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Generation and Characterization of *Lhx3*^{GFP} reporter knock-in and *Lhx3*^{loxP} conditional knockout Mice

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Abstract

LHX3, a LIM-homeodomain transcription factor, is broadly expressed in the developing pituitary, spinal cord, medulla, retina and inner ear, and plays essential roles during embryonic development. Mice with homozygous *Lhx3* null mutation exhibit failure in the formation of pituitary gland and die perinatally. To facilitate the functional study of *Lhx3* in mice, we engineered and characterized two novel *Lhx3* mouse strains: *Lhx3*^{GFP} reporter knock-in and *Lhx3*^{loxP} conditional knockout mice. Co-immunolabeling of LHX3 and GFP shows that the expression pattern of the knock-in GFP reporter recapitulates that of endogenous LHX3 in cochlea, vestibule, retina, and spinal cord. By crossing *Lhx3*^{loxP} mice with the ubiquitous *CMV-Cre* mice, we have demonstrated a high efficiency of Cre recombinase-mediated removal of exons 3 to 5 of *Lhx3*, which encode the second LIM-domain and the HD domain of LHX3, resulting global knockout of *Lhx3*. Thus, *Lhx3*^{GFP} and *Lhx3*^{loxP} mice serve as valuable genetic tools to dissect the tissue-specific roles of *Lhx3* at late-gestation and postnatal stages in mice.

Keywords

LIM-homeodomain; transcription factor; pituitary; inner ear; retina; spinal cord

Introduction

LIM-homeobox genes encode a series of LIM-homeodomain (LIM-HD) transcription factors (TFs) that contain two LIM domains in their amino termini and a centrally located HD. The two LIM domains are tandem repeats of two cysteine-rich, double-zinc finger motifs that can interact with other TFs, and the HD binds to specific DNA elements in target genes (Hobert and Westphal, 2000). LHX3 belongs to the LHX3/4 subfamily of LIM-HD proteins and is phylogenetically conserved from *C. elegans* to mammals (Freyd *et al.*, 1990); (Taira *et al.*, 1993); (Zhadanov *et al.*, 1995a). In addition to the LIM domains and HD domain, LHX3 contains a trans-activation carboxyl terminus (Bach *et al.*, 1995). *Lhx3* is composed of six exons and alternative transcriptional initiation generates two isoforms,

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Lhx3a and *Lhx3b*, which encode different amino termini from alternative first exons. These isoforms target different pituitary genes besides possessing different amino-termini (Sloop *et al.*, 2001); (Zhadanov *et al.*, 1995b).

Published studies have reported that *Lhx3* is expressed in hindbrain, pineal gland, spinal cord and pituitary (Seidah *et al.*, 1994) as well as in retinal bipolar cells (Elshatory *et al.*, 2007); (Balasubramanian *et al.*, 2014); (Blixt *et al.*, 2017) and the inner ear (Hume *et al.*, 2007); (Huang *et al.*, 2008); (Rajab *et al.*, 2008). Deletion of the two LIM domains and a part of the HD of LHX3 by conventional knockout approach in mice leads to abnormal Rathke's pouch and embryonic death at birth or neo-natal death within 24 hours after birth and abolishes the expression of *Pit-1*, *TSH*, *GSU* and *GH* (Sheng, 1996). During spinal cord development, LHX3 is involved in the specification of motor neuron and interneuron by interacting with NLI or NLI and ISL1 (Thaler *et al.*, 2002).

In human, *LHX3* is associated with the combined pituitary hormone deficiency type 3 (CPHD3) disease. Patients with CPHD3 carry recessive *LHX3* mutations and present deficiencies in the thyroid stimulating hormone, growth hormone, prolactin, follicular stimulating hormone, luteinizing hormone, and abnormal pituitary. Some CPHD3 patients also exhibit adrenocorticotrophic hormone deficiency, cervical abnormalities and sensorineural hearing loss (Rajab *et al.*, 2008); (Kristrom *et al.*, 2009); (Bonfig *et al.*, 2011); (Ramzan *et al.*, 2017). Most of the LHX3 mutations are located in the LIM or HD sequences, resulting in a truncated protein without part or entire LIM domain or HD or causing a loss-of-function mutation (Ramzan *et al.*, 2017).

In this study, we generated and characterized the *Lhx3* conditional knockout and the GFP reporter knock-in mouse lines for further study of the role of *Lhx3*. Our data demonstrated the effective Cre recombinase-mediated ablation of *Lhx3* with the conditional knockout allele and the faithful GFP expression in our *Lhx3-GFP* reporter line. Thus, these two mouse lines provide useful tools to facilitate studies of *Lhx3* during development and in adults.

Results and discussion

To generate *Lhx3* conditional knockout and GFP knock-in alleles, we designed a targeting construct by using two loxP sites to flanking exons 3–5 of *Lhx3*, and a FRT flanked En2SA-IRES-GFP (GFP)-loxP-Neo-pA (Neo) cassette to drive the expression of the GFP reporter gene from *Lhx3* locus (Fig. 1a). The *Lhx3^{ckoGFP}* targeting construct was electroporated into embryonic stem cells (ESCs). The targeted ESC clones were confirmed by Southern blotting genotyping (Fig. 1b) and injected into C57BL/6J blastocysts to generate mouse chimeras. By crossing the chimeras with FLPeR mice, the GFP/Neo cassette was removed to create the *Lhx3^{loxP}* allele and the GFP reporter mouse line was generated by crossing the chimeras with *CMV-Cre* mice to remove the exons 3–5 and Neo (Fig. 1a). In addition to Southern genotyping, PCR genotyping was used to identify the wild type allele, the floxed allele, and the GFP knock-in allele (Fig. 1c). The viability and fertility of *Lhx3^{loxP/+}*, *Lhx3^{loxP/loxP}*, *Lhx3^{GFP/+}* and *Lhx3^{loxP/GFP}* mice were observed normal, suggesting that the insertion of loxP should not affect *Lhx3* and that mice with *Lhx3* heterozygous deletion do not display overt phenotype.

To assess the expression of GFP knock-in reporter, we compared the expression pattern of LHX3 and GFP in the developing inner ear, retina, and spinal cord by co-immunolabeling with anti-GFP and anti-LHX3 in heterozygous *Lhx3^{GFP/+}* mice. In the cochlea, the onset of hair cell differentiation starts at the basal cochlear turn and progresses toward the apical turn. In addition, the inner hair cells (IHCs) are generated prior to the outer hair cells (OHCs). Anti-LHX3 and anti-GFP immunolabeling showed that the expression of LHX3 and GFP was undetectable at E14.5 (Fig. 2a). At E15.5, both LHX3 and GFP were first observed in the IHCs in basal cochlea and they were co-expressed in the IHCs and OHCs of basal-middle and basal cochlea at E16.5 (Fig. 2b–c, asterisk). At E17.5, the expression of LHX3 was detected in the IHCs and OHCs of basal to apical-middle cochlea along with the expression of GFP (Fig. 2d, upper-right, asterisk). At P0, the expression of LHX3 and GFP intensified in the IHCs and OHCs (Fig. 2e, upper-right, asterisk).

We further compared the expression of LHX3 and GFP in the developing vestibule in heterozygous *Lhx3^{GFP/+}* mice. The macules of saccule and utricle and the ampoules of the three semicircular canals, which make up the five sensory organs responsible for the vestibular function in the inner ear, contain sensory hair cells. Anti-LHX3 immunolabeling showed that while LHX3 expression was not detected in these receptive organs at E13.5 (Fig. 3a), it first appeared in the hair cells of the macules of saccule and utricle, and of the posterior cristae at E14.5 (Fig. 3b and data not shown). The expression of LHX3 in the hair cells was maintained at E15.5 and in adults (Fig. 3c and data not shown). Expectedly, co-immunolabeling with anti-GFP and anti-LHX3 revealed that GFP was expressed in the hair cells of the macules and cristae from E14.5 to adult (Fig. 3a–c, data not shown), completely overlapping the expression of LHX3.

Our previous studies have shown that LHX3 is expressed in developing retinal bipolar cells and its expression persists in adult retinas (Elshatory *et al.*, 2007); (Balasubramanian *et al.*, 2014). We thus compared the expression of LHX3 and GFP in *Lhx3^{GFP/+}* retinas. Anti-LHX3 immunolabeling showed that LHX3 expression was not detected at P4 (Fig. 4a) but was first observed in differentiating bipolar cells in the inner nuclear layer (INL) starting at P5 (Fig. 4b). The expression of LHX3 in the bipolar cells built up at P9–15 (Fig. 4c–d) and was later down-regulated at P30 (Fig. 4e). Co-labeling of GFP and LHX3 showed that LHX3 and GFP were clearly co-expressed in the bipolar cells. Similar to LHX3 expression, GFP expression was low, albeit detectable, in the INL at P5, intensified at P9–15 (Fig. 4c–d'), and weakened at P30 (Fig. 4e, e').

LHX3 is one of the key factors regulating the specification of motor neurons and interneurons in the spinal cord. We analyzed the co-expression of LHX3 and GFP in developing spinal cord from E13.5 to E17.5 (Fig. 4f–j). In spinal cord, neurogenesis of motor neurons starts at ventricular zone and continues as developing neurons migrating from ventricular zone towards the surrounding intermediate zone. Neuronal progenitors and postmitotic precursors of different neuronal subtypes are arranged in a specific order along the dorsoventral axis as the results of the dorsoventral (dv) patterning in the neural tube. Most of the cells co-expressing LHX3 and GFP were positioned in ventral motor neuron (mn) group and ventral interneuron group 2 (v2) at E13.5 to E17.5 (Fig. 4f–j). Thus, these

spatiotemporal co-expression results demonstrate that the expression of GFP knockout reporter recapitulates that of endogenous LHX3 in vivo.

Finally, we tested whether loxP-flanked exons 3–5 in *Lhx3^{loxP}* conditional knockout allele could be efficiently excised by Cre recombinase to generate the *Lhx3*-null mutation as designed (Fig. 1a). We crossed *Lhx3^{loxP}* conditional knockout mice with *CMV-Cre* mice, which express Cre recombinase in all tissues including germ cells under the control of a human cytomegalovirus (CMV) promoter (Schwenk *et al.*, 1995), to delete exons 3–5 in germline and to generate *Lhx3^{+/-}* heterozygotes. Interbreeding of *Lhx3^{+/-}* mice resulted in *Lhx3*-null (*Lhx3^{-/-}*) homozygous mice that were stillborn or died within 24 hours after birth (data not shown), consistent with published studies (Sheng, 1996). To confirm the inactivation of *Lhx3*, we surveyed LHX3 expression in cochlea, vestibule and spinal cord in *Lhx3^{-/-}* mice at E16.5. Cre recombinase-mediated recombination removed exons 3–5, which encode LHX3's second LIM domain and HD domain, and also resulted in a frame-shift mutation immediately into exon 6. Anti-LHX3 immunolabeling of the control *Lhx3^{loxP/loxP}* mice showed that LHX3 expression was seen in spinal cord neurons in ventral motor neuron and v2 interneuron group (Fig. 5a) and in hair cells of cochlea (Fig. 5b), posterior cristae (Fig. 5c) and macule of saccule (Fig. 5d) and utricle (Fig. 5e). In contrast, anti-LHX3 immunolabeling of *Lhx3*-null mice detected no LHX3 expression in spinal cord neurons (Fig. 5a'), cochlear hair cells (Fig. 5b'), and vestibular hair cells (Fig. 5c'–e'). Thus, the *Lhx3^{loxP}* allele allows the efficient ablation of *Lhx3* by Cre recombinase in vivo, demonstrating that *Lhx3^{loxP}* mouse line serves as a valuable tool for the study of *Lhx3*'s novel roles in mice.

Materials and methods

Animals

To generate the *Lhx3* conditional knockout and GFP knock-in alleles, the *Lhx3^{ckoGFP}* targeting construct was constructed containing 1.2 kb floxed exons 3–5 fragment, 4.9 kb 5' arm, 5.8 kb 3' arm, a FRT flanked En2SA-IRES-GFP (GFP)-loxP-Neo-pA (Neo) cassette, and diphtheria toxin fragment A (DTA) cassette for negative selection. Linearized *Lhx3^{ckoGFP}* targeting construct was electroporated into C57BL/6J-129S6 hybrid embryonic stem cells (ESCs). The targeted ESC clones were confirmed by Southern blotting genotyping using an external 3' probe to identify the 8.8 kb wild type and 7.4 kb targeted DNA fragments from *XbaI* digested genomic DNA and injected into C57BL/6J blastocysts to generate mouse chimeras. By crossing the chimeras with flippase-expressing FLP^{er} mice (Jackson Laboratory Stock Number 009086), GFP and Neo were removed to produce *Lhx3^{loxP}* mouse line. Likewise, crossing the chimeras with Cre recombinase-expressing *CMV-Cre* mice (Jackson Laboratory Stock Number 006054) removed exons 3–5 and Neo and generated the *Lhx3^{GFP}* mouse line. PCR strategy was used to identify the wild type allele and *loxP* allele with the following primers: Lhx3ckoF1 (5'-CCA AGT CCG ACA AGG ACA GCA TCC A -3') and Lhx3ckoR1 (5'-GCT GTG CTA GTA ACC GAG GTG ACA ATA CA -3'). GFP knock-in allele was identified with primers: IRES F1 (5'-AAT GGC TCT CCT CAA GCG -3') and GFP R1 (5'-GCG GAT CTT GAA GTT CAC CTT GAT GCC A -3'). PCR genotyping of *CMV-Cre* transgene was performed according to

protocols provided by Jackson Laboratory. University Committee of Animal Resources at the University of Rochester and Hangzhou Normal University approved all animal procedures described here. The mouse strains were maintained in the C57BL/6J and 129S6 mixed background. The corresponding authors will make these *Lhx3^{loxP}* and *Lhx3^{GFP}* mouse strains available to the research community upon acceptance of this manuscript.

Immunocytochemistry

Embryonic day 0.5 (E0.5) was defined as the day when the vaginal plug was detected. Embryos at E13.5 and heads of embryos at E14.5 and later stages were harvested and immediately fixed in pre-chilled 4% paraformaldehyde (PFA) in PBS overnight. Samples were rinsed with cold PBS three times for 15 minutes each, cryopreserved in sucrose gradient (10%, 20% and 30%), embedded and frozen in OCT compound (TissueTek). Cryosections were cut at 16 μ m thickness and dried on slides at 37°C for 1 hour before immunostaining. After two 15-minute rinses with PBS and two 15-minute rinses with 0.3% Triton-X100 in PBS (PBST), sections were blocked in 10% horse serum in 0.3% PBST for 1 hour at room temperature and incubated with primary antibodies overnight at 4°C. Then, slides were rinsed four times for 15 minute each with PBST and incubated with Alexa-conjugated secondary antibodies (Invitrogen) in PBST for one and half hour. After washing with PBST and PBS, sections were nuclear counter-stained with DAPI, mounted and imaged on a Zeiss LSM 510 META confocal microscope. Antibodies used were: chicken anti-GFP (1:500, Aves Labs: GFP-1020) and rabbit anti-LHX3 (1:500, Abcam: ab1455).

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FIG. 1. Generation of *Lhx3^{GFP}* and *Lhx3^{loxP}* mice

(a) *Lhx3* restriction enzyme map and targeting strategy. FRT (black arrow) flanked GFP/Neo cassette was inserted in intron 2–3 and loxP (open arrow) sequences were used to flank exons 3–5. Thick bars represent DNA sequences of the 5' and 3' arms for homologous recombination. The 3' Southern probe is marked as the hatched box. Abbreviations: FRT, flippase recognition target sequence; Neo, PGK-Neo cassette; DTA, diphtheria toxin A. (b) Genotyping examples of Southern blotting screening of ESC clones using the external 3' probe to identify 8.8 kb wild type and 7.4 kb targeted *Lhx3^{ckoGFP}* DNA fragments from *XbaI* digested genomic DNA. (c) PCR genotyping of a typical litter from the crossing of

Lhx3^{loxP/+} and *Lhx3^{GFP/+}* heterozygotes detects the 330 bp wild type, 410 bp *Lhx3^{loxP}* product and 700 bp *Lhx3^{GFP}* alleles.

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FIG. 2. Comparison of LHX3 and GFP expression in developing cochlea
Sagittal sections of *Lhx3^{GFP/+}* cochleae were immunolabeled with antibodies against GFP (green) and LHX3 (red) at E14.5 (a), E15.5 (b), E16.5 (c), E17.5 (d) and P0 (e), showing the spatiotemporal expression pattern of GFP resembling that of LHX3. LHX3 signals were first detected in the inner hair cells (arrow) of basal turn (asterisk) at E15.5 (b). At E16.5 and later stages (c–e), all hair cells (arrow, bracket) express LHX3 and GFP at the basal and basal-middle turns (asterisk). Abbreviations: A, apex; A-M, apical-middle; B-M, basal-middle; B, base; IHC, inner hair cell; OHC, outer hair cell. Scale bars equal to 50 μ m.

FIG. 3. Co-expression of LHX3 and GFP in the hair cells of the developing vestibule
Sagittal sections of *Lhx3^{GFP/+}* inner ears were immunolabeled with antibodies against GFP (green) and LHX3 (red). **(a)** No specific labeling of LHX3 and GFP were detected at E13.5. **(b)** The co-expression of LHX3 and GFP appeared in the hair cells of the posterior cristae (PC), macule of utricle (U) and saccule (S) at E14.5. **(c)** At E15.5, the co-expression of LHX3 and GFP remained in the hair cells of the developing vestibule. Scale bars equal to 100 μ m in left panels and 50 μ m in right panels.

FIG. 4. Overlapping expression of LHX3 and GFP in the developing retina and spinal cord
Cryosections of mouse retinas and spinal cord were immunolabeled with antibodies against GFP (green) and LHX3 (red). (**a-e**) No specific labeling of LHX3 and GFP was observed in the developing retina at P4 (**a**); LHX3 and GFP expression started in the INL at P5 (**b**) and intensified at P9 (**c**) and P15 (**d**) but lessened at P30 (**e**). Enlarged panels in **c'-e'** show the co-expression of LHX3 and GFP in the bipolar cells. (**f-j**) Immunolabeling experiments revealed that LHX3 and GFP were co-expressed in the neurons of developing spinal cord from E13.5-E17.5 (**f-j**). Abbreviations: v2, ventral interneuron 2; mn, motor neuron. Scale bars equal to 50 μm (**a-j**, **f'**) and 25 μm (**c'-e'** lower panel).

FIG. 5. Effective inactivation of *Lhx3* by Cre recombinase-mediated removal of the loxP flanked exons 3–5 in *Lhx3*^{loxP} mice

Crossing *CMV-Cre* mice with *Lhx3*^{loxP} mice resulted in the germline deletion of exons 3–5 and the generation of *Lhx3*^{+/-} heterozygotes. By interbreeding *Lhx3*^{+/-} mice, *Lhx3*-null (*Lhx3*^{-/-}) mice were generated and analyzed for the loss of LHX3 expression. (**a** and **a'**) Compared to the control *Lhx3*^{loxP/loxP} mice, anti-LHX3 immunolabeling showed the loss of LHX3 expression in the *Lhx3*-null spinal cord at E16.5. Similarly, while the expression of LHX3 was detected in the hair cells in the cochlea (**b**), cristae (**c**), saccule (**d**) and utricle (**e**) of the control mice, it was ablated in *Lhx3*-null mice (**b'**-**e'**). Scale bars equal to 100 μ m.