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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.

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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

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