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Generation and characterization of *Lhx9* – *GFPCreER^{T2}* knock-in mouse line

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Summary

LHX9 is a LIM-homeodomain transcription factor essential for the development of gonads, spinal cord interneurons, and thalamic neurons to name a few. We recently reported the expression of LHX9 in retinal amacrine cells during development. In this study, we generated an *Lhx9* - *GFPCreER^{T2}* (GCE) knock-in mouse line by knocking-in a GCE cassette at the *Lhx9* locus, thus inactivating endogenous *Lhx9*. *Lhx9^{GCE/+}* mice were viable, fertile, and displayed no overt phenotypical characteristics. *Lhx9^{GCE/GCE}* mice were all phenotypically female, smaller in size, viable, but infertile. The specificity and efficacy of the *Lhx9*-GCE mouse line was verified by crossing it to a *Rosa26* - *tdTomato* reporter mouse line, which reveals the Cre recombinase activities in retinal amacrine cells, developing limbs, testis, hippocampal neurons, thalamic neurons, and cerebellar neurons. Taken together, the *Lhx9*-GCE mouse line could serve as a beneficial tool for lineage tracing and gene manipulation experiments.

Keywords

LIM-homeodomain; *Lhx9*; transcription factor; retina; amacrine cells; Cre recombinase

INTRODUCTION

LHX9 belongs to the Apterous (*Drosophila*) group in the LIM-homeodomain (HD) family of transcription factors (Hobert and Westphal 2000). Its expression has been described in various central nervous system structures such as spinal cord interneurons (Wilson et al. 2008), developing diencephalic neurons (Nakagawa and O'Leary 2001; Peukert et al. 2011), subset of hypothalamic neurons (Lakina et al., 2013), cerebellar neurons (Bertuzzi et al. 1999) and other structures such as the developing limbs (Bertuzzi et al. 1999) and urogenital system (Birk et al. 2000). *Lhx9* function has been extensively studied for its crucial role during the development of gonads. In mice lacking *Lhx9*, somatic cells of the gonadal ridge

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fail to proliferate, causing a failure of formation of discrete gonads. The resulting absence of testosterone and anti-Müllerian hormone causes genetically male mice to be phenotypically female (Birk et al. 2000). The function of *Lhx9* has also been described during development of the caudal forebrain. *Lhx9* along with *Lhx2*, a related member in the *Apterous* group of LIM-homeobox gene family, is required for thalamic neuronal differentiation (Peukert et al. 2011). We have previously described expression of LHX9 in retinal amacrine interneurons (Balasubramanian et al. 2014). In order to perform lineage tracing experiments and other forms of genetic manipulation to study the role of *Lhx9* in retinal amacrine cell subtype development and specification, we generated an *Lhx9* - *GFPCreERT²* (GCE) knock-in mouse line by knocking in the GCE cassette (a GFP and CreER^{T2} in-frame fusion gene) at the *Lhx9* locus, allowing for spatiotemporal gene manipulations in *Lhx9*-expressing cells. The GCE knock-in resulted in a complete inactivation of *Lhx9* in the *Lhx9^{GCE/GCE}* mice. After crossing the *Lhx9*-GCE mice to a *Rosa26-tdTomato* reporter line and upon tamoxifen administration, there is an efficient recombination in *Lhx9*-expressing cells and the spatiotemporal expression pattern of *Lhx9*-GCE in the developing retina recapitulates that of endogenous *Lhx9*. Current *Lhx9* mouse lines include a straight knock-out of *Lhx9* (Birk et al. 2000) or targeted reporter knock-in at the *Lhx9* locus (Wilson et al. 2008). To our knowledge, there are no *Lhx9* mouse lines that drive the expression of Cre or CreER^{T2} allowing for spatiotemporal gene manipulations. The *Lhx9*-GCE mice we describe here serves as a suitable inducible Cre recombinase mouse strain for gene manipulation in the retinal amacrine cells as well as other *Lhx9*-expressing tissues.

RESULTS AND DISCUSSION

We generated the *Lhx9*-GCE mouse line by inserting the GCE cassette at the initiation site of Exon 4 coding region of the *Lhx9* locus (Fig. 1A). The GCE cassette contains a polyA tail to terminate transcription at the *Lhx9* locus. The targeted knock-in was confirmed by Southern blotting (Fig. 1B) using an external 5'-probe (Wilson et al. 2008) to identify the 4.8 kb wild type band and 5.6 kb GCE knock-in band. PCR was also performed to identify the 441 bp wild type fragment and 483 bp GCE fragment (Fig. 1C). Generated *Lhx9^{GCE/+}* heterozygous mice were viable, fertile, and did not display any gross abnormalities. *Lhx9^{GCE/GCE}* homozygous mice were viable, but were smaller compared to their heterozygous and wild type littermates (Fig. 1D). Moreover, *Lhx9^{GCE/GCE}* mice were born phenotypically female (100%) and infertile. LHX9 expression in *Lhx9^{GCE/+}* retina and its absence in *Lhx9^{GCE/GCE}* retina confirmed the efficiency of the targeted knock-in (Fig. 2A). While we were unable to visualize endogenous GFP fluorescence and GFP signal with an antibody (data not shown), anti-ER immunolabeling revealed the faithful expression of GCE in *Lhx9*-expressing retinal cells in *Lhx9^{GCE/+}* mice (Fig. 2B). We then assessed GCE expression and activities in the *Lhx9^{GCE/+}* retina. We crossed the *Lhx9^{GCE/+}* line to the *Rosa26-tdTomato* reporter mouse line. We induced GCE recombinase activity in *Lhx9*-expressing retinal cells by tamoxifen administration in *Lhx9^{GCE/+}; Rosa26^{tdTomato/+}* mice (Fig. 2C). In *Lhx9^{+/+}; Rosa26^{tdTomato/+}* and *Lhx9^{GCE/+}* control mice, and uninduced *Lhx9^{GCE/+}; Rosa26^{tdTomato/+}* mice, we found little or no tdTomato reporter gene expression, suggesting that there was no significant leaky expression of CreER^{T2} in the absence of tamoxifen (data not shown). In the *Lhx9^{GCE/+}; Rosa26^{tdTomato/+}* retina, *Lhx9*-GCE is

expressed in a subset of amacrine cells (Fig. 2), consistent with previously described expression of LHX9 specifically in retinal amacrine cells (Balasubramanian et al. 2014). Using an antibody against RFP that also recognizes tdTomato, a variant of RFP, we noticed that the tdTomato expression was restricted to retinal cells in the inner nuclear layer (INL) and ganglion cell layer (GCL) (Fig. 2). Immunostaining with POU4F1 and POU4F2 ruled out the possibility that these tdTomato+ cells in the GCL were retinal ganglion cells (Fig. 2A). Immunostaining with SOX2 ruled out the possibility that they were Müller glial cells, the layer of retinal cells lying adjacent to amacrine cells in the INL (Fig. 2B). Co-staining with CHX10 - a marker for bipolar cells revealed that they were not bipolar cells (Fig. 2C). These cells however co-labeled with PAX6 – a marker for amacrine cells and retinal ganglion cells (Fig. 2D). Co-labeling with Syntaxin and AP2 α – markers only for amacrine cells confirmed that LHX9 expressing cells were indeed a subtype of amacrine cells (Fig. 2E, F).

To test the efficacy of tamoxifen-induced recombination in other structures where *Lhx9* expression has been documented, we looked at several structures in adult mice. Strong expression of tdTomato was seen in the hippocampal neurons (Fig. 4A), thalamic neurons (Fig. 4B), and cerebellar neurons (Fig. 4D). We also found strong tdTomato staining in interstitial cells of Leydig of the male gonadal organ, the testis (Fig. 4C). We further assessed the efficiency of recombination in embryonic tissues. We performed tamoxifen administration to pregnant females once at E15.5, harvested embryos at E17.5, and observed tdTomato expression in the developing limbs and brain (Fig. 4D-F).

In summary, we have developed an *Lhx9-GCE* mouse line which will be useful for gene manipulation in retinal amacrine neurons and other tissues including but not limited to the testis, hippocampus, thalamus and developing limbs, at both embryonic and postnatal time points.

MATERIALS AND METHODS

Animals

To make the *Lhx9-GCE* targeting construct, a 3.7 kb 5' homologous region in a *NheI* fragment containing most of the intron sequences between Exons 3 and 4 was subcloned into the *AvrII* site of the modified eGFP-CreER vector (pGCE). A 6.7 kb *FseI-NheI* fragment containing the 3.7 kb 5' homologous region and the 3 kb GCE cassette was released and subcloned into the pKO targeting vector (L. Gan., unpublished). A 3.9 kb 3' homologous region in a *NotI* fragment containing the LIM1 (Exon 5) and LIM2 (Exon 6) domains was subcloned at the *NotI* site of the vector. To generate *Lhx9-GCE* knock-in mice, the *AscI* linearized targeting construct was electroporated into embryonic stem (ES) cells derived from C57BL/6J/129S6 hybrid line. Nine targeted ES clones were identified from 192 G418-resistant clones. The positive clones were identified by Southern blotting performed by digestion of genomic DNA with *BamHI* and detection of fragments was done using an external 5'-probe. The targeted clones were then injected into C57BL/6J blastocysts. Chimeric mice were generated and bred with C57BL/6J WT mice. Heterozygous *Lhx9^{GCE/+}* mice (mixed 129S6 and C57BL/6J) were generated and the following PCR primers were used to identify wild type and mutant alleles: *Lhx9* WT-F

(TGCAGTTAGAATCGCCTGCTT) and *Lhx9* WT-R (TTGAGCTGAGTGCCTTTGGT) to amplify a 441 bp wild type fragment, and *Lhx9* GCE-F (GCCCCGCGCTGGAGTTTCAATAGC) and *Lhx9* GCE-R (GGAGCGCCAGACGAGACCAATCAT) to amplify a 483 bp knock-in fragment. *Lhx9*^{GCE/+} mice will be made available to the research community upon acceptance of this manuscript. *Rosa26-tdTomato* reporter (*Gt(ROSA)26Sor^{tm14}(CAG-tdTomato)Hze) mice were obtained from Jackson Laboratory (Stock number: 007908). Genotyping for the *Rosa26-tdTomato* mice were performed as described in the Jackson Laboratory protocols.*

Tamoxifen administration

Timed mating was setup and noon of the first day plug was visualized was designated as E0.5. Noon of the first day pups were born was designated as P0. Tamoxifen was prepared by dissolving 10 mg of tamoxifen (Sigma-Aldrich T5648) in 1 ml of corn oil. Tamoxifen was administered intraperitoneally at 5 mg/40 g body weight to pregnant females (for embryos), once at the required time point. For adult mice, tamoxifen was administered intraperitoneally at 5 mg/40 g body weight for five consecutive days. All animal procedures performed were approved by the University Committee of Animal Resources at the University of Rochester.

Immunohistochemistry

Animals were perfused transcardially with 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS). Harvested tissues were then fixed in PFA overnight (brain, embryo) or 2 hours (eye). Following fixation of eye tissue, the anterior segment and lens was removed to expose the retina. Retina, brain, and embryo samples were then equilibrated in 30% (w/v) sucrose in PBS and embedded in Tissue Freezing Medium (TFM) compound for sectioning. Retina sections were obtained on slides at 20 µm thickness while brain and embryo sections were obtained as floating sections in cold PBS at 40 µm thickness. For whole mount imaging, embryos were fixed and collected in PBS containing DAPI. The following primary antibodies were used: Guinea Pig Anti-LHX9 (1:20,000; Gift from Dr. Jane Dodd, Columbia University), Chick Anti-GFP (1:1000; Abcam), Rabbit Anti-ER (1:200; Thermo Scientific), Rabbit Anti-RFP (1:1,000; Rockland), Mouse Anti-PAX6 (1:200; DSHB), Mouse Anti-POU4F1 (1:500; SantaCruz), Goat Anti-POU4F2 (1:500; SantaCruz), Mouse Anti-Syntaxin (1:1,000, Sigma Aldrich), Goat Anti-SOX2 (1:500; Chemicon). Alexa-conjugated secondary antibodies (Molecular probes) were used at 1:1,000. Images were obtained on a Zeiss LSM510 confocal microscope or Zeiss Lumar Stereoscope.

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A **B** **C** **D** **FIG. 1.**

Generation of *Lhx9-GCE* mice. (A) *Lhx9* targeting strategy and restriction map. Black boxes are Exons 4, 5 and 6. Thick bars are the sequences used to generate the homologous arms in the targeting vector. Also indicated are the sizes of the expected wild type and targeted *Bam*HI fragments detected by using an external 5'-probe (660 bp fragment; striped box). Abbreviations: DTA: diphtheria toxin A; Neo: PGK Neo cassette; GCE: GFPCreER^{T2}. (B) Representative Southern blot for selection of ES cells with site-specific insertion of GCE cassette using a 5'-probe that identifies a 4.8 kb wild type band and 5.6 kb GCE insert band. (C) Representative PCR amplification identifies 441 bp wild type fragment and 483 bp GCE fragment. (D) *Lhx9*^{GCE/GCE} mice are smaller in size compared to their heterozygous *Lhx9*^{GCE/+} littermates.

FIG. 2.

Validation of and functional recombination in *Lhx9*-GCE mice. (A) LHX9 is expressed in *Lhx9*^{GCE/+} retinas but not in *Lhx9*^{GCE/GCE} retinas. (B) LHX9 co-localizes with ER in *Lhx9*^{GCE/+} retinas. (C) Faithful recombination in *Lhx9*-expressing cells as seen by the complete co-localization of tdTomato reporter with LHX9 and ER in retinal neurons. Scale bars = 200 μ m, time point = P30, n=6.

FIG. 3.

Lhx9-GCE driven reporter expression in retinal amacrine neurons. (A) tdTomato does not co-localize with POU4F1 or POU4F2, markers for retinal ganglion cells. (B) tdTomato is not co-expressed with SOX2, marker for Müller glial cells and cholinergic amacrine cells. (C) tdTomato is not co-expressed with CHX10, marker for bipolar cells. (D) tdTomato is expressed in retinal neurons in the INL and GCL and co-localizes with PAX6, marker for amacrine cells and retinal ganglion cells. (E) tdTomato expression co-localizes with

Syntaxin, marker for retinal amacrine neurons. (F) tdTomato expression co-localizes with AP2 α , marker for retinal amacrine neurons. Scale bars = 200 μ m, time point = P30, n=4.

FIG. 4.

Lhx9-GCE driven reporter expression in other structures. The expression of tdTomato reporter (n=3) driven by *Lhx9* in P30 hippocampal neurons (A), thalamic neurons (B), testicular cells (C) and cerebellar neurons (D). Embryonic expression (E17.5) of TdTomato reporter in developing limbs and brain (arrow heads) (n=3) (E) Expression of TdTomato reporter in the developing diencephalon (E17.5) (F). Abbreviations: DG: Dentate gyrus, DCN: Deep cerebellar nuclei, MH: Medial habenula, PVT: Paraventricular nucleus of thalamus, LC: Leydig cells, ST: Seminiferous tubules, 3V: Third ventricle, PHN: Paraventricular hypothalamic nucleus, PHA: Posterior hypothalamic area. Scale bars = 200 μm (A-D) and 1 mm (E, F).