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Generation and characterization of *Atoh1-Cre* knock-in mouse line

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Summary

Atoh1 encodes a basic helix-loop-helix (bHLH) transcription factor required for the development of the inner ear sensory epithelia, the dorsal spinal cord, brainstem, cerebellum, and intestinal secretory cells. In this study to create a genetic tool for the research on gene function in the ear sensory organs, we generated an *Atoh1-Cre* knock-in mouse line by replacing the entire *Atoh1* coding sequences with the *Cre* coding sequences. *Atoh1*^{Cre/+} mice were viable, fertile, and displayed no visible defects whereas the *Atoh1*^{Cre/Cre} mice died perinatally. The spatiotemporal activities of Cre recombinase were examined by crossing *Atoh1-Cre* mice with the *R26R-lacZ* conditional reporter mice. *Atoh1-Cre* activities were detected in the developing inner ear, the hindbrain, the spinal cord, and the intestine. In the inner ear, *Atoh1-Cre* activities were confined to the sensory organs in which lacZ expression is detected in nearly all of the hair cells and in many supporting cells. Thus, *Atoh1-Cre* mouse line serves as a useful tool for the functional study of genes in the inner ear. In addition, our results demonstrate that *Atoh1* is expressed in the common progenitors destined for both hair and supporting cells.

Keywords

Math1; *Atoh1*; Cre recombinase; inner ear; hair cells; supporting cells

INTRODUCTION

Atoh1 plays an essential role in the development of the central and peripheral nervous systems and intestine (Akazawa et al., 1995; Ben-Arie et al., 1997; Ben-Arie et al., 2000; Yang et al., 2001). During ear development, the expression of *Atoh1* starts in the otic vesicle at E12.5 but is not observed in the cochlear duct until E13.5 and its expression is limited to a specific group of cells within the cochlear sensory region (Bermingham et al., 1999; Chen et al., 2002). Absence of *Atoh1* results in apoptosis of this specific group of cells and in the failure to form the cochlear sensory epithelium (Ben-Arie et al., 2000; Bermingham et al., 1999; Chen et al., 2002; Woods et al., 2004). Forced expression of *Atoh1* leads to the ectopic formation of hair cells and supporting cells in nonsensory regions of the cochlea (Woods et al., 2004) or in the formation of additional hair cells (Gubbels et al., 2008; Izumikawa et al., 2005; Kawamoto et al., 2003; Zheng and Gao, 2000). Thus, *Atoh1* is necessary and

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sufficient to direct hair cell differentiation. The function of *Atoh1* appears to differ from that of *Ngn1*, another bHLH-class proneural gene during ear development. While *Ngn1* expression biases the progenitor cells to the neural fate, *Atoh1* expression irreversibly drives these progenitor cells to the hair cell fate. It is thought that *Ngn1* and *Atoh1* function antagonistically (Raft et al., 2007).

The early onset of *Atoh1* expression in the ear sensory epithelium and the absence of hair and supporting cell differentiation in the *Atoh1*-null mice suggest that *Atoh1* locus could be ideal for the expression of Cre recombinase at the very beginning of hair cell differentiation. In this study, we have generated the *Atoh1-Cre* knock-in mouse line. By comparing *Atoh1* expression with the expression of the conditional *R26R-lacZ* reporter gene, we have demonstrated that the spatiotemporal expression pattern of *Atoh1-Cre* in the developing ear recapitulates that of endogenous *Atoh1*. Furthermore, we have shown that *Atoh1-Cre* specifically and efficiently deletes loxP-floxed regions in nearly all of the inner ear hair cells as well as in supporting cells of all types though partially. Therefore, the *Atoh1-Cre* knock-in mouse is a suitable Cre-expression strain for gene deletion in the ear sensory epithelia before the segregation of hair and supporting cell fates.

RESULTS AND DISCUSSION

We generated an *Atoh1-Cre* mouse line by replacing the entire *Atoh1* coding sequences with the *Cre* coding sequences (Fig. 1a). The *Atoh1-Cre* mice were confirmed by Southern blotting using an external 3'-probe to identify the 12.5 kb wild type (Fig. 1b, black arrow) and the 7.0 kb *Atoh1-Cre* (Fig. 1b, open arrow) DNA fragments from *EcoRI*-digested genomic DNA. PCR genotyping was also used to identify the 488 bp and 502 bp products in wild type and *Atoh1-Cre* mice, respectively (Fig. 1c). In situ hybridization experiments confirmed the absence of *Atoh1* expression in the sensory region of *Atoh1*-null (*Atoh1*^{Cre/Cre}) cochleae compared to the control cochleae (Fig. 1d, asterisks). The heterozygous *Atoh1*^{Cre/+} mice were viable, fertile and displayed no visible developmental defects though *Atoh1*^{Cre/Cre} mice died soon after birth, which is consistent with previous reports (Ben-Arie et al., 1997; Bermingham et al., 1999; Chen et al., 2002; Woods et al., 2004).

To evaluate the *Atoh1-Cre* recombinase activity and specificity in vivo, we used the *R26R-lacZ* reporter line (Soriano, 1999) to reveal the expression of *Atoh1-Cre* recombinase. Wholemount X-Gal staining for β -galactosidase activities showed the activation of *lacZ* expression by *Atoh1-Cre* recombinase in the hindbrain (Fig. 2a-d, arrows), spinal cord (Fig. 2a-d, arrowheads), intestine (Fig. 2e, arrowhead) and inner ear (Fig. 2f). Within the inner ear, *Atoh1-Cre* recombinase activity was detected in the sensory organs of both the cochlea (Fig. 4c', asterisk) and vestibule (Fig. 2f, arrowheads).

We further investigated the temporal and spatial characteristics of *Atoh1-Cre* activity in developing inner ears by comparing the expression of *Atoh1* and *lacZ* in *Atoh1*^{Cre/+}; *R26R-lacZ* mice. In the developing vestibular sensory regions, *Atoh1* expression was initially detected at E12.5 but no *lacZ* expression was detected at this stage (data not shown). The lag in *lacZ* expression is likely due to the Cre recombinase-mediated recombination event to activate the expression of *lacZ* reporter gene. At E13.5, strong *Atoh1* expression was seen in the cristae of the semicircular canals (Fig. 3a and data not shown) and the maculae of the utricle (Fig. 3b) and saccule (Fig. 3c). From E14.5 to P0, the high level of *Atoh1* expression was maintained in the above vestibular sensory regions (Fig. 3d-l). Correspondingly, the expression of *lacZ* was first detected in the sensory regions of these cristae and maculae at E13.5 (Fig. 3a'-c'). From E14.5 to P0, *lacZ* expression intensified and became more widespread to overlap the regions of *Atoh1* expression (Fig. 3d'-l' and data not shown).

In the developing cochlea, no *Atoh1* and *lacZ* expression was seen at E12.5 (data not shown). At E13.5, faint *Atoh1* signals were first detected in the sensory region of the cochlea at the basal turn (Fig. 4a) while *lacZ* expression was undetectable at this stage (Fig. 4a'). Neither *Atoh1* nor *lacZ* expression was detected at the middle and apical turns of the cochlea at E13.5 (data not shown). At E14.5, *Atoh1* signals were obvious in the sensory regions of the basal and middle turns of the cochlea (Fig. 4b and data not shown), whereas *lacZ* expression was detected only in the basal cochlea (Fig. 4b' and data not shown), a lag likely due to the Cre-mediated activation of *lacZ* reporter. At E15.5, *Atoh1* signals appeared in the sensory region at the apex of the cochlea in addition to the basal and mid-cochlea (Fig. 4c and data not shown) while weak *lacZ* expression became detectable in the mid-cochlea but not in the apex (Fig. 4c' and data not shown). From E16.5 to P0, both *Atoh1* and *lacZ* expression was clearly visible in the sensory region throughout the entire cochlea (Fig. 4d'-f' and data not shown).

To determine the extent of *Atoh1*-Cre recombinase-mediated recombination in the hair cell population, we co-labeled the cochlear and vestibular sections of heterozygous *Atoh1*^{Cre/+}; *R26R-lacZ* mice at P0 with anti-lacZ (green) and anti-MYO6 (red) for the hair cells (Fig. 5). Quantification analysis indicated that the Cre-mediated recombination (*lacZ*+) is observed in hair cells at the percentages of 92.07% ± 5.35% (n=4) in the cochlea, 90.11% ± 3.35% (n=4) in the utricle, 89.88±3.90% (n=4) in the saccule, and 89.06 ± 2.81% (n=4) in the cristae. Additionally, Cre recombinase-mediated recombination events were also detected in Deiter, Pillar, and phalangeal cells in the cochlea (Fig. 5a-a'', arrowheads), and in the supporting cells of the utricle (Fig. 5b-b'', arrowheads), cristae (Fig. 5c-c'', arrowheads), and saccule (Fig. 5d-d'', arrowheads). Quantitative analysis reveals that for every 100 hair cells counted, the number of *lacZ* supporting cells are 59.75 ± 5.25 in the cochlea (n=4), 6.73 ± 2.04 in the utricle (n=4), 15.48 ± 4.23 in the saccule (n=4), and 42.65 ± 9.20 in the cristae (n=4). The significant number of *lacZ* supporting cells suggesting that the onset of *Atoh1*-Cre expression could occur in the common progenitor cells giving rise to hair and supporting cells as well as in progenitors destined for hair cells.

We also asked whether the cells of the *Atoh1*-expressing lineage (*lacZ*+) could give rise to the cochleovestibular ganglion (CVG) neurons. We analyzed the expression of *Atoh1* and *lacZ* in the developing CVG. Similar to *Atoh1* expression pattern detected by in situ hybridization, X-Gal staining of the *Atoh1*^{Cre/+}; *R26R-lacZ* cochlea showed the expression of *lacZ* only in the cochlear sensory region but not in the spiral or vestibular ganglion cells from E13.5 to P9 (Fig. 5e, f and data not shown). Further co-labeling of the *Atoh1*^{Cre/+}; *R26R-lacZ* cochlea section with anti-lacZ (green) and anti-calretinin (red), a marker for spiral ganglion cells and inner hair cells, confirmed this observation (Fig. 5g). Thus, our data suggest that *Atoh1*-expressing cells do not contribute to the CVG neurons. In conclusion, our results demonstrate that the *Atoh1*-Cre knock-in mouse line will be useful in research focused on gene function in inner ear development and disorders as well as the potential uses similarly in spinal cord, cerebellum and intestine.

MATERIALS AND METHODS

Animals

To generate the *Atoh1*^{Cre} knock-in mice, *Atoh1* genomic DNA sequences were isolated from a mouse 129S6 (formally 129SvEvTac) BAC library (CHORI) using *Atoh1* coding sequences as a probe. The *Atoh1*^{Cre} targeting construct was generated by inserting the 2.7 kb of 5'-flanking sequences that ends immediately upstream of the translational initiation codon ATG, and the 3.8 kb of 3'-flanking sequences into the *Bam*HI-*Xba*I and the *Eco*RI-*Not*I sites of the pKII-Cre vector, respectively (Fig. 1). The knock-in construct replaced the entire *Atoh1* coding sequences with those of *Cre* and placed *Cre* under the control of *Atoh1*

regulatory sequences. To generate *Atoh1^{cre}* knock-in mice, a *NotI*-linearized *Atoh1^{cre}* targeting construct was electroporated into W4 embryonic stem (ES) cells (Auerbach et al., 2000). Two targeted ES clones were identified from a total of 288 G418-resistant ES clones. The targeted clones were confirmed by Southern blotting genotyping and injected into C57BL/6J blastocysts to generate mouse chimeras. The heterozygous *Atoh1^{Cre/+}* mice were generated and maintained in a 129S6 and C57BL/6J mixed background. PCR methods were used to genotype mice from subsequent breeding of *Atoh1^{Cre/+}* heterozygotes. The PCR primers used to identify the wild type *Atoh1* mice were Atoh1wt-F1 (5'-TGA CGC CAC AGC CAC CTG CTA-3') and Atoh1wt-R1 (5'-GGA CAG CTT CTT GTC GTT GTTG-3'), and the PCR primers used to identify the cre knock-in allele were Atoh1 Cre-F (5'-GCG CAG CGC CTT CAG CAAC-3') and Atoh1 Cre-R (5'-GCC CAA ATG TTG CTG GAT AGT-3'). *R26R-LacZ* conditional reporter mice were obtained from the Jackson Laboratory (Stock Number 003310) and PCR genotyping of the reporter mice was performed according to protocols provided by the Jackson Laboratory. Embryonic day 0.5 (E0.5) was defined as the day when the vaginal plug was detected. University Committee of Animal Resources at the University of Rochester approved all animal procedures described here. The mouse strains were maintained in the C57BL/6J and 129S6 mixed background.

Immunohistochemistry, In Situ Hybridization and X-Gal Staining

Staged embryos and tissue samples were harvested and immediately fixed in 4% paraformaldehyde (PFA) in PBS for 1-2 hours. The samples were then embedded and frozen in OCT compound (TissueTek) for cryosectioning. For immunohistochemistry staining, cryosections were cut at 18 μ m thickness. The working dilutions and sources of antibodies used in this study were: chicken anti-lacZ (1:500, Abcam #ab9361-250), and rabbit anti-myosin-6 (anti-MYO6) (1:500, Proteus Biosciences Inc., #25-6791). Alexa-conjugated secondary antibodies (Invitrogen) were used at a concentration of 1:1,000. Immunolabeled sections were scanned and photographed under a Zeiss LSM 510 META confocal microscope. For in situ hybridization experiments, 20 μ m thick cryosections were used as previously described (Li and Joyner, 2001). A fragment of ORF (nt178-1233) of *Atoh1* cDNA was used as an in situ hybridization probe. Detection of β -galactosidase activities was performed by X-Gal staining (Gan et al., 1999). Briefly, cryosections were prepared at 20 μ m thickness and stained overnight at 30°C in staining solution containing 1 mg/ml X-Gal, 4mM K₄Fe(CN)₆, 4 mM K₃Fe(CN)₆, and 2 mM MgCl₂ in PBS. For wholemount X-Gal staining, embryos were fixed in 4% PFA in PBS overnight and stained at 30°C overnight in the staining solution.

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FIG. 1.

Generation of *Atoh1-Cre* knock-in mice. (a) Restriction enzyme map and targeting strategy. The *Atoh1* open reading frame (ORF) is shown as the box. Thick bars represent the DNA sequences used as the 5' and 3' arms for homologous recombination. The external 3'-Southern probe is shown as the hatched box. Arrows indicate the approximate positions of the PCR genotyping primers. Abbreviations: Cre, *Cre* recombinase gene and SV40 polyA cassette; Neo, PGK-neo cassette; TK, MC1-TK cassette. (b) Southern genotyping of a typical litter from the cross of *Atoh1-Cre* heterozygotes using the 3'-probe identifies the 12.5 kb wild-type (black arrow) and the 7.0 kb targeted (open arrow) DNA fragments from *EcoRI*-digested genomic DNA. (c) PCR genotyping of the same litter as (b) identifies the 488 bp product in wild type (black arrow) and the 502 bp product in *Atoh1*^{Cre} (open arrow) mice. (d) In situ hybridization of the cochlear cryosections at E14.5 confirms the absence of *Atoh1* expression in the *Atoh1*^{Cre/Cre} ear compared to the contro. Scale bars equal 50 μ m.

FIG. 2.

Functional recombination in *Atoh1*-Cre mouse line. (a-f) Wholemount X-Gal staining of the *Atoh1*^{Cre/+}; *R26R-lacZ* embryos at E13.5 (a,b) and E15.5 (c-f). The expression of *lacZ* is readily detectable in the hindbrain (a-d, arrow), spinal cord (a-d, arrowhead), intestine (e, arrowhead), and inner ear (f). Within the E15.5 inner ear, *lacZ* expression is easily detected in the vestibular sensory organs (arrowheads) including the cristae, utricle, and saccule, and weak *lacZ* expression is also observed in the cochlear duct. Abbreviations: lc, lateral semicircular canal; pc, posterior semicircular canal; u, utricle; s, saccule; cd, cochlear duct.

FIG. 3.

Expression of *Atoh1-Cre* in the developing vestibule. (a-l) In situ hybridization of *Atoh1* shows its expression in the sensory region of the control vestibule at E13.5 (a-c), E14.5 (d-f), E15.5 (g,h), E16.5 (i), E17.5 (j,k), and P0 (l). (a'-l') X-Gal staining for *lacZ* expression in vestibular sections reveals the *Atoh1-Cre*-mediated recombination events in the *Atoh1^{Cre/+}; R26R-lacZ* ear at E13.5 (a'-c'), E14.5 (d'-f'), E15.5 (g',h'), E16.5 (i'), E17.5 (j',k') and P0 (l'). The expression of *lacZ* becomes detectable in the vestibule at E13.5 (a'-c') and is confined to the sensory regions through vestibular development (a'-l'). Abbreviations: lc, lateral semicircular canal; mu, macula of utricle; s, saccule. Asterisks Scale bars equal 50 μ m.

FIG. 4.

Expression of *Atoh1-Cre* in the developing cochlea. (a-f) In situ hybridization results show that *Atoh1* expression in the cochlear sensory region starts at E13.5 (a) and that its confined expression in the cochlear sensory region persists throughout the embryonic development (b-f). (a'-f') X-Gal staining experiments reveal the activity of *Atoh1-Cre* in *Atoh1*^{Cre/+}; *R26R-lacZ* cochleae. No expression of *lacZ* is detected in the cochlea at E13.5 (a'). From E14.5 to P0 (b'-f'), *lacZ* expression first appears in the sensory region of the basal cochlea and gradually extends in the sensory region from cochlear base to apex during embryogenesis. Scale bars represent 50 μ m.

FIG. 5. Efficient *Atoh1*-Cre-mediated recombination is restricted to both hair cells and supporting cells. (a-d'') Co-immunolabeling of the *Atoh1*^{Cre/+}; *R26R-lacZ* cochlea at P0 with anti-lacZ (green) and anti-myosin-6 (anti-MYO6, red) for the inner ear hair cells. In the cochlea, the activation of *lacZ* expression is observed in nearly all hair cells (MYO+) as well as in some supporting cells (arrowheads) of all types. Abbreviations of cell types: D, Deiter cell; IHC, inner hair cell; OHC, outer hair cell; P, Pillar cell; and Ph, Phalangeal cell. (b-d'') Similarly, co-immunolabeling with anti-lacZ and anti-MYO6 shows the expression of *lacZ* in most hair cells and a few supporting cells of the vestibular sensory regions including utricle (b-b''), crista (c-c''), and saccule (d-d''). (e-g) Compared to the limited expression of *Atoh1* to the sensory region (asterisks) in the control cochlea (e) at E18.5, the expression of *lacZ* is similarly confined in the sensory region of *Atoh1*^{Cre/+}; *R26R-lacZ* cochlea (g, f). However,

no *Atoh1* or lacZ expression is detected in spiral ganglion (SG, arrows) cells by the *Atoh1* in situ hybridization (e) or X-Gal staining (f). (g) Double immunolabeling with anti-lacZ (green) and anti-calretinin (red) shows no lacZ expression in spiral ganglion cells (arrow) at P9 in *Atoh1*^{Crel/+}; *R26R-lacZ* mice. Asterisk indicates the sensory region of cochlea. SG, the spiral ganglion cells. Scale bars equal 10 μ m in (a-a''), 30 μ m in (c-c''), and 50 μ m in (b-b'', d-d'', e-g).