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CTCF is required for maintenance of auditory hair cells and hearing function in the mouse cochlea

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ABSTRACT

Auditory hair cells play an essential role in hearing. These cells convert sound waves, mechanical stimuli, into electrical signals that are conveyed to the brain via spiral ganglion neurons. The hair cells are located in the organ of Corti within the cochlea. They assemble in a special arrangement with three rows of outer hair cells and one row of inner hair cells. The proper differentiation and preservation of auditory hair cells are essential for acquiring and maintaining hearing function, respectively. Many genetic regulatory mechanisms underlying hair-cell differentiation and maintenance have been elucidated to date. However, the role of epigenetic regulation in hair-cell differentiation and maintenance has not been definitively demonstrated. CTCF is an essential epigenetic component that plays a primary role in the organization of global chromatin architecture. To determine the role of CTCF in mammalian hair cells, we specifically deleted *Ctcf* in developing hair cells by crossing $Ctcf^{fl/fl}$ mice with $Gfi1^{Cre/+}$ mice. $Gfi1^{Cre}$; $Ctcf^{fl/fl}$ mice did not exhibit obvious developmental defects in hair cells until postnatal day 8. However, at 3 weeks, the *Ctcf* deficiency caused intermittent degeneration of the stereociliary bundles of outer hair cells, resulting in profound hearing impairment. At 5 weeks, most hair cells were degenerated in $Gfi1^{Cre}$; $Ctcf^{fl/fl}$ mice, and defects in other structures of the organ of Corti, such as the tunnel of Corti and Nuel's space, became apparent. These results suggest that CTCF plays an essential role in maintaining hair cells and hearing function in mammalian cochlea.

1. Introduction

Mammalian hearing relies on sound transduction by the organ of Corti, a highly specialized structure in the spiraled cochlea of the inner ear. The organ of Corti consists of various cell types, including three rows of outer hair cells (OHC) and one row of inner hair cells (IHC). OHCs mechanically amplify sound, increasing the responsiveness of sensory epithelium to the frequency selection. IHCs excite the spiral ganglion neurons. The development and function of auditory hair cells involves multiple processes, including differentiation, maturation, and maintenance. While most hair cells are formed during embryonic development of the cochlea, the hair cells in the organ of Corti undergo maturation after birth. Accurate gene expression is essential for various biological processes, such as cell differentiation and cell survival, so hair cells can properly develop and mature [1]. Congenital and acquired defects in hair cells due to misregulated genes are a primary cause of the hearing impairment. Genetic studies have broadened our understanding of the development and dysfunction of hair cells.

Epigenetic mechanisms control chromatin structure without altering DNA sequences (e.g., DNA methylation, histone modification, chromatin conformation) and may affect the expression of spatiotemporal genes essential for development and disease [2]. Researchers have identified the specific combination of epigenetic modifiers, such as DNA methylation and histone modification, and transcription factors that affect early inner ear development. For example, DNA methyltransferase, DNMT3A, and histone demethylase, KDM4B, regulate transcription factors, such as *Pax2*, *Gbx2*, and *Dlx3*. These factors play a crucial role in the formation and invagination of the otic placode [3,4]. In the mammalian inner ear, the chromatin structure at the *Atoh1* locus was altered via histone

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modification during hair-cell differentiation [5]. However, the role of epigenetic regulations in the differentiation, maturation, and maintenance of auditory hair cells remains unclear.

CCCTC-binding factor (CTCF) is a central player that regulates gene expression by mediating the organization of chromatin architecture in the nucleus. CTCF is an important factor governing the development of various tissues (including neural, muscle, and cardiac cells) [6–8]. Aberrant CTCF expression in humans is involved in several diseases and disorders, including Silver-Russell syndrome, Beckwith-Wiedemann syndrome, mental retardation, and various cancers [2,9]. Patients with Silver-Russell syndrome have corresponding sensorineural hearing loss [10]. However, it is unclear if chromatin architecture regulation plays a role in the development and functional maintenance of auditory hair cells.

In order to investigate the role of CTCF in auditory hair cells, we deleted *Ctcf* specifically in hair cells. Loss of *Ctcf* did not affect the differentiation and morphological maturation of auditory hair cells. However, *Ctcf* deficiency led to the progressive degeneration of stereociliary bundles starting at 3 weeks, followed by loss of hair cells and finally resulting in profound deafness. To our knowledge, this study is the first to elucidate the pathological role of CTCF in the maintenance of the hair cells responsible for hearing function.

2. Materials and methods

2.1. Mice generation and breeding

$Ctcf^{fl/fl}$ mice were crossed with $Gfi1^{Cre/+}$ mice to generate inner-ear-specific *Ctcf* cKO mice [11,12]. All animal protocols were approved by the Institutional Animal Care and Use Committee at Yonsei University College of Medicine.

2.2. In situ hybridization, immunostaining, auditory brainstem response (ABR), and scanning electron microscopy (SEM)

In situ hybridization, immunostaining, ABR, and SEM were performed as described previously [13].

2.3. Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical significance was determined using the Student's t-test, with $p < 0.05$ being considered significant.

3. Results

3.1. Differentiation and morphological maturation of hair cells in the inner ear of Ctcf cKO mice does not differ from control mice

To investigate the role of CTCF in auditory hair cells of the cochlea, *Ctcf* was deleted in developing hair cells by breeding $Ctcf^{fl/fl}$ mice with $Gfi1^{Cre/+}$ mice (Fig. 1A). $Gfi1^{Cre/+}$ mice express Cre recombinase in hair cells during both development and adulthood [12]. We examined the expression pattern of *Atoh1*, a gene that is necessary and sufficient for the progression of hair-cell specification and differentiation, at postnatal (P) 0 to determine whether CTCF is involved in hair-cell differentiation [14]. *Atoh1* is expressed on *Sox2*-expressing cells, which are supporting cells in the P0 cochlea. We found no significant difference in *Atoh1* expression between $Gfi1^{Cre/+}$; $Ctcf^{fl/+}$ (control), and $Gfi1^{Cre/+}$; $Ctcf^{fl/fl}$ (*Ctcf* cKO) mice in *Sox2*-expressing cells (Fig. 1B–U). Furthermore, after E14.5, when *Gfi1* expression was first observed in nascent hair cells, *Atoh1* was not detected in control or *Ctcf* cKO mice (Supplementary Fig. 1). These results indicate that CTCF does not affect the competency of *Atoh1* for hair-cell differentiation.

The organ of Corti has one row of IHCs and three rows of OHCs displaying a typical V-shape at P4 [15]. In order to assess the maturation and organization of hair cells and detect any defects, we performed whole-mount immunostaining of the organ of Corti from control and *Ctcf* cKO mice using Myo7a antibody and phalloidin staining at P5. As indicated by the hair-cell marker Myo7a, the location of the hair cells were visualized in both control and *Ctcf* cKO tissue (Fig. 2A–F, green). The morphological maturation of the hair cell is completed from the basal region to the apical region, which consists of three rows of OHCs and one row of IHCs (Fig. 2D–F), similar to the control (Fig. 2A–C). A previous study found that the angle of the V-shaped hair bundles in organized OHCs has a morphological gradient that is wider at basal hair cells and smaller at apical hair cells [15]. Based on the phalloidin staining (Fig. 2, red), the gradient V-shape of the OHC bundles were typically formed from the base to the apex at P5 in control and *Ctcf* cKO tissues (Fig. 2A–F). These results suggest that CTCF does not regulate the morphological maturation and base-to-apex gradient of hair bundles.

3.2. Ctcf deficiency causes profound hearing loss through progressive degeneration of auditory hair cells and the structure of the organ of Corti

Because the lack of *Ctcf* did not affect the morphology and arrangement of the IHCs and OHCs (Fig. 2), we next assessed the contribution of CTCF to the ability of hair cells to detect sound by measuring ABR using click stimuli corresponding to low frequencies at 2–7 kHz and tone-burst stimuli at 8, 12, 18, 24, and 30 kHz. We first tested hearing at 3 weeks, once the hearing system had matured. *Ctcf* cKO mice exhibited significantly higher ABR thresholds for click stimuli and tone bursts compared with control mice (Fig. 3A). Then, we tested ABR in 5-week-old mice to examine whether hearing function deteriorated over time. The ABR threshold in control mice at 5 weeks of age was lower than that of control mice at 3 weeks of age. *Ctcf* cKO mice did not respond to click stimuli or tone bursts at 5 weeks, suggesting profound hearing impairment (Fig. 3B). These results demonstrate that the deletion of *Ctcf* causes progressive and profound hearing loss in mice.

We next examined whether stereocilia degeneration was associated with hair-cell loss in *Ctcf* cKO mice using SEM. Stereociliary bundles displayed three rows of OHCs with a 'V' shape and one row of IHCs with a linear arrangement (Fig. 4A–C). At P8, the overall structure of the cochlea showed a typical arrangement of stereociliary bundles in both genotypes (Fig. 4A–F). At 3 weeks, the arrangement of the cochlea was typical from base to apex in control mice (Fig. 4G–I), but the stereociliary bundles of OHCs in the base, mid, and apex regions started to degenerate in *Ctcf* cKO mice (Fig. 4J–L). At 5 weeks, the OHCs and IHCs of control mice had a well-organized overall arrangement of the stereociliary bundles (Fig. 4M–O), whereas the OHCs and IHCs of *Ctcf* cKO mice showed severe degeneration of stereociliary bundles. The numbers of degenerating hair cells appeared to be much higher in the basal turn than in the middle and apical turns (Fig. 4S–U). Specifically, no stereociliary bundles were detected in the OHCs and IHCs of the basal turn in the cochlea (Fig. 4S). Furthermore, columnar pillar cells, one of the supporting cells that form the tunnel of Corti and Nuel's space, were also degenerated in the basal turn. In *Ctcf* cKO mice, the few hair cells that remained and the remnant stereocilia in hair cells showed a compromised arrangement and shape (Fig. 4P–R). In order to identify any histological alterations in *Ctcf* cKO mice, we hematoxylin and eosin (H & E) stained paraffin sections from 5-week-old control and *Ctcf* cKO mice. Control sections displayed normal hair cells and Deiter's cells (Fig. 4P–R). By contrast, H & E staining of the *Ctcf* cKO sections showed complete

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