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Research paper

Hair cell regeneration or the expression of related factors that regulate the fate specification of supporting cells in the cochlear ducts of embryonic and posthatch chickens

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ABSTRACT

Hair cells in posthatch chickens regenerate spontaneously through mitosis or the transdifferentiation of supporting cells in response to antibiotic injury. However, how embryonic chicken cochleae respond to antibiotic treatment remains unknown. This study is the first to indicate that unlike hair cells in posthatch chickens, the auditory epithelium was free from antibiotic injury (25–250 mg gentamicin/kg) in embryonic chickens, although FITC-conjugated gentamicin actually reached embryonic hair cells. Next, we examined and counted the cells and performed labeling for BrdU, Sox2, Atoh1/Math1, PV or $p27^{kip1}$ (triple or double labeling) in the injured cochlea ducts after gentamicin treatment at 2 h (h), 15 h, 24 h, 2 days (d), 3 d and 7 d after BrdU treatment in posthatch chickens. Our results indicated that following gentamicin administration, proliferating cells (BrdU+) were labeled for Atoh1/Math1 in the damaged areas 3d after gentamicin administration, whereas hair cells (PV+) renewed through mitosis (BrdU+) or direct transdifferentiation (BrdU-) were evident only after 5 d of gentamicin administration. In addition, Sox2 expression was up-regulated in triggered supporting cells at an early stage of regeneration, but stopped at the advent of mature hair cells. Our study also indicated that $p27^{kip1}$ was expressed in both hair cells and supporting cells but was down-regulated in a subgroup of the supporting cells that gave rise to hair cells. These data and the obtained dynamic changes of the cells labeled for BrdU, Sox2, Atoh1/Math1, PV or $p27^{kip1}$ are useful for understanding supporting cell behaviors and their fate specification during hair cell regeneration.

1. Introduction

Recent WHO data indicate that 360 million people (328 million adults, 32 million children) have disabling hearing loss, a major part of which is induced by damage to and loss of sensory hair cells that convert sound vibrations into electrical signals; this process is primarily caused by aging, exposure to noise and ototoxic drugs (Wu et al., 2001; Taylor et al., 2008). Hair cells in mammals are primarily produced during embryonic development and are practically irreplaceable after they are lost, resulting in permanent hearing loss. Therefore, hair cell regeneration has become a crucial issue in deafness therapy (Stone and Rubel, 1999; Wu et al., 2001; Taylor et al., 2008). Fortunately, it has long been known that the hair cells of non-mammalian vertebrates such as birds (Cruz et al., 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Adler and Raphael, 1996), amphibians (Balak et al., 1990; Jones and Corwin, 1996; Baird et al., 1996) and bony fish (Harris et al., 2003; López-Schier and Hudspeth, 2006) can regenerate a complete set of hair cells after injury, thereby restoring auditory function. However, the developmental processes involved in the formation of the inner ear and gene expression during embryogenesis in mammals are essentially similar to those in birds (Cotanche and Kaiser, 2010), and thus, birds have become a significant model for studying the mechanism of hair cell regeneration, which represents great hope

List of Abbreviations: PV, parvalbumin; E, embryonic day; PG, post-gentamicin; PB, post-BrdU; FITC, fluorescein isothiocyanate

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for millions of people with hearing loss (Stone and Rubel, 1999; Hawkins et al., 2003; Roberson et al., 2004; Izumikawa et al., 2005). To date, hair cell regeneration has been well studied in posthatch chickens, but it is not yet known whether the auditory epithelium can be restored after hair cell loss in embryonic chickens. The issue is worthy of study for the following considerations: (1) if hair cells can regenerate after loss, then embryonic chickens would be an excellent model for studying the regeneration mechanisms, and the results could be used to prevent congenital deafness; and (2) if hair cells are not damaged, there must be a mechanism for protecting the cochlea from ototoxic drugs. Regardless of which circumstance occurs, embryonic chicken cochleae represent a useful model for studying congenital deafness in human fetuses.

The cochlear duct sensory epithelium of the inner ear is composed of hair cells and supporting cells in both mammals and birds. In posthatch chickens, once hair cell loss occurs, the adjacent surviving supporting cells immediately generate new hair cells through mitosis and direct transdifferentiation (Stone et al, 1996; Roberson et al., 2004; Cafaro et al., 2007). To date, several factors have been shown to be closely involved in the regulation of progenitor cells during the process of hair cell regeneration, including Sox2, a high-mobility group transcription factor (Kiernan et al., 2005; Dabdoub et al., 2008; Neves et al., 2011; Pan et al., 2013), Atoh1, a basic helix-loop helix transcription factor, a homolog of the *Drosophila* gene atonal or mammalian Math1 (Bermingham et al., 1999), and $p27^{kip1}$, a cyclin-dependent kinase inhibitor (Chen and Segil, 1999; Löwenheim et al., 1999; Oesterle et al., 2011). Sox2 is expressed in all of the supporting cells, but not in the hair cells, or embryonic progenitor cells in the developing mammalian organ of Corti (Kiernan et al., 2005). Atoh1/Math1 is necessary and sufficient for the specification of developing hair cells (Bermingham et al., 1999; Zheng and Gao, 2000; Chen et al., 2002). In embryonic mice, Atoh1/Math1 is expressed in proliferating progenitor or postmitotic precursor cells (Zine et al., 2001; Woods et al., 2004; Matei et al., 2005), and the supporting cells that undergo direct transdifferentiation in the lesion region are Atoh1/Math1-positive (Stone and Rubel, 1999; Zheng and Gao, 2000; Chen et al., 2002; Kiernan et al., 2005). Elevated Atoh1/Math1 triggers dividing supporting cells to exit the cell cycle and to be transdifferentiated into hair cells (Cafaro et al., 2007). Further studies have shown that Sox2 acts as an upstream regulator of Atoh1/Math1 to specify hair cell fate (Kiernan et al., 2005; Ahmed et al., 2012) or of $p27^{kip1}$ to maintain the quiescent state of postmitotic supporting cells (Liu et al., 2012). Following hair cell injury, Atoh1/Math1 is up-regulated, but $p27^{kip1}$ is down-regulated (Torchinsky et al., 1999; Daudet et al., 2009; Oesterle et al., 2007). However, to date, the detailed dynamics of the expression of the above-described genes during hair cell regeneration are still unclear.

In this study, we first examined whether embryonic auditory epithelia of chick were damaged after treatment with the aminoglycoside antibiotic, gentamicin. Our results indicated that although FITC-conjugated gentamicin actually reached the embryonic hair cells, the cochleae were free from any injury. We then examined and counted the cells labeled for BrdU, Sox2, Atoh1, PV or $p27^{kip1}$ in the epithelium of the injured cochlear ducts in posthatch chickens from 2 h to 7 d after BrdU treatment, to elucidate the detailed dynamic changes of these cells during hair cell regeneration after gentamicin treatment.

2. Materials and methods

2.1. Animal care

Fertilized eggs and new hatchlings of Beijing fatty chickens (*Gallus gallus domesticus*) were purchased from the Chinese Academy of Agricultural Science. The eggs were stored in a humidified incubator at 37.5 °C until embryo harvesting. The posthatch chickens were raised in a heated brooder and were provided with ample food and water at all times. All experiments performed in this study were conducted in accordance with the Beijing Laboratory Animal Welfare & Ethics Review guidelines, and all of the procedures were approved by the Animal Management Committee of the College of Life Sciences, Beijing Normal University.

2.2. Drug injections

All chicken embryos received a single injection of the aminoglycoside antibiotic gentamicin (75, 150 or 250 mg/kg egg weight) into the yolk sac on embryonic day (E) 12 (E12), by which time, hair-cell production is completed (Katayama and Corwin, 1989). A single injection of 10 μL 5-bromo-2'-deoxyuridine (BrdU, Sigma; an analog of thymidine, Cafaro et al., 2007) dissolved in sterile physiological saline (5 mg/mL) was administered on E13, E14 or E15.

The posthatch chickens received a single subcutaneous injection of gentamicin (250 mg/kg weight) per day on 2 consecutive days between 6 and 8 days posthatch (25–40 g). The treated chickens were allowed to recover in a heated brooder, and the first gentamicin administration was considered to be time zero for survival. At 3 d after the gentamicin administration, the chickens received one intraperitoneal injection of BrdU (100 mg/kg; Sigma) in sterile physiological saline to label a subset of dividing cells. This time point was chosen because a large number of dividing cells are in S phase at that time (Stone and Rubel, 1999; Cafaro et al., 2007).

To examine whether gentamicin can enter the hair cells, FITC-conjugated gentamicin (bsF-0972, Bioss, China) was injected into the yolk sac on E12. As a control, a subcutaneous injection of unconjugated FITC (c-0028, Bioss, China) was administered to the chickens between 6 and 8 days posthatch. The injection dosage was 5 mg/kg weight (for the fertilized eggs or chickens) with reference to a previous report (Tapaneya-Olarn et al., 1999).

2.3. Tissue dissection

The chickens were anesthetized by ethyl carbamate (intraperitoneal; 1250 mg/kg), and sacrificed by decapitation. For the fixative buffer to reach the inner ear, both the external and middle ear were sufficiently opened (Cafaro et al., 2007). The heads of the chickens were immersed in 4% buffered paraformaldehyde overnight at 4 °C. The cochlear ducts were dissected from the temporal bone and fixed in 4% paraformaldehyde for 2 h (h) at 4 °C, dehydrated in a gradient of ethyl alcohol and stored in 70% ethyl alcohol at 4 °C. Before whole-mount immunoreaction, the tegmentum vasculosum overlying the sensory epithelia was dissected off, and the remaining tissues were used. Some cochlear ducts were dehydrated in 30% sucrose (in 0.1 M PB, pH 7.4) at 4 °C. They were then cut into 10-μm-thick sections with a freezing microtome (Leica, model CM1850), and a total of ten sets of sections were collected and used for each cochlear duct.

2.4. Immunohistochemistry

The whole-mount preparations or the sections of cochlear ducts were processed for indirect or direct immunofluorescence. The following primary antibodies and dilutions were used: rat anti-BrdU (1:2000, ABD Serotec, OBT0030CX), goat anti- Atoh1/Math1 (1:600, Santa Cruz Biotechnology, sc-19249), goat anti-Sox2 (1:600, Santa Cruz Biotechnology, sc-17320), mouse anti-parvalbumin (PV) (1:1500, Chemicon, MAB1572) and mouse anti-$p27^{kip1}$ (1:150, BD, Cat. 610241). The following secondary antibodies were used: Alexa

594 conjugated donkey anti-rat IgG for BrdU labeling (1:400, Jackson ImmunoResearch, Cat. 712-585-150), DyLight 488 conjugated rabbit anti-sheep IgG for Math1 labeling (1:400, Jackson ImmunoResearch, Cat. 313-486-003), and Alexa 647 conjugated donkey anti-mouse IgG for PV or $p27^{kip1}$ labeling (1:400, Jackson ImmunoResearch, Cat. 715-606-151). For the detection of F-actin, direct immunofluorescence was performed with fluoresce conjugated phalloidin (5 units/ml, Invitrogen, Cat. A12379).

Before all of the immunoreactions, the tissues were inhibited with blocking solution (5% normal horse, rabbit, or goat serum in 0.05% Triton X-100 in PBS, pH 7.4) for 30 min at room temperature, for tissue permeation and nonspecific antibody binding. The primary antibodies were diluted in blocking solution, and the secondary antibodies were dissolved in PBS. The primary antibody incubations were performed overnight at 4 °C followed by 1.5 h at room temperature, whereas the secondary antibody incubation was for 2 h at room temperature. For BrdU detection, the tissues were treated with 2 N HCl for 3 h, followed by an incubation with

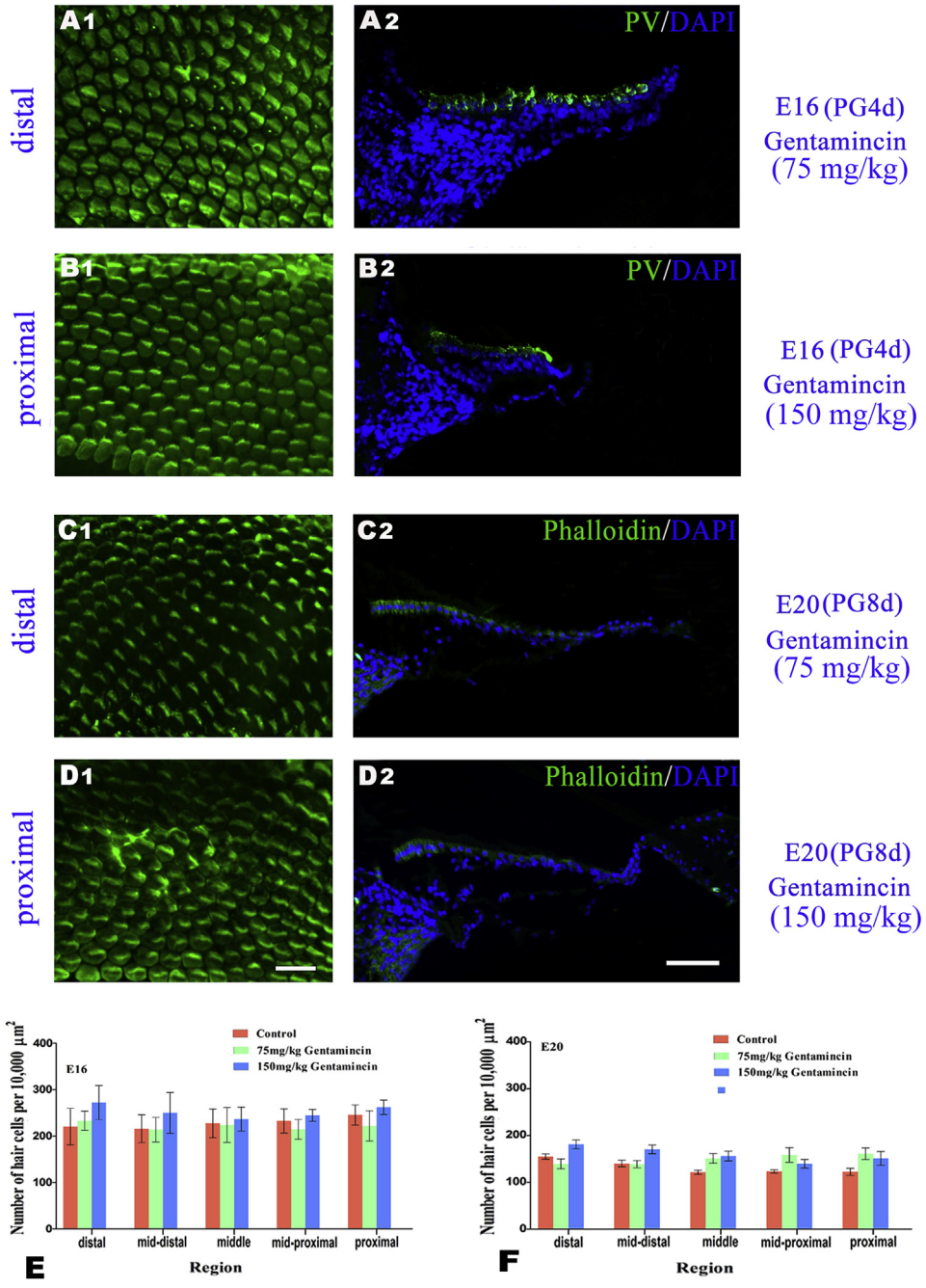

Fig. 1. Hair cell survival in the cochleae of embryonic chicken treated with gentamicin. A1-D1: Hair cells are labeled for parvalbumin (PV) in the whole-mount cochleae (A1, B1, C1 and D1), and for PV (A2 and B2) or phalloidin (C2 and D2) in the cross-sections of cochlea (A2, B2, C2 and D2), in which the nuclei of cells are counter-stained with DAPI. Hair cells are kept intact under treatment with two different dosages of gentamicin (mg/kg fertilized egg weight), 75 (A1, A2, C1 and C2) and 150 (B1, B2, D1 and D2), at embryonic day (E) 16 or 4 d post-gentamicin (PG) treatment (A1-B2), and E20 or PG 8d treatment (C1-D2). E and F: Statistical data show no significant differences between the control and gentamicin treatment in the densities of hair cells, the ratio of the number of hair cells in the examined areas (μm^2) within the five equally divided sections from the apex to base along the whole-mount cochlear duct E16 (E) and E20 (F) groups. Scale bar in D1 = 20 μm for A1-D1, and in D2 = 50 μm for A2-D2.

boric acid borax buffer solution (pH 8.4) for 30 min, prior to the blocking solution. Some of the samples were counterstained with DAPI (5 mg/mL, Invitrogen, Cat. D3571) to label nucleic acids. The whole-mount cochlear ducts were mounted onto sticky microscope slides, and all of the samples of the whole-mount cochlear ducts and the sections were cover-slipped with Antifade Solution (Applygen).

The specificity of the antibodies used above was verified in previous reports involving chicks, including anti-PV (Heller et al., 2002), anti-Sox2 (Neves et al., 2011), and anti-p27^{kip1} (Liu et al., 2012). The other antibodies were recommended for use in chickens by commercial suppliers.

Based on previous reports, the markers for labeling different cell types in the cochlear duct were chosen as follows. 1) Sox2 is expressed in all of the supporting cells, but not in the hair cells (Kiernan et al., 2005). It was therefore chosen as a marker of the supporting cells. 2) PV is a significant mobile Ca^{2+} buffer in hair cells, and has been regarded as a marker of early mature hair cells in

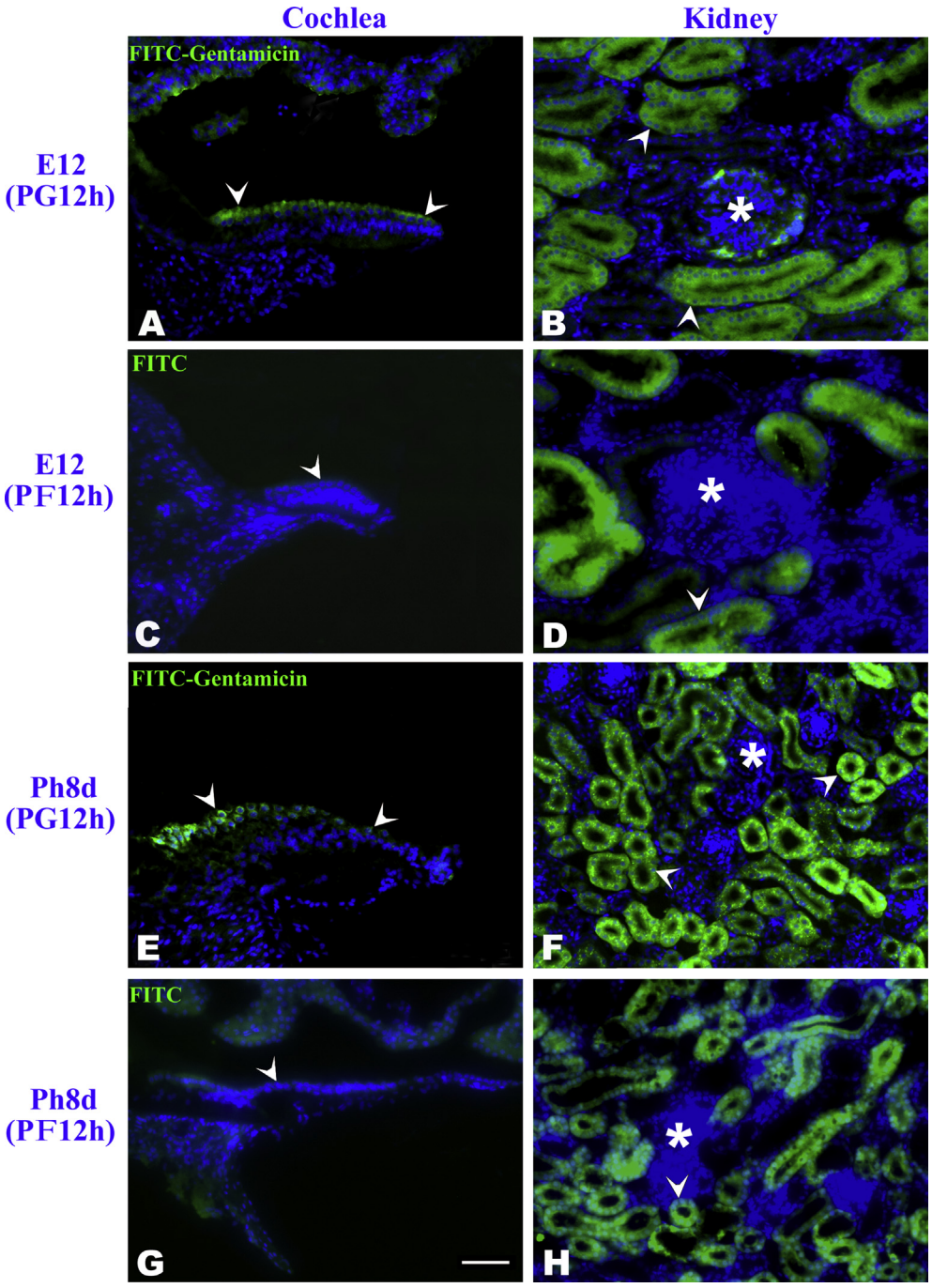

Fig. 2. The distribution of FITC fluoresce in the kidney and cochlea at 12 h post- FITC-conjugated gentamicin (PG 12 h) or unconjugate FITC (PF 12 h) injection. A–D: An injection of FITC-conjugated gentamicin (A and B) or unconjugated FITC (C and D) into the yolk sac at embryonic day 12 (E12). E–H: An injection of FITC-conjugated gentamicin (E and F) or unconjugated FITC (G and H) into the chickens at posthatch day 8 (Ph8d). The distributional patterns of FITC fluoresce are similar among the groups receiving an injection of FITC-conjugated gentamicin or unconjugated FITC, except that FITC fluoresce is not observed in hair cells after unconjugated FITC injections. Arrow heads indicate hair cells (A, C, E and G) or the renal tubule (B, D, F and H), and asterisks indicate the glomerulus (B, D, F and H). The sections are counter-stained with DAPI. Scale bar in G = 100 μm for A-H.

zebra fish, frogs, chickens and mammals (Heller et al., 2002; Oesterle et al., 2007). 3) Phalloidin labels filamentous actin intensely in intercellular adherens junctions between sensory epitheliums (Steyger et al., 1997) and is thus used as a marker for mature hair cells. 4) Atoh1/Math1 triggers the dividing supporting cells to exit the cell cycle and initiates the transdifferentiation of supporting cells into hair cells (Stone and Rubel, 1999; Zheng and Gao, 2000; Cafaro et al., 2007). It is thus used as an earlier marker of hair cells.

2.5. Microscopic imaging

The immunolabelled whole-mount cochlear ducts and the sections were imaged using an inverted fluorescence microscope with a 20 × objective (Axio Observer Z1, Zeiss) that was equipped with a monochromatic digital camera (AxioCam MRm, Zeiss). AxioVision Rel.4.8 acquisition and processing software was used to acquire uniform digital images for each antibody throughout the experiments, and the images were converted to TIFF files. ImageJ and Adobe Photoshop were used to analyze and manage the TIFF files.

2.6. Quantitative analyses

The whole-mount embryonic cochlea was divided into five equal successive regions, and in four non-overlapping scale boxes with dimensions 100 × 100 μm, chosen randomly, all of the hair cells in each box were counted. To calculate the post−hatch chicken hair cells, the whole cochlea was divided into 10 equal successive parts from apex to base, and all of the phalloidin-positive cells in the sections with 6 μm thickness were counted in each region divided above. The ratio of the number of hair cells to the length of the sensory epithelium (the linear density of the hair cells) was further determined in each experiment group.

Relative staining intensities were assessed based on the optical densities of the measured areas, which were obtained using Image Pro Analysis Software (Media Cybernetics, L.P., Silver Spring, Maryland). The relative optical density of positively labeled cells (Sox2+ or $p27^{kip1}$+) is the optical density of the measured areas compared to that of a randomly chosen surrounding blank area. These subtracted optical densities were averaged in each studied cochlea to obtain a mean optical density. Statistical analyses were performed using the SPSS 13.0 software package. Student's *t* test

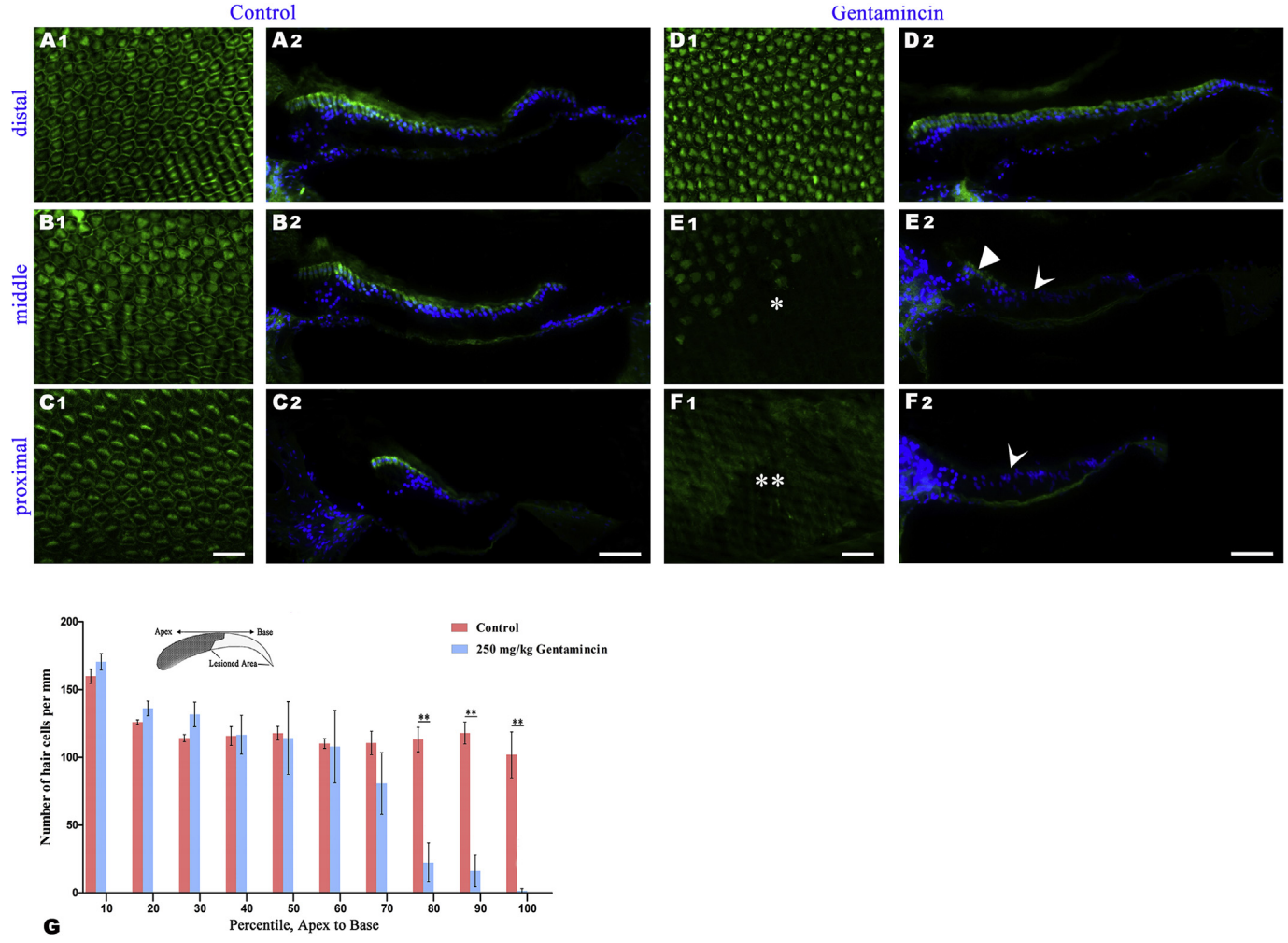

Fig. 3. Hair cell loss in the cochleae of posthatch chickens at 4 days after gentamicin treatment. A1-F1: Hair cells labeled for phalloidin in the control (the whole-mount: A1, B1 and C1; the cross-sections: A2, B2 and C2), and gentamicin treatment (the whole-mount: D1, E1 and F1; the cross-sections: D2, E2 and F2) groups. Hair cells in the control groups are intact, but they are damaged in the middle (E, the transition zone of injury is marked by "*") and proximal (F) parts of the cochlear duct. Toward the basal end of the cochlear ducts, no hair cells are observed toward the basal end of the cochlear ducts (marked by "**" in F). Cell nuclei are counter-stained with DAPI in the cross-sections. G: Statistical data show the differences in the linear density of the hair cells (the ratio of the number of hair cells to the length of sensory epithelium) within the ten equally divided parts from apex to base along the whole-mount cochlear duct between the control and gentamicin treatment groups. Scale bar in C1 and F1 = 20 μm for A1-F1, and in C2 and F2 = 50 μm for A2-F2.

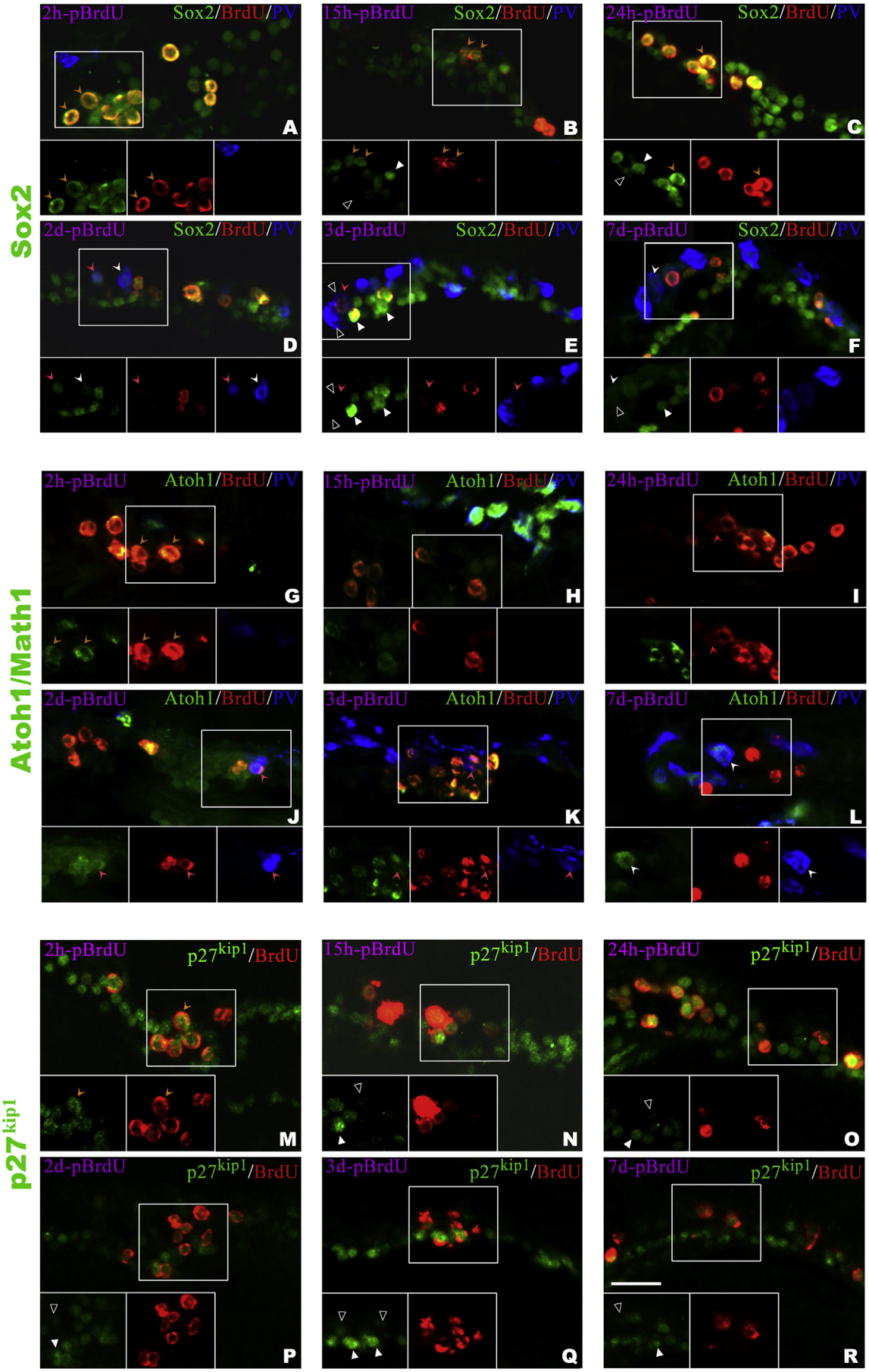

Fig. 4. Sox2, Atoh1 and p27^{kip1} expression in the damaged basilar papilla during hair cell regeneration. A–F: Triple-labeling for Sox2 (green), BrdU (red) and parvalbumin (PV, blue) in the surviving supporting cells. Some Sox2+/BrdU + cells are indicated by yellow arrows at 2 (A), 15 (B) and 24 (C) hrs after BrdU injection. A high Sox2 expression is indicated by a solid arrowhead, and a low Sox2 expression is indicated by a hollow arrowhead (B, C, E and F). Some Sox2+/BrdU+/PV+ and Sox2+/BrdU-/PV + cells are indicated by pink and

was used to compare the differences between the two groups under the same experimental conditions. One-way ANOVA with Tukey's *post hoc t* tests was used to compare the differences among more than two groups. The significance was set at $p < 0.05$.

3. Results

3.1. Cochlear ducts of embryonic chickens free from gentamicin treatment

To study whether gentamicin administration to chicken embryos injures hair cells, we first determined the gentamicin dosages. Based on human medicinal doses, we chose several concentrations, including 25, 50, 75, 150 and 250 mg/kg egg weight. We found that the hatching rates were much lower in the groups that received gentamicin at 25–250 mg/kg compared to the control groups that received an injection of sterile physiological saline solution ($F_{(5, 18)} = 13.989$, $p = 0.000$; $n = 30$ for each dosage or control group). In addition, the hatching rates were noted to decrease with increasing doses of gentamicin. At a dose of 25 mg gentamicin/kg, the hatching rate was 90.5 ± 8.3%, compared to 82.5 ± 14.2% at 50 mg/kg, 66.5 ± 12.2% at 75 mg/kg, 52.7 ± 10.3% at 150 mg/kg, and only 30.1 ± 16.5% at 250 mg/kg. Based on the above study, the three higher dosages of gentamicin (75, 150, and 250 mg/kg egg weight) were used in the subsequent study.

The hair cells in the cochlear ducts were checked at post-gentamicin (PG) 4d (corresponding to E16) or at PG8d (corresponding to E20, towards the end of hatching). The results showed that, as in the control groups, PV-labeled hair cells were arranged in an orderly pattern in the whole-mount cochleae (Fig. 1A1–D1) or in the cross-sections of the cochleae (Fig. 1A2–D2) in both the PG4d and PG8d groups at a dosage of 75 or 150 mg/kg. The density of the hair cells in each of the divided parts along the cochlear duct did not show significant differences between the control and gentamicin treatment groups (Fig. 1E and F). To exclude the possibility that hair cells can regenerate immediately after injury and that the generation could have been completed before the time points examined above, BrdU was injected after PG1d, PG2d or PG3d ($n = 3$, for each group), and no BrdU-labeled cells were observed (data not shown).

To directly test whether gentamcin reached the hair cells, FITC-conjugated gentamicin was injected into the yolk sac at E12. Following the injection, FITC fluorescence was observed in the hair cells (indicated by arrow heads, Fig. 2A and B) or stria vascularis (arrows), but not in the supporting cells in the cochlea, 12 h after the injection (Fig. 2A). FITC fluoresce was also observed in the cells in the renal tubule, another tissue that is readily injured by the aminoglycoside antibiotic, gentamicin (Nagai and Takano, 2004) (indicated by arrow heads, Fig. 2B), but not in the glomerulus (asterisks). Similar results were also observed after an injection of FITC-conjugated gentamicin into the chickens on posthatch day 8 (Fig. 2E and F).

As a control, unconjugated FITC was also injected into the embryonic or posthatch chickens by using the same method described above. We did not observe any obvious differences of distribution in FITC fluorescence in the kidney between the unconjugated FITC (Fig. 2D and H) and FITC-conjugated gentamicin (Fig. 2B and F) groups in either embryonic (Fig. 2D) or posthatch (Fig. 2H) groups, and FITC fluoresce was observed in the renal tubule, but not in the glomerulus. However, we did not observe FITC fluorescence in any parts of the cochlea (Fig. 2C and G), including the hair cells, in both the embryonic (Fig. 2C) and posthatch (Fig. 2G) groups.

3.2. Injury of cochlear ducts of posthatch chickens after gentamicin treatment

Compared with the control groups of posthatch chickens (Fig. 3A1–C2), the hair cells were partially lost in the middle (Fig. 3E1 and E2) or almost thoroughly eliminated in the proximal part (Fig. 3F1 and F2) of the cochlear duct, although those in the distal part were intact without any lesions after PG4d (Fig. 3D1 and D2). In contrast, supporting cells in all parts of the cochlear ducts survived and remained intact (Fig. 3D1–F2), which was similar to those in the control groups (Fig. 3A1–C2). The linear densities of phalloidin-positive hair cells in the cross-sections of the 10 successive parts that were divided equally along the cochlear duct are shown in Fig. 3G. They decreased significantly starting from the seventh part, and almost no hair cells were present in the last proximal part (Fig. 3F1–F2).

3.3. Gene expression in the cochlear ducts during hair cell regeneration

Following gentamicin administration to the posthatch chickens, BrdU-labeled cells were found in the proximal part of the cochlear duct in which nearly all of the original hair cells were lost (Fig. 4) but not in the distal part or in any parts of the control cochlear ducts. The cells labeled for BrdU, Sox2, Atoh1/Math1, PV or $p27^{kip1}$ (triple or double labeling) in the proximal part were examined and counted. The dynamic changes in the above labeled cells from 2 h to 7 d after BrdU treatment ($n = 3$–5 for each group) are shown in Fig. 5, and the percentages of the above labeled cells are shown in Table 1.

3.3.1. Triple labeling for Sox2, BrdU and PV

In the earlier stages (2–24 h post-BrdU treatment), all of the BrdU-labeled cells were Sox2 positive, indicating that all of the newborn cells (BrdU+) originated from supporting cells (Sox2+). As shown in Fig. 5A, Sox2+ cells labeled at 2 h post-BrdU treatment had the following fate specifications. 1) Some underwent mitosis and finally became the newly generated hair cells (PV+). Although some of this cell type were labeled by BrdU (Sox2+/BrdU+, Fig. 5A-a1), others were not (Sox2+/BrdU-, Fig. 5A-a2), as the valid period for BrdU incorporation into the proliferating cells is approximately 6 h from a single BrdU injection in posthatch chickens (Stone and Cotanche, 1994), and BrdU is not incorporated into the proliferating cells beyond this period. 2) The other Sox2+ support cells included those undergoing direct transdifferentiation to produce hair cells (Sox2+/BrdU-/PV+) (Fig. 5A-b) and those still remaining as support cells (Sox2+/BrdU-/PV-) (Fig. 5A-c).

Along the a1 route in Fig. 5A, the percentage of BrdU+/Sox2+ cells compared to the single-labeled cells for Sox2+ increased between 2 and 24 h post-BrdU treatment. However, these percentages decreased and changed by approximately 10% from 2 to 7 d

white arrows, respectively, at 2 (D), 3 (E) and 7 (F) days after BrdU injection. G–L: Triple labeling for Atoh1 (green), BrdU (red) and parvalbumin (PV, blue). G–I: Some Atoh1+/BrdU+, Atoh1-/BrdU+ and Atoh1+/BrdU- cells are indicated by yellow, red and green arrows, respectively, at 2 (G), 15 (H) and 24 (I) hrs after BrdU injection. Some Atoh1+/BrdU+/PV+ and Atoh1+/BrdU-/PV + cells were indicated by pink and white arrows, respectively, at 2 (J), 3 (K) and 7 (L) days after BrdU injection. M–R: Double-labeling for $p27^{kip1}$ (green) and BrdU (red) at 2 h (A), 15 h (B), 24 h (C), 2 days (D), 3 days (E) and 7 days (F) after BrdU injection. A $p27^{kip1}$+/BrdU + cell is shown by a yellow arrow. Some cells with high or low $p27^{kip1}$ expression are indicated by solid or hollow arrowheads, respectively in N-R. To better illustrate the labeling pattern, each color channel is shown separately in insets. Scale bar in R = 20 μm for A-R. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

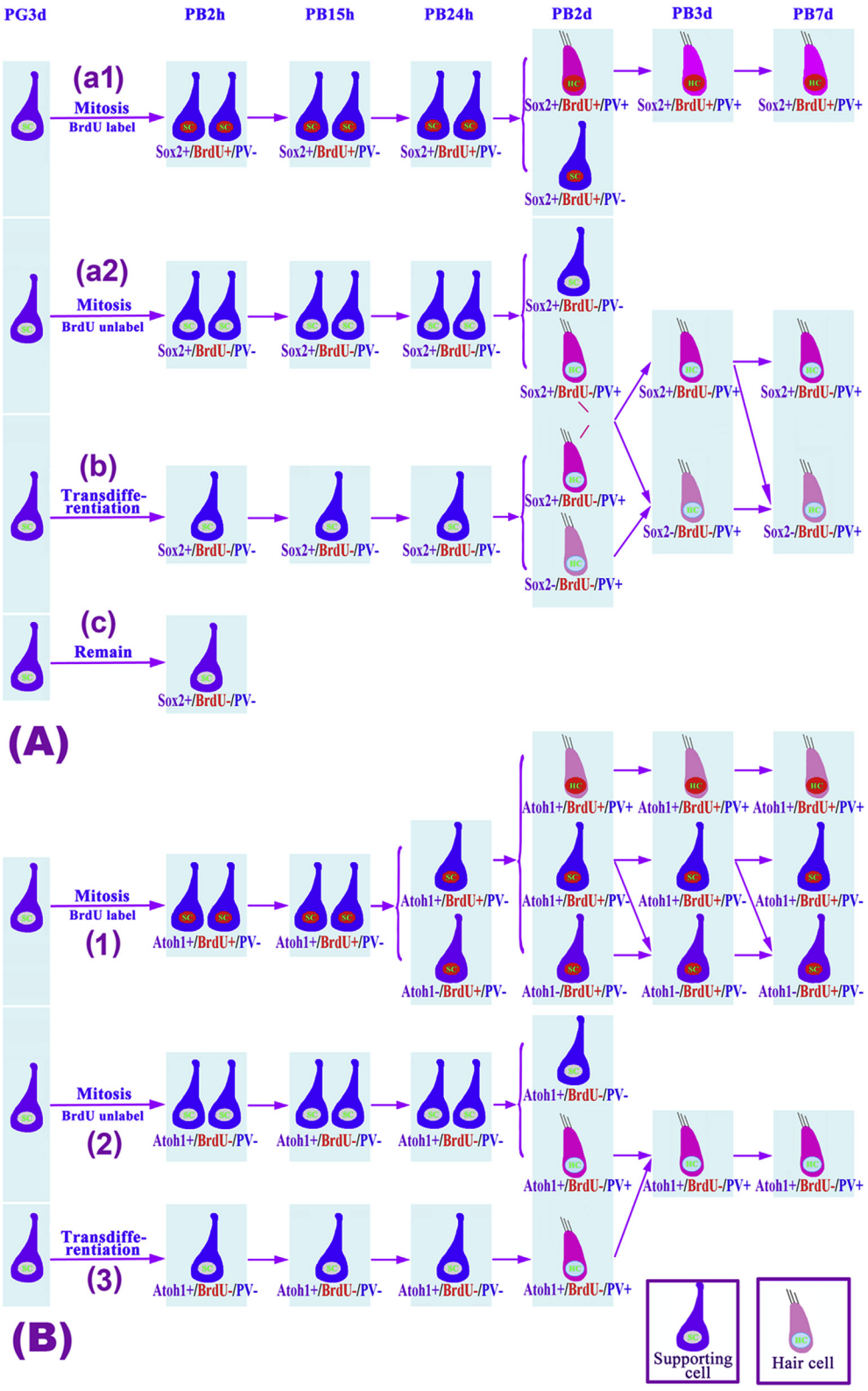

Fig. 5. The lineage of supporting cells in the damaged basilar papilla from post-gentamicin 3 days (PG3d) to 7 days post-BrdU (PB) injection 7d. A: Sox2-positive supporting cells have several fate specifications during hair cell regeneration. a1 and a2: Supporting cells undergoing mitosis are labeled by BrdU (a1) or not by BrdU (a2) because the valid period of BrdU incorporation into the proliferating cells is only approximate 6 h post-BrdU injection. b and c: Some supporting cells undergo transdifferentiation (b), while the others still remain the status of supporting cells (c). B: The types of Atoh1 positive cells. (1) and (2): Atoh1 positive cells undergoing mitosis are labeled by BrdU (Atoh1+/BrdU+) (1), and not by BrdU (Atoh1+/BrdU-) (2). (3): Atoh1 positive cells undergoing transdifferentiation (Atoh1+/BrdU-).

Table 1
The percentages of the labeled cells forSox2, Atoh1/Math1, $p27^{kip1}$, PV or BrdU (triple or double labeling) in the injured proximal part of cochlea after gentamicin administration.

	Cell types	PB2h	PB15h	PB24h	PB2d	PB3d	PB7d	F*	p*
Sox2+	Sox2+/BrdU+vs. Sox2+	4.8 ± 1.0	7.3 ± 1.2	16.7 ± 5.5	13.7 ± 7.2	9.2 ± 3.6	10.0 ± 1.7	4.228	0.017
	Sox2+/BrdU+/PV+ vs. PV+	–	–	–	35.7 ± 15.5	7.0 ± 6.2	8.3 ± 9.7	6.305	0.034
	Sox2+/BrdU-/PV+vs. PV+	–	–	–	41.7 ± 21.2	56.0 ± 15.5	23.0 ± 7.9	3.267	0.110
	Sox2+/PV+vs. PV+	–	–	–	77.3 ± 13.1	62.7 ± 9.7	31.7 ± 5.5	16.584	0.004
Atoh1/Math1+	Atoh1/Math1+/BrdU+vs. Atoh1/Math1+	16.7 ± 3.2	30.0 ± 8.9	36.0 ± 8.9	23.7 ± 6.5	17.3 ± 13.1	14.7 ± 0.6	3.399	0.038
	Atoh1/Math1+/BrdU+vs. BrdU+	100.0 ± 0	100.0 ± 0	95.3 ± 5.0	93.7 ± 11.0	83.3 ± 25.5	80.1 ± 28.8	0.641	0.673
	Atoh1/Math1+/PV+vs. Atoh1/Math1+	–	–	–	17.0 ± 8.9	25.7 ± 11.0	47.7 ± 13.8	6.945	0.028
	Atoh1/Math1+/BrdU+/PV+ vs. BrdU+	–	–	–	15.0 ± 10.1	19.7 ± 5.5	42.0 ± 14.7	9.062	0.040
$p27^{kip1+}$	$p27^{kip1}$+/BrdU+vs. $p27^{kip1}$+	6.7 ± 1.5	11.3 ± 3.2	17.0 ± 3.6	10.3 ± 3.2	7.0 ± 2.8	10.7 ± 0.6	5.956	0.004

PB: Post-BrdU treatment; h: hour; d: day; -: No data; F: F values from One-way ANOVA with Tukey's *post hoc t* tests, sample numbers (n) = 3–5 for each group.
p: the significance was set at $p < 0.05$.

post-BrdU treatment.

PV + cells first appeared in the 2-d post-BrdU treatment groups, and some of them were arranged in the same manner as those in the control groups (Fig. 4D). Within the newly generated hair cells (PV+), triple-labeled cells for Sox2+/BrdU+/PV + originated from mitosis (Fig. 5A-a1), whereas Sox2+/BrdU-/PV + cells originated from direct transdifferentiation (Fig. 5A-b) or from mitosis but were not labeled by BrdU (Fig. 5A-a2) (Fig. 4E and F). The percentages of Sox2+/BrdU-/PV + cells compared to the total PV + cells were significantly higher than those of Sox2+/BrdU+/PV + cells to the total PV + cells from 2 to 7 d post-BrdU treatment (2d post-BrdU treatment: $t = 6.785$, $p < 0.05$; 3d post-BrdU treatment: $t = 1.785$, $p < 0.001$; 7d post-BrdU treatment: $t = 3.432$, $p < 0.001$). However, both percentages decreased substantially 3 d after post-BrdU treatment (Table 1).

We noted that some double-labeled cells for Sox2+/BrdU + showed a relatively higher expression of Sox2 in the gentamicin treatment groups than those with a single positive labeling for Sox2 in the control groups at 2 h post-BrdU treatment. To evaluate the difference, we measured and compared the mean optical densities of all of the Sox2+/BrdU + double cells in each cochlear duct in the gentamicin treatment group (2 h post-BrdU treatment) with the cells with single positive labeling for Sox2 (approximately 50 cells examined in each cochlear duct, and 3 cochlear ducts examined in each group). The results indicated that the above mean optical densities were significantly different between the gentamicin treatment and the control ($t = 3.234$, $p = 0.023$).

3.3.2. Triple labeling for Atoh1/Math1, BrdU and PV

Atoh1/Math1 was found in the damaged areas even at 2 h post-BrdU treatment (Fig. 4G), indicating that direct transdifferentiation of supporting cells into hair cells (Atoh1/Math1+) had begun at this time point. Based on the double labeling for Atoh1/Math1+ and BrdU+, there were three types of Atoh1/Math1+ cells, including two types from mitosis (Atoh1/Math1+/BrdU+/PV-, Fig. 5B-1, and Atoh1/Math1+/BrdU-/PV-, Fig. 5B-2), and one from direct transdifferentiation (Atoh1/Math1+/BrdU-/PV-, Fig. 5B-3).

From 2 to 24 h post-BrdU treatment, the percentages of Atoh1/Math1+/BrdU + cells to that of the total Atoh1/Math1+ cells increased, but decreased from 2 to 7 d post-BrdU treatment (Table 1). The percentages of Atoh1/Math1+/BrdU + cells to that of the total BrdU + cells decreased from 2 h to 7 d post-BrdU treatment, but did not show any significance (Table 1). In addition, the percentages of Atoh1/Math1+/PV + cells to that of the total Atoh1/Math1+ cells increased significantly from 2 to 7 d post-BrdU treatment (Table 1). The percentages of Atoh1/Math1+/BrdU+/PV + cells to that of total BrdU + cells increased from 2 to 7 d post-BrdU treatment (Fig. 4G–L, Table 1). Because the primary antibody for Atoh1/Math1 and Sox2 were obtained from the same animal species and a replacement antibody is not available, the corresponding double labeling was not performed in the present study.

3.3.3. Double labeling for $p27^{kip1}$ and BrdU

In addition to hair cells and supporting cells in control groups, all of the BrdU + cells in the damaged areas were also labeled for $p27^{kip1}$ (Fig. 4M–R). However, $p27^{kip1}$ expression was not uniform among the supporting cells and was expressed at lower levels in some of them, as evaluated by optical density, compared to those in the control groups. The mean optical densities examined in approximately 50 cells in each cochlear duct (3 cochlear ducts for each group) were significantly lower in the gentamicin treatment than those in the control groups ($t = -7.886$, $p = 0.001$). At an earlier stage of regeneration (2–24 h post-BrdU), the percentages of the number of BrdU+/$p27^{kip1}$+ cells to that of the total $p27^{kip1}$+ cells increased significantly from 2 to 24 h post-BrdU treatment, but then decreased and remained at approximately 10% thereafter (Table 1).

4. Discussion

4.1. Embryonic chickens represent a useful model for studying hair cell protection against antibiotic injury

Our study shows that gentamicin treatment caused hair cell loss in posthatch chickens, which is consistent with previous reports (Stone and Rubel, 1999; Cafaro et al., 2007). However, no hair cell loss was observed in the embryonic chickens that received gentamicin at the three doses. It is noteworthy that the drug levels that we used were sufficiently high, especially compared to the human deafness pathopoiesis dosage (Kahlmeter and Dahlager, 1984; Prins et al., 1993; Tapaneya-Olarn et al., 1999). The assertion that gentamicin delivered to the yolk sac must have caused some damage to the embryonic chickens is supported by our two results: a close correlation between the dosages chosen (25–250 mg gentamicin/kg egg weight) and the hatching rates (30.1–90.5%), and the much lower hatching rates in fertilized eggs that received gentamicin treatment in contrast to those that received an injection of sterile physiological saline solution.

In addition, following an injection of FITC-conjugated gentamicin into the yolk sac at E12 or into the chickens at posthatch 6–8 days, we detected FITC fluorescence in the hair cells (but not in supporting cells) in the cochlea as well as in the renal tubule (but not in the glomerulus). However, unconjugated FITC was detected in the renal tubule but not in any part of the cochlea, including hair cells in both the embryonic and posthatch groups. These data suggest that FITC-conjugated gentamicin was selectively transported into the hair cells of E12 chick embryos.

Compared to the vulnerability of posthatch chicken or mammalian cochleae (Stone and Rubel, 1999; Wu et al., 2001; Cafaro et al., 2007; Daudet et al., 2007; Taylor et al., 2008), the invulnerability of embryonic chicken cochleae reveals that hair cells are effective against gentamicin injury. The mechanism for hair cell protection against antibiotic damage in embryonic chicken cochleae might be similar to that observed in bacteria: glycosylation catalyzed by glycosyltransferases affects the stability, chemical polarity and water-solubility of antibiotics, largely decreasing their bioactivities (Baltz, 2002; Walsh et al., 2003). However, this issue needs to be further addressed in future studies.

Following a single injection of [^{3}H]thymidine or BrdU into the yolk sac of the fertilized eggs, unbound [^{3}H]thymidine rapidly decreased by 90% within 48 h due to degradation by thymidine phosphorylase and/or synthesis of other macromolecules (Yurkewicz et al., 1981). Our study indicated that no BrdU-labeled cells appeared in the cochleae at E16 or E20, after BrdU was injected into the eggs on PG1d to PG3d. Based on the previous report (Yurkewicz et al., 1981), BrdU should be available for incorporation into newly synthesized DNA for proliferating cells at least for 48 h after injection. Therefore, the failure to observe BrdU-labeled cells on PG1d to PG3d should not be attributed to the lack of BrdU to be incorporated into the newly synthesized DNA.

In addition, our study and previous reports have shown that BrdU-labeled cells are found in the damaged areas of the cochlear duct on 3 d after gentamicin administration to posthatch chickens (Cruz et al., 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Adler and Raphael, 1996), which suggests that hair cell regeneration through cell mitosis must have lasted until 3 d after gentamicin administration. Thus new cell generation was not completed before the examined time points (PG1d to PG3d). Embryonic chickens could be used to study how hair cells are effectively free from antibiotic injury.

4.2. Expression of Sox2, Atoh1/Math1 and p27^{kip1} during the fate specification of supporting cells in hair cell regeneration in post–hatch chickens

By using triple or double labeling, we obtained the dynamic changes in the cells labeled for Sox2, Atoh1/Math1, p27^{kip1}, PV or BrdU at different time points covering almost the entire hair cell regeneration process. This is very helpful for determining how supporting cells behave during hair cell regeneration.

4.2.1. Fate specification of Sox2 + cells

The present study shows that Sox2 expression was limited to the supporting cells, but not to the hair cells, which is consistent with previous reports (Kiernan et al., 2005; Neves et al., 2007; Dabdoub et al., 2008). Along the route towards the newly generated hair cells from mitosis (Fig. 5A-a1), the percentages of BrdU+/Sox2+ cells to the total Sox2+ cells increased from 2 to 24 h post-BrdU treatment, but decreased thereafter. This suggests that Sox2+ cells undergoing mitosis incorporated BrdU within 24 h post-BrdU treatment, which is consistent with the report that show that the effective period for BrdU incorporation into proliferating cells is within 24 h after a single BrdU injection into posthatch chickens (Stone and Cotanche, 1994). From 2 to 7 d post-BrdU treatment, some Sox2+ cells may renew themselves through mitosis to compensate for the lost supporting cells (for example, owing to transdifferentiation into hair cells) (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Girod et al., 1989; Raphael, 1992; Hashino and Salvi, 1993; Stone and Cotanche, 1994; Stone and Rubel, 2000). The increasing number of Sox2+/BrdU- cells causes the percentage of double-positive cells (BrdU+/Sox2+) as a function of the entire number of Sox2+ cells to decrease from 2 to 7 d post-BrdU treatment.

In the 2-d post-BrdU treatment groups, PV first appeared in the supporting cells undergoing mitosis (Sox2+/BrdU+/PV+, Fig. 5A-a1; or Sox2+/BrdU-/PV+, Fig. 5A-a2) or direct transdifferentiation (Sox2+/BrdU-/PV+, Fig. 5A-b). From 2 to 7 d post-BrdU treatment, Sox2+/BrdU-/PV + cells were much more numerous than Sox2+/BrdU+/PV + cells, suggesting that hair cell regeneration from transdifferentiation exceeds that from mitosis. In the late stage of hair cell regeneration, some cells that originally expressed Sox2 stopped expressing it and completed the transdifferentiation from supporting cells (Sox2+) to hair cells (Sox2-). This may cause the percentages of Sox2+/PV + cells of the total PV + cells to be decreased (Table 1).

Our results indicated that the expression level of Sox2, based on optical density, was clearly higher in the BrdU-labeled cells than in some of the BrdU-unlabeled cells. We hypothesized that supporting cells with a high Sox2 expression level tend to produce hair cells, whereas those with adequate Sox2 expression tend to maintain their progenitor state or supporting cell attributes. It is necessary to note that Sox2 is not affected by drug damage in mammals (Oesterle et al., 2007), which might be an important reason for the differences in hair cell regeneration that exist between birds and mammals.

4.2.2. Fate specification of Atoh1/Math1 + cells

Atoh1/Math1+ cells could be observed in 2 h post-BrdU treatment groups, as reported previously (Cafaro et al., 2007). There were two types of Atoh1/Math1+ i.e., Atoh1/Math1+/BrdU+/PV- from mitosis and Atoh1/Math1+/BrdU-/PV- from transdifferentiation. As shown above, renewed PV + cells, unlike Atoh1/Math1+ cells, was observed only in 2 d post-BrdU treatment groups, supporting that Atoh1/Math1 is an earlier marker of hair cells (Stone and Rubel, 1999; Zheng and Gao, 2000; Cafaro et al., 2007). It is necessary to note that of the Atoh1/Math1+/BrdU-/PV- cells, some might originate from mitosis and would not incorporate BrdU as their DNA replication was beyond the valid period of BrdU availability (Stone and Cotanche, 1994). Due to the limits of the methods used in the present study, the cells that originated from mitosis could not be distinguished from those that originated from transdifferentiation.

The percentage of Atoh1/Math1+ and BrdU + cells to the total BrdU + cells was much higher in the present study than in a previous report (Cafaro et al., 2007). The percentage difference might be caused by the various methods used. In the present study, the labeled cells were counted in 10-μm sections, but they were counted in the whole-mount cochlear ducts in the previous report, and some labeled cells might be neglected in such a large image.

Our study indicated that the percentages of Atoh1/Math1+ and BrdU + cells to the total Atoh1/Math1+ cells increased from 2 to 24 h post-BrdU treatment. Such an increase might be caused by the persistent incorporation of BrdU into the proliferating Atoh1/Math1+ cells. However, towards the late stage of hair cell regeneration, with increasing supporting cells producing hair cells through transdifferentiation, they began to express Atoh1/Math1. This could result in an increase in the number of Atoh1/Math1+ cells but a decrease in the percentage of Atoh1/Math1+/BrdU + cells to the total Atoh1/Math1+ cells from 3 d post-BrdU treatment. In addition, with increasing Atoh1/Math1+/PV- cells turning into hair cells (Atoh1/Math1+/PV + or Atoh1/Math1+/PV+/BrdU+), the proportion of Atoh1/Math1+/PV + or Atoh1/Math1+/PV+/BrdU + cells to the total Atoh1/Math1+ or BrdU + cells increased from 2 to 7 d post-BrdU treatment (Table 1).

Previous reports have shown that Atoh1/Math1 expression is not observed in normal quiescent auditory epithelium in the basilar papilla, but it is abundant in the normal vestibular epithelium of posthatch chicks (Cafaro et al., 2007). However, the present results

indicated that Atoh1/Math1 was also detected in the hair cells of the normal chicken auditory epithelium. This staining difference might be caused by the two different antibodies used, one a rabbit polyclonal antibody against Atoh1/Math1 (not for commercial use) (Cafaro et al., 2007) and the other a goat anti-Math1 from Santa Cruz (Cat.19249). Here, the antibody for Math1 (Santa Cruz, Cat.19249) is recommended for detection in chickens, and our staining pattern was similar to previous reports using immunohistochemistry (Cafaro et al., 2007) or *in situ* hybridization (Dabdoub et al., 2008). Given that only hair cells and not supporting cells were stained, our staining might not be non-specific.

4.2.3. Fate specification of $p27^{kip1}$ + cells

Our work showed that $p27^{kip1}$ was expressed in both hair cells and supporting cells in normal chicken cochlea, consistent with a previous report (Torchinsky et al., 1999). However, it has been reported to be expressed only in hair cells but not in supporting cells in mammals (Chen and Segil, 1999; Oesterle et al., 2011). Our study further indicated that in response to hair cell loss, $p27^{kip1}$ was expressed in all of the BrdU-positive cells, and in some of these cells, it was expressed at lower levels, based on the optical density.

Previous studies have shown that during cochlear development, $p27^{kip1}$ is induced in the primordial cochlea between E12 and E14 as an inhibitor of cell cycle progression (Sherr and Roberts, 1995, 1999; Elledge et al., 1996), which correlates with the terminal division of hair cells and supporting cells (Chen and Segil, 1999; Torchinsky et al., 1999; Oesterle et al., 2011). In addition, in mice with $p27^{Kip1}$ deletion, cell proliferation still continues after normal mitosis has ceased in the organ of Corti, which results in supernumerary hair cells and supporting cells (Chen and Segil, 1999; Löwenheim et al., 1999). Thus, the persistence of $p27^{kip1}$ expression in the auditory epithelium contributes to the maintenance of the quiescence of this tissue, and the down-regulation of $p27^{kip1}$ could trigger supporting cells to re-enter the cell cycle, initiating hair cell regeneration (Torchinsky et al., 1999).

It has been recently shown that Sox2 acts as a key upstream regulator of $p27^{kip1}$ to maintain the quiescent state of postmitotic supporting cells in mammals (Liu et al., 2012). It is probable that the co-expression of Sox2 with Atoh1/Math1 significantly stimulates hair cell regeneration through cell division and conversion, as in the development of the cochlea (Ahmed et al., 2012; Neves et al., 2012). Once the hair cells differentiate, Sox2 is limited to the supporting cells, and Atoh1/Math1 is restricted to the hair cells to keep the progenitor state or terminal differentiation stage. Our study indeed indicated that the staining intensity of both $p27^{kip1}$ and Sox2 varied among the supporting cells, which suggests that the expression changes in Sox2 and $p27^{kip1}$ might synergistically regulate hair cell regeneration. These issues should be further investigated in future studies.

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