SoxC transcription factors are essential for the development of the inner ear

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Hair cells, the mechanosensory receptors of the inner ear, underlie the senses of hearing and balance. Adult mammals cannot adequately replenish lost hair cells, whose loss often results in deafness or balance disorders. To determine the molecular basis of this deficiency, we investigated the development of a murine vestibular organ, the utricle. Here we show that two members of the *SoxC* family of transcription factors, *Sox4* and *Sox11*, are down-regulated after the epoch of hair cell development. Conditional ablation of *SoxC* genes in vivo results in stunted sensory organs of the inner ear and loss of hair cells. Enhanced expression of *SoxC* genes in vitro conversely restores supporting cell proliferation and the production of new hair cells in adult sensory epithelia. These results imply that *SoxC* genes govern hair cell production and thus advance these genes as targets for the restoration of hearing and balance.

auditory system | cochlea | hair cell | utricle | vestibular system

Unlike mammals, nonmammalian vertebrates can regenerate hair cells effectively throughout life and thus recover from hearing and balance deficits (1). The discovery of the ear's regenerative potential in avian species (2–4) initiated a wave of studies directed toward understanding the molecular basis of hair cell regeneration and the deficiency of this process in mammals. Two distinct mechanisms of regeneration have emerged (5). The first involves the production of hair cells by the transdifferentiation of supporting cells, which are the epithelial cells that separate and provide metabolic support for hair cells (6–8). A rudimentary form of this process occurs in mammals (9, 10). A limitation of this pathway, however, is that transdifferentiation depletes the population of supporting cells and thereby interferes with the ability of sensory organs to function properly (11). The second mode of regeneration involves supporting cell proliferation, which restores both hair cells and supporting cells. Prevalent in the auditory sensory epithelia of nonmammalian species, this mechanism allows functional recovery (5). The corresponding mechanism is absent in mammals, however, and little is known about the molecular events involved (12, 13).

In the sensory epithelia of the mammalian inner ear, the ability to restore hair cells after trauma declines late in development, largely as a result of the diminished proliferative capacity of supporting cells (10). Reasoning that this transition should be reflected by differences in the expression of genes involved in proliferation, differentiation, and regeneration, we investigated the genes expressed late in the development of the murine utricle. With a simple architecture and just under 4,000 hair cells in an adult animal (14), the sensory epithelium of the utricle—the macula—represents a useful model system. Although gene expression has been characterized in early otic development (15, 16) and in the neonatal organ of Corti (17, 18), corresponding data are lacking for the developing utricle.

Results

Chronology of Diminishing Proliferative Capacity in the Ear. To assess the time course of the ear's decline in proliferative ability, we dissected utricles from mice at embryonic day 17.5 (E17.5) through postnatal day 9 (P9). By culturing the organs in medium supplemented with 5-ethynyl-2′-deoxyuridine (EdU), we labeled the proliferating cells at each stage. In accord with previous results (14), we observed proliferating cells at the periphery of the utricular macula and in the striola, the organ's central region (Fig. S1 *A*–*C*). In keeping with previous reports, we term these proliferating cells "supporting cells"; however, we cannot exclude the possibility that proliferation was confined to sensory-progenitor cells or a subset of the morphologically defined supporting cells. The number of proliferating cells decreased by 80% at P2 and by 93% at the end of the first week of life (Fig. S1*D*). These results indicate that the proliferative potential of supporting cells in the utricle is largely lost within 48 h after birth.

Genes Involved in the Development of the Utricular Macula. To identify the transcriptional changes responsible for silencing proliferation and hair cell production, we conducted RNA sequencing to compare the genes expressed before and after the cessation of cellular proliferation and hair cell differentiation. We developed a method for isolating utricular sensory epithelia and prepared sequencing libraries at E17.5, P0, and P9 (Fig. 1*A*). To determine the consistency of our results, we analyzed three independent samples at each developmental stage. Principal-component analysis demonstrated a high degree of similarity among the samples at each stage but a significant difference between stages (Fig. 1*B*). These distinctions also were reflected upon hierarchical clustering of the differentially expressed genes (Fig. 1*C*), which revealed that groups of genes were up- or down-regulated to similar levels in all three samples at each stage and that the samples from P0 exhibited expression patterns intermediate between those from E17.5 and P9 mice. Gene-ontology

Significance

Hair cells, the sensory receptors of the inner ear, underlie our ability to hear and maintain balance. In mammals, these cells are formed by birth, and they cannot be restored through regeneration. Mammals therefore lack the capacity to recover hearing and balance after the loss of hair cells. By assessing gene expression during inner ear development in mice, we identified several genes that are downregulated at the cessation of hair cell production. We demonstrated that two of these genes—*Sox4* and *Sox11*—are necessary and sufficient for the production of hair cells in the sensory epithelia of the inner ear. Our data suggest that *Sox4* and *Sox11* represent targets in the development of therapies for deafness and disequilibrium.

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Data deposition: The sequences reported in this paper have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc (accession no. GSE72293).

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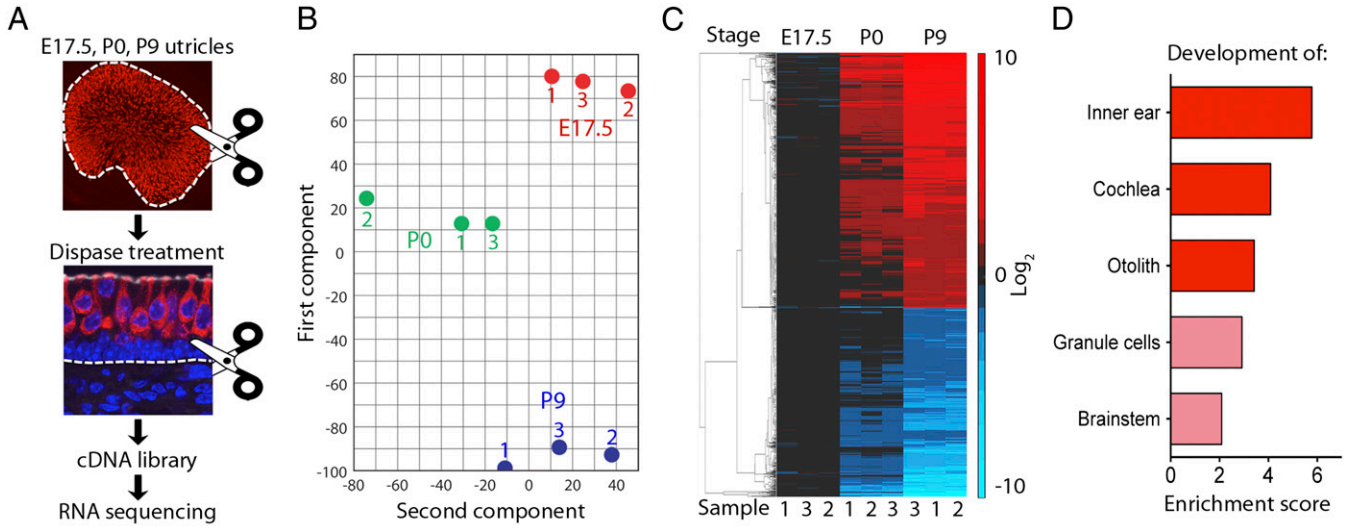

Fig. 1. Transcriptional changes in late utricular development. (*A*) In a schematic representation of the protocol for isolation of the utricular macula and preparation of cDNA libraries, the white dashed lines represent the borders between the macular epithelium and the nonsensory tissues that were removed by microdissection and enzymatic digestion. Hair cells are immunolabeled for Myo7A (red) and nuclei are stained blue. (*B*) Principal-component analysis of the RNA-sequencing results for nine samples reveals that the first two components capture 56% (ordinate) and 14% (abscissa) of the variance in the set of samples from E17.5, P0, and P9 mice. (*C*) In a hierarchical-clustering analysis of transcriptional changes in the developing utricle, the vertical rows represent the nine samples in *B*. Each horizontal stripe represents a gene whose level of expression changed at least twofold between E17.5 and P9 ($P < 0.05$). Each expression level is normalized to the average value for that gene at E17.5. Increased expression is indicated in shades of red and decreased expression in blue. (*D*) According to gene-ontology analysis, the principal categories up-regulated between E17.5 and P9 are associated with the ear's development. $P < 0.05$ for the top three categories.

analysis revealed that the greatest enrichment occurred for transcripts known to be involved in inner ear development (Fig. 1*D*).

Many of the genes that showed at least a twofold change in expression between E17.5 and P9 already have been found to play roles in hair cell development. The cognate proteins include constituents of the Notch pathway such as Hey1 and Hes6 (19, 20) and members of the Wnt cascade such as the G-protein–coupled receptor Lgr5 (21, 22). On the basis of gene function—stem cell maintenance, cell-cycle progression, tumor suppression, and neurogenesis—we selected 31 transcription factors that might be expected to influence the regenerative capacity of sensory epithelia (Fig. S2*A*). To validate the RNA-sequencing data, we performed quantitative PCR (qPCR) for selected genes with RNA isolated from E17.5 and P9 mice. Because of its declining expression in developing sensory epithelia (21, 22), we chose *Lgr5* as a positive control for our validation. qPCR confirmed changes in the level of

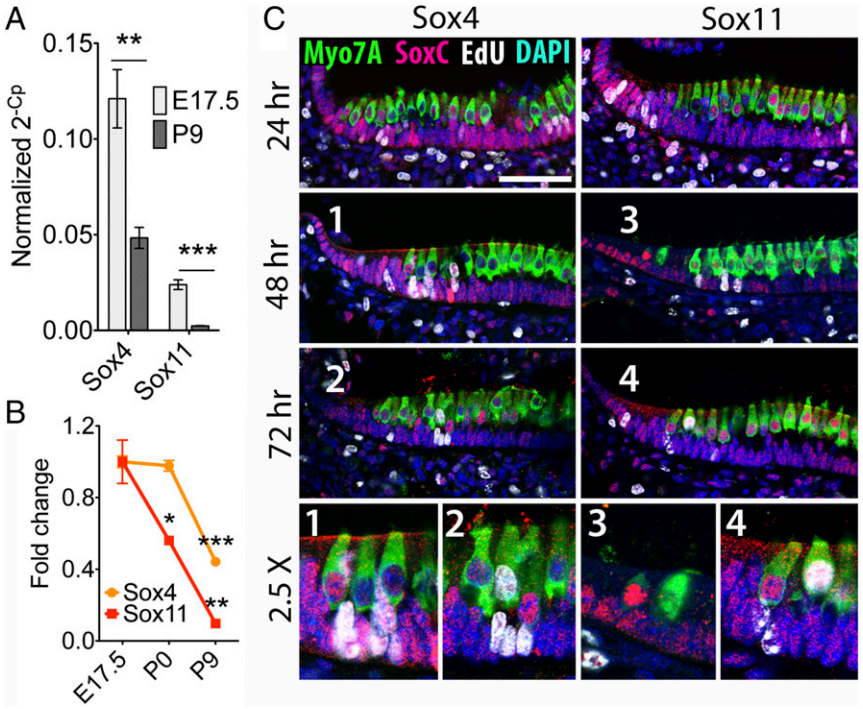

Fig. 2. *SoxC* expression in the developing sensory organs of the inner ear. (*A*) qPCR confirms the decrease in expression of *SoxC* genes between E17.5 and P9. The significance of the change in expression is $^{**}P = 0.0012$ for *Sox4* ($n = 6$) and $^{***}P = 0.0001$ for *Sox11* ($n = 6$). (*B*) Normalized to the RNA-sequencing data from E17.5 mice, the fold changes in the number of reads per kilobase of exon per million fragments mapped emphasize the delay in the down-regulation of *Sox4* relative to *Sox11*. The change is significant at P0 for *Sox11* ($^{*}P = 0.023$, $n = 3$) and at P9 for both *Sox4* ($^{***}P = 0.0001$, $n = 3$) and *Sox11* ($^{**}P = 0.0017$, $n = 3$). (*C*) Twenty-four hours after injection, EdU labeling and antibody labeling for Sox4 and Sox11 (red) demonstrate that these proteins occur in actively proliferating supporting cells (white) at the periphery of each utricular macula. Some labeled hair cells (green) are newly formed by the criteria of EdU labeling, peripheral localization, absence of mature hair bundles, and weak immunolabeling for Myo7A. Representative *SoxC*-expressing hair cells are shown in the bottom images at a further enlargement of 2.5×. Nuclei are labeled blue. (Scale bar, 50 μm.)

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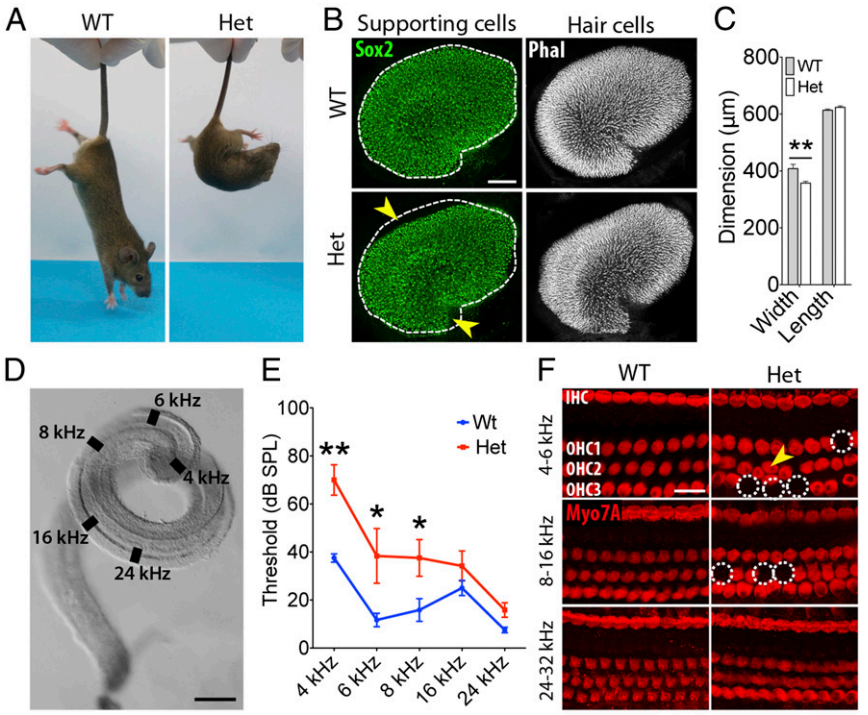

Fig. 3. Vestibular dysfunction and deafness in heterozygous *SoxC* CKO mice. (*A*) In the tail-suspension test, a 4-wk-old WT mouse reaches downward for the substrate, whereas a heterozygous *SoxC* CKO animal (Het) displays ataxic curling. (*B*) In utricles from 4-wk-old WT and Het animals, supporting cells are immunolabeled with anti-Sox2 (green), and hair bundles are revealed by phalloidin labeling (white). Fitting the outline of the WT macula (dashed line) to the mutant's macula demonstrates a decrease in the organ's width, which was quantified by measuring the distance across the organ at the notch on its neural edge and perpendicular to its long axis. (Scale bar, 100 μm.) (*C*) Measurements confirm that the difference observed in *B* is statistically significant and corresponds to a 13% decrease in the organ's width (**$P = 0.0076$) (WT $n = 5$, Het $n = 6$). (*D*) A dissected murine cochlear duct shows the anatomical positions of the hair cells most sensitive to the frequencies at which auditory brainstem responses were measured. (Scale bar, 250 μm.) (*E*) Heterozygous *SoxC* CKO mice ($n = 6$) display a significant elevation in their thresholds for auditory brainstem responses with respect to WT animals ($n = 6$). *$P < 0.05$, **$P < 0.001$. (*F*) Immunolabeling of a WT ear for Myo7A (red) reveals a regular hair cell array with one row of inner hair cells (IHC) and three rows of outer hair cells (OHC1, OHC2, and OHC3) in the apical (4–6 kHz and 8–16 kHz) and middle (16–24 kHz) cochlear turns. In a heterozygous *SoxC* CKO animal, in contrast, some hair cells in the apical turn are missing (dotted circles), and others are misaligned (yellow arrowhead). (Scale bar, 25 μm.)

expression for 25 of the transcription factors, suggesting that they participate in inner ear development (Fig. S2*B*).

Expression of *Sox4* and *Sox11* in the Mammalian Inner Ear. As shown by RNA sequencing and confirmed by qPCR analysis, two *SoxC* genes were highly expressed in the developing utricular macula at E17.5. Their expression was strongly down-regulated by P9, *Sox4* by 50% and *Sox11* by 90% (Fig. 2*A*). In situ hybridization demonstrated a similar pattern of expression for both genes. At E10.5, *Sox4* and *Sox11* were present throughout the prosensory domains of the otic vesicle and in the hindbrain (Fig. S3*A*). The intense expression also was detected at E14.5 in all the developing sensory organs of the inner ear and in the spiral ganglion (Fig. S3 *B* and *C*). At E17.5 the intense signals were restricted to the actively growing edges of the vestibular maculae (Fig. S3*B*). The cochlea manifested strong expression of *SoxC* genes throughout the organ of Corti and in the spiral ganglion (Fig. S3*C*). Although *Sox4* expression remained robust until P2, *Sox11* expression was markedly down-regulated in the utricular macula and the organ of Corti. Both genes remained highly expressed in the spiral ganglion during this period. The difference in the timing of down-regulation for *Sox4* and *Sox11* was corroborated by RNA sequencing: When normalized to E17.5 expression levels, *Sox4* expression remained unchanged at P0, whereas *Sox11* expression declined significantly by 50% (Fig. 2*B*). By P9 we could no longer detect RNA for either gene by in situ hybridization.

To investigate the relationship between the expression of *Sox4* and *Sox11* and the production of new hair cells, we injected pregnant mice with EdU at E16.5 and analyzed the inner ears of progeny 24, 48, and 72 h later. Using antibody labeling for SoxC proteins, we found that 24 h after EdU injection the periphery of the macula and the striolar region contained actively proliferating progenitor cells that robustly expressed both Sox4 and Sox11 (Fig. 2*C*). Young EdU$^+$ hair cells, which also strongly expressed the two transcription factors, appeared by 48 h after EdU injection. The expression of SoxC proteins was down-regulated in mature hair cells. In light of the neurogenic role of *SoxC* genes (23, 24), these findings suggest that Sox4 and Sox11 are involved in the regulation of cell proliferation and hair cell formation in the developing sensory organs of the mammalian inner ear.

Vestibular Dysfunction and Deafness in *SoxC*-Deficient Mice. The overlapping expression patterns and functional redundancy of *SoxC* genes allow some members of the gene family to compensate in part for deficiencies in others (25). We therefore generated mice with inner ear-specific conditional knockout (CKO) of both *Sox4* and *Sox11* genes by crossing *Pax2-Cre* mice with *Sox4*$^{fl/fl}$ *Sox11*$^{fl/fl}$ *Sox12*$^{-/-}$ animals (26–28). The phenotypic effects of Sox12 are known to be weak (29), and our RNA-sequencing data showed an insignificant expression of *Sox12* in the sensory epithelia of the inner ear. We therefore disregarded the *Sox12* genotype in our subsequent analysis.

By 4 wk of age, heterozygous *Sox4 Sox11* double-CKO animals repetitively demonstrated signs of vestibular dysfunction, including circling, head tossing and bobbing, and loss of balance (Movie S1). To characterize this phenotype further, we performed a battery of vestibular and hearing tests (30, 31). In tail-suspension tests we observed frequent trunk curling and propelling behavior in the

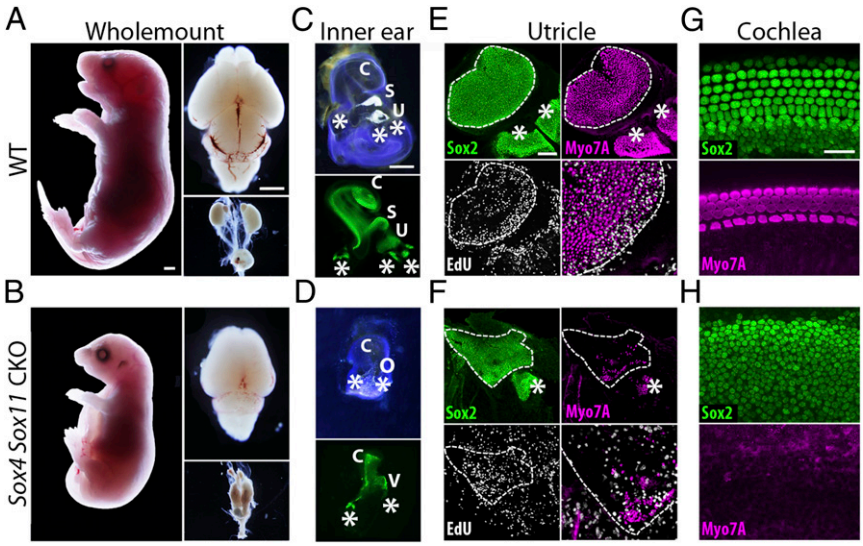

Fig. 4. Anatomical defects in homozygous *Sox4 Sox11* double-CKO mice. (*A*) An E17.5 WT embryo (*Left*), its brain (*Upper Right*), and its kidneys and bladder (*Lower Right*) are presented for reference. (Scale bars, 1 μm.) (*B*) An E17.5 *Sox4 Sox11* double-CKO embryo is substantially smaller and displays microcephaly as well as malformations of the brain and kidneys. (*C*, *Upper*) A whole-mount preparation of the inner ear from a WT mouse shows the cochlea (C), saccule (S), utricle (U), and ampullae (white asterisks) of the posterior (left asterisk), horizontal (middle asterisk), and anterior (right asterisk) semicircular canals. (*Lower*) Immunolabeling for Sox2 (green) reveals the size and position of the sensory epithelia within these organs. (Scale bar, 500 μm.) (*D*) Identical preparations of the inner ear of a *Sox4 Sox11* double-CKO mouse highlight the underdevelopment of the cochlea (C), a single otolithic organ (O), and only two ampullae (white asterisks). (*E*, *Upper Left*) Supporting cells in a WT utricular macula (white outline) and cristae of the anterior and horizontal semicircular canals (white asterisks) are immunolabeled for Sox2 (green). (*Upper Right*) Hair cells are immunolabeled for Myo7A (magenta). (*Lower Left*) The proliferating supporting cells labeled for EdU (white) include a prominent band at the abneural edge of the utricle, shown in 2× enlargement at *Lower Right*. (Scale bar, 100 μm.) (*F*) Identical labeling of the single otolith organ (white outline) and rudimentary crista of a semicircular canal (white asterisk) in a *Sox4 Sox11* double-CKO animal reveals that proliferating supporting cells are not concentrated at the organ's periphery and that hair cells are nearly absent. (*G*) In the organ of Corti of an E17.5 WT embryo, supporting cells are immunolabeled for Sox2 (green), and the four rows of hair cells are immunolabeled for Myo7A (magenta). (Scale bar, 50 μm.) (*H*) The cochlea of an identically labeled *Sox4 Sox11* double-CKO embryo of the same age contains a field of disordered supporting cells and no hair cells.

heterozygous CKO animals but not in their WT littermates (Fig. 3*A* and Movie S2). This result suggests that there are defects in the utricle and saccule, the organs that detect static position. In contrast, we detected no difference in contact righting; the organs that sense rotation, the cristae of the semicircular canals, apparently remained functional. Although the gross appearance of the inner ear was relatively normal in the heterozygous CKO animals, we noticed an aberrant shape and a minor but statistically significant reduction in the size of the vestibular organs (Fig. 3*B*). The utricles of heterozygous CKO mice were 6% smaller in area than those of WT littermates, a difference caused by a consistent reduction in organ width (Fig. 3*C*).

We evaluated the hearing of heterozygous CKO mice by recording auditory brainstem responses to stimulation at 4–24 kHz, frequencies represented in the apex and middle turn of the organ of Corti (Fig. 3*D*) (30). The mutant mice displayed a significant 30-dB elevation of hearing thresholds for low-frequency sounds (Fig. 3*E*), implying that they could detect only sounds more than 30-fold louder than the faintest stimuli perceived by their WT littermates. The difference in threshold was smaller at 8 kHz and was insignificant for frequencies of 16–24 kHz. The increased hearing thresholds in the low-frequency range were mirrored by aberrations in the cochlear structure of SoxC-deficient mice: Many hair cells in the apical turn were misaligned or absent, but the defects were less severe in the region subserving 8–16 kHz and were absent from the basal organ of Corti (Fig. 3*F*).

Role of *Sox4* and *Sox11* During Inner-Ear Development. We obtained CKO mice homozygously deficient for various combinations of *SoxC* genes by crossing *Pax2-Cre* $Sox4^{fl/+}$ $Sox11^{fl/+}$ $Sox12^{-/+}$ and $Sox4^{fl/fl}$ $Sox11^{fl/fl}$ $Sox12^{-/-}$ animals (Fig. S4 *A* and *B*). Homozygous *Sox4* or *Sox11* CKO animals exhibited respiratory distress, became cyanotic, and died shortly after birth. In accord with the reported pattern of Cre activity in *Pax2-Cre* mice (26), in situ hybridization demonstrated an absence of *Sox4* and *Sox11* expression in most of the hindbrain and midbrain (Fig. S4*C*). Because the relevant nuclei are located in these brain regions, cardiopulmonary dysfunction likely underlay the neonatal mortality of the homozygous *Sox4* or *Sox11* CKO mutants.

Homozygous *Sox4* or *Sox11* CKO mice were small but appeared anatomically normal (Fig. S5 *A* and *B*). The Pax2-Cre–expressing organs of these mutants—the kidney, midbrain, and hindbrain—were grossly intact. *Sox4 Sox11* double-CKO mice were born dead. Much smaller than WT mice, these animals displayed severe defects including microcephaly and gross anatomical malformations of the brain (Fig. 4 *A* and *B*). They also manifested severe edema, likely caused by greatly reduced kidneys.

We next assessed the development of the inner-ear structures in each variety of CKO mouse. At E18.5 the inner ear of a homozygous *Sox4* or *Sox11* CKO embryo had a relatively normal structure. The cochlea, utricle, saccule, and all three cristae of the semicircular canals were present, but the shapes of the utricle and saccule and the patterns of their otoconia were altered (Fig. 4*C* and Fig. S5 *C* and *D*). The inner ear of a homozygous *Sox4 Sox11* double-CKO animal exhibited severe malformations. The size of the cochlea was markedly reduced in comparison with that of a WT animal, all three semicircular canals were absent, and there were no otoconia in either the utricle or the saccule (Fig. 4 *C* and *D*). Sox2 labeling revealed further irregularities: The organ of Corti was short and broad, only a single sensory patch was seen at the expected location of the utricle and saccule, and the cristae of the anterior and horizontal semicircular canals were replaced by a single rudimentary crista (Fig. 4 *E* and *F*). However, the crista of the posterior semicircular canal appeared only slightly smaller than normal (Fig. 4*D*).

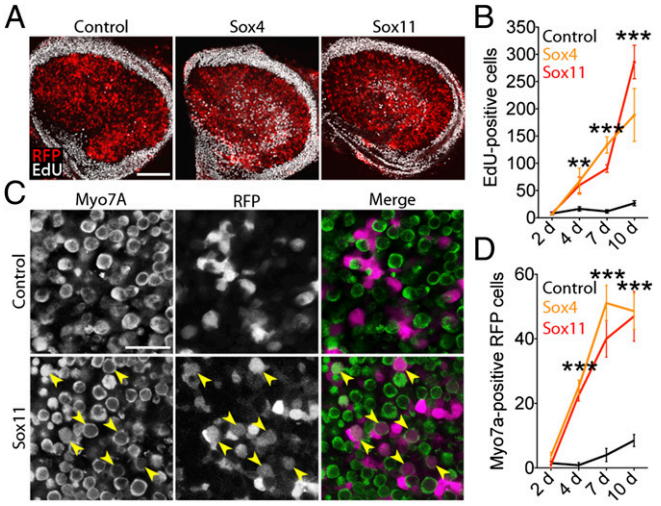

Fig. 5. Effects of viral overexpression of *SoxC* genes in adult utricles. (*A*) In representative images of cultured utricles transfected 7 d previously with control, *Ad-Sox4-RFP*, or *Ad-Sox11-RFP* virus, transfected cells are marked by RFP (red) and proliferating cells are labeled by EdU (white). (Scale bar, 100 μm.) (*B*) The number of EdU$^+$ cells rises significantly in *SoxC*-transfected utricles between 2 d and 10 d in culture. $^{**}P < 0.01$ $^{***}P < 0.001$. (Control $n = 4, 7, 8, 8$; *Sox4* $n = 3, 8, 10, 4$; *Sox11* $n = 3, 3, 10, 4$.) (*C*) In transfected utricles after 7 d in culture, transfected cells are marked by RFP, and hair cells are immunolabeled for Myo7A. Newly formed hair cells displaying both labels occur in the *Sox11*-overexpressing culture (yellow arrowheads) but not in the control preparation. (Scale bar, 20 μm.) (*D*) The number of newly formed hair cells, which are doubly positive for RFP and Myo7A, increases significantly during culture ($^{***}P < 0.001$). (Control n = 5, 6, 5, 5; *Sox4* $n = 5, 5, 7, 5$; *Sox11* $n = 6, 5, 9, 7$.)

To ascertain whether the morphological abnormalities of the vestibular organs in the *SoxC* CKO mice arose from deficiencies in supporting cell proliferation, we labeled proliferating cells with EdU and analyzed the inner ears of E18.5 embryos. As shown previously (14), a band of proliferating supporting cells occurred along the convex abneural periphery of a WT utricle (Fig. 4*E*). In contrast, the utricle of a homozygous *Sox4* or *Sox11* CKO mice was smaller and had a concave abneural edge with a dearth of proliferating cells (Fig. S5 *E* and *F*). The density of hair cells appeared normal. Although the single observable macula of a *Sox4 Sox11* double-CKO mouse was not much smaller than the utricle of a homozygous *Sox4* or *Sox11* CKO animal, the structure of this putative vestibular organ was inconsistent with that of either a utricle or a saccule. In addition, proliferating cells were lacking from the periphery of the organ and hair cells were nearly absent (Fig. 4*F*).

The structure of the organ of Corti in a homozygous *Sox4* or *Sox11* CKO animal was relatively normal but less regular than that of a WT control (Fig. S5 *G* and *H*). The organ's distinctive supporting cells—pillar cells, Deiters's cells, and Hensen's cells—were present but occurred in a disordered array. The hair cells were misaligned, with ectopic hair cells among the inner hair cells and gaps between the outer hair cells. These defects were more prominent in the apical and middle turns and were nearly absent from the basal turn. As in the vestibular macula, the supporting cells were completely disorganized in homozygous *Sox4 Sox11* double-CKO animals; no hair cells or distinctive subtypes of supporting cells were detected (Fig. 4 *G* and *H*).

Restorative Effect of *Sox4* and *Sox11* Overexpression. To address the functional relationship between *SoxC* expression, supporting cell proliferation, and the formation of new hair cells, we overexpressed the two principal *SoxC* genes in the utricular maculae of young adult mice. We developed adenoviral overexpression vectors carrying *Sox4* (*Ad-Sox4-RFP*) and *Sox11* (*Ad-Sox11-RFP*) and confirmed their efficacy in human embryonic kidney cells (Fig. S6). Cultured utricles of P12–P14 mice were infected with either vector and were compared with those infected with the control construct *Ad-RFP* (Fig. 5*A*). After 4 d of culture, the average number of proliferating cells in a macula overexpressing *Sox4* or *Sox11* was fourfold that in a control preparation (Fig. 5*B*). After 10 d a *Sox4*-overexpressing utricle and a *Sox11*-overexpressing macula showed proliferation seven- and 11-fold higher, respectively, than in a control. A similar 10-fold increase in the number of proliferating supporting cells was observed in the utricles of 6-wk-old mice when *Sox4* or *Sox11* was overexpressed for 10 d.

To test whether the mitogenic effect of SoxC proteins was specific, we overexpressed a member of the related SoxE family of transcription factors—Sox9—in P12 utricles in vitro. No effect on supporting cell proliferation was observed 2, 4, or 7 d after the infection (Fig. S7). Although after 10 d Sox9-overexpressing utricles showed slightly elevated levels of proliferation (Fig. S7*A*), they contained significantly fewer proliferating cells than SoxC-overexpressing cultures (Fig. S7*B*).

To determine whether overexpression of *SoxC* genes yielded new hair cells, we also analyzed the transfected utricles for expression of Myo7A, a marker of mature hair cells. Because adenovirus of serotype 5 preferentially infects supporting cells (32), any cell doubly positive for RFP and Myo7A likely represented a hair cell newly derived from a transfected supporting cell. Two days after infection utricles contained virtually no doubly positive cells in cultures exposed to *Ad-Sox4-RFP*, *Ad-Sox11-RFP*, or *Ad-RFP* viruses (Fig. 5*C*). By 4 d, however, RFP$^+$ hair cells were seen in the *Sox4*- or *Sox11*-overexpressing utricles but not in the control preparations. After 7 d the number of doubly positive hair cells increased to 13-fold the control value in Sox4-overexpressing cultures and to 10-fold the control value in Sox11-overexpressing cultures (Fig. 5*D*).

Discussion

Here we show that SoxC proteins play an integral role in the development of the vestibular and auditory receptor organs. Our results indicate that, by guiding the proliferation of progenitor cells, SoxC proteins are necessary for the growth of the sensory epithelia of the inner ear. Deletion during development of an increasing number of alleles for the cognate genes produced a progressive decrease in the size of the vestibular organs, ranging from a modest reduction in heterozygous CKO animals to diminutive or absent sensory organs in homozygous *Sox4 Sox11* double-CKO mutants. The decrease in organ size was accompanied by defects in cell proliferation; conversely, overexpression of *SoxC* genes restored proliferation in the sensory epithelia of adult utricles.

Our observations on the mitogenic effects of SoxC proteins are congruent with previously known roles for these transcription factors. *Sox4* and *Sox11* are highly expressed early during murine embryonic development and in the stem cells of adult neural tissues (24, 25). Additionally, the cognate proteins control the survival and proliferation of neural progenitor cells (27). In the hippocampal subgranular zone, for example, Sox4 and Sox11 are strongly expressed in proliferating progenitor cells and newly generated neuroblasts (23). The loss of the two proteins during early neurogenesis results in major cortical defects and a severely stunted or absent hippocampus (23).

Our data additionally suggest that *SoxC* genes play a prosensory role by directing the differentiation of supporting cells into sensory receptors. In the periphery of the utricular macula, *Sox4* and *Sox11* were highly expressed in proliferating progenitor cells and in newly generated hair cells, but their activity declined sharply in maturing sensory receptors. Although severely underdeveloped, vestibular and auditory organs nonetheless were present in homozygous *Sox4 Sox11* double-CKO mice; however, these organs completely lacked hair cells. We also found that the overexpression of *Sox4* or *Sox11* in adult murine utricles resulted in hair cell production through the direct differentiation of infected supporting cells.

Previous research has demonstrated a prosensory role for *SoxC* genes. For example, it has been shown that the activation of *SoxC* expression in the eye increases the production of retinal ganglion cells (24). SoxC transcription factors also directly foster the expression of neuron-specific genes during neurogenesis in the adult hippocampus (23). SoxC proteins support the flexion of DNA and thereby promote the formation of transcriptional enhancer complexes (25). It is possible that Sox4 and Sox11 proteins function upstream of the transcription factor Atoh1, a master regulator of hair cell differentiation (33). We note the presence of multiple SoxC-binding sites in the 3′ enhancer of *Atoh1*, the principal regulatory region for this gene (34).

Our findings demonstrate that the proper functioning of *SoxC* genes is necessary and sufficient for hair cell production in the sensory epithelia of the murine inner ear. Although further investigation of the mechanism of action of SoxC transcription factors in the inner ear is required, these proteins hold great potential as therapeutic targets for hair cell regeneration.

Materials and Methods

Experiments were conducted in accordance with the policies of Rockefeller University's Institutional Animal Care and Use Committee. After dissection of murine utricles (35) and isolation of total RNA (RNeasy Micro Kit; Qiagen), samples were analyzed by single-end, 100-bp sequencing (Illumina HiSeq 2500). In situ hybridization, immunohistochemistry, and qPCR were conducted by standard protocols. The AdEasy Adenoviral Vector System (36) was used to create adenoviral vectors containing the full-length coding sequence of murine *Sox4* or *Sox11* under the control of a cytomegalovirus promoter. The experimental procedures are detailed in *SI Materials and Methods*. The RNA-sequencing data have been deposited under accession code GSE72293 in the Gene Expression Omnibus of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE72293). The primers were designed with PrimerQuest (Integrated DNA Technologies) and are listed in Table S1.

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