CTCF Regulates Otic Neurogenesis via Histone Modification in the Neurog1 Locus

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The inner ear is a complex sensory organ responsible for hearing and balance. Formation of the inner ear is dependent on tight regulation of spatial and temporal expression of genes that direct a series of developmental processes. Recently, epigenetic regulation has emerged as a crucial regulator of the development of various organs. However, what roles higher-order chromatin organization and its regulator molecules play in inner ear development are unclear. CCCTC-binding factor (CTCF) is a highly conserved 11-zinc finger protein that regulates the three-dimensional architecture of chromatin, and is involved in various gene regulation processes. To delineate the role of CTCF in inner ear development, the present study investigated inner ear-specific Ctf knockout mouse embryos (Pax2-Cre; Ctcffl/fl). The loss of Ctf resulted in multiple defects of inner ear development and severely compromised otic neurogenesis, which was partly due to a loss of Neurog1 expression. Furthermore, reduced Neurog1 gene expression by CTCF knockdown was found to be associated with changes in histone modification at the gene’s promoter, as well as its upstream enhancer. The results of the present study demonstrate that CTCF plays an essential role in otic neurogenesis by modulating histone modification in the Neurog1 locus.

Keywords: CTCF, inner ear development, neurogenesis, Neurog1

INTRODUCTION

The inner ear is a complex sensory organ composed of five sensory patches specialized for hearing and balance. The organ of Corti in the cochlea is responsible for hearing. In the vestibule, three cristae (anterior, lateral, and posterior) detect angular movements and two maculae (utricle and saccule) detect linear acceleration. These five sensory patches house mechanosensory hair cells that recognize and convert the mechanical stimuli originating from sound or angular/linear movements into electrical signals. These signals are then conveyed to neurons of the cochlear and vestibular ganglia, which transmit the signals to the brain. Formation of a functional inner ear requires tight regulation of genes important to developmental processes, including fate determination, differentiation, and morphogenesis (Wu and Kelley, 2012).

Recently, epigenetic regulations have been shown to play important roles in development and disease (Ong and Corces, 2014). Epigenetic mechanisms influence gene expression patterns by modulating DNA methylation status and histone modifications. An essential epigenetic component, CCCTC-binding factor (CTCF) plays a primary role in the global organization of chromatic architecture. CTCF has been implicated in various cellular processes, including transcriptional regulation, insulation, X chromosome inactivation, and RNA
splicing (Ong and Corces, 2014; Phillips and Corces, 2009). Furthermore, CTCF has been found to play crucial roles in limb and brain development: Specific deletion of Ctcf in the mesenchyme of a developing limb was found to cause massive cell death, resulting in a loss of limb structures (Soshnikova et al., 2010). In addition, CTCF was shown to play essential roles during early cortex formation by balancing cell proliferation and differentiation and by promoting cell survival of neuroprogenitors (Watson et al., 2014). Further, in postmitotic neurons in the telencephalon, Ctcf deficiency elicited alterations in Pcdh gene expression that led to defects in dendritic arborization and spine density (Hirayama et al., 2012). The function of CTCF, however, in inner ear development has not been examined.

In this study, we applied a conditional knockout (cKO) mouse system to determine the role of CTCF in inner ear development. Our results highlight an essential association between CTCF and otic neurogenesis and posit CTCF as a crucial homeostatic regulator of inner ear by regulating a master neurogenic regulator, Neurog1.

**MATERIALS AND METHODS**

**Animals**

The mice utilized in this study carried a conditional Ctcf allele (Ctcf<sup>fl/fl</sup>) was described in our previous study (Kim et al., 2015). Ctcf<sup>fl/fl</sup> mice were bred with Tg(Pax2-Cre<sup>1Akg/Mmnc</sup>) (Pax2-Cre; MMRRC, NC) mice to obtain inner ear specific conditional knockout mutants (Ohyama and Groves, 2004). All animal protocols were approved by the Institutional Animal Care and Use Committee at Yonsei University College of Medicine.

**Paint-fill injection, in situ hybridization, and immunostaining**

Paint-fill analysis, in situ hybridization, and immunostaining were performed as described previously (Morsli et al., 1998; Watson et al., 2014).

**Microarray**

Total RNA was extracted from control and Ctcf<sup>cKO</sup> otocysts at E10.5 using TRIZOL reagent according to the manufacturer's instructions (Invitrogen, USA). Sample preparation and
microarray data analyses were performed as described previously (Kim et al., 2015). The microarray data were validated by qPCR. The primers used for qPCR are listed in Supplementary Table S3.

Cell culture
P19 cells were maintained in alpha minimum essential medium (Welgene, Korea) supplemented with 10% heat-inactivated fetal bovine serum (Welgene) and 100 U/ml of penicillin/streptomycin (Welgene).

Chromatin immunoprecipitation (ChIP)
ChiP assays were performed as described previously (Park et al., 2016). The sequences of the primers for the Neurog1 locus are listed in Supplementary Table S4.

RESULTS
Ctcf deficiency causes severe developmental defects of the inner ear
To investigate the role of CTCF in inner ear development, we conditionally deleted Ctcf in the otic epithelium by crossing Ctcffl/fl mice with Pax2-Cre mice (Ohyama and Groves, 2004). The inner ears of Pax2-Cre; Ctcffl/fl (Ctcf cKO) embryos were severely malformed, such that no discernible vestibular structures were observed, except for the posterior canal/ampulla, and the cochlear duct was shortened without its typical coiled structure (Figs. 1A-1C vs Figs. 1D-1F).

As stated above, inactivation of Ctcf in the developing limb and brain has been shown to cause massive cell death, leading to loss of tissue and structures (Soshnikova et al., 2010; Watson et al., 2014). We, thus, sought to determine whether the morphological defects observed in Ctcf-deficient inner ears (Fig. 1) were due to increased cell death. Compared to controls, TUNEL-positivity (marker of apoptosis) greatly increased in the absence of functional CTCF, as reflected by fewer thymidine analog 5-ethynyl-2'-deoxyuridine (EdU)-positive cells among the otocysts (Figs. 1J and 1N, white dotted line). Moreover, cell proliferation decreased in the absence of functional CTCF, as reflected by lower thymidine analog 5-ethynyl-2'-deoxyuridine (EdU)-positive cells among the otocysts (Figs. 1J and 1N, white dotted line). These results suggest that CTCF plays an important role in the promotion of cell survival and cell proliferation during inner ear development, which may account for the morphological defects observed in the Ctcf cKO inner ears.

Spiral ganglion neurons are lost in the inner ears of Ctcf-deficient mouse embryos
We then examined whether development of the sensory patches or spiral ganglion neurons residing within the inner ear are compromised in the malformed inner ears of Ctcf cKO mouse embryos. We used Bone morphogenetic protein 4 (Bmp4) and Lunatic fringe (Lfng) as molecular markers for cristae and maculae in the developing vestibule, respectively (Figs. 2A and 2E) (Morsli et al., 1998). In Ctcf cKO ears, Bmp4 expression was observed in the posterior crista region (Fig. 2D, arrow), and Lfng expression was observed in the anterior edge (Fig. 2G). We also used Bmp4 and Lfng to delineate the lateral epithelial ridge and the organ of Corti in the cochlea, respectively (Figs. 2B and 2F). We found that Bmp4 and Lfng were expressed in severely malformed mutant cochlea (Figs. 2D and 2H, arrowheads). We also observed that Atoh1, a master regulator for hair cell specification, was expressed in the sensory patches of malformed inner ears (Figs. 2K and 2L). These results suggest that although loss of CTCF severely affects inner ear morphogenesis, sensory specification is preserved.

Next, we sought to determine whether the development of spiral ganglion neurons is affected by the loss of CTCF. Foxg1, which is expressed in the delaminating spiral ganglia (Fig. 2N, arrows) and the medial side of cochlear epithelium (Fig. 2N, arrowheads), was used as a neuronal marker (Pauley et al., 2006). In Ctcf cKO ears, while Foxg1 expression in the cochlear epithelium was detected (Fig. 2P, arrowheads), it was completely downregulated in the mesenchymal region where spiral ganglion neurons are normally localized (Fig. 2P, red asterisk), indicating a complete loss of spiral ganglion neurons in the absence of CTCF. These results suggest that CTCF is required for the differentiation or survival of spiral ganglion neurons in the developing inner ear.

Changes in gene expression upon Ctcf deletion in otocysts
The complete lack of spiral ganglion neurons in the inner ears of Ctcf-deficient mouse embryos prompted us to perform microarray-based gene expression analyses to identify genes regulated by CTCF in otocysts at E10.5. Among 279 genes exhibiting significantly different expression levels between control and mutant otocysts (≥ two-fold, p < 0.01), 269 genes were downregulated and 10 genes were upregulated (Fig. 3A; Supplementary Table S1). When subjected to DAVID functional annotation clustering analysis, genes differentially expressed in the otocysts from Ctcf-deficient embryos were enriched primarily in neurogenesis-related clusters, such as neuron projection (clusters 1 and 2), neuron differentiation (clusters 2 and 4), and neuron development (clusters 2 and 4) (Supplementary Table S2). Consistent with our microarray results, quantitative real-time PCR (qPCR) revealed significant reductions in the expression levels of selected genes, including Ctcf, Neurog1, and Neurod1, but not Tbx1, in Ctcf cKO otocysts, compared to controls (Figs. 3B-3E).

Additionally, we analyzed the expression domains of the genes significantly altered by loss of Ctcf with immunofluorescence and in situ hybridization. Immunofluorescence signals for CTCF were specifically decreased in the otic epithelium of Ctcf cKO otocysts, but not the surrounding mesenchyme (Figs. 3F and 3G). The expression domains of Neurog1 and Neurod1 were greatly decreased in Ctcf cKO otocysts (Figs. 3H-3K). Moreover, the expressions of Nhlh1, 6tm2, and Dcx, which were expressed in delaminating neuroblasts, were also greatly reduced in Ctcf cKO otocysts (Supplementary Fig. S1). These results suggest that CTCF plays an essential role in neurogenesis in the otocyst by regulating genes important for early neurogenesis.

CTCF is required for the expression of neurogenic, but not prosensory, genes
In light of the above, we wondered whether the gene
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Expression changes arising in the absence of CTCF are specific to neurogenic genes. When examined by whole-mount in situ hybridization, the expression domains of Neurog1 and Neurod1, which are normally observed in the anteroventral aspects of the otocyst at E9.5 (Figs. 3N and 3P; black bracket and white dotted lines), were greatly decreased in the Ctcf cKO otocysts (Figs. 3O and 3Q, red brackets and dotted lines). In contrast, expression of Tbx1, which is normally expressed in the posterior otocyst and inhibits neurogenesis by suppressing the expression of neurogenic genes (Raft et al., 2004), was not altered in the mutant otocysts (Figs. 3R and 3S). This result indicates that the compromised neurogenesis noted in Ctcf-deficient otocysts is not due to misregulation of Tbx1. In addition, the expressions of genes important for sensory specification, such as Sry-related HMG-box 2 (Sox2) and Lfng, were also generally unaffected in Ctcf cKO otocysts (Figs. 3T-3W; arrows and dotted lines). These results indicate that, in the Ctcf-deficient otocyst, otic neurogenesis is affected most likely by specific downregulation of neurogenic genes, such as Neurog1 and Neurod1, but not by global misregulation of genes important for inner ear development.

Regulation of Neurog1 gene expression by CTCF is associated with changes in histone modification

Our analyses of the inner ear from Ctcf cKO mouse embryos showed that CTCF is required for the expression of genes associated with neurogenesis, including Neurog1. To investigate the role of CTCF in the regulation of Neurog1 during neuronal differentiation, we knocked down CTCF expression in P19 embryonal carcinoma cells, which differentiate into a neuronal fate upon treatment with RA through Neurog1 induction (Kim et al., 2004). In the Ctcf-diminished P19 cells, upregulation of Neurog1 expression by RA treatment was significantly compromised, compared to control cells (Fig. 4B, P < 0.01). These results indicated that CTCF is required for
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Fig. 3. Otic neurogenesis is decreased in Pax2-Cre; Ctcffl/fl otocysts. (A) Heat map represents the relative mRNA expression levels analyzed by microarray analyses from Pax2-Cre; Ctcffl/+ (control) and Pax2-Cre; Ctcffl/fl (Ctf cKO) otocysts at E10.5. (B-E) Validation of gene expression levels by quantitative real-time PCR. *p < 0.05; ***p < 0.001. (F, G) Immunostaining with anti-CTCF antibody in E10.5 otocysts. CTCF-positive cells (green) were greatly reduced in Pax2-Cre; Ctcffl/fl, compared with Pax2-Cre; Ctcffl/+ otocysts. White asterisk indicates loss of otic neuroblasts. (H-M) Reduced expression of Neurog1 and Neurod1 (I, K; red asterisk), but not Tbx1 (M), in Pax2-Cre; Ctcffl/fl otocysts was confirmed by in situ hybridization at E10.5. Red dotted line in the left upper diagram indicates section plane for in situ hybridization. (N-W) Expression of Neurog1, Neurod1, Tbx, Sox2, and Lfng was examined by whole-mount in situ hybridization in Pax2-Cre; Ctcffl/+ (N, P, R, S, T, V) and Pax2-Cre; Ctcffl/fl (O, Q, S, U, W) embryos at E9.5. Expressions of Neurog1 and Neurod1, which were observed in the anterior otic vesicle and delaminating neuroblasts in Pax2-Cre; Ctcffl/+ otocysts (N, P; black bracket and dotted line), were severely reduced in Pax2-Cre; Ctcffl/fl otocysts (O, Q; red bracket and dotted line). (R-W) Expression of Tbx1 in the posterior otocyst (R, S) and Sox2 and Lfng in the anterior otocyst was observed in both Pax2-Cre; Ctcffl/+ (T, V; arrow and dotted line) and Pax2-Cre; Ctcffl/fl (U, W; arrow and dotted line) embryos. Scale bars (F-M), 100 μm; (N-W), 50 μm.
Neurog1 expression in P19 cells in vitro and that the P19 neuronal differentiation model is suitable for studying the regulatory mechanism of CTCF in relation to Neurog1 expression.

Genome-wide CTCF occupancy patterns, which have been mapped across more than 100 mammalian cell types, revealed prominent CTCF binding to the -14.4 kb and -7.6 kb transcription start sites of Neurog1 (Fig. 4A). We performed ChIP-qPCR analysis to validate the in vivo binding of CTCF in P19 cells, and observed strong occupancy of CTCF to these binding sites (Fig. 4C). RA treatment did not abolish this pattern of CTCF binding to the Neurog1 locus, suggesting that CTCF binds to these binding sites constitutively, regardless of Neurog1 expression (Fig. 4C).

As histone modification contributes to distinct chromatin states and gene expression, we attempted to determine whether CTCF is involved in histone modifications of the Neurog1 locus during neuronal differentiation of P19 cells. In control cells, the enrichment of H3K27Ac, an active enhancer marker, was significantly increased at the Neurog1 promoter, as well as other enhancers, upon RA treatment. However, under the same conditions, H3K27Ac levels at the Neurog1 promoter prominently decreased in CTCF-deficient P19 cells (Fig. 4D). These results suggest an epigenetic dependence on Neurog1 expression and that neuronal differentiation is regulated by H3K27 acetylation in the Neurog1 locus.

**DISCUSSION**

In this study, by analyzing inner ear-specific Ctcf knockout mouse embryos, we discovered that CTCF plays essential roles in inner ear development. The Ctcf-deficient inner ears exhibited severe morphological defects both in the vestibule and cochlea. Interestingly, we observed that, while sensory specification was preserved in the malformed inner ears,
neuronal specification was greatly reduced. The compromised neurogenesis was due to downregulation of neurogenic gene expression, particularly Neurog1 and Neurod1, in the inner ear primordium. Moreover, we found that CTCF regulates Neurog1 expression by directly binding to enhancer sequences at the Neurog1 locus and by modulating histone modifications.

Neurosensoy specification in the anterior aspect of the inner ear primordium is one of the earliest and most important processes in inner ear development (Wu and Kelley, 2012). This specification establishes the anteroposterior axis of the developing inner ear, which guides the subsequent development of sensory patches of the inner ear. The anterior crista and utricular macula are specified within the neurosensory domain, whereas the posterior crista is specified outside the domain. This neurosensory specification is directed by anteriorly restricted expression of genes important for sensory specification, such as Sox2 and Lfng, and for neurogenesis, such as Neurog1 and Neurod1 (Bok et al., 2007). Since the expression domains for these neurogenic and prosensory genes overlap in the anterior aspect of the otocyst and since the two specification processes occur simultaneously during early stages of inner ear development, one could postulate that the expressions of these genes would be regulated by a common mechanism. However, our results demonstrated that, in Ctcf-deficient otocysts, while the expressions of neurogenic genes, such as Neurog1, Neurod1, Stmn2, and Dcx, were greatly reduced, those important for sensory specification, such as Sox2 and Lfng, were generally unaffected (Figs. 1 and 3; Supplementary Fig. S1). These results suggest that these two specification processes are regulated independently from one another and occur as separate events.

Epigenetic regulations in inner ear development have recently been reported. In the inner ear of chickens, epigenetic modifiers, such as the DNA methyltransferase DNMT3A and the histone demethylase KDM4B, were shown to be important for proper formation and invagination of the otic placode, respectively (Roellig and Bronner, 2016; Uribe et al., 2015). In the present study, we demonstrated that the chromatin architecture protein CTCF also plays a critical role in otic neurogenesis by regulating Neurog1 gene expression. In particular, histone acetylation in the promoter and enhancer sequences of the Neurog1 locus was found to be closely associated with CTCF. Given the constitutive binding of CTCF in the Neurog1 locus, we suggest that the observed induction of Neurog1 expression upon RA treatment would require other regulatory factors, which remains to be elucidated in future studies.

In our Ctcf cKO mutants, the shapes of the otocysts were relatively normal, from which we discerned that CTCF does not seem to be involved in placodal induction and invagination. The normal otocyst formation could be due to late onset of Cre recombinase expression in the developing inner ear, which is driven by the Pax2-Cre transgene in the Ctcf cKO mutants. However, since Cre recombinase activity is detected in the otic placode from the 6-7 somite stages in Pax2-Cre mice (Ohyama and Groves, 2004), CTCF does not appear to play a major role in placodal specification and invagination. In addition, in the mammalian inner ear, the epigenetic states of histone modifications in the Atoh1 locus are dynamically changed during hair cell differentiation and maturation (Stojanova et al., 2015). However, we observed Atoh1 expression in the malformed Ctcf cKO inner ears, suggesting that CTCF does not play a major role in Atoh1 expression in the developing inner ear.

Depletion of Ctcf has been shown to increase cell death in the limb and brain (Hirayama et al., 2012; Soshnikova et al., 2010; Watson et al., 2014). Consistent therewith, we also observed massive cell death among the otocysts of Ctcf cKO mouse embryos (Fig. 2). Meanwhile, although upregulation of p53-dependent transcriptional activation of Puma has been observed in the Ctcf-deficient brain (Watson et al., 2014), the levels of Puma in the present study were not altered in the Ctcf cKO embryos upon microarray analysis, suggesting that the mechanism of cell death elicited by the Ctcf loss is tissue specific. In addition to increased cell death, we also observed decreased cell proliferation in the Ctcf-deficient otocyst (Fig. 2). This could be secondarily caused by downregulation of Neurog1, which was previously shown to promote cell proliferation by regulating Cdk2 in immortalized multipotent otic progenitor cells (Song et al., 2017). These results suggest that CTCF is required for cell survival and proliferation in the otocyst, which may account for the abnormal morphogenesis noted in the inner ear of Ctcf-deficient mouse embryos.

In summary, we identified a role for CTCF during inner ear development by analyzing developing inner ears of Ctcf-deficient mouse embryos. Further study is required to fully elucidate the underlying mechanism of how CTCF controls inner ear development by modulating chromatin architecture and also to examine the possible roles of CTCF in the maintenance and hearing functions of the mature inner ear.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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