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Abstract

Following injury to the adult mammalian cochlea, hair cells cannot be spontaneously replaced. Nonetheless, the postnatal cochlea contains progenitor cells, distinguished by the expression of nestin, which are able to proliferate and form neurospheres in vitro. Such resident progenitors might be endowed with reparative potential. However, to date little is known about their behaviour in situ following hair cell injury. Using adult mice and ex vivo cochlear cultures, we sought to determine whether: (i) resident cochlear progenitors respond to kanamycin ototoxicity and compensate for it; and (ii) the reparative potential of cochlear progenitors can be stimulated by the addition of growth factors. Morphological changes of cochlear tissue, expression of nestin mRNA and protein and cell proliferation were investigated in these models. Our observations show that ototoxic injury has modest effects on nestin expression and cell proliferation. On the other hand, the addition of growth factors to the injured cochlear explants induced the appearance of nestin-positive cells in the supporting cell area of the organ of Corti. The vast majority of nestin-expressing cells, however, were not proliferating. Growth factors also had a robust stimulatory effect on axonal sprouting and the proliferative response, which was more pronounced in injured cochleae. On the whole, our findings indicate that nestin expression after kanamycin ototoxicity is related to tissue reactivity rather than activation of resident progenitors attempting to replace the lost receptors. In addition, administration of growth factors significantly enhances tissue remodelling, suggesting that cochlear repair may be promoted by the exogenous application of regeneration-promoting substances.

Introduction

Following injury to the inner ear of birds and other non-mammalian species, a subpopulation of progenitor cells or supporting cells can re-enter the cell cycle and differentiate into new hair cells, or, in the case of supporting cells, acquire a hair cell fate without dividing (Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Cafaro et al., 2007). In contrast, in the mammalian cochlea, there is no evidence for spontaneous hair cell regeneration, and the degeneration of auditory receptors leads to irreversible sensorineural hearing loss.

Recent studies have shown that supporting cells of the mammalian organ of Corti can be induced to divide after specific gene manipulation, including downregulation of p27, p19, or retinoblastoma, or overexpression of Math1 (Lowenheim et al., 1999; Mantela et al., 2005; Sage et al., 2005; White et al., 2006; Laine et al., 2007). However, it is still not clear whether all supporting cells are able to proliferate, or whether this ability is restricted to a specific cell subset, endowed with stem cell properties. Furthermore, recent evidence suggests that the postnatal mammalian inner ear contains progenitor cells, which are able to proliferate in vitro or form otospheres and differentiate into multiple phenotypes (Zhai et al., 2005; White et al., 2006; Martinez-Monedero et al., 2007; Oshima et al., 2007; Savary et al., 2007; Chai et al., 2012).

Nestin is a class IV intermediate filament protein expressed during development in the central nervous system (CNS) and commonly associated with neural progenitors (Frederiksen & McKay, 1988; Lendahl et al., 1990; Reynolds & Weiss, 1992; Morshead et al., 1994; Dahlstrand et al., 1995). During CNS embryogenesis, nestin is expressed in many proliferating and migrating cells, whereas in the adult it is usually restricted to astrocytes close to injury sites. These findings suggest that nestin expression is related to the ability of neural cells to proliferate and initiate differentiation during physiological development or repair (Lendahl et al., 1990; Dahlstrand et al., 1995; Yamaguchi et al., 2000; Mignone et al., 2004). In the inner ear, nestin has been detected in progenitors isolated from the newborn organ of Corti that can proliferate and differentiate into hair cells and supporting cells in vitro (Malgrange et al., 2002; Savary et al., 2007). Moreover, downregulation of nestin in supporting cells has been related to the lack of regenerative ability of the adult sensory epithelia (Smeti et al., 2011).

The proliferation and differentiation of progenitor cells that reside in the adult mammalian CNS can be enhanced by infusion of growth factors (Craig et al., 1996; Kuhn et al., 1997; Benraiss et al., 2001; Pencea et al., 2001; Grimaldi & Rossi, 2006). Likewise, following appropriate growth factor stimulation, progenitor cells contained in the ear sensory epithelia can re-enter the cell cycle and undergo self-renewal (Lambert, 1994; Zheng et al., 1997; Lin et al., 2009). In light of these findings, in the present study we used an in vivo and an in vitro approach to investigate whether: (i) a population of progenitor cells, which may be distinguished by the expression of nestin, reside in the postnatal and adult cochlea and/or can be activated in response to kanamycin-induced hair cell degeneration; and (ii) the reparative potential of cochlear progenitors in the postnatal cochlea can be boosted by the addition of growth factors.

Materials and methods

Ototoxic injury

The experimental plan was formulated according to the European Communities Council Directive (86/609/EEC), the NIH guidelines, and the Italian law for care and use of experimental animals (DL116/92), and was approved by the Italian Ministry of Health and the Ethical Committee at the University of Turin. To induce ototoxic injury, young adult C57BL/6 mice (aged 4–5 weeks; Harlan, San Pietro al Natisone, Italy; n = 14) received a single subcutaneous injection of kanamycin sulphate (1 g/kg; Sigma-Aldrich, St. Louis, MO, USA) dissolved in phosphate-buffered saline (PBS), followed 30 min later by a single intraperitoneal injection of the loop diuretic furosemide (400 mg/kg; Mayne Pharma, Napoli, Italy) (Oesterle et al., 2008). The mice were killed 6 days after treatment. Age-matched untreated mice were used as controls (n = 14).

Auditory brainstem response measurement

To assess the extent of the ototoxic injury, we performed auditory brainstem response (ABR) tests on untreated mice (n = 7) and treated mice (n = 7). Treated mice were tested both before kanamycin–furosemide injection and 4 days after treatment. Briefly, mice were anaesthetized with a mixture of ketamine (100 mg/kg; Ketavet; Bayern, Leverkusen, Germany) and xylazine (5 mg/kg; Rompun; Bayer, Milan, Italy), and placed in an audiometric testing box (sound booth), where sound stimuli were administered. Evoked responses were measured by means of three subcutaneous needle electrodes: the positive one at the vertex, the negative one at the mastoid, and the ground at the tail. We used clicks at alternating polarity (duration, 100 μs; 11 Hz), and for each stimulus the electroencephalographic activity was recorded for 12 ms. ABR waveforms were recorded in 10-dB sound pressure level (SPL) intervals down from the maximum amplitude. The threshold was defined as the lowest stimulus level at which response peaks for waves I–V were clearly and repeatably present on visual inspection. Measurements were performed with the TDT BioSig III system (TDT, Alachua, FL, USA). Both ears were tested, because aminoglycosides occasionally produce unilateral, and not bilateral, hearing loss (Roeser et al., 2007).

5-Bromo-2-deoxyuridine administration

To monitor cell proliferation, the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) (50 mg/kg; Sigma-Aldrich) was intraperitoneally injected twice daily from day 2 to day 5 following treatment with kanamycin–furosemide (n = 7) or in control mice (n = 3). Control and treated mice were killed 24 h after the last BrdU injection.

Cochlear dissection

Control and treated adult mice were deeply anaesthetized (as above) and transcardially perfused with 500 mL of 4% paraformaldehyde in 0.2 m phosphate buffer (pH 7.4). The cochleae were isolated from the temporal bone, further perfused through the finestra ovalis, and post-fixed by immersion in the same fixative for 24 h. The dissected cochleae were incubated in 0.5 m EDTA solution for 6 days for bone decalcification. After being rinsed in PBS, decalcified cochleae were embedded in 3% agarose (Biorad, Segrate, Milan, Italy) and cut on a vibratome into 150-μm-thick sections (VP1000S; Leica, Wetzlar, Germany). Sections were collected in PBS and processed for immunohistochemistry (see below).

Cochlear epithelial cultures

Inner ear organ cultures were established from postnatal day (P)6 C57BL/6 mice. After removal of the cochlear bone and dissection out of the lateral wall in Hank's balanced salt solution supplemented with 10 mm Hepes buffer (pH 7.3) on ice, the sensory epithelia were transferred onto a sterile cell culture insert (pore size, 0.4 μm; BD Falcon; BD Biosciences Discovery Labware, Bedford, MA, USA) placed into a 12-well culture plate containing 1 mL of Dulbecco's modified Eagle's medium–F12 (1 : 1), supplemented with glutamine (2 mm), 1% N2, 1% B27, and 1 μg/mL ampicillin (all reagents from Gibco, Life Techologies, Monza, Italy). The slices were covered with 100 μL of medium, and then kept at 37 °C in 5% CO2 for 5 days.

Hair cell degeneration was induced by 24 h of exposure to kanamycin sulphate (2.5 mm; Sigma-Aldrich), added to the culture medium 1 day after the establishment of cultures. Growth factors [epidermal growth factor (EGF) 20 ng/mL plus basic fibroblast growth factor (bFGF) 50 ng/mL] were added after kanamycin removal, and the medium was incubated for 72 h. To monitor cell proliferation, after kanamycin treatment the explants were incubated for 24 h in basal medium supplemented with 10 μm BrdU. The cultures were fixed for 20 min in 2% paraformaldehyde in PBS (pH 7.4) and processed for histological analysis.

Immunohistochemistry

Immunohistochemistry was carried out with the following primary antibodies (see Table 1 for details): anti-nestin (1 : 250), anti-Sox2 (1 : 300), anti-p27 Kip 1 (1 : 200), anti-calbindin (1 : 1500), anti-BrdU (1 : 250), anti-Ki67 (1 : 1000), anti-myosin VIIa (1 : 400), and anti-neurofilament (NF) H (1 : 4000). Primary antibodies were dissolved in PBS with 1% bovine serum albumin and 0.25% Triton X-100. Incubation was carried out overnight at room temperature. The sections were then incubated with secondary antibodies: Alexa-488-conjugated or Alexa-546-conjugated anti-mouse, Alexa-488-conjugated anti-chicken, Alexa-488-conjugated or Alexa-555-conjugated anti-rabbit (1 : 500; Invitrogen, Paisley, UK), anti-rat fluorescein isothiocyanate (1 : 200; Vector Laboratories, Burlingame, CA, USA), anti-rat aminomethylcoumarin acetate (1 : 200; Jackson ImmunoResearch, Newmarket, UK), and anti-rat Cy3 (1 : 200; Jackson ImmunoResearch). Tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (1 : 500; Invitrogen) was used to visualize actin filaments, and 4’,6-diamidino-2-phenylindole (DAPI; Fluka, Milan, Italy) was used to visualize nuclei. For BrdU detection, the sections were treated with 2 m HCl for 30 min at 37 °C before incubation with the primary antibody. Positive controls for BrdU incorporation in the in vivo experiments were coronal sections of the subventricular zone. Negative controls were performed by omitting primary antibodies. After processing, sections were mounted on microscope slides with Tris–glycerol supplemented with 10% Mowiol (Calbiochem, La Jolla, CA, USA).

Table 1. Primary antibodies used in this study

Antibodies	Supplier and cat. no.	Species of origin	Immunogen	Purification	Controls
Anti-nestin, clone rat-401	Millipore MAB353	Mouse	Nestin purified from embryonic rat spinal cord	Protein A purified	Immunohistochemistry on mouse embryonic brain tissue (see data sheet)
Anti-p27 Kip 1	Abcam 7961	Rabbit	Synthetic peptide corresponding to C-terminal amino acids 181–198 of human p27 Kip 1	Not reported	Immunocytochemistry on HEK293 (see data sheet)
Anti-Sox2	Millipore AB5603	Rabbit	Synthetic peptide corresponding to human Sox2	Immunoaffinity purified	Western blotting on mouse or human embryonic stem cell lysate Immunocytochemistry on human embryonic stem cells (see data sheet)
Anti-calbindin D-28k	Swant CB38	Rabbit	Recombinant rat calbindin D-28k	Not reported	Western blotting on brain homogenate of various species (see data sheet)
Anti-BrdU	Abcam AB6326	Rat	Not available	Not reported	Immunohistochemistry on mouse brain tissue sections (see data sheet)
Anti-Ki67	Leica Novocastra NCL-Ki67p	Rabbit	Prokaryotic recombinant fusion protein corresponding to 1087-bp motif-containing cDNA fragment	Not reported	Immunohistochemistry on tonsil epithelium (see data sheet)
Anti-myosin VIIa	Proteus Biosciences 25-6790	Rabbit	Amino acids 880–1077 from the tail region of human myosin VIIa	Immunoaffinity purified	Western blotting and immunohistochemistry Hasson *et al*. (1995)
Anti-NF H	Millipore AB5539	Chicken	Purified bovine NF H	Ammonium sulphate precipitation	Immunohistochemistry on cultured neonatal rat forebrain cells (see data sheet)

All images were collected by means of a confocal Leica TCS SP5 microscope (Leica Microsystems) under a × 20 [numerical aperture (NA), 0.70] × 40 (NA, 1.25) or × 63 (NA, 1.40) objective lens. Confocal stacks spanned the entire thickness of the cochlear epithelium (slice separation, 1 µm). adobe photoshop 6.0 (Adobe Systems, San Jose, CA, USA) was used to adjust image contrast and assemble the final plates.

BrdU quantification

For quantification of BrdU-positive cells, we selected four 5000-µm2 areas within the inner spiral sulcus (ISS) and the outer spiral sulcus (OSS) in the different cochlear turns. The BrdU labelling density was expressed as number of BrdU-positive nuclei per 0.01 mm2.

Real-time reverse transcription polymerase chain reaction

For quantitative analysis of nestin mRNA expression, total RNA was isolated from cochlear epithelial cultures (RNeasy Micro Kit; Qiagen, Milan, Italy) and used to synthesize first-strand cDNA (High-Capacity cDNA Archive Kit; Applied Biosystems, Life Technologies), according to the recommended procedure. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed with SYBR Green Master Mix (QuantiTect SYBR Green PCR Kit; Qiagen) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), with the following oligonucleotide primers: c-Nest (forward), AGCAACTGGCACACCTCAA; and c-Nest (reverse), CCAAGAGAAGCCTGGGAACT. Blank controls, consisting of no template (water) or reverse transcription-negative reactions, were performed for each run. Data extracted from each real-time RT-PCR run were analysed with 7000 v1.1 sds instrument software (Applied Biosystems), which gives a threshold cycle number (CT). For the relative quantification, CT data were normalized to the endogenous β-actin housekeeping gene, and then used to calculate fold changes in gene expression relative to untreated conditions, according to the 2−ddCT method (Livak & Schmittgen, 2001).

Morphometric analysis of NF-positive neurites

Morphometric analysis of NF-immunolabelled neurites was carried out with neurolucida software (MicroBrightField, Williston, VT, USA). For each condition, we selected four slices and superimposed an unbiased counting frame with 20 × 20-µm squares. We counted the density (number/0.01 mm2) of NF-positive fibres crossing the grid under a × 40 objective. For each condition, we averaged the crossing density values.

Data analysis

Data were analysed with graphpad prism 6.00 (GraphPad Software, La Jolla, CA, USA).We used Student's t-test for comparisons between two groups, or one-way anova followed by Tukey's multiple comparison for multiple group comparisons. All values are represented by means ± standard errors of the mean, and a P-value of < 0.05 was considered to be significant.

Results

Histological features of the ototoxic injury in adult mice

In uninjured adult mice, phalloidin and calbindin staining of the inner ear sensory epithelium revealed the characteristic cytoarchitecture of the organ of Corti, organized in three rows of outer hair cells (OHCs) and one row of inner hair cells (IHCs) (Fig. 1A and B). In contrast, administration of kanamycin–furosemide induced an almost complete loss of OHCs, as previously described for ototoxic antibiotic treatment (Prieve

& Yanz, 1984; Taylor et al., 2008, 2012). In particular, OHCs were generally preserved at the apex (Fig. 1E), partially lost in the middle turn (Fig. 1F), and totally absent from the basal turn (Fig. 1C, D and G). Moreover, in the lower turns, the lamina reticularis was also severely altered, and showed a process of tissue reorganization, highlighted by the phalloidin labelling of the junctional actin bands between Deiters' cells (Fig. 1C and G). IHCs were generally less affected by the treatment (Fig. 1C and D). Morphological alterations were correlated with hearing functional deficit, as shown by the ABR threshold, which was consistently higher in treated mice than in control mice (> 80 dB SPL vs. 30–50 dB SPL; Table 2).

Table 2. ABR thresholds of the experimental animals

Condition	**Animal**	**ABR threshold (dB SPL)**
	1	50
	2	40
	3	40
Untreated	4	40
	5	50
	6	40
	7	30
	1	80
	2	> 100
	3	> 100
Treated	4	> 100
	5	> 100
	6	> 100
	7	80

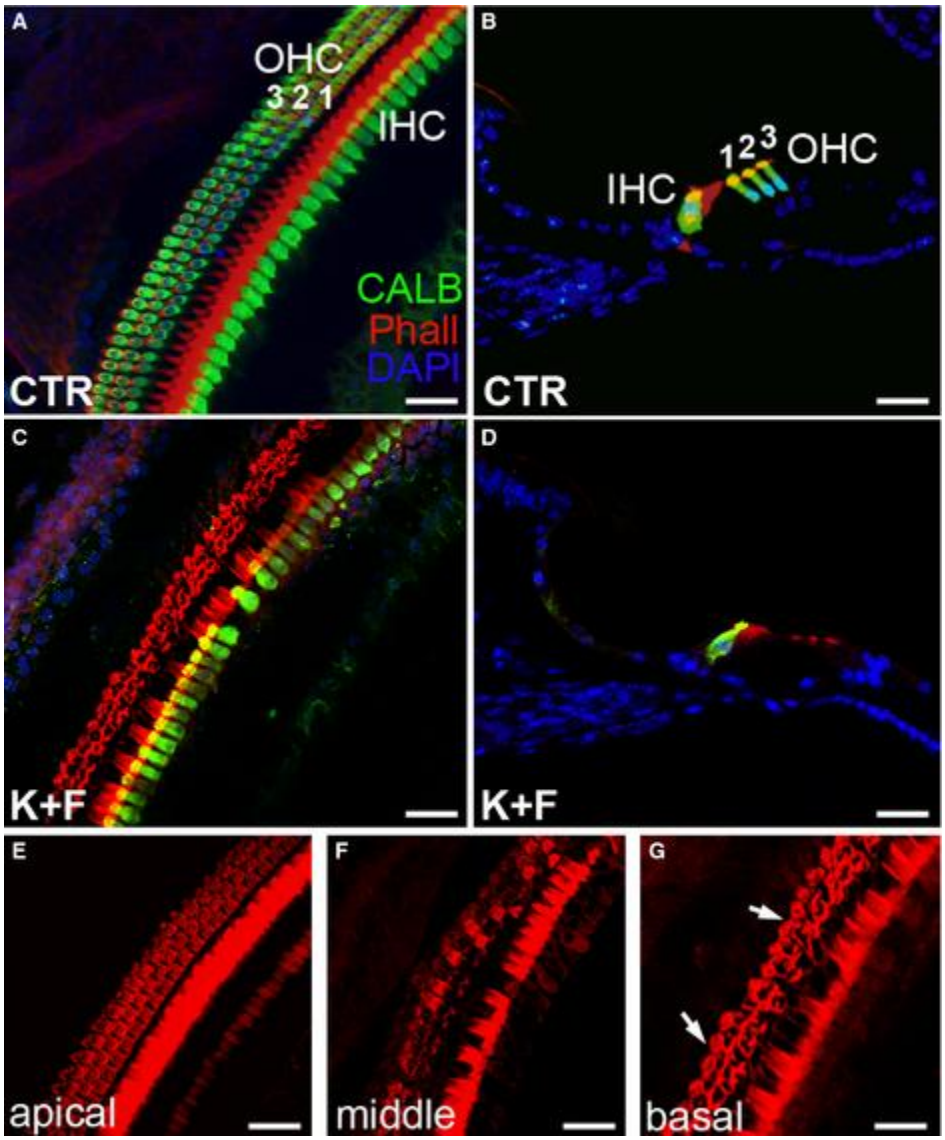

Figure 1.

Morphological alterations induced in vivo by kanamycin–furosemide treatment in the adult mouse cochlear sensory epithelium. (A and B) Confocal images of horizontal (A) and vertical (B) sections of the mid-basal turn from an undamaged cochlea. Intact OHCs (three rows, 1–3) and IHC (one row), labelled with calbindin (green), are visible. The heads of the inner and outer pillar cells, between the IHC and the OHCs, were heavily stained with phalloidin (red). (C and D) Images of horizontal (C) and vertical (D) sections of the mid-basal turn of the cochlea after kanamycin–furosemide treatment show that OHCs are missing, whereas IHCs are partly preserved. Calbindin (green) was used to label hair cells, TRITC–phalloidin (red) to label actin filaments, and DAPI (blue) to counterstain nuclei (A–D). (E–G) Phalloidin labelling of the reticular lamina of a damaged cochlea shows the variable process of hair cell degeneration through the different cochlear turns. The apical turn of the cochlea shows a normal organization of the organ of Corti (E). At the medial turn, the integrity of the lamina reticularis is variably affected, and only a few OHCs remain (F). In the more basal turns, OHCs are replaced by scars, composed of junctional actin bands between Deiters' cells (G). Scale bars: 30 µm. CALB, calbindin; CTR, control; K+F, kanamycin–furosemide; Phall, phalloidin.

Nestin is not upregulated in the organ of Corti of adult mice after ototoxic injury

To investigate whether the tissue response following the ototoxic damage could lead to activation of progenitor cells in the adult cochlea, we compared the expression pattern of nestin in treated and control mice. In all untreated mice, nestin immunoreactivity was evident in some cells located in the spiral ganglion and in the stria vascularis, as well as in fibroblasts of the spiral ligament and basilar membrane [see also Coppens et al. (2003)]. No nestin-positive cells were apparent in the organ of Corti (Fig. 2A). The labelling in the tectorial membrane reflected unspecific staining, as shown by a negative control with omission of the primary antibody (Fig. S1). This pattern of expression was not substantially altered by kanamycin–furosemide treatment (Fig. 2B and C). Nonetheless, in three of 14 mice, nestin-positive filaments appeared at the more apical turns in some cells of the OSS as well as in the inner border cells that surround the IHCs in the ISS (Fig. 2C–D). Nestin-positive cells in the OSS were supporting cells, as shown by the expression of p27 (Fig. 2D and D'), an inhibitor of cell cycle progression that is expressed at high levels in differentiated supporting cells of the mature sensory epithelium, including Deiters', Hensen's, Claudius' and pillar cells (Chen & Segil, 1999). However, when we carried out a BrdU proliferation assay, we found that nestin-positive cells in the OSS and ISS did not incorporate the nucleotide analogue, suggesting that the nestin increase in these areas after injury is not associated with cell proliferation (Fig. 3A). In general, BrdU-positive cells, which were not detected throughout the entire cochlea in untreated mice, were also very rare in the injured cochleae. Namely, in six of 14 treated mice, sparse BrdU-positive nuclei were apparent in the stria vascularis (1.5 ± 0.42 nuclei/0.01 mm2), in the spiral ligament (0.9 ± 0.39 nuclei/0.01 mm2), and in the OSS (1.27 ± 0.31 nuclei/0.01 mm2; Fig. 3B and C). The remaining eight mice showed occasional immunopositive nuclei also in the organ of Corti (0.59 ± 0.17 nuclei/0.01 mm2; Fig. 3B and C), in line with previous observations concerning the effects of kanamycin injury in mature guinea pigs (Yamasoba & Kondo, 2006). BrdU/nestin-double-labelled cells were present only in the area of the stria vascularis (Fig. 3D). Sections of brain used as positive controls showed numerous BrdU-positive nuclei in the SVZ along the lateral ventricle area (Fig. S2).

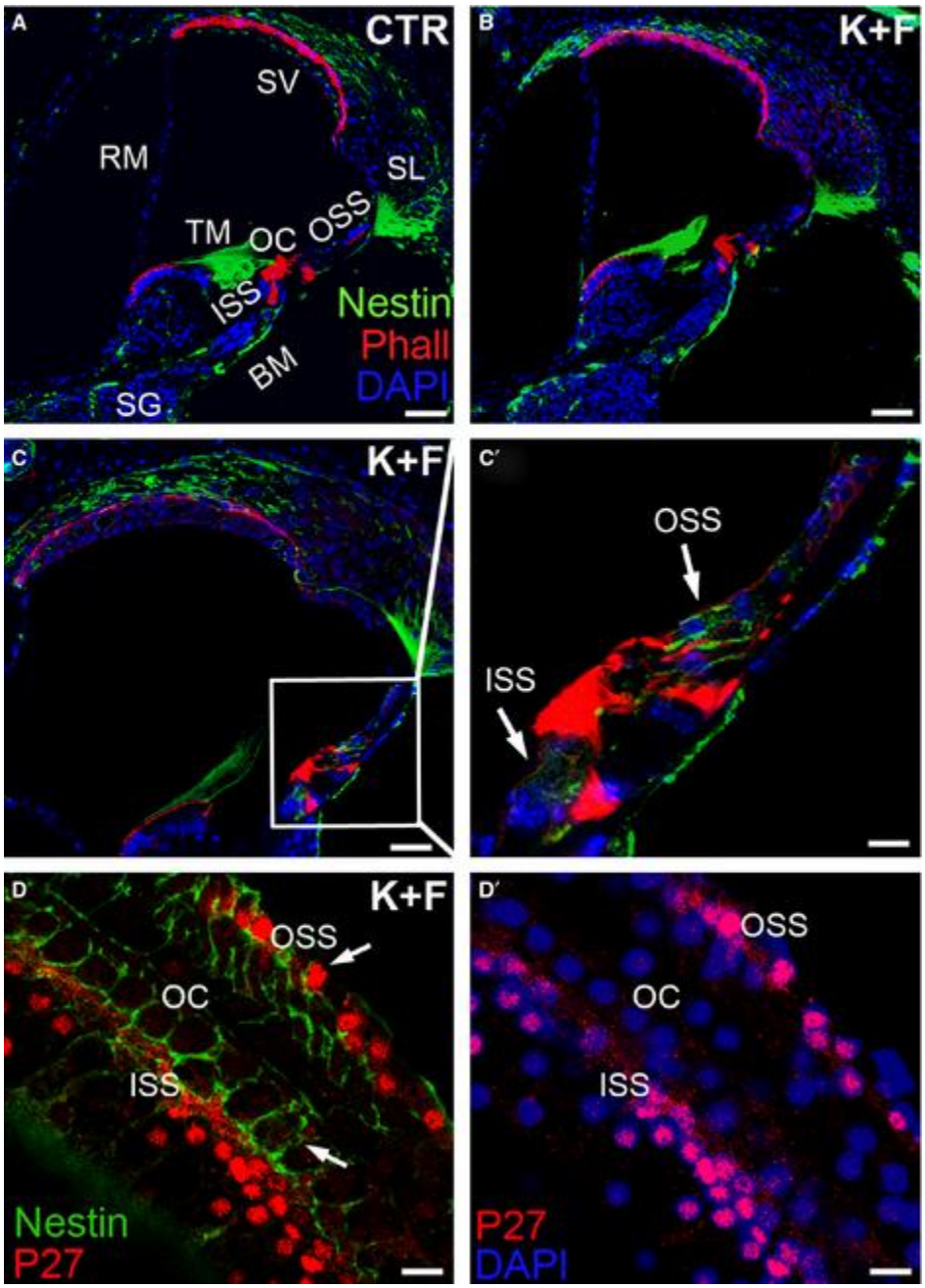

Figure 2.

Effect of kanamycin–furosemide treament on nestin expression in the adult cochlea. (A) Image of a cochlear cross-section from an untreated mouse showing expression of nestin (green) in the spiral ganglion (SG), the stria vascularis (SV), fibroblasts of the spiral ligament (SL), and the basilar membrane (BM). Staining of the tectorial membrane (TM) reflects non-specific binding of the secondary antibodies (Fig. S1). (B) The pattern of nestin expression is not altered by kanamycin–furosemide treatment. (C–D′) In a few mice (three of 14), nestin filaments are also apparent in the OSS and inner hair border cells in the ISS of the apical turn (C, C′, and D, arrows). (D and D′) are images of horizontal sections, in which nestin-positive cells (green) in the OSS show expression of p27 in their nuclei (red; D). Blue indicates DAPI (D′). Scale bars: 70 µm in A–C; 20 µm in C′, D, and D′. CTR, control; K+F, kanamycin–furosemide; OC, organ of Corti; Phall, phalloidin; RM, Reissner membrane.

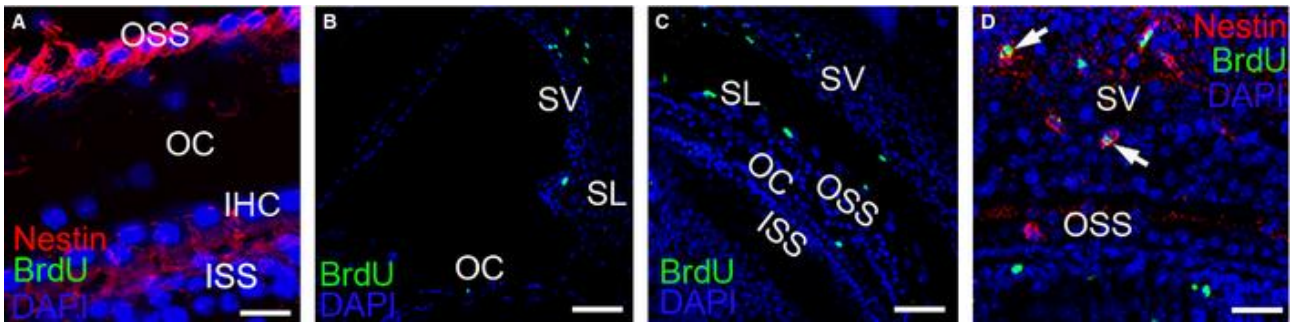

Figure 3.

Proliferative response in the kanamycin–furosemide-injured adult cochlea. (A) Nestin-positive cells (red) in the OSS and ISS of the damaged cochlea do not show BrdU staining (green). A section from the apical turn is shown. (B and C) Images of a vertical (B) and a horizontal (C) section showing the presence of a few BrdU-positive nuclei (green) throughout the damaged cochlea, i.e. in the stria vascularis (SV), the spiral ligament (SL), the OSS, and, occasionally, the organ of Corti (OC). (D) Some BrdU-positive cells in the SV express nestin (arrows). Scale bars: 20 µm in (A); 100 µm in (B and C); 50 µm in (D).

Reactive phenomena in postnatal cochlear cultures after kanamycin ototoxicity and growth factor application

Although upregulation of nestin in the adult organ of Corti after kanamycin ototoxicity was evident in only a few cases and was not related to proliferative activity, previous reports have shown that cells of the organ of Corti from the postnatal mouse cochlea, dissociated and cultured in vitro in the presence of EGF and bFGF, produce otospheres, express nestin, and retain properties of developmental inner ear progenitor cells (Savary et al., 2007; Oiticica et al., 2010). This ability to form spheres decreases ~100-fold after P7 (Oshima et al., 2007). Therefore, we sought to examine whether resident cochlear progenitors in situ are able to respond to injury during postnatal development. Because, in postnatal mice or rats, hair cells are generally resistant to aminoglycosides, and typically require very high doses before any toxicity is evident, causing a high mortality rate (Marot et al., 1980; Chen & Saunders, 1983), we shifted to organotypic cochlear explants. In this model, we also investigated whether the application of EGF and bFGF could stimulate their reactivity. We employed organotypic cultures of cochleae isolated from P6 mice and examined after 5 days in vitro. At P6, the development of the sensory structures is nearly complete (Anniko, 1983; Bulankina & Moser, 2012), but progenitor cells are still present, making it possible to investigate their behaviour following injury and the application of growth factors.

Untreated cultures showed the distinctive morphological features of the cochlea in situ, with the typical arrangement of IHCs and OHCs (Fig. 4A and E). This organization was consistent throughout the cochlear turns, and it was not overtly modified following the addition of growth factors that are known to mitogenically or trophically stimulate inner ear progenitors in otospheres (EGF and bFGF), except for a slight enlargement of the tunnel of Corti, the ISS, and the OSS (Fig. 4B and F). This expansion was probably caused by the outgrowth of fibrocytes from the tissue underlying the organ of Corti in response to growth factors.

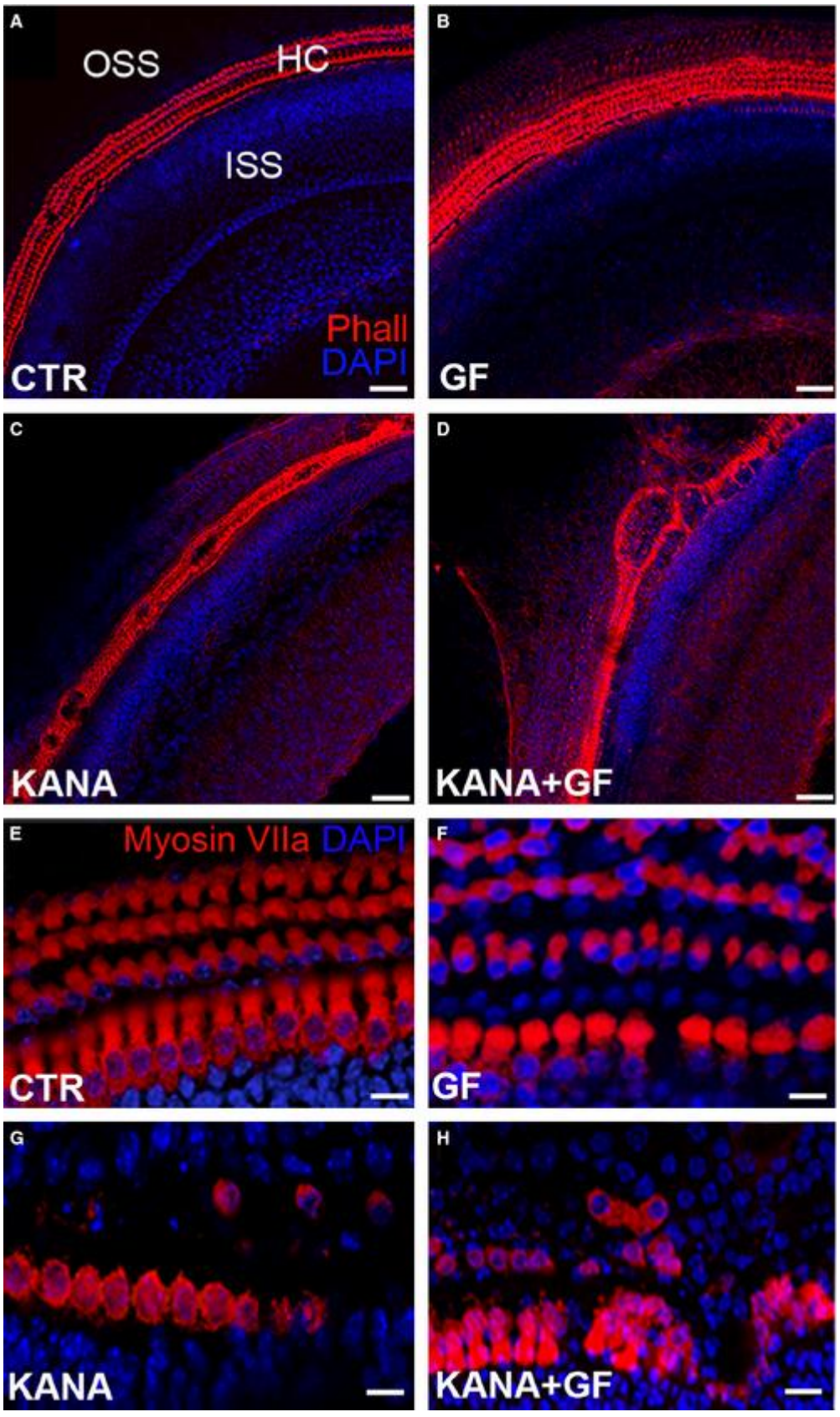

Figure 4.

Effects of kanamycin and growth factors on organotypic cultures of cochlea. Untreated control explants show the typical cytoarchitecture of the organ of Corti, with three rows of OHCs and one row of IHCs, as shown by phalloidin (red, A) or myosin VIIa (red, E) labelling. Addition of EFG and bFGF to untreated

explants causes a slight expansion of the organ of Corti, the ISS, and the OSS (B and F). After kanamycin application, the sensory epithelium is variably disarrayed (C) and most hair cells are missing, as shown by myosin VIIa staining (G). Addition of growth factors to kanamycin-treated cultures induces significant changes in the middle turn of the sensory epithelium, namely an expansion of the OSS and visible enlargements of the lamina reticularis, stained by phalloidin (D). Myosin VIIa labelling shows that most hair cells are lost (H). Scale bars: 50 μm in (A–D); 25 μm in (E–H). CTR, control; GF, growth factor; HC, hair cell; KANA, kanamycin; Phall, phalloidin.

Following kanamycin treatment, most hair cells were lost or showed signs of ongoing degeneration (Fig. 4C and G). Notably, in the basal turn, the architecture of the sensory epithelium was severely disrupted, with complete loss of IHCs and OHCs and the appearance of a flat epithelium (Fig. S3A). In the middle turn, the lamina reticularis also showed profound alterations. OHCs were always missing, whereas some IHCs were preserved (Fig. 4C and G). Inner pillar cells were still recognizable. Actin filaments accumulated strongly at the junctions between Hensen's cells and the last row of Deiters' cells (Fig. S3B). In the apical turn, the damage was less severe, and the supporting cell layer was more preserved (Fig. S3C). Addition of growth factors to the kanamycin-treated cultures induced some significant changes in the middle turn of the sensory epithelium (Figs 4D and H, and S3D), while not overtly affecting the other turns. At the level of the organ of Corti, in the upper part of the middle turn, the lamina reticularis showed some enlargements resulting from the combination of concomitant degenerative phenomena and reactive processes. In correspondence with those expansions, the OSS region was extended (Fig. 4D). Such enlargements were composed of degenerating supporting cells and other unidentified elements, probably coming from the underlying basilar membrane. Strong actin filament accumulations were also detected, which could have derived from ISS cells migrating towards the OSS, or from Hensen's and Claudius' cells spreading towards inner pillar cells (Fig. S3D).

Nestin expression in organotypic cultures of the cochlea

To investigate whether the tissue response to injury and/or growth factor application is associated with activation of progenitor cells, we analysed nestin espression in the cochlear cultures. In uninjured cultures, nestin immunofluorescence was localized in the OSS of the upper turns (Fig. 5A) and in the basilar membrane. Nestin-positive cells located in the OSS at the border with the organ of Corti were supporting cells (Hensen's/Claudius' cells), as shown by their expression of p27 (Fig. 5A’).

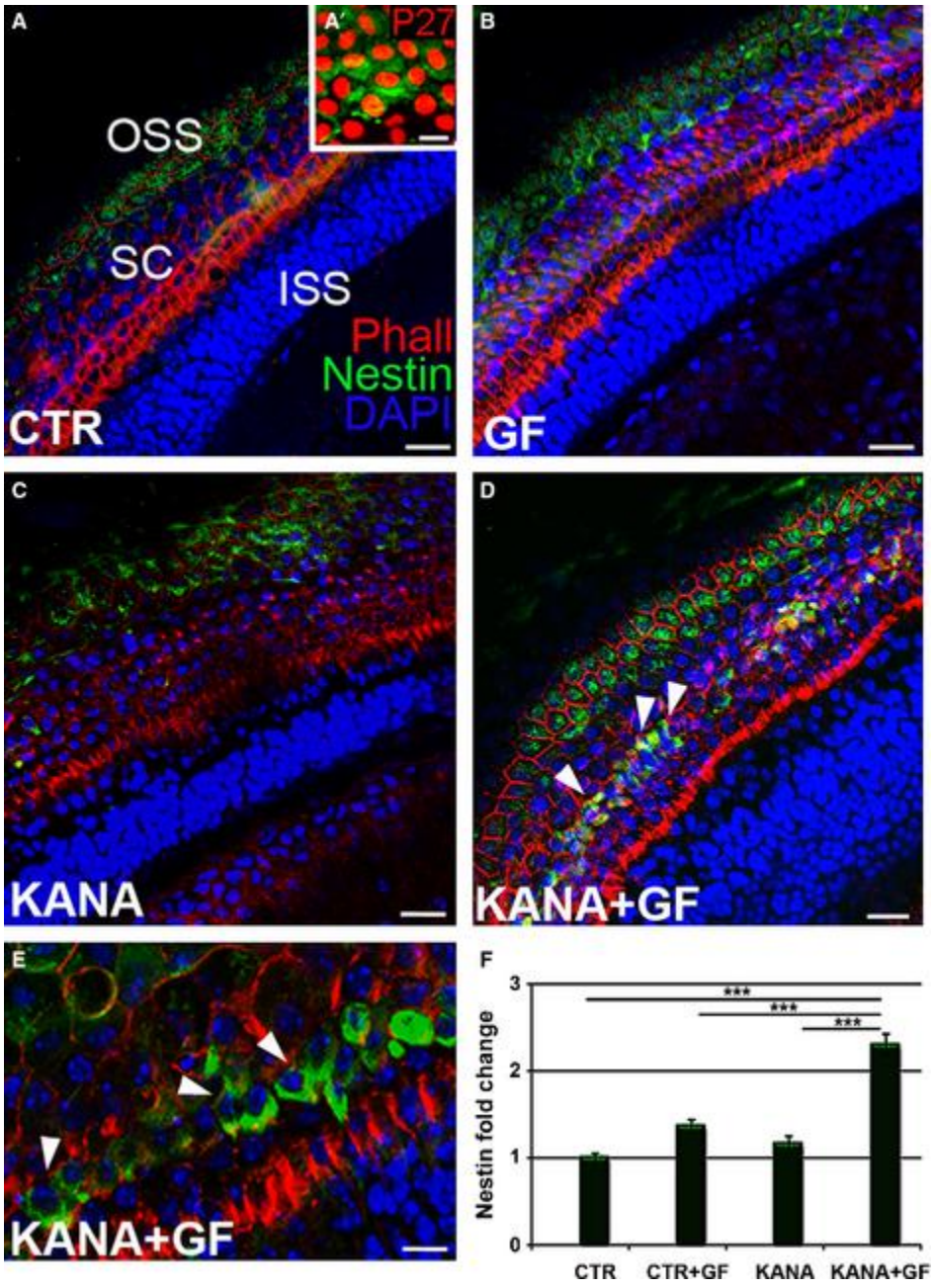

Figure 5.

Nestin expression in cochlear organotypic cultures. (A–E) Confocal images of mid-apical turns of cultured cochleae in different conditions taken at the supporting cell level. (A) In control cultures, nestin (green) is absent in the area of the organ of Corti, whereas it is present in the OSS, specifically in p27-positive supporting cells (A'). The addition of growth factors does not change the pattern of nestin expression observed in untreated cultures (B). In kanamycin-treated cultures, nestin expression remains confined to the OSS (C). In kanamycin-treated cultures stimulated with growth factors, nestin immunoreactivity is present in the OSS and in some cells localized in the supporting cell area of the organ of Corti (arrowheads in D and E). (F) Expression levels of nestin mRNA in cochlear cultures in the different conditions. In all images, nestin is labelled in green, actin with TRITC-conjugated phalloidin (red), and nuclei with DAPI (blue). ***P < 0.001. Scale bars: 30 µm in (A–D); 15 µm in (A' and E). CTR, control; GF, growth factor; KANA, kanamycin; Phall, phalloidin; SC, supporting cell.

The distribution of nestin was not modified after the addition of growth factors (Fig. 5B). Indeed, quantitative analysis of nestin mRNA did not reveal any significant change in the growth factor-treated cultures as compared with the untreated ones (Fig. 5F; $F_{3,12} = 39.22$; one-way anova, $P > 0.05$).

In the kanamycin-treated cultures, nestin expression remained confined to the upper turns, but the area covered by immunoreactive cells was slightly expanded, probably because of the tissue rearrangement in response to damage (Fig. 5C). RT-PCR did not reveal significant differences in the expression of nestin mRNA as compared with uninjured cultures (Fig. 5F; one-way anova, $P > 0.05$).

However, when growth factors were added to kanamycin-treated cultures, the pattern of nestin distribution changed. Nestin immunoreactivity was still present in supporting cells of the OSS, but additional nestin-positive cells appeared in the organ of Corti (Fig. 5D and E). The characterization of these cells with specific markers revealed that they were supporting cells (i.e. Deiters' cells), as demonstrated by their expression of p27 (Fig. 6A and A') and Sox2 (Fig. 6B and B'), a transcription factor expressed in supporting cells of the postnatal and adult mouse organ of Corti, including after kanamycin-induced damage (Hume et al., 2007; Oesterle et al., 2008). In line with these morphological observations, the injured cultures treated with growth factors showed a conspicuous increase in the content of nestin mRNA as compared with uninjured cultures (Fig. 5F; 2.3-fold ± 0.09-fold; one-way anova, $P < 0.001$).

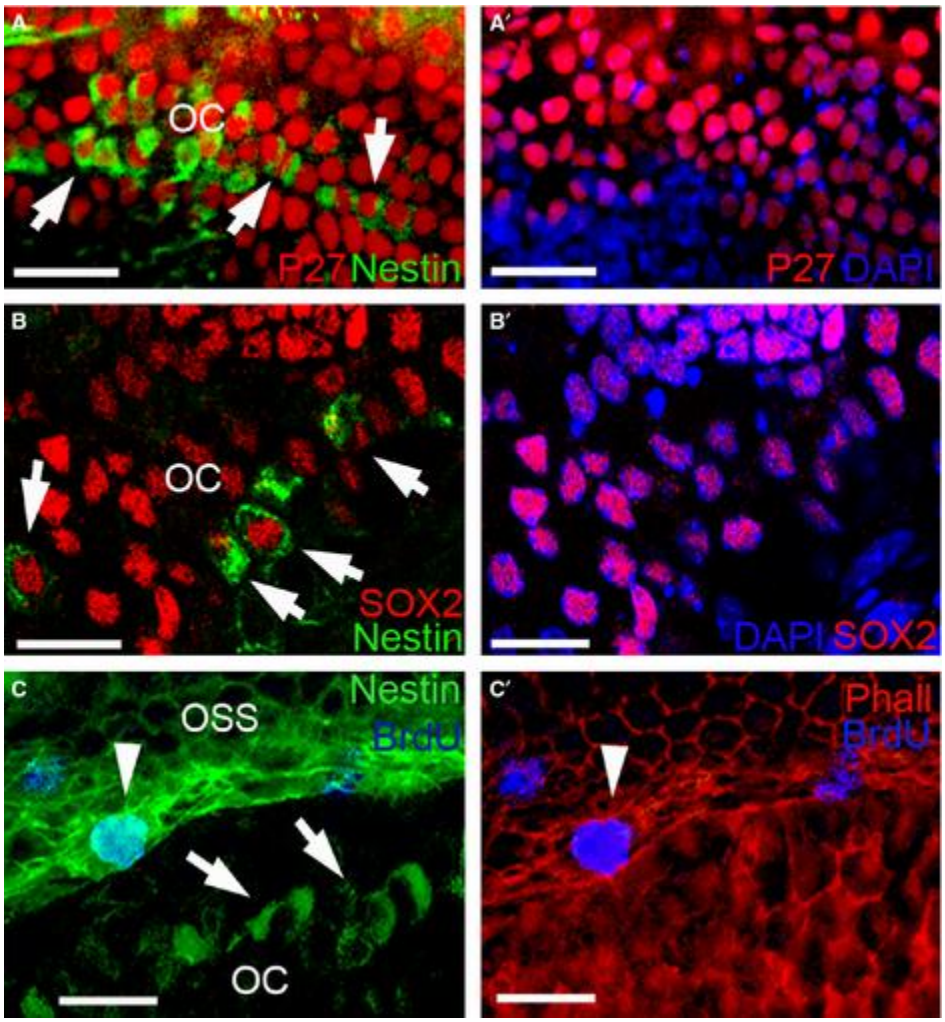

Figure 6.

Identity of nestin-positive cells in injured explants after growth factor application. Cells immunoreactive for nestin in the organ of Corti of injured explants after growth factor application are supporting cells, as revealed by the expression of p27 (A and A', arrows) and Sox2 (B and B', arrows) in their nuclei (counterstained with DAPI). C and C' show that nestin-positive cells in the organ of Corti do not incorporate BrdU (arrows in C), whereas a few cells in the OSS show BrdU incorporation (arrowheads in C and C'). Scale bars: 40 µm in (A and A'); 30 µm in (B–C'). OC, organ of Corti; Phall, phalloidin.

To elucidate whether nestin-positive cells were endowed with proliferative potential, we assessed BrdU incorporation. In the organ of Corti of the injured cultures treated with growth factors, no nestin-positive supporting cells incorporated BrdU, and only rare nestin-positive cells in the OSS region showed BrdU labelling (Fig. 6C and C'). In general, the distribution of BrdU-incorporating cells, regardless of whether they expressed nestin or not, varied according to the experimental condition. The uninjured cultures showed rare BrdU-labelled cells scattered mainly throughout the OSS (ISS, 0.31 ± 0.15 nuclei/0.01 mm2; OSS, 0.94 ± 0.25 nuclei/0.01 mm2; t-test, $P < 0.05$; Fig. 7A and E). Addition of growth factors stimulated the proliferation of supporting cells located in the OSS (ISS, 1.31 ± 0.40 nuclei/0.01 mm2; OSS, 3.06 ± 0.51 nuclei/0.01 mm2; one-way anova for OSS, $F_{3,58} = 59.98$, $P < 0.05$; Fig. 7B and E). On the other hand, whereas kanamycin treatment induced only a slight increase in the number of BrdU-labelled nuclei in the ISS as compared with the basal condition (ISS, 1.94 ± 0.40; OSS, 1.56 ± 0.36; one-way anova for ISS, $F_{3,55} = 59.45$, $P < 0.05$; Fig. 7C and E), the addition of growth factors to the damaged cultures dramatically increased cell proliferation in both the ISS and OSS (ISS, 7.35 ± 0.60 nuclei/0.01 mm2; OSS, 8.79 ± 0.62 nuclei/0.01 mm2; one-way anova, $P < 0.001$; Fig. 7D and E). The proliferative activities observed in the ISS and OSS in the different experimental conditions were confirmed by staining for Ki67 (Fig. 7F–M), a nuclear protein expressed by proliferating cells.

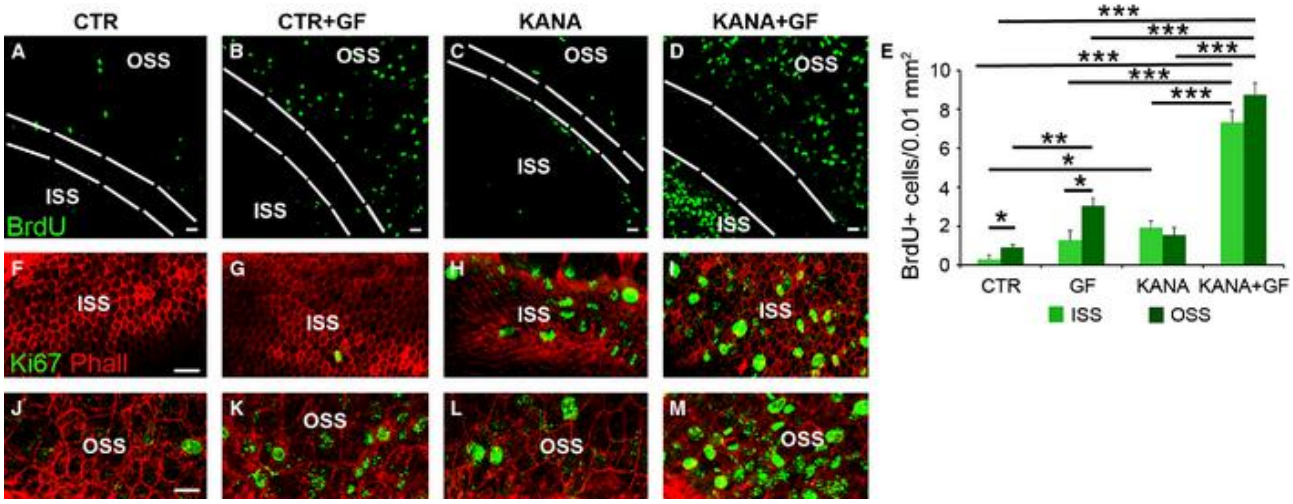

Figure 7.

Cell proliferation in organotypic cultures of cochleae. BrdU staining shows that rare BrdU-positive nuclei are present in control cultures (A). The addition of growth factors increases the number of BrdU-positive nuclei in the OSS of intact cultures (B). In kanamycin-treated cultures, a slight increase in the number of BrdU-positive cells is observed in the ISS (C). The addition of growth factors to injured cultures induces a strong increase in the number of BrdU-positive cells in both the OSS and the ISS (D). (E) shows quantification of the density of BrdU-labelled cells in the different conditions. (F–I) and (J–M) show the presence of Ki67-positive cells in the ISS (F–I) and the OSS (J–M) in the four experimental conditions. Note the increased number of Ki67-positive nuclei in the ISS after kanamycin treatment (H) and growth factor administration

to injured cultures (I) as compared with the control condition (F) and the growth factor-treated condition (G). An increased number of Ki67-positive nuclei is also apparent in the OSS after growth factor application to intact (K) or injured (M) cultures. *P < 0.05, **P < 0.01, ***P < 0.001. Dashed lines denote the region of the organ of Corti in (A–D), in which all images are projections of sections through an 18-μm depth of the cochlea. Scale bars: 20 μm in A–D and J (also applies to K–M); 30 μm in F (also applies to G–I). CTR, control; GF, growth factor; KANA, kanamycin; Phall, phalloidin.

Addition of growth factors to the injured cochlea induces profuse axonal sprouting

As application of neurotrophins to the damaged cochlea has been previously shown to induce sprouting of peripheral sensory axons (Wise et al., 2005), we also investigated whether, in our model, tissue reaction after growth factor application would induce sprouting of peripheral acoustic nerve axons towards the damaged areas. Morphometric analysis of NF-labelled profiles showed that, in the uninjured cultures, addition of growth factors induced clear increases in the number and length of NF-positive neurites (control, 2.43 ± 0.23 grid crossings/0.01 mm2, n = 3; growth factor-treated, 3.83 ± 0.11 grid crossings/0.01 mm2, n = 3; one-way anova, $F_{3,12} = 44.80$, P < 0.01; Fig. 8A, B and E). In the kanamycin-treated cultures, there was an apparent reduction in the number of labelled axons (1.82 ± 0.14 grid crossings/0.01 mm2, n = 3; Fig. 8C and E), which just failed to reach statistical significance as compared with intact cultures (one-way anova, P = 0.07). Most interestingly, however, when ototoxic injury was combined with growth factor administration, there were dramatic increases in the number and extension of NF-positive axons, which were particularly numerous in the enlarged portions of the lamina reticularis (5.04 ± 0.31 grid crossings/0.01 mm2, n = 3; Fig. 8D and E). Statistical comparisons confirmed that the number of axons observed in kanamycin/growth factor-treated cochleae was significantly higher than the number observed in all of the other conditions (one-way anova: vs. intact cultures, P < 0.001; vs. growth factor-treated intact cultures, P < 0.01; vs. kanamycin-treated cultures, P < 0.001).

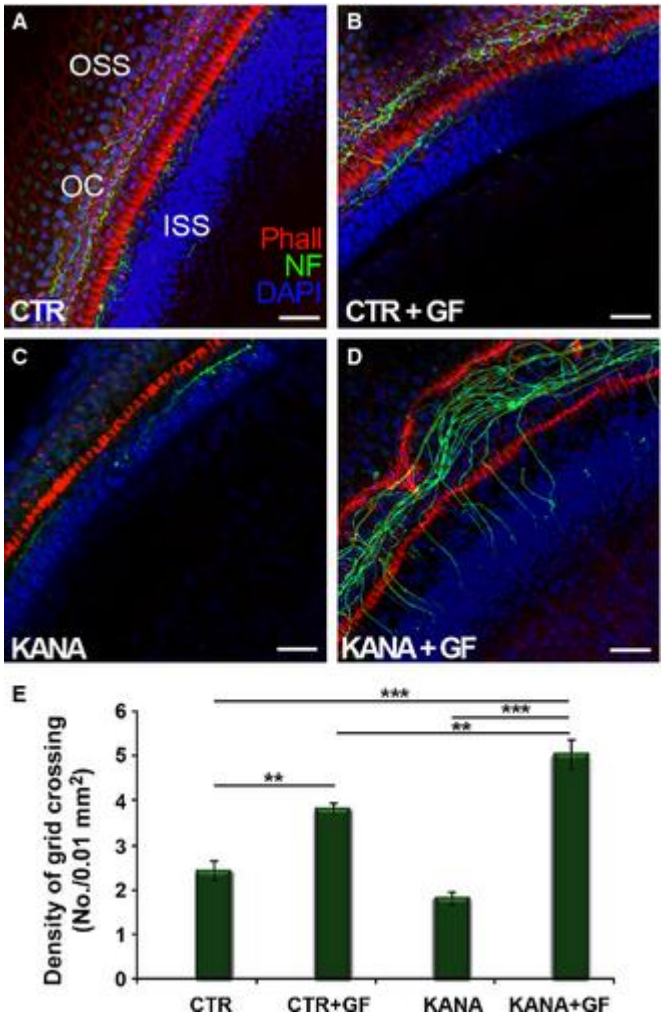

Figure 8.

Effects of growth factors on NF distribution in damaged organotypic cultures. (A) NF immunoreactivity (green) in control cultures, which is evident in acoustic nerve processes in the ISS and the organ of Corti. (B) The addition of growth factors causes a slight but significant increase in the number of NF-positive fibres, both in the ISS and in the organ of Corti. (C and D) Following kanamycin treatment, the number of NF-positive fibres is reduced (C), whereas growth factor treatment of injured cultures induces profuse sprouting, which is particularly abundant in the lamina reticularis enlargements (D). (E) Quantification of the density of NF-positive axons in the different conditions. In all images, red is TRITC–phalloidin, green is anti–NF, and blue is DAPI. All images were projections of sections spanning the region below the hair cells. Scale bars: 50 μm. **P < 0.01, ***P < 0.001. CTR, control; GF, growth factor; KANA, kanamycin; OC, organ of Corti; Phall, phalloidin.

Discussion

Therapeutic strategies to treat cochlear damage are focused on the use of cells that can be induced to differentiate and generate new hair cells, or harvested and used for transplantation approaches. However, both in vivo and ex vivo strategies require procedures and criteria to unequivocally identify cells with stem or progenitor properties. In this context, several studies performed on dissociated inner ear cells and ex vivo analyses have demonstrated a correlation between nestin expression and the initiation of neurosphere formation, suggesting that this protein could be a distinctive marker for cells that are able to differentiate into sensory hair cells (Malgrange et al., 2002; Li et al., 2003; Chen et al., 2011). Nevertheless, the

specificity of nestin as a marker for stem cells is still debated, and, to our knowledge, there is no evidence for the involvement of nestin-expressing cells in the repair of the inner ear epithelium in situ after kanamycin-induced damage. Only one recent study has reported an increase in nestin expression after traumatic noise exposure (Watanabe et al., 2012). Therefore, we examined two different experimental models (adult mice in vivo and postnatal cochlear explants) to assess whether a population of progenitor cells, which may be distinguished by expression of nestin, resides in the cochlea and is able to respond to ototoxic damage.

Our results show that exposure to kanamycin induces a process of hair cell degeneration, with analogous features in the adult and the postnatal cochlea. After drug treatment, the loss of hair cells followed a typical progression along the organ of Corti, reflecting the differential sensitivity to kanamycin toxicity of receptor cells located at different positions along the cochlea. The injury procedure also triggered a significant tissue reaction, leading to disruption of the organ of Corti cytoarchitecture [see also Taylor et al. (2008, 2012), and Oesterle (2013)]. These changes, however, were not accompanied by any significant upregulation of nestin or proliferation of nestin-positive cells.

Nestin expression in the adult cochlea

In line with previous reports (Kojima et al., 2004; Smeti et al., 2011; Taniguchi et al., 2012), our present analysis of uninjured cochleae in vivo shows that nestin is not expressed in the sensory epithelia of the organ of Corti. After ototoxic injury, a slight increase in nestin filaments occurs in some animals in the adjacent areas, but not within the damaged sensory epithelium. Furthermore, BrdU incorporation experiments showed that these cells were not dividing. It is known that intermediate filament proteins, such as glial fibrillary acidic protein, vimentin, and nestin, are developmentally regulated and may be re-expressed in astrocytic scars following CNS injuries (Frisén et al., 1995; Pekny et al., 1999; Pekny & Lane, 2007). Similar regulation of nestin expression during inner ear development has also been described (Lopez et al., 2004; Smeti et al., 2011). Therefore, following ototoxic injury, the appearance of nestin filaments in non-dividing cells adjacent to the adult organ of Corti is suggestive of a tissue reaction to injury rather than activation of resident progenitors attempting to replace the lost receptors.

Nestin expression in the postnatal cochlea

In spite of the low expression of nestin in the adult organ of Corti, several studies have described the presence of nestin cells responding to growth factor signals in otospheres deriving from enzymatic cell dissociation. Namely, cells isolated from postnatal mouse cochleae and maintained in vitro in the presence of EGF and bFGF produce otospheres that retain the properties of inner ear progenitor cells (Wang et al., 2006; Savary et al., 2007; Oiticica et al., 2010). Starting from this evidence, we chose to investigate the effects of ototoxic injury on P6 cochlear explants, namely when the development of the sensory structures is nearly complete (Anniko, 1983; Bulankina & Moser, 2012), but progenitor cells are still present (Oshima et al., 2007). Cochlear explants largely preserve the original tissue architecture, allowing the precise localization of progenitors and the analysis of their behaviour following injury. In addition, in this condition we could also test whether the endogenous regenerative properties of cochlear cells can be boosted by the addition of growth factors used to produce otospheres (EGF and bFGF).

In the uninjured cultures, nestin was expressed by some supporting cells located in the apical region of the OSS, but not in the organ of Corti. This observation is consistent with the young age of the cochleae at the time of dissection, as well as with the base-to-apex progression of inner ear maturation (Ruben, 1967; Montcouquiol & Kelley, 2003; Kelley et al., 2009).

Neither ototoxic damage nor the addition of growth factors alone induced any overt change in the expression of nestin mRNA and protein. In contrast, the applied substances strongly enhanced nestin transcription in the injured cultures, where nestin immunolabelling also appeared in some supporting cells of the organ of Corti, particularly in the apical turns. Therefore, the in vitro experiments show that damaged cochlear tissues are sensitive to exogenous application of growth factors. The unequal response of supporting cells to growth factors according to their position along the cochlear turns can be related to the different degrees of maturation, but also to the extent of damage, as the latter factors appear to be less efficient when applied to a severely disrupted tissue. Indeed, in the apical turns of the kanamycin-treated cultures, the damage was less severe and the supporting cell layer was more preserved.

Significant upregulation of nestin required the concurrent effects of tissue damage and growth factors. It is plausible that, in the cochlear regions undergoing moderate tissue disruption, kanamycin toxicity disrupts the cell-to-cell interactions needed to maintain inner ear cells in a differentiated state. Indeed, the impairment of Notch pathways following the loss of cell-to-cell contacts can induce nestin expression (Shih & Holland, 2006). In this context, the injury-induced loss of local regulatory mechanisms, together with the stimulation of growth factors, may induce nestin upregulation as sign of dedifferentiation. Also in this case, however, these nestin-expressing cells did not show any proliferative behaviour. Therefore, either these cells were attempting to replace the lost hair cells without dividing, or they were actually engaged in a process of tissue scarring.

Cell proliferation and sprouting response in the postnatal cochlea

Our data on cell proliferation show that ISS cells appear to be more sensitive to damage-induced signals than OSS cells, whereas growth factors preferentially stimulate OSS cells. The different proliferative abilities of ISS and OSS cells [see also Sinkkonen et al. (2011)] may depend on different expression patterns of genes regulating hair cell differentiation (Zhai et al., 2005; Zhang et al., 2007). Moreover, ototoxic injury alone did not stimulate neuritic growth; rather, it induced some retraction, which was probably related to target loss or direct damage to spiral ganglion cells (Rossi et al., 1993; Hinojosa et al., 2001). In contrast, growth factors had a moderate, but significant, neurite growth-promoting effect in the untreated cultures, which was further potentiated in the damaged preparations. A similar outcome was observed for BrdU incorporation, the strongest effect being found when kanamycin injury and growth factors were associated, in both the ISS and the OSS. Altogether, these observations indicate that, although there is no evidence for spontaneous activation of precursor cells, the application of growth factors significantly enhanced the tissue reaction to ototoxic damage.

Conclusion

In conclusion, nestin does not appear to be a distinctive marker for cochlear cell progenitors in situ. However, the evidence for nestin upregulation in a subset of supporting cells in the damaged cochlea upon growth factor administration may stimulate the exploration of strategies for dedifferentiating or reprogramming those cells into progenitors to regenerate the damaged organ of Corti. Moreover, our observations on cell proliferation, nestin expression and neuritic growth in the different conditions suggest that cells in the injured cochlea may be sensitive to repair-promoting cues, which can be provided from exogenous sources, thus opening interesting avenues for novel therapeutic approaches.

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Abbreviations

ABR: auditory brainstem response

bFGF: basic fibroblast growth factor

BrdU: 5-bromo-2-deoxyuridine

DAPI: 4’,6-diamidino-2-phenylindole

EGF: epidermal growth factor

IHC: inner hair cell

ISS: inner spiral sulcus

NA: numerical aperture

NF: neurofilament

OHC: outer hair cell

OSS: outer spiral sulcus

P: postnatal day

PBS: phosphate-buffered saline

RT-PCR: reverse transcription polymerase chain reaction

SPL: sound pressure level

TRITC: tetramethylrhodamine isothiocyanate

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