Developmental Biology 354 (2011) 144–150

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

Repression of *Rx* gene on the left side of the sensory vesicle by Nodal signaling is crucial for right-sided formation of the ocellus photoreceptor in the development of *Ciona intestinalis*

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ARTICLE INFO

Article history:
Received for publication 1 November 2010
Revised 18 February 2011
Accepted 4 March 2011
Available online 11 March 2011

Keywords:
Nodal
Left–right asymmetry
Ocellus
Rx
Ciona intestinalis

ABSTRACT

Nodal signaling plays an essential role in the establishment of left–right asymmetry in various animals. However, it is largely unknown how Nodal signaling is involved in the establishment of the left–right asymmetric morphology. In this study, the role of Nodal signaling in the left–right asymmetric ocellus formation in the ascidian, *Ciona intestinalis* was dealt with. During the development of *C. intestinalis*, the ocellus pigment cell forms on the midline and moves to the right side of the midline. Then, the photoreceptor cells form on the right side of the sensory vesicle (SV). *Ci-Nodal* is expressed on the left side of the SV in the developing tail bud embryo. When Nodal signaling is inhibited, the ocellus pigment cell form but remain on the midline, and expression of marker genes of the ocellus photoreceptor cells is ectopically detected on the left side as well as on the right side of the SV in the larva. Furthermore, *Ci-Rx*, which is essential for the ocellus differentiation, turns out to be negatively regulated by the Nodal signaling on the left side of the SV, even though it is required for the right-sided photoreceptor formation. These results indicate that Nodal signaling controls the left–right asymmetric ocellus formation in the development of *C. intestinalis*.

Introduction

Left–right asymmetry is a fundamental characteristic of vertebrates and many invertebrates (Brown and Wolpert, 1990; Levin, 2005). In vertebrates, Nodal signaling plays an essential role in the establishment of left–right asymmetry by regulating a homeodomain transcription factor, *Pitx2*, in the left lateral plate mesoderm. *Nodal-Pitx* cascade is essential for left–right asymmetric morphogenesis, such as unidirectional looping of heart and gut in vertebrate development (Capdevila et al., 2000; Hamada et al., 2002; Shiratori et al., 2001). The left–right asymmetric *Nodal-Pitx* cascade can also be found in deuterostome animals, such as ascidians, amphioxus and sea urchins (Boorman and Shimeld, 2002a; Duboc and Lepage, 2008; Morokuma et al., 2002; Yoshida and Saiga, 2008; Yu et al., 2002), and even in snails (Grande and Patel, 2009). However, it is not well understood how left–right asymmetric morphology is established from the asymmetric Nodal signaling and what are the downstream effector genes of asymmetric morphogenesis.

Ascidians belong to the subphylum Urochordata regarded as the closest relative of vertebrates (Putnam et al., 2008). Their larvae share a characteristic body plan of chordates with a hollow dorsal neural tube, a notochord and paraxial mesoderm. Left–right asymmetry can also be seen in the development of ascidians (Boorman and Shimeld, 2002a). Morphological asymmetry is apparent in unidirectional coiling of the elongation of the tail in the chorion, right-sided positioning of the two sensory pigment cells in the sensory vesicle (SV), which is the anterior most part of the larval central nervous system (CNS), and unidirectional gut looping in the juvenile ascidians. SV contains two sensory organs, the ocellus and the otolith. In an ascidian species, *Ciona intestinalis*, the ocellus, a light-sensing organ consisting of a pigmented cell, three lens cells and about 20 photoreceptor cells, is located on the right side of SV (Nicol and Meinertzhagen, 1991). The otolith consists of a single pigmented cell and is considered to be involved in geotactic response. The otolith and the ocellus pigment cells are aligned anterior–posteriorly on the midline at the early tail bud stage, and then the two pigment cells shift from the midline to the right side by the late tail bud stage (stages E75–E85 in Cole and Meinertzhagen, 2004). Although the otolith pigment cell shifts to the right side during the tail bud stage, the right-sided shift of the otolith is less clear than the shift of ocellus in the larvae.

In ascidians, *C. intestinalis* and *Halocynthia roretzi*, *Nodal* gene is expressed on the left side of the epidermis and it regulates the expression of *Pitx* in the left epidermis during tail bud stages (Morokuma et al., 2002; Yoshida and Saiga, 2008). In *C. intestinalis*, expression of *Ci-Nodal* starts at the 32-cell stage in three pairs of vegetal blastomeres and a pair of blastomeres designated b6.5. In the b6.5 descendants, expression of *Ci-Nodal* continues until the gastrula

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doi:10.1016/j.ydbio.2011.03.006

stage (Imai et al., 2004), which is required for the determination of fates of a subset of muscle and notochord cells, patterning of the neural plate across the medio-lateral axis, and formation of the neural tube (Hudson and Yasuo, 2005, 2006; Mita and Fujiwara, 2007). However, the functional aspect of left-sided Nodal signaling in the left–right asymmetric morphogenesis in the ascidian development has not been clarified so far, except for *Pitx* genes. Furthermore, it is still not known which genes are targets in the establishment of morphological left–right asymmetry in ascidians.

Our study has revealed the new role of the left-sided *Nodal* expression in the establishment of the left–right asymmetric morphogenesis of *C. intestinalis*. We have shown that Nodal signaling is required for the proper right-sided localization of the ocellus pigment cell and for the right-sided formation of photoreceptor cells. Also, *Ci-Rx*, which is known to be essential for ocellus formation and is expressed on the right side of SV at the late tail bud stage (D'Aniello et al., 2006), has been identified as a downstream gene of the asymmetric Nodal signaling. *Ci-Nodal* represses expression of *Ci-Rx* on the left side of SV, which, in turn, leads to the right-sided photoreceptor formation. These results demonstrate that Nodal signaling plays an essential role in the left–right asymmetric ocellus formation during the development of *C. intestinalis*.

Materials and methods

Ascidians

Adult ascidians of *C. intestinalis* were provided by the Maizuru Fisheries Research Station of Kyoto University and Misaki Marine Biological Station of University of Tokyo through the National Bio-Resource Project (NBRP) of the MEXT, Japan. Eggs and sperm were obtained surgically from the gonoducts. After insemination, embryos were raised in filtered seawater at 18 °C. Embryos were allowed to develop within the chorion, then dechorionated immediately prior to the fixation.

Treatment with Nodal signaling pathway inhibitor

SB431542 (Sigma) was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 5 mM and stored as stock solution at −20 °C. The stock solution was diluted with the filtered seawater immediately prior to use. Embryos were treated with 5 μM SB431542 or 0.1% (vol./vol.) DMSO from the neurula stage (7 hours post fertilization [hpf]).

Whole-mount in situ hybridization and histochemical staining

Whole-mount in situ hybridization (WISH) was carried out using digoxigenin or fluorescein labeled RNA probes as described previously (Ikuta and Saiga, 2007; Ikuta et al., 2004). RNA probes for *Ci-Tyrosinase*, *Ci-opsin1*, *Ci-arrestin* and *Ci-Cralbp* were synthesized using EST clones, citb041l04, cilv041m16, cilv038l24 and cilv037n16, respectively, as templates, obtained from *C. intestinalis* Gene Collection release 1 (http://www.ghost.zool.kyoto-u.ac.jp/indexr1.html). A DNA fragment for probe synthesis of *Ci-Rx* was obtained through RT-PCR using total RNA prepared from tail bud stage embryos and cloned into the *EcoRI*/*ClaI* sites of the pBluescript KS + vector. A template for RNA probe synthesis of *Ci-Nodal* was prepared as described previously (Yoshida and Saiga, 2008).

Phalloidin staining was carried out as described previously (Christiaen et al., 2005). Images were captured using a Zeiss LSM710 confocal scanning system. Signals of Alexafluor-546-phalloidin (Molecular Probes) and DAPI were transformed into pseudo-green and pseudo-magenta, respectively, using ZEN 2009 Light Edition (Zeiss).

Preparation of plasmid DNA constructs and microinjection

A genomic DNA fragment of 5′ flanking region of *Ci-Cralbp* was amplified by PCR using the primers 5′-TTTTCTAGATGAATCT-TAAAATGTACAGCGTT-3′ and 5′-TTAGGATCCATGGTTGCAGG-GAAATG-3′. To create *Cralbp-GFP* reporter construct, the isolated DNA fragment was inserted into the *XbaI*/*BamHI* sites within the pPD-GFP vector generated by replacing the *lacZ* gene with the *GFP* gene coding sequence at the *KpnI*/*EcoRI* sites of pPD46.21 vector, a variant of pPD1.27 (Fire et al., 1990). The *GFP* gene has been isolated from pβGFP/RN3P vector (Zernicka-Goetz et al., 1996). A *Ci-Nodal* cDNA fragment was obtained through RT-PCR using total RNA prepared from tail bud stage embryos. The primers used for the RT-PCR were 5′-AAAGGTACCACCGGAAGTTACAAAATATTCG-3′ and 5′-AAAGAATT-CAACAATTGGAACTTTATGACGTA-3′. To create pPD-*Nodal* plasmid, the *lacZ* gene was replaced by the *Ci-Nodal* cDNA at the *KpnI*/*EcoRI* sites of the pPD46.21 vector. To create the construct of *Cralbp-Nodal*, 5′ flanking region of *Ci-Cralbp* was isolated from the *Cralbp-GFP* construct and inserted into the *XbaI*/*SmaI* sites within the pPD-*Nodal* plasmid.

Construct plasmid DNAs of a circular form were dissolved in 0.1× TE buffer. Constructs of *Cralbp-Nodal* and *Cralbp-GFP* were injected together into fertilized eggs with the chorion to give final concentrations of 0.16 pg/μl (*Cralbp-Nodal*) and of 0.08 pg/μl (*Cralbp-GFP*), respectively. For control experiments, *Cralbp-GFP* was injected into fertilized eggs with the chorion to give the final concentration of 0.24 pg/μl. Embryos positive for GFP expression in SV were used for WISH analysis.

Results

Left–right asymmetric expression of Ci-Nodal

Prior to the functional analysis of asymmetric Ci-Nodal signaling, we examined expression of *Ci-Nodal*. Although it has been reported that *Ci-Nodal* is expressed in the left epidermis at the early tail bud stage (Yoshida and Saiga, 2008), asymmetric expression of *Ci-Nodal* in later stages, when morphological asymmetry becomes evident, has not been reported. Therefore, we examined the expression pattern of *Ci-Nodal* in the later tail bud stages. Left–right asymmetric expression of *Ci-Nodal* became detectable by WISH in the left epidermis at the early tail bud stage (Fig. 1A; Yoshida and Saiga, 2008). This expression continued until the mid tail bud stage (Fig. 1B). As development proceeded, *Ci-Nodal* expression in the left epidermis became down regulated. At the late tail bud stage, expression of *Ci-Nodal* was detected on the left side of the SV and on the left side of the posterior endoderm (Fig. 1C). This asymmetric expression of *Ci-Nodal* in the SV raises a possibility that Nodal signaling is involved in the asymmetric morphogenesis of the SV.

Nodal signaling is required for asymmetric ocellus formation

To investigate the function of the asymmetric Nodal signaling in the SV development, the effect of Nodal signaling inhibition upon the morphology of the SV was examined using SB431542, which is an inhibitor of ALK4/5/7 (Inman et al., 2002). Normally developing embryos were treated with SB431542 from the neurula stage and allowed to develop to the swimming larva stage. Larvae treated with SB431542 hatched at the same timing as the control larvae. However, in the SB431542 treated larvae, the ocellus pigment cell, which is positioned on the right side of the SV in normal development (Fig. 2D), was located on the midline and it appeared as two pigment cells (Fig. 2A). Confocal imaging with Phalloidin and DAPI staining confirmed the existence of a single pigment cell located on the midline, in which melanin granules are located bilaterally, and are separated by the midline (Fig. 2B and C).