and endogenous cells.

Our present findings show surprisingly few human-derived cells in the cochlea, yet the administration of HSC appeared to be correlated with tissue regeneration and repair. Stem cells are deeply influenced by their microenvironment (or niche), which plays a fundamental role in the morphogenesis of complex structures, tissues, and organs. It has been shown that purified multipotent primate embryonic stem cells, cultured in a 3D matrix, synthesize their own endogenous repertoire of molecules and contribute to form their own niche (i.e., a microenvironment vital to their survival and differentiation). Small variations in their microenvironment may affect the type and amount of stem cell-related factors (i.e., receptors, cell–cell adherent molecules, and other factors released by the cells) favoring stem cell commitment and lineage differentiation (29). This suggests that trophic effects mediated by the secretion of stem cell-related neurotrophic factors and other environmental factors should also be considered to exert an influence and accelerate recovery of mesenchymal cells, fibrocytes, inner and outer hair cells, and any other support cells in the OC of deafened mice after HSC transplantation.

CONCLUSION

In this article we show dramatic repair of damage induced in vivo in mice treated with kanamycin and noise, after IV transplantation with human $CD133^+$ HSC. The results of the present study lead us to suggest that similar to the cells found in the liver, circulating human $CD133^+$ HSC, originally derived from umbilical CB, once migrated to the inner ear, contributed somehow to stimulating resumption of damaged tissue. We have shown unequivocally that a fraction of circulating HSC fused with resident cells, generating hybrids. These chimeric cells or their progeny may have contributed to resumption of the cochlear phenotype. Given the diversity of potential precursors that generate the inner ear in these mice, the chimeric cells may multiply and differentiate in vivo into functional cells along different lineages or, via cell–cell adhesion or the secretion of growth factors, activate endogenous neuron precursor cells, which normally do not divide, stimulating their expan-

sion and differentiation, playing a fundamental role in piloting the resumption of the mouse inner ear phenotype and recovery of the cochlear sensorineural elements after damage (31). Hair cell and neuron regeneration in the OC present important challenges in addition to the initial stem cell differentiation, like growth of neuronal processes with the capacity to grow to a specific target and resumption of functional activity (6). We have demonstrated after transplantation of HSC to the oto-injured mice a significant recovery and reorganization in the OC of hair and support cells expressing specific Math1 and Myosin VIIa proteins, and the development of neuronal processes expressing β-III-tubulin and PGP 9.5, reminiscent of neuronal connections between spiral ganglion and hair cells.This approach might emerge as a potential strategy for hearing rehabilitation.

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