Developmental Biology 347 (2010) 53–61

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Dynein axonemal intermediate chain 2 is required for formation of the left–right body axis and kidney in medaka

Yusuke Nagao [a], Jinglei Cheng [b], Keiichiro Kamura [c], Ryoko Seki [d], Aya Maeda [a], Daichi Nihei [c], Sumito Koshida [c], Yuko Wakamatsu [a,d], Toyoshi Fujimoto [b], Masahiko Hibi [a,d,e], Hisashi Hashimoto [a,d,*]

[a] Department of Biological Sciences, Graduate School of Science, Nagoya University, Nagoya, Japan
[b] Department of Anatomy and Molecular Cell Biology, Graduate School of Medicine, Nagoya University, Nagoya, Japan
[c] Department of Biological Sciences, Graduate School of Science, the University of Tokyo, Tokyo, Japan
[d] Bioscience and Biotechnology Center, Nagoya University, Nagoya, Japan
[e] Laboratory for Vertebrate Axis Formation, RIKEN Center for Developmental Biology, Kobe, Japan

ARTICLE INFO

Article history:
Received for publication 17 March 2010
Revised 14 July 2010
Accepted 3 August 2010
Available online 11 August 2010

Keywords:
Ciliary disease
Dynein arms
Kidney cyst
Nodal flow
Left–right asymmetry
Medaka

ABSTRACT

Ciliary defects lead to various diseases, such as primary ciliary dyskinesia (PCD) and polycystic kidney disease (PKD). We isolated a medaka mutant *mii*, which exhibits defects in the left–right (LR) polarity of organs, and found that *mii* encodes *dynein axonemal intermediate chain 2a* (*dnai2a*). Ortholog mutations were recently reported to cause PCD in humans. *mii* mutant embryos exhibited loss of nodal flow in Kupffer's Vesicle (KV), which is equivalent to the mammalian node, and abnormal expression of the left-specific gene. KV cilia in the *mii* mutant were defective in their outer dynein arms (ODAs), indicating that Dnai2a is required for ODA formation in KV cilia. While the *mii* mutant retained motility of the renal cilia and failed to show PKD, the loss of *dnai2a* and another *dnai2* ortholog *dnai2b* led to PKD. These findings demonstrate that Dnai2 proteins control LR polarity and kidney formation through regulation of ciliary motility.

Introduction

Primary ciliary dyskinesia (PCD; OMIM accession 242650) is a group of autosomal-recessive genetic disorders of the motile cilia which affects approximately one in 20,000–60,000 humans. Motile cilia dysfunction results in pleiotrophic phenotypes in diverse organs of PCD patients (Zariwala et al., 2007). For instance, multiple motile cilia covering the upper and lower respiratory tracts are known to be required for mucociliary clearance, and thus their dysfunction leads to recurrent respiratory infections and inflammation of the respiratory tract in PCD patients. Male PCD patients often manifest reduced fertility, which can be accounted for by the dysmotility of the sperm flagella. These motile cilia in respiratory systems and sperm exhibit a 9+2 axonemal orientation with dynein arms on each peripheral microtubule doublet, and exhibit a beating activity (Satir and Christensen, 2007).

A portion of PCD patients exhibits *situs inversus* (heterotaxy), which is referred to as Kartagener syndrome (OMIM accession 244400). The abnormal organ laterality seen in PCD patients is caused by monocilia dysfunction in the embryonic node. The nodal cilia are solitary and have a 9+0 axonememal structure with dynein arms. The nodal cilia are motile and generate a leftward flow across the node (nodal flow) by rotational movement (Okada et al., 2005; Satir and Christensen, 2007). Studies in mouse mutants revealed that the nodal flow is critical for the proper formation of LR body axis (reviewed in (Wagner and Yost, 2000)).

Polycystic kidney disease (PKD; OMIM accession 173900), a common genetic disease characterized by distension of the renal tubules (and/or correcting ducts), is now categorized as a ciliary disease (Igarashi and Somlo, 2007). Most PKD patients carry a mutation in *PKD1* or *PKD2*, which encode polycystin 1 and polycystin 2, respectively, which localize at the ciliary membrane. Among the mouse PKD models, the *Tg737 (Polaris)* mutant, which is defective in a component of the intraflagellar transport (IFT) system and lacks ciliary structures, displays PKD and abnormal LR polarity formation. Although both PKD and PCD are caused by defective cilia, PCD does not usually accompany PKD. Renal cilia in the lumen of the tubules, which consist of 9+0 axonemes without dynein arms in mammals, are immotile, and are called "primary cilia" (Satir and Christensen, 2007). Recent advances in the understanding of PKD pathogenesis suggest that immotile primary cilia in the renal tubules function as passive mechanosensors in detecting the fluid flow rate in the tubule and thereby sense the lumen size (reviewed in (Yoder, 2007)). It is thought that abnormality of primary cilia mechanoreceptor function

* Corresponding author. Bioscience and Biotechnology Center, Nagoya University, Nagoya, Japan. Fax: +81 52 789 5053.
E-mail address: hsshsmt@bio.nagoya-u.ac.jp (H. Hashimoto).

0012-1606/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2010.08.001

results in impaired regulation of the renal lumen size and eventually leads to PKD.

Fish, such as medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), are emerging model animals for study of developmental genetics (Furutani-Seiki et al., 2004; Ishikawa, 2000; Kasahara et al., 2007; Wittbrodt et al., 2002). Accumulating data have revealed that the mechanisms underlying LR polarity formation are conserved between fish and other vertebrates (Essner et al., 2002; Levin, 2005). In fish, initial breaking of symmetry is achieved in Kupffer's vesicle (KV), which is functionally equivalent to the mouse node, where the nodal flow is generated by rotational movement of the cilia with 9 + 0 axonemes and dynein arms, as is the case in mammals (Essner et al., 2005; Okada et al., 2005). In contrast to the mammalian kidney, the maintenance of the renal lumen size requires active beating of the renal cilia, which produces the driving force for intratubular urine flow; loss or reduced motility of the cilia causes PKD in medaka and zebrafish (Kramer-Zucker et al., 2005; Omran et al., 2008). Consistent with this, the renal cilia in fish consist of 9 + 2 axonemes with dynein arms, which are features of motile cilia. The species-specific difference in phenotypes associated with defective renal ciliary motility was recently shown in mutants of Kintoun/PF13, which function in the pre-assembly of axonemal dyneins (Omran et al., 2008). The Medaka *kintoun* mutant exhibits organ laterality defects and PKD, while human patients having a mutation in the ortholog gene exhibit defective LR polarity formation, but not the PKD phenotype (Omran et al., 2008).

We carried out mutant screening in medaka and isolated a mutant, *mii* (mirror image of the internal organs), which displayed defects in organ laterality. By positional cloning, we found that the *mii* locus encodes a medaka ortholog (Dnai2a) of dynein axonemal intermediate chain 2 (Dnai2), which is a component of the outer dynein arms (ODAs). We demonstrated that *dnai2a* is expressed in the KV and that *mii* mutants display loss of nodal flow and randomization of the LR organ polarity. We have found that *dnai2b*, another *dnai2* ortholog, is expressed in the prospective kidney, and that inhibition of Dnai2a and Dnai2b resulted in PKD. The human *DNAI2* gene has been recently identified as a causative gene of PCD (Loges et al., 2008). Our findings reveal a conserved role for Dnai2 in ciliary motility and nodal flow-mediated LR polarity formation, and the involvement of Dnai2a and Dnai2b in the motility of the renal cilia as well as kidney formation in medaka. We demonstrate that the *mii* mutant is a medaka disease model for human PCD.

Materials and methods

Medaka strains

The orange-red variety (OR) of the medaka fish *Oryzias latipes* was used as a wild type strain. Mutagenesis screen was carried out using the see-through II (STII) strain (Wakamatsu et al., 2001). The HNI-I inbred strain (Hyodo-Taguchi, 1996) was used for crossing in genetic mapping. The *mii* mutant STII was repeatedly crossed with the OR for more than 5 times to change the genetic background. The *mii* heterozygous fish was identified by the production of homozygous offspring in pair mating, and used to maintain the mutant line.

Whole mount in situ hybridization

In situ hybridization was performed as previously described (Hashimoto et al., 2004). A digoxygenin-labeled riboprobe was made from a cloned template in pDrive (Qiagen) or pCRII-TOPO (Invitrogen) using SP6 or T7 polymerase after restriction enzyme digestion. The embryos were treated with Proteinase K (10 μg/ml) in PBS for 5 min at 30 °C before hybridization. Detection signals were developed in BM purple (Roche).

Immunohistochemistry

Immunohistochemistry was performed as described previously (Hashimoto et al., 2009). The cilia were visualized with anti-acetylated-α-tubulin antibody (Sigma) and goat anti-mouse Alexa 488 (Invitrogen). Images were obtained with a laser-scanning inverted microscope (LSM 700, Carl Zeiss). At least 15 KV cilia length was measured for each individuals in three embryos per group according to the method described previously (Hashimoto et al., 2009).

Positional cloning

The *mii* heterozygous fish were crossed with wild type HNI fish. We obtained 150 embryos of their F2 offspring displaying reversed internal organs. Bulked segregant analysis with the M-marker system (Kimura et al., 2004) using these putative homozygotes mapped the *mii* locus on the medaka LG 17 (data not shown). By further recombination analysis, the *mii* locus was narrowed down to the 480 kb region between the flanking markers MF01SSB034M20 and AU169106. Detailed information on the markers used is available on request.

Antisense-mediated knockdown and rescue by synthetic RNA injection

Antisense gripNA (Active motif) for *dnai2a* and *dnai2b* was designed to prevent *dnai2* mRNA from being spliced at exon4/intron4 donor site and morpholino for *dnai2b* at exon1/intron1. The gripNA sequence is: 5'-CTGACCCACCAACATCCC-3' for *dnai2a* (grip-*dnai2a*), 5'-TTGACCCACCAATGTCCC-3' for *dnai2b* (grip-*dnai2b*). The morpholino sequence is: 5'-ACATTTTCAGGTTAACTCACCTGTT-3' for *dnai2b* (MO-*dnai2b*). The gripNA and morpholino were resuspended and diluted with HPLC water. A gripNA 5'-GGCACTGACCTTCAGGCT-3' (recognizes the gene which is unrelated to *dnai2a*/*b*) was used as a control for the knockdown experiments described in Fig. 5.

Wild type *dnai2a* and *dnai2b* mRNAs were synthesized from a cloned cDNA with the open reading frame in pCS2 vector as previously described (Hashimoto et al., 2004). Wild type OR embryos and offspring from crossed heterozygous *mii* were used for knockdown and for rescue, respectively. gripNA or synthetic RNA was microinjected in one blastomere at the 1 or 2-cell stage embryos.

Transmission electron microscopy

Embryos were dechorionated with hatching enzyme and incubated until the 6-somite stage. Kidneys were obtained from OR fish and *mii* homozygous fish, which were 3–4 months old. Samples were fixed with 2% glutaraldehyde, 2% paraformaldehyde, 0.2% tannic acid, and 10% sucrose in 0.1 M cacodylate buffer (pH7.4) at 4 °C overnight, followed by postfixation with 1% OsO4 and 10% sucrose in 0.1 M cacodylate buffer (pH7.4) for 1 h and dehydrated with ethanol. Then the samples were stained with 2% uranyl acetate in ethanol for 30 min and embedded in Quetol812 resin.

Detection of nodal flow

To detect nodal flow, fluorescent beads were injected into Kupffer's vesicle of 2-somite embryos as previously described (Hojo et al., 2007), and the movement of the fluorescent beads was observed as an indicator of nodal flow. Sample embryos were prepared by artificial insemination with homozygous eggs and sperm.

Histological analysis

Paraffin sections were prepared according to the method previously described (Fedorova et al., 2008). Briefly, fish were fixed with Bouin's solution, dehydrated with an alcohol series, and then embedded in paraffin. The samples were transversely sectioned at a thickness of 6 μm and stained with hematoxylin and eosin.

Observation of renal cilia

Kidneys were excised from OR fish and *mii* homozygous fish. In Yamamoto's Ringer solution (128 mM NaCl, 5 mM KCl, 1.4 mM CaCl2 and 2.4 mM NaHCO3, pH7.3), kidneys were finely cut up with a scalpel and then the tissues were additionally broken up with trypsinization and voltex mixing. Samples were mounted on a slide glass and observed under microscopy.

Results

Medaka mii mutant displays abnormal left–right patterning

We carried out N-ethyl-N-nitrosourea (ENU)-mediated mutagenesis to screen medaka mutants with defects in embryonic and adult organogenesis (the detailed information will be published elsewhere). Two mutations exhibited abnormal organ laterality, and they turned out to be non-allelic. We named one mutant '*mii*' (mirror image of the internal organs) after the mutant phenotype and further analyzed it in this study. *mii* mutants were found to be viable and grew to adulthood, although they exhibited a severely wavy trunk and did not spawn by natural mating (Fig. 1A and B). In the wild type medaka, the heart ventricle is positioned on the right side and the gallbladder and liver are on the left side. This laterality was retained at a high rate ($n = 164/165$)