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Research Paper

Transcriptomic analysis of chicken cochleae after gentamicin damage and the involvement of four signaling pathways (Notch, FGF, Wnt and BMP) in hair cell regeneration

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ABSTRACT

Unlike mammalian hair cells, which are essentially unable to regenerate after damage, avian hair cells have a robust capacity for regeneration. The prerequisite for understanding the above difference is knowing the genetic programming of avian hair cell regeneration. Although the major processes have been known, the precise molecular signaling that induces regeneration remains unclear. To address this issue, we performed a high-throughput transcriptomic analysis of gene expression during hair cell regeneration in the chick cochlea after antibiotic injury *in vivo*. A total of 16,588 genes were found to be expressed in the cochlea, of which about 1000 genes were differentially expressed among the four groups studied, i.e., 2 days (d) or 3 d post-treatment with gentamicin or physiological saline. The differentially expressed genes were distributed across approximately one hundred signaling pathways, including the Notch, MAPK (FGF), Wnt and TGF-β (BMP) pathways that have been shown to play important roles in embryonic development. Some differentially expressed genes (2–3 in each pathway) were further verified by qRT-PCR. After blocking Notch, FGF or BMP signaling, the number of regenerating hair cells and mitotic supporting cells increased. However, the opposite effect was observed after suppressing the Wnt pathway or enhancing BMP signaling. To our knowledge, the present study provided a relatively complete dataset of candidate genes and signaling pathways most likely involved in hair cell regeneration and should be a useful start in deciphering the genetic circuitry for inducing hair cell regeneration in the chick cochlea.

1. Introduction

Millions of people have permanent hearing deficits, which can be caused by genetic disorders, acoustic trauma, aging process or ototoxic drugs (Yorgason et al., 2006; Oishi and Schacht, 2011). Therapy for deafness is challenging because hair cells in mature mammals are essentially unable to regenerate after damage (Selimoglu, 2007; Taylor et al., 2008). However, there is a strong capacity for hair cell regeneration in the cochlea and utricle of lower vertebrates and a limited regenerative ability in the mammalian vestibular organ (Cruz et al., 1987; Baird et al., 1996; Harris et al., 2003; Cafaro et al., 2007; Dror and Avraham, 2010). The molecular signals that guide cell regeneration in non-mammalian ears are of great biological and clinical interest, as they suggest that once we understand how regeneration is triggered by the molecular signals, we could induce similar hair cell regeneration in the mammalian cochlea (Cotanche and Kaiser, 2010; Dror and Avraham, 2010; Oishi and Schacht, 2011).

Although the major anatomical events that occur during regeneration in the avian ear have been known (Roberson et al.,

Abbreviations: MAPK, mitogen-activated protein kinase; FGF, fibroblast growth factor; TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; SAGE, serial analysis of gene expression; BrdU, 5-bromo-2-deoxyuridine; FPKM, fragments per kilobase of exons per million fragments mapped; DEG, differentially expressed genes; FDR, false discovery rate; PV, parvalbumin

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1996, 2004; Daudet et al., 2009; Cotanche and Kaiser, 2010), the precise molecular signaling that initiates regenerative proliferation remains unclear. However, it is assumed that many of the genes and signaling pathways required for the production of embryonic hair cells are also needed during the regeneration of hair cells (Cotanche and Kaiser, 2010; Groves et al., 2013). Many studies have shown that the genes and signaling pathways involved in the production of embryonic hair cells are highly conserved among vertebrates (Cotanche and Kaiser, 2010; Groves et al., 2013) and are complex with a multitude of interacting signaling pathways, including the Notch, FGF, Wnt and BMP pathways (Lewis, 1998; Bryant et al., 2002; Daudet and Lewis, 2005; Li et al., 2005, 2015; Millimaki et al., 2007; Jayasena et al., 2008; Daudet et al., 2009; Su et al., 2015). Some studies have investigated the roles of these pathways in the regulation of cell regeneration (Bermingham-McDonogh et al., 2001; Ma et al., 2008; Daudet et al., 2009; Mizutari et al., 2013; Wu et al., 2016). Nevertheless, how hair cell regeneration is induced after damage and what/how signaling pathways are involved in hair cell regeneration still remain unknown.

To know the signaling circuitry of hair cell regeneration after damage, comparing the transcriptional profiles of regenerating hair cells is regarded as a significant starting point (Chen and Corey, 2002; Tao and Segil, 2015; Schimmang and Maconochie, 2016). By using cDNA microarrays, SAGE or RNA-seq, several studies have investigated the changes in the transcriptional profiles during hair cell regeneration in the basilar papilla and utricle of chick or zebrafish (Hawkins et al., 2003, 2007; Schuck et al., 2011; Liang et al., 2012; Jiang et al., 2014; Ku et al., 2014; Schimmang and Maconochie, 2016). These experiments generate large amounts of data. However, no consensus has been reached regarding changes in gene expression patterns, probably due to the different methods applied. To date, quite a lot of studies have been reported on the roles of some signaling pathways, including Notch, FGF, Wnt and Bmp in the embryonic development of hair cells (Li et al., 2005, 2015; Millimaki et al., 2007; Petrovic et al., 2015; Munnamalai and Fekete, 2016). However, the precise molecular mechanisms by which the signaling pathways mediate hair-cell regeneration are less known. Notably, the comprehensive gene expression of *in vivo* cochlea has not been analyzed during regeneration after damage. Such a study is necessary, considering that proliferating cells (BrdU-labeled) can be detected in the chick cochlea in *in vitro* culture, even without any treatment with ototoxic drugs (Shang et al., 2010), but are not found in the undamaged *in vivo* cochlea (Jiang et al., 2016). These data suggest that cell regeneration is actually initiated in *in vitro* culture even without drug treatment. Thus, to obtain the genetic expression profile necessary to induce hair cell regeneration, the transcriptional differences between the cochlea undergoing regeneration after damage and the cochlea lacking regeneration completely need to be compared.

To address the above issue, RNA-seq was employed to analyze the transcriptomic profiles of *in vivo* basilar papillae after 2 d or 3 d of treatment with gentamicin or physiological saline (for control). According to previous reports, nearly all hair cells were damaged, and the peak of dividing cells in S phase and the onset of hair cells regenerated via direct transdifferentiation occur in the proximal part of cochlea after 2 or 3 d gentamicin treatment (Daudet et al., 2009; Shang et al., 2010). The genes involved in initiating cell proliferation and transdifferentiation during cell regeneration should be included in this period of time (2–3 d after gentamicin treatment). The results indicated that about 1000 genes were differentially expressed in the chicken basilar papillae in the studied groups, including some members of the Notch, FGF, Wnt and TGF-β (BMP) signaling pathways. By pharmacological inhibition or activation of the four pathways, we further studied the roles of the four signaling pathways in hair cell regeneration in the chicken basilar papilla.

2. Materials and methods

2.1. Animal care and treatment

Neonatal chickens (*Gallus gallus domesticus*) were obtained from the Chinese Academy of Agricultural Sciences. They were raised in a heated brooder and provided with adequate food and water at all times. Posthatch chicks aged between 6 and 8 d (30–45 g) received a single subcutaneous injection of gentamicin per day for 2 consecutive days, and the injection dosage was 0.25 mg/g body weight. Control chicks were treated with physiological saline. This level of gentamicin treatment kills nearly all of the hair cells in the proximal part of the cochlear duct (hair cells in the control animals are intact) (Jiang et al., 2016). Animal experiments were performed according to the Beijing Laboratory Animal Welfare and Ethics Review guidelines, and all experimental procedures were approved by the Animal Management Committee of the College of Life Sciences, Beijing Normal University.

2.2. RNA isolation and preparation

RNA samples were obtained from the sensory epithelia of 70–80 chickens from each group. After the chickens were treated with gentamicin or physiological saline, they were allowed to survive for 2 d or 3 d (the first injection of gentamicin or physiological saline was considered time zero for survival). The study included four total groups: 2 and 3 d post-treatment with physiological saline (PS) (PS2d/PS3d), and 2 and 3d post-treatment with gentamicin (PG) (PG2d/PG3d).

To obtain the inner ears, the chickens were anesthetized by administration of ethyl carbamate (intraperitoneal, 1.25 mg/g body weight) and then euthanized by decapitation. The heads were immersed in alcohol for a few seconds, and the external or middle ears were progressively opened. After the cochlear ducts were dissected from the inner ear, the tegmentum vasculosum overlying the sensory epithelium was dissected away. The sensory epithelia in the proximal half of the whole cochlear duct, in which the hair cells were nearly completely damaged, were pooled for each time point and treatment group. The pooled tissue was then dissolved in Trizol (GeneCopoeia, America, E01010A) and stored at −80 °C for RNA isolation (Ku et al., 2014). Total RNA was extracted using a Total RNA Kit (TIANGEN, Beijing, DP419), and the quality was checked. RNA was stored at −80 °C for RNA-seq or qRT-PCR.

2.3. RNA-seq and data analysis

Enriched mRNA was obtained from the RNA sample of each group by using an NEB Next Poly(A) mRNA Magnetic Isolation Module (NEB, E7490). Before RNA-seq, cDNA libraries were constructed according to the manufacturer's instructions for Illumina mRNA-seq. Briefly, the enriched mRNA was fragmented and used to generate first-strand cDNA with random primers. Second-strand synthesis, end repair, poly-A tail addition and adaptor ligation were then conducted. Finally, each RNA-seq library was sequenced by using the Illumina HiSeq™ 2500 system with paired-end (2 × 125 bp) sequencing at Beijing Biomarker Biotechnology. The average correlation coefficient between technical replicates was >0.998.

Raw reads were purified by removing the adaptor sequence and low-quality unknown sequences. The clean reads in the FASTQ format were mapped to the Ensembl *Gallus gallus* reference genome (//ftp.ensembl.org/pub/release-75/fasta/gallus_gallus/)

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