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Evolution of Developmental Control Mechanisms

The formation and positioning of cilia in *Ciona intestinalis* embryos in relation to the generation and evolution of chordate left–right asymmetry

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ABSTRACT

In the early mouse embryo monocilia on the ventral node rotate to generate a leftward flow of fluid. This nodal flow is essential for the left-sided expression of *nodal* and *pitx2*, and for subsequent asymmetric organ patterning. Equivalent left fluid flow has been identified in other vertebrates, including *Xenopus* and zebrafish, indicating it is an ancient vertebrate mechanism. Asymmetric *nodal* and *Pitx* expression have also been identified in several invertebrates, including the vertebrates' nearest relatives, the urochordates. However whether cilia regulate this asymmetric gene expression remains unknown, and previous studies in urochordates have not identified any cilia prior to the larval stage, when asymmetry is already long established. Here we use Scanning and Transmission Electron Microscopy and immunofluorescence to investigate cilia in the urochordate *Ciona intestinalis*. We show that single cilia are transiently present on each ectoderm cell of the late neurula/early tailbud stage embryo, a time point just before onset of asymmetric *nodal* expression. Mapping the position of each cilium on these cells shows they are posteriorly positioned, something also described for mouse node cilia. The *C. intestinalis* cilia have a 9+0 ring ultrastructure, however we find no evidence of structures associated with motility such as dynein arms, radial spokes or nexin. Furthermore the 9+0 ring structure becomes disorganised immediately after the cilia have exited the cell, indicative of cilia which are not capable of motility. Our results indicate that although cilia are present prior to molecular asymmetries, they are not motile and hence cannot be operating in the same way as the flow-generating cilia of the vertebrate node. We conclude that the cilia may have a role in the development of *C. intestinalis* left–right asymmetry but that this would have to be in a sensory capacity, perhaps as mechanosensors as hypothesised in two-cilia physical models of vertebrate cilia-driven asymmetry.

Introduction

Many animals have asymmetric positioning of organs which differentiates the left side of the organism from the right. In humans, the failure to establish correct organ positioning results in heterotaxy (Kosaki and Casey, 1998) which is a symptom of a number of genetic diseases including primary ciliary dyskinesia (PCD), also known as immotile cilia syndrome. PCD has a 50% incidence of *situs inversus*, the complete mirror image reversal of organs, as well as defects in respiratory cilia and sperm motility (Afzelius, 1976; Noone et al., 1999).

Morphological asymmetry in mice is preceded by molecular asymmetry, with the left-sided expression of *nodal* observed adjacent to the mouse node (Collignon et al., 1996; Lowe et al., 1996). Left-sided *nodal* expression is later transferred from the node to the Lateral Plate Mesoderm, which is essential for downstream morphological asymmetries (Brennan et al., 2002; Kawasumi et al., 2011), and is followed by asymmetric expression of the *nodal* antagonists *lefty-1* and *−2* (Meno et al., 1996) and the transcription factor *pitx2* (Ryan et al., 1998).

Scanning Electron Microscopy (SEM) of mouse embryos from this developmental time shows the presence of cilia, ~5 μm in length and 0.3 μm in diameter (Hirokawa et al., 2009), on the mouse ventral node, with one cilium projection per cell (Sulik et al., 1994). The microtubule-based cytoskeleton known as the axoneme can provide strength and motility for cilia, and cilia are generally separated into two groups on the basis of axoneme structure; motile (9+2 with dynein arms, radial spokes and nexin) and immotile (9+0 without dynein arms). However the cilia at the mouse ventral node do not categorise to either type, instead having an unusual 9+0 with dynein arms structure (Fig. 1A) (Hirokawa et al., 2006).

Cilia at the mouse ventral node are motile and beat in a vortical fashion which generates a net leftward fluid flow across the node (Nonaka et al., 1998). The flow, termed nodal flow, generated by cilia is able to create a net flow as a result of the posterior position and 40°±10° tilt of each cilium on the cells of the node (Hashimoto et al., 2010; Hirokawa et al., 2006; Nonaka et al., 2005; Okada et al., 2005). Disruption to nodal flow or the positioning of cilia leads to

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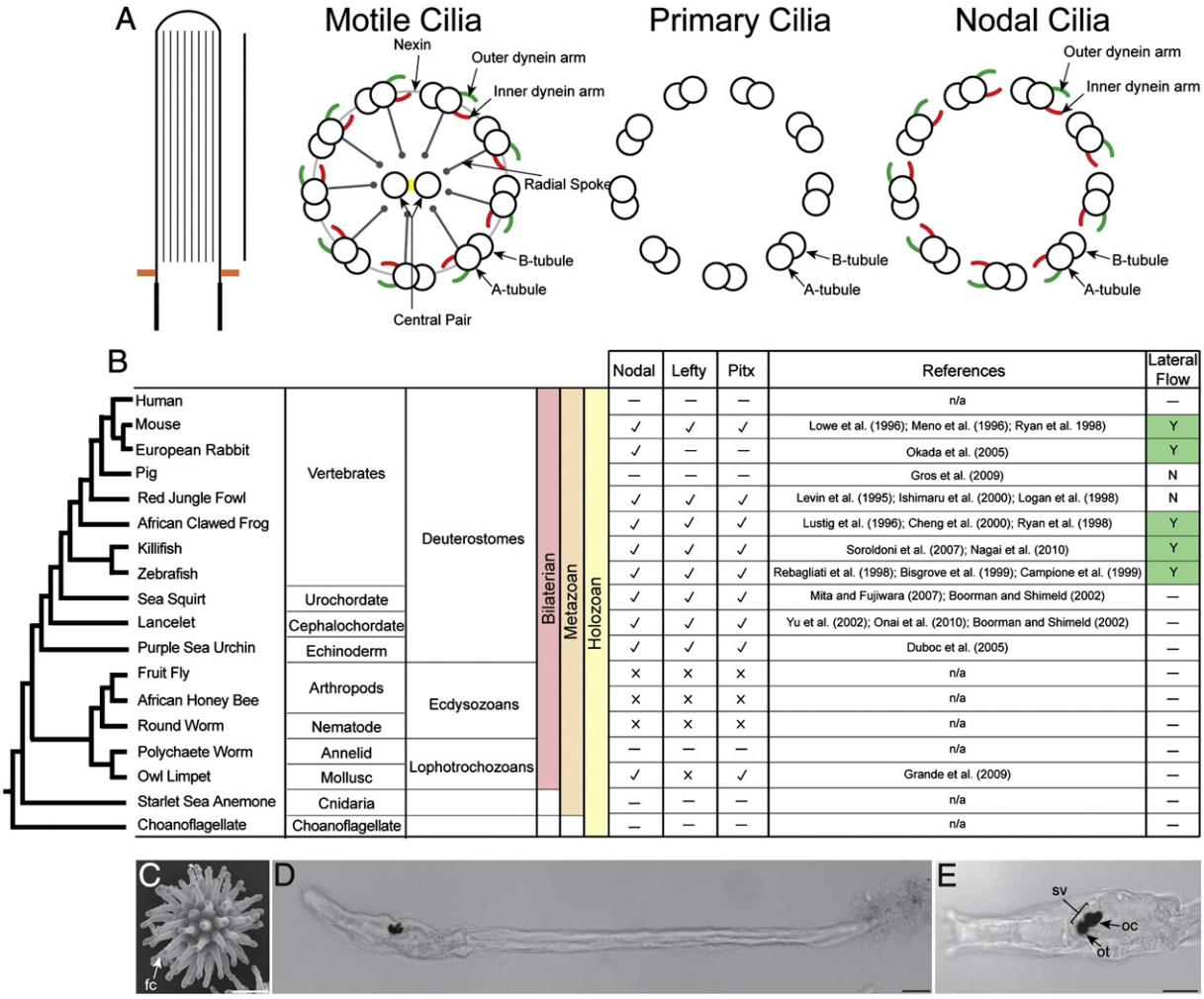

Fig. 1. Mechanisms and asymmetric gene expression involved in left–right asymmetry across the holozoa, including *Ciona intestinalis*. A: The ultrastructure of motile cilia (9 + 2), primary cilia (9 + 0) and nodal cilia (9 + 0 with dynein arms). Motile cilia have a 9 + 2 structure in which the nine doublet microtubules surround a central pair whereas primary cilia do not possess a central pair. Additionally, motile cilia also possess dynein arms, radial spokes and nexin connections which are essential for motility. Nodal cilia have the same structure as primary cilia but also possess dynein arms which provide the motor to enable the cilia to rotate. B: The Holozoan phylogeny of selected genomes and including the presence of asymmetric localisation of genes important in the development of the left–right axis (asymmetric localisation through experimentation is depicted with a tick). The presence and asymmetric localisation of the genes has not been tested in some species (−) or is absent (cross). Some species which are known to require lateral flow for the generation of left–right asymmetry are indicated with a green box and Y. Experimentation has shown that lateral flow is not present on the chick (red jungle fowl) or pig. Compared to chordates the purple sea urchin has reversed asymmetric expression, as do some molluscs. Relevant references for gene expression are (Bisgrove et al., 1999; Boorman and Shimeld, 2002; Campione et al., 1999; Cheng et al., 2000; Duboc et al., 2005; Grande and Patel, 2009; Ishimaru et al., 2000; Levin et al., 1995; Logan et al., 1998; Lowe et al., 1996; Lustig et al., 1996; Meno et al., 1996; Mita and Fujiwara, 2007; Nagai et al., 2010; Okada et al., 2005; Onai et al., 2010; Rebagliati et al., 1998; Ryan et al., 1998; Soroldoni et al., 2007; Yu et al., 2007). C: SEM of a *C. intestinalis* embryo within a chorion with emanating follicle cells (fc). D: A *C. intestinalis* larvae. E: A dorsal view of the head of a *C. intestinalis* larva, showing the asymmetric positioning of the ocellus (oc) and otolith (ot) in the sensory vesicle (sv). Scale bars = 100 μm (C); 50 μm (D); 20 μm (E).

randomised and bilateral expression of normally left-sided expressed genes, and compromised downstream left–right development. While a role for cilia in the development of left–right asymmetry is now generally accepted, the precise mechanism whereby nodal flow sets up the left–right axis remains controversial. Two main hypotheses have been invoked to explain this: the chemical hypothesis and the physical hypothesis. The former is based on the idea that a morphogen is transported to the left of the node in nodal vesicular parcels (NVPs) by the nodal flow, hence producing a concentration gradient in the cavity of the ventral node (Nonaka et al., 1998; Okada et al., 2005). The latter assumes that the leftward flow is mechanically detected by immotile cilia on the periphery of the ventral node, resulting in an intracellular Ca^{2+} cascade (Fliegauf et al., 2007; Hamada, 2008; Hirokawa et al., 2009; McGrath et al., 2003; Shiratori and Hamada, 2006; Vandenberg and Levin, 2010).

Nodal flow is conserved in some other vertebrates, with cilia driven lateral fluid flow identified in the homologous organiser structures in rabbit, *Xenopus* and zebrafish, but has not been identified in invertebrates. However the asymmetric expression of *nodal* and *pitx* has been found in invertebrate chordates, echinoderms and molluscs, suggesting it is of ancient evolutionary origin (Fig. 1B). Here, we focus on the vertebrates nearest invertebrate relative, the urochordates (Delsuc et al., 2006), to test whether cilia may be involved in the regulation of left–right asymmetry outside the vertebrates.

Urochordates, together with cephalochordates and vertebrates, constitute the phylum Chordata. At some point in their lifecycle all chordates share the same distinctive characteristics including a notochord, dorsal neural tube, endostyle and postanal tail. The two most frequently studied urochordate systems are the ascidians *Ciona intestinalis* and *Halocynthia roretzi*, both sessile filter feeders as adults but which during embryogenesis develop a motile tadpole larva with clear similarities to vertebrates. The *C. intestinalis* larva has left–right asymmetry, with asymmetric positioning of two sensory pigment spots and adjacent regions of the brain, while in the adult there is an asymmetrically folded gut (Fig. 1C–E) (Boorman and Shimeld, 2002). *Nodal* has been described in *H. roretzi* (Morokuma et al., 2002), *C. intestinalis* (Mita and Fujiwara,