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ScienceDirect
Developmental Biology 306 (2007) 480–492
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Mouse homologues of *Shisa* antagonistic to Wnt and Fgf signalings

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Received for publication 20 November 2006; revised 12 March 2007; accepted 19 March 2007
Available online 27 March 2007

Abstract

In an effort to identify Otx2 targets in mouse anterior neuroectoderm we identified a gene, *mShisa*, which is homologous to *xShisa1* that we previously reported as a head inducer in *Xenopus*. *mShisa* encodes an antagonist against both Wnt and Fgf signalings; it inhibits these signalings cell-autonomously as *xShisa1* does. The *mShisa* expression is lost or greatly reduced in *Otx2* mutant visceral endoderm, anterior mesendoderm and anterior neuroectoderm. However, *mShisa* mutants exhibited no defects in head development. *Shisa* is composed of five subfamilies, but normal head development in *mShisa* mutants is unlikely to be explained in terms of the compensation of *mShisa* deficiency by its paralogues or by known Wnt antagonists in anterior visceral endoderm and/or anterior mesendoderm.

Keywords: *Shisa*; Head development; Anterior visceral endoderm; Anterior mesendoderm; Anterior neuroectoderm; Wnt; Fgf; Antagonist; Mutant mouse

Introduction

Otx2 plays essential roles in each step and site of mouse head development. First, *Otx2* is essential to the anterior movement of distal visceral endoderm cells to form anterior visceral endoderm (Kimura et al., 2000). A gene encoding Wnt antagonist, *Dkk1*, was identified as a direct target of *Otx2* gene product (Otx2) in visceral endoderm (Kimura-Yoshida et al., 2005). The *Dkk1* expression is lost in $Otx2^{-/-}$ mutants, and the transgenic *Dkk1* expression in visceral endoderm cells restores the anterior movement of distal visceral endoderm cells in $Otx2^{-/-}$ mutants. However, the transgenic *Dkk1* is not sufficient to restore the development of anterior neuroectoderm. This suggests that, in anterior visceral endoderm and/or anterior mesendoderm, Otx2 targets another gene to suppress posteriorizing signals for head development. A series of antagonists against Wnt, Fgf, Nodal and BMP signalings are expressed in anterior visceral endoderm and anterior mesendoderm. However, except for *Dkk1*, all of them are expressed in $Otx2^{-/-}$ distal visceral endoderm.

Otx2 is also one of the genes expressed earliest in anterior neuroectoderm induced by anterior visceral endoderm and anterior mesendoderm (Simeone et al., 1992; 1993; Ang et al., 1994). We previously identified the AN enhancer responsible for this *Otx2* expression (Kurokawa et al., 2004). In the mutants that lack this enhancer ($Otx2^{\Delta AN/-}$), *Six3*-positive anterior neuroectoderm is once induced, but subsequently caudalized into *Gbx2*-positive posterior neuroectoderm. It is speculated that Otx2 also targets antagonists against posteriorizing signals in anterior neuroectoderm. In contrast to anterior visceral endoderm and anterior mesendoderm, however, signal antagonists known to be expressed in anterior neuroectoderm are scarce. We have made an effort to identify Otx2 targets in this tissue by a microarray analysis between embryonic day (E) 7.5 $Otx2^{\Delta AN/+}$ and $Otx2^{\Delta AN/-}$ embryos. The analysis identified a gene, *mShisa*, which is homologous to *xShisa1* that we previously reported as a head inducer in *Xenopus* (Yamamoto et al., 2005).

xShisa1 encodes an antagonist against both Wnt and Fgf signalings; it inhibits these signalings cell-autonomously by

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0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.03.028

suppressing the maturation and cell surface expression of Frizzled and Fgf receptor (Yamamoto et al., 2005). Here we report that *mShisa* also encodes a cell-autonomous antagonist against Wnt and Fgf signalings. The *mShisa* expression is lost or greatly reduced in $Otx2^{-/-}$ visceral endoderm and $Otx2^{\Delta AN/-}$ anterior mesendoderm and anterior neuroectoderm. However, *mShisa* mutants exhibited no defects in head development. *Shisa* consists of five subfamilies, and a series of Wnt antagonists are expressed in anterior visceral endoderm and anterior mesendoderm. Normal head development in *mShisa* mutants, however, is unlikely to be explained in terms of the compensation of *mShisa* deficiency by its paralogues or by known functionally related genes.

Materials and methods

Isolation of mShisa

Total RNAs were isolated from E7.5 or 5–8 somite stage $Otx2^{\Delta AN/+}$ and $Otx2^{\Delta AN/+}$ embryos, in which one allele lacks the AN enhancer and the other allele is either wild-type or null mutant (Kurokawa et al., 2004). Five micrograms of total RNAs was subjected to the One-Cycle Target Labeling method to synthesize biotin-labeled cRNA probes. The cRNA probes were subsequently fragmented and hybridized to the GeneChip Mouse Genome 430 2.0 array (Affymetrix), according to the manufacturer's instruction. The microarray image data were generated with GeneChip Scanner3000 (Affymetrix) and analyzed with GeneChip Operating Software (GCOS). The signal intensities of each transcript on the chip were compared between $Otx2^{\Delta AN/-}$ and $Otx2^{\Delta AN/+}$ embryos, and the transcripts with reduced signals in the $Otx2^{\Delta AN/-}$ embryos at both E7.5 and 5–8 somite stages were selected for further analyses. One of these transcripts was an unknown IMAGE clone, *Tmem46*, which turned out to be the mouse homologue of *xShisa1* we previously isolated in *Xenopus* (Yamamoto et al., 2005). *mShisa* full-length cDNA (GenBank accession no. AB280737) was isolated from mouse E8.0–8.5 cDNA libraries (Murata et al., 2004); *mShisa3*, *mShisa4* and *mShisa5* (GenBank accession no. AK077061, AK039457 and AK033545, respectively) cDNAs were obtained from the FANTOM II libraries (Okazaki et al., 2001).

Generation of mutant mice

mShisa, *mShisa3*, *mShisa4*, *mShisa5* and *Dkk1* mutant mice (Accession no.: CDB0044K, CDB0045K, CDB0046K, CDB0047K and CDB0030K, respectively) were generated by gene targeting in TT2 embryonic stem (ES) cells as described (Yagi et al., 1993; Murata et al., 2004). Genomic DNA fragments were isolated by long amplification PCR with C57BL/6 BAC clones to construct targeting vectors (TV) and control vectors (CV) (Murata et al., 2004). To disrupt *mShisa*, *mShisa3* and *mShisa4*, the TV cassette used was *LacZ/Neo-DTA*, and the CV cassette was *LacZ/Neo*. To disrupt *mShisa5*, the *lacZ* gene in the *LacZ/Neo-DTA* cassette was capped with an internal ribosomal entry site (IRES) (Furushima et al., 2005). To disrupt *Dkk1*, the *lacZ* gene in *LacZ/Neo-DTA* and *LacZ/Neo* cassettes was replaced with *Venus* (Nagai et al., 2002) to construct TV and CV, respectively. IDs of BAC clones used, primer sequences to amplify the DNA fragments and to identify homologous recombinant ES clones, and restriction enzyme sites to which each DNA fragment was inserted in the *LacZ/Neo-DTA* or *LacZ/Neo* cassette are described in Supplementary Tables 1 and 2. Details of the vector construction will be provided upon request.

Three mutant mouse strains were established from independent homologous recombinant ES clones on each gene; no difference in phenotype was apparent among these mutant strains of the same gene. Chimeras were mated and mutant mice were maintained by mating with C57BL/6 mice. Southern hybridization was performed as described (Suda et al., 1999); genotyping of mice or embryos was routinely performed with tail, yolk sac or a part of embryonic tissue specimens by PCR using a mixture of two or three primers (Nagy et al., 2003). In some experiments, embryos after whole-mount in situ hybridization were genotyped after the overnight treatment with protease K in PCR buffer (Martinez Barbera et al., 2000). The sequences of primers used and size of PCR products are shown in Supplementary Table 2.

Sfrp1 mutant mice were previously generated and genotyped as described (Satoh et al., 2006). *Cerl*, *Cerl-2*, *Lefty1* and *Lefty2* mutant mice were the kind gift of Dr. J.A. Belo (Instituto Gulbenkian de Ciência, Portugal) and Dr. H. Hamada (Osaka University, Japan), respectively, and genotyped as described (Belo et al., 2000; Marques et al., 2004; Meno et al., 1998, 1999). The mutants maintained by mating with C57BL/6 mice were subjected to the generation of compound mutants with *mShisa* mutants.

Mice were housed in environmentally controlled rooms of the Laboratory Animal Housing Facility of the Center for Developmental Biology (CDB), RIKEN Kobe, under the Institute guidelines for animal and recombinant DNA experiments.

In situ hybridization, βGal staining and histology

Whole-mount and section in situ hybridization analyses were performed using digoxigenin-UTP-labeled (Roche) probes as described (Wilkinson, 1998; Suda et al., 2001). Probes used were as follows: *Otx2* (Matsuo et al., 1995), *Pax6* (Walther and Gruss, 1991), *Six3* (Oliver et al., 1995) and *Cer1* (Belo et al., 1997). The probe for the *mShisa3* expression was a partial cDNA, and those for *mShisa4* and *mShisa5* expression were full-length cDNA; they were obtained from the FANTOM II library (Okazaki et al., 2001). *Dkk1* (GenBank accession no. BC050189) full-length clone was obtained from IMAGE clone (http://image.llnl.gov).

βGal expression was determined as previously described (Kimura et al., 1997, 2000). E9.5 and E14.5 embryos were fixed in 4% formaldehyde and 0.2% glutaraldehyde in PBS for 5 min and 60 min, respectively. Histological analysis was performed as described (Suda et al., 2001); embryos were fixed with Bouin's fixative solution, serial sections were prepared at 10 μm thickness and stained with hematoxylin and eosin.

RT-PCR and Northern blot analysis

One microgram of total RNAs isolated with the ISOGEN (Nippon gene) from embryos at appropriate stages and of each genotype was subjected to the RT-PCR analysis, using Advantage RT-for-PCR (Clontech), according to the protocol provided by the manufacturer. The purity and quantity of cDNAs were analyzed with *Hprt* specific primers. Primers used to detect each gene transcript and lengths of amplified products are: RT-S1, 5′-TGGCTACAGACCAGTG-CAGC-3′; RT-S2, 5′-GGAGACACACACAGACACGC-3′; RT-H1, 5′-AGG-TTGCAAGCTTGCTGG-3′; RT-H2, 5′-GTAGGCTGGCCTATAGGCTC-3′. The locations for each primer are indicated in the construction figure (Fig. 6A). Northern blot analysis was conducted with total RNAs isolated from each genotype of E9.5 embryos using a probe covering *mShisa* exon 2 and 3′ UTR as previously described (Sambrook et al., 1989).

Skeletal analysis

Cartilage and bones were stained with alcian blue and alizarin red as described (Kelly and Bryden, 1983; Hide et al., 2002).

Assays with Xenopus embryos and Hek293T cells

Frog care, fertilization, and embryonic culture were carried out as described (Sive et al., 2000). mRNA injection into *Xenopus* embryos and animal cap assay were performed as described (Yamamoto et al., 2005; Nagano et al., 2006). Luciferase assay with Hek293T cells was also performed as described (Yamamoto et al., 2005; Nagano et al., 2006). To detect cellular localization of the *mShisa* gene product, the open reading frame was subcloned into pCS2 (*mShisa*/pCS2) and HA-tag sequence was added to the C-terminals of the coding sequences by PCR, yielding *mShisa*-HA/pCS2.

Results

Mouse orthologue of Xenopus Shisa1 as a potential Otx2 target

The *Otx2* expression in anterior neuroectoderm at presomite and early somite stage is regulated by an enhancer designated as