Developmental Biology 338 (2010) 38–49

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Requirement for *Lmo4* in the vestibular morphogenesis of mouse inner ear

Min Deng [a], Ling Pan [a], Xiaoling Xie [a], Lin Gan [a,b,c,*]

[a] University of Rochester Flaum Eye Institute, University of Rochester, Rochester, NY 14642, USA
[b] Center for Neural Development and Disease, University of Rochester, Rochester, NY 14642, USA
[c] Department of Neurobiology and Anatomy, University of Rochester, Rochester, NY 14642, USA

ARTICLE INFO

Article history:
Received for publication 26 November 2008
Revised 4 November 2009
Accepted 4 November 2009
Available online 10 November 2009

Keywords:
LIM
LMO
LMO4
Otic vesicle
Otocyst
Vestibular morphogenesis
Inner ear development
Transcription factor

ABSTRACT

During development, compartmentalization of an early embryonic structure produces blocks of cells with distinct properties and developmental potentials. The auditory and vestibular components of vertebrate inner ears are derived from defined compartments within the otocyst during embryogenesis. The vestibular apparatus, including three semicircular canals, saccule, utricle, and their associated sensory organs, detects angular and linear acceleration of the head and relays the information through vestibular neurons to vestibular nuclei in the brainstem. How the early developmental events manifest vestibular structures at the molecular level is largely unknown. Here, we show that LMO4, a LIM-domain-only transcriptional regulator, is required for the formation of semicircular canals and their associated sensory cristae. Targeted disruption of *Lmo4* resulted in the dysmorphogenesis of the vestibule and in the absence of three semicircular canals, anterior and posterior cristae. In *Lmo4*-null otocysts, canal outpouches failed to form and cell proliferation was reduced in the dorsolateral region. Expression analysis of the known otic markers showed that *Lmo4* is essential for the normal expression of *Bmp4*, *Fgf10*, *Msx1, Isl1, Gata3*, and *Dlx5* in the dorsolateral domain of the otocyst, whereas the initial compartmentalization of the otocyst remains unaffected. Our results demonstrate that *Lmo4* controls the development of the dorsolateral otocyst into semicircular canals and cristae through two distinct mechanisms: regulating the expression of otic specific genes and stimulating the proliferation of the dorsolateral part of the otocyst.

Introduction

The compartmentalization process plays an important role in the development of the vertebrate nervous systems. In the developing central nervous system (CNS), the cytologically homogeneous sheet of neural epithelial cells is initially partitioned along the anterior–posterior axis into regions representing the anlagen of forebrain, midbrain, hindbrain, and the spinal cord in the posterior (Lumsden and Krumlauf, 1996). Within each region, neuronal cell types are generated with unique identities assessed by morphology, physiological properties, and axon projections. Moreover, each of these regions is further patterned into distinct subregions such that in the forebrain, compartments are formed to develop into unique functional regions processing cognitive, motor, and sensory information (O'Leary and Nakagawa, 2002).

The compartmentalization process is similarly important in the development of the peripheral nervous system. The mammalian inner ear is a complex structure containing two functional parts, cochlea and vestibule. The cochlea is a coiled structure and is responsible for auditory function. The vestibular system consists of a central vestibule, three semicircular canals, and an endolymphatic duct and sac. It is essential for balance by sensing gravity, linear and rotational motion. The entire inner ear structure is derived from the otic placode, a thickening ectoderm near the hindbrain (Fritzsch et al., 2002). Previous grafting and lineage tracing experiments in chick embryos have demonstrated that specific parts of the inner ear are derived from distinct compartments of the early otocyst (Baker and Bronner-Fraser, 2001; Fekete, 1996). While the cochlea arises from a ventrally extending region of the ventral otocyst, vestibular structures develop from the dorsolateral otocyst and the endolymphatic duct and sac come from the small dorsomedial projection of the otocyst (Morsli et al., 1998). Recent fate mapping experiments in chicken otic placodes have shown that otic neurons and their sensory targets come from a common proneural domain, in which different precursors are spatially segregated. The otic placode is spatially partitioned along the dorsal/ventral axis with dorsal (cristae) located in the anterior and lateral domain, ventral (cochlea) in the posterior and medial region, and the maculae in an intermediate position (Bell et al., 2008). These otic compartments are demarcated early by the expression of specific regulatory genes, namely the otic patterning genes (Bober et al., 2003; Fekete, 1996). Genetic alteration of the otic patterning genes often leads to defective morphogenesis of the inner ear. For example, the paired-class homeobox gene, *Pax2*, is mostly expressed in the medioventral part of the otocyst. Inactivation of *Pax2* in mice resulted in either agenesis or severe malformation of the cochlea whereas the

* Corresponding author. Department of Ophthalmology, University of Rochester, Box 659, 601 Elmwood Avenue, Rochester, NY 14642, USA. Fax: +1 585 276 2432.
E-mail address: lin_gan@urmc.rochester.edu (L. Gan).

0012-1606/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2009.11.003

development of the vestibule is unaffected (Burton et al., 2004; Torres et al., 1996). The *Hmx* homeobox genes, *Hmx2* and *Hmx3* (*Nkx5.2* and *Nkx5.1*, respectively), are co-expressed in the dorsolateral otic epithelium (Wang et al., 2001, 2004b). Targeted disruption of *Hmx2* results in agenesis of all semicircular canals and a severe loss in the three cristae and the macula utriculus (Wang et al., 2001). A null mutation in *Hmx3* causes a significant loss of sensory cells in the fused utriculosaccular cavity and an absence of the lateral crista (Wang et al., 1998). A compound null mutation of *Hmx2* and *Hmx3* results in a complete loss of the entire vestibular structures, demonstrating their redundant and distinct role in vestibular development (Wang et al., 2004b). The *distal-less* class homeobox genes, *Dlx5* and *Dlx6*, are expressed in the dorsal otic epithelium and mice deficient for these two genes fail to form dorsal otic derivatives including the semicircular ducts, utricle, saccule, and endolymphatic duct (Acampora et al., 1999; Merlo et al., 2002). Despite our knowledge about the contribution of these transcription factors in establishing patterns of growth and differentiation within distinct otocyst compartments, the regulatory relationship among these factors is not fully understood.

In addition to transcription factors, signaling pathways are critical in the induction of the otic placode and in the subsequent morphogenesis of the otocyst. The mesenchymal fibroblast growth factors, FGF8 and FGF10, are required together with FGF3 from hindbrain for the expression of otic placode genes and for otic placode induction and vesicle formation (Adamska et al., 2001; Alvarez et al., 2003; Hatch et al., 2007; Ladher et al., 2005; Wright and Mansour, 2003a,b; Zelarayan et al., 2007). WNT signals from the hindbrain are necessary to limit the region of ectoderm that forms the otic placode and are sufficient to maintain the expression of dorsal otic genes (Ohyama et al., 2006; Riccomagno et al., 2005). WNT1 and WNT3a from the dorsal hindbrain are required redundantly for the formation of the vestibular structures (Riccomagno et al., 2005). FGF3 prevents the ventral expansion of WNT3a and restricts WNT signals on the dorsal otocyst (Hatch et al., 2007). Sonic hedgehog (SHH) secreted from the notochord is required for the specification of ventral otic fate (Bok et al., 2007; Riccomagno et al., 2002). The restricted expression of WNT target genes to the dorsal otocyst is also influenced by SHH (Riccomagno et al., 2005). Thus, a balance between FGF/WNT (dorsal) and SHH (ventral) signaling activities is important in determining the vestibular and auditory cell types. FGF signals are also important for otocyst morphogenesis. Gain- and loss-of-function experiments show that FGFs in the sensory cristae promote non-sensory canal development by upregulating *Bmp2* (Chang et al., 2004). Studies in chickens and mice have shown that the formation of semicircular canals and their cristae requires BMP signals (Chang et al., 2008, 1999; Gerlach et al., 2000).

Here, we show that targeted disruption of *Lmo4*, a nuclear LIM-domain-only transcription regulator, results in a profound defect in the vestibular system of mouse inner ears including the absence of three semicircular canals, anterior and posterior cristae. Without *Lmo4*, canal outpouches fail to form and cell proliferation is significantly reduced in the dorsolateral otocyst. Interestingly, loss of *Lmo4* does not affect the initial compartmentalization of the otocyst. Rather, *Lmo4* is required for the normal expression of *Bmp4*, *Fgf10*, *Msx1, Isl1*, *Gata3*, and *Dlx5* in the dorsolateral otic vesicle. Our results demonstrate that *Lmo4* plays an essential role in vestibular morphogenesis by regulating cell proliferation and maintaining otic gene expression in the dorsolateral otocyst.

Materials and methods

Generation of Lmo4-null mice

The *Lmo4-lacZ* ($Lmo4^{lacZ}$) knock-in mutation was generated by inserting the *Lmo4* 4.3 kb 5′- and 3.0 kb 3′-flanking sequences into the 5′ and 3′ multiple cloning sites of pKII-*lacZ* knock-in vector (L. G., unpublished), respectively. In $Lmo4^{lacZ}$ knock-in construct, the coding region of Exon 2 and a part of Intron 2 were replaced by *lacZ* reporter gene and SV40 polyA sequences (Fig. 1A). The construct placed *lacZ* under the control of *Lmo4* regulatory sequences and allowed the study of *Lmo4* expression. $Lmo4^{lacZ}$ knock-in construct was linearized by *Not*I-digestion and introduced into W4 embryonic stem (ES) cells by electroporation (Auerbach et al., 2000). We obtained 11 targeted ES clones from 192 G418-resistant clones screened by Southern blot analysis and generated $Lmo4^{lacZ}$ knock-in mice from two targeted ES cell clones. To generate *Lmo4* conditional knockout allele ($Lmo4^{cko}$), we created the targeting construct by using two loxP sequences to flank Exon 2 (Fig. 1B). After electroporation of W4 ES cells with $Lmo4^{cko}$ construct, we obtained 2 positive ES clones from 144 G418-resistant clones by Southern blot screening. Both targeted ES clones were used to generate the $Lmo4^{cko}$ mice. The FRT-flanked neomycin resistance gene in $Lmo4^{cko}$ mice was removed by crossing $Lmo4^{cko}$ mice with ROSA26-FLPe mice (The Jackson Laboratory, Stock Number: 003946) to generate heterozygous $Lmo4^{lox/+}$ mice. We then crossed $Lmo4^{lox/+}$ with tissue-specific $Foxg1^{Cre}$ deleter mice (Hebert and McConnell, 2000) to remove *Lmo4* specifically in the inner ear (Fig. 1B). Southern genotyping confirmation of $Lmo4^{lacZ}$, $Lmo4^{cko}$, and $Lmo4^{lox}$ alleles was performed by using a 3′-probe on *Eco*RI-digested genomic DNA to detect 3.8, 9.8, and 6.7 kb fragments in wild type, $Lmo4^{lacZ}$, and $Lmo4^{lox}$ mice, respectively (Fig. 1C). Additionally, PCR methods were used to genotype mice from subsequent breeding of $Lmo4^{lacZ}$ mice. The PCR primers used to identify wild type *Lmo4* allele were 5′-TGCCGGCGAGCCTCCCTTCTTC-3′ and 5′-GGCAGCCCGACTTACCTA-3′, and $Lmo4^{lacZ}$ allele 5′-AGGGCCGCAAGAAAACTATCC-3′ and 5′-ACTTCGGCACCTTACGCTTCTTCT-3′. All analyses were done on a mixed C57BL/6J and 129S6 background. Embryos were identified as E0.5 at noon on the day at which vaginal plugs were observed. The University Committee of Animal Resources (UCAR) at University of Rochester approved all animal procedures used in this study.

Paint-filling analysis

Paint-fill injections were performed as described (Morsli et al., 1998). Briefly, mouse embryos at E11.5 to E15.5 were harvested and fixed overnight in Bodian's fixative. Embryos were then dehydrated in ethanol and cleared in methyl salicylate. The inner ears were visualized by injecting 0.1% white latex paint in methyl salicylate into the membranous labyrinth. At E11.5, the injection micropipette was inserted in the lateral surface of the otocyst. For E15.5 inner ears, latex paint was injected into the cochlea. Five or more inner ears were injected for each stage examined.

Immunohistochemistry, BrdU labeling, X-Gal staining, and in situ hybridization

For immunolabeling, cryosections were cut at a thickness of 14 µm. Primary antibodies and concentrations used for this study were: mouse anti-bromodeoxyuridine (BrdU) (Becton Dickson, 1:200), rabbit anti-Caspase3 (R&D system, 1:500), goat anti-LMO4 (Santa Cruz, 1:200), rabbit polyclonal anti-Pax2 (Covance, 1:200) and mouse anti-β-Tubulin (TuJ1) (Covance, 1:500). Alexa-conjugated secondary antibodies were obtained from Molecular Probes, Inc. and were used at a concentration of 1:1,000. Images were captured with a Zeiss 510 META confocal microscope.

Detection of β-galactosidase activity was determined by X-Gal staining (Gan et al., 1999). Briefly, embryos were fixed in 4% paraformaldehyde in PBS at 4 °C for 30 min. Whole-mount embryos or 20 µm frozen sections were stained overnight at room temperature with 0.1% X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS. For bromodeoxyuridine (BrdU) (Sigma) pulse-labeling experiments, pregnant females were injected intraperitoneally with 100 µg BrdU/gram body weight one hour before they were sacrificed. Embryo processing and anti-BrdU labeling were performed on serial sections as previously described (Mishina et al., 1995).