Developmental Biology 348 (2010) 67–75

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

ankAT-1 is a novel gene mediating the apical tuft formation in the sea urchin embryo

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ARTICLE INFO

Article history:
Received for publication 20 July 2010
Revised 16 September 2010
Accepted 16 September 2010
Available online 27 September 2010

Keywords:
Ankyrin
Cilia
Ciliogenesis
FoxQ2
NK2.1
Nodal
Animal plate

ABSTRACT

In sea urchin embryos, the apical tuft forms within the neurogenic animal plate. When FoxQ2, one of the earliest factors expressed specifically in the animal plate by early blastula stage, is knocked down, the structure of the apical tuft is altered. To determine the basis of this phenotype, we identified FoxQ2-dependent genes using microarray analysis. The most strongly down-regulated gene in FoxQ2 morphants encodes a protein with ankyrin repeats region in its N-terminal domain. We named this gene *ankAT-1*, Ankyrin-containing gene specific for Apical Tuft. Initially its expression in the animal pole region of very early blastula stage embryos is FoxQ2-independent but becomes FoxQ2-dependent beginning at mesenchyme blastula stage and continuing in the animal plate of 3-day larvae. Furthermore, like FoxQ2, this gene is expressed throughout the expanded apical tuft region that forms in embryos lacking nuclear β-catenin. When AnkAT-1 is knocked-down by injecting a morpholino, the cilia at the animal plate in the resulting embryos are much shorter and their motility is less than that of motile cilia in other ectoderm cells, and remains similar to that of long apical tuft cilia. We conclude that AnkAT-1 is involved in regulating the length of apical tuft cilia.

Introduction

Cilia or flagella are conserved structures observed in almost all animal cells at least once during their lives. Because of their characteristic appearances and behaviors, they have been studied extensively to examine the structural components and signaling pathways driving beating forces especially in unicellular organisms or sperm of multicellular organisms. Motile cilia assumed to produce liquid current around cells or individual organisms have elementally conserved molecular sets, such as 9 + 2 microtubules, radial spokes, and inner and outer dyneins (Inaba, 2007). The precise orientation of these fundamental sets is required for cilia to produce and control their beating behaviors. To form precisely constructed cilia, the components of axoneme are transferred towards the tip of cilia along the doublet microtubules by intraflagellar transport, IFT, dependent on kinesin-II and cytoplasmic dyneins (Kozminski et al., 1995; Pazour et al., 1998; Rosenbaum and Witman, 2002). Some of the non-motile cilia are used for sensing the external environment, for instance, at photoreceptor cells or olfactory epithelia. Chemical or mechanical receptors are transferred by the IFT system to the membrane of those non-motile cilia, and their defects cause behavioral disorders (Insinna and Besharse, 2008; Kuhara et al., 2008; Satir et al., 2010). Thus, for both motile and immotile cilia it is essential to produce the precise structure to achieve normal function.

Some invertebrate embryos living in water that use motile cilia instead of muscles to move have long immotile cilia at the anterior end of the body. These cilia, as a group, are called the apical tuft. Compared with the motile cilia normally covering the entire body of the embryo and beating synchronously to produce a water current, the apical tuft cilia are longer and almost immotile. As shown in Fig. 1A, the presence of an apical tuft has been reported not only in triploblastic animals such as echinoderms (Hörstadius, 1939), hemichordates (Urata and Yamaguchi, 2004), mollusca (Dictus and Damen, 1997) and annelids (Arenas-Mena et al., 2007) but also in diploblastic animals like sea anemones (Pang et al., 2004). In those embryos, the apical tuft has been thought to be part of a sensory organ because the long cilia seldom show standard beating behavior and the anterior end of the embryonic body contains many nerves (Bisgrove and Burke, 1987; Page, 2002). However, very little is understood about the composition or the mechanisms of formation of these cilia.

In the sea urchin embryo, the long and almost immotile apical tuft forms during blastula stages at the animal plate, which is a neurogenic ectoderm, persists to prism stage and disappears at pluteus stage (Fig. 1B–G; supplemental movie 1). Although its existence as part of the apical organ has been known for several decades (Hörstadius, 1939), its function and the molecules that confer its unusual length and lack of motility remain unknown. Dunn et al. (2007) showed that a transcription factor, NK2.1, is involved in inducing genes encoding ciliary components that are expressed around the apical tuft and NK2.1 morphants lack the long cilia and have reduced swimming

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doi:10.1016/j.ydbio.2010.09.011

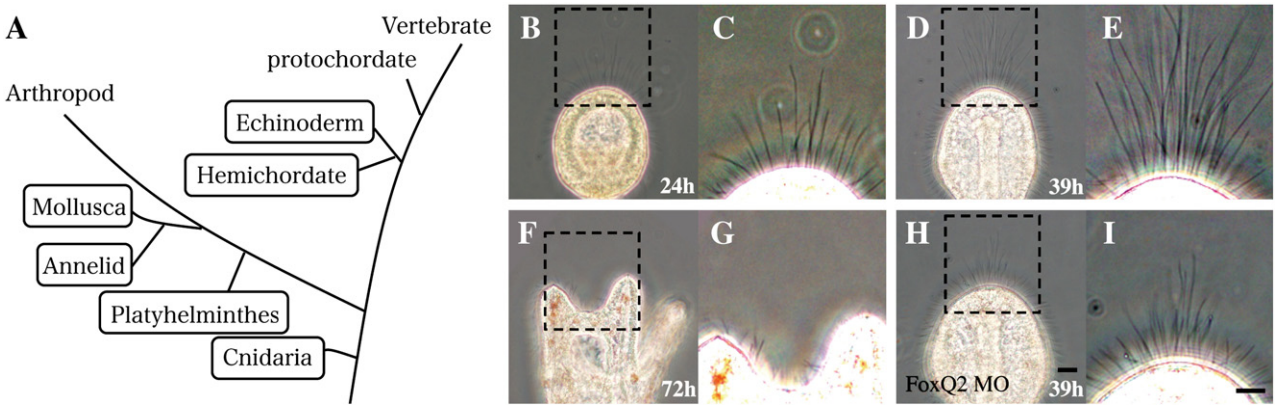

Fig. 1. The apical tuft is present in the animal plate of the sea urchin embryos. (A) The phylogenetic position of animals that has been reported as having an apical tuft. The embryos/larvae of phyla enclosed with the square have an apical tuft at the anterior end. (B) The apical tuft observed in early gastrula. (C) Magnified image of dashed-lined rectangle in (B). (D) The apical tuft observed in prism larva. (E) The magnified image of (D) shows the apical tuft cilia increase in number and length compared to those in (C). (F) At the 72-h pluteus stage, the embryo loses the apical tuft. (G) Magnified image of (F). (H) FoxQ2 morphant does not have a long apical tuft. Prism larva is shown here. (I) Magnified image of the rectangle region of (H). Bars in (H) and (I) = 25 μm and 20 μm, and refer to panels (B, D, and F) and (C, E, and G), respectively.

activity in *Strongylocentrotus purpuratus*. This is the only gene known to be required for the formation of the apical tuft in sea urchin embryos so far. However, the role of NK2.1 and its target genes in the structure and function of the apical tuft is not clear. First, the NK2.1-dependent genes examined are expressed maternally and their functions related to these long cilia are unknown. Second, NK2.1 probably has many additional functions based on its expression in other parts of the embryo (Takacs et al., 2004). In this study, to understand the mechanisms controlling the formation of the long immotile cilia, we focused on identifying genes specific for the apical tuft. We used a microarray approach to identify genes whose expression depends on FoxQ2, because this factor is encoded by one of the earliest regulatory genes expressed only at the animal plate where the apical tuft forms and has been implicated in animal plate specification (Tu et al., 2006; Yaguchi et al., 2008). We show here that one of the genes downstream of FoxQ2 function during mesenchyme blastula and gastrula stages is specific for apical tuft formation, and could be a key factor for further investigating the apical tuft function.

Materials and methods

Animals and embryo culture

Adult sea urchins, *S. purpuratus* obtained from The Cultured Abalone, Goleta, CA were maintained in seawater at 10 °C and the embryos were used for microarray experiment. For all of other experiments, the embryos of the sea urchin, *Hemicentrotus pulcherrimus* collected around Shimoda Marine Research Center, University of Tsukuba, around Research Center for Marine Biology, Tohoku University, and around Marine and Coastal Research Center, Ochanomizu University were used. The gametes were collected by intra-blastocoelar injection of 0.5 M KCl and the embryos were cultured by standard methods with filtered natural seawater (FSW) at 15 °C.

Microinjection of morpholino antisense oligonucleotides (MO) and synthetic mRNAs

Dejellied eggs were arrayed in rows on a 35-mm plastic dish coated with 1% protamine sulfate (Sigma). After insemination in FSW containing 3-amino-1,2,4-triazole (Sigma), microinjection was performed with micromanipulators (Narishige) and an injector (Femtojet; Eppendorf). All control embryos shown in this study were injected with 24% glycerol. We used the following morpholinos with a concentration in final 24% glycerol in injection needles and injected them about 1% volume of egg: SpFoxQ2-MO1 (800 μM; Yaguchi et al., 2008), AnkAT-1-MO1 (1.6–2.0 mM), AnkAT-1-MO2 (0.4–3.8 mM), FoxQ2-MO (200 μM), NK2.1-MO (1.0 mM), Nodal-MO (200 μM), and Lefty-MO (400 μM). All morpholinos are designed against *H. purcherrimus* genes except for SpFoxQ2-MO1 against *S. purpuratus* FoxQ2 in microarray experiment and the morphants for which published phenotypes exist in other urchin species showed the same phenotypes (Duboc et al., 2004, 2008; Dunn et al., 2007; Yaguchi et al., 2008). The morpholino sequences were the following:

AnkAT-1-MO1: 5′-AGTGACCGTCTGTATCAAATACCAT-3′,
AnkAT-1-MO2: 5′-TACGAGCTGTGTTGTCACGCAGAAC-3′,
FoxQ2-MO: 5′-TCATGATGAAATGTTGGAACGAGAG-3′,
NK2.1-MO: 5′-TGGTCTGTTTAGGGCTATATGACAT-3′,
Nodal-MO: 5′-AGATCCGATGAACGATGCATGGTTA-3′, and
Lefty-MO: 5′-AGCACCGAGTGATAATTCCATATTG-3′.

mRNAs were synthesized from linearized plasmids using mMessage mMachine kit (Life Technologies), and injected at the following concentrations in injection needles with final 24% glycerol: Δ-cadherin, 0.3–0.6 μg/μl (Logan et al., 1999), green fluorescent protein (GFP)-tagged AnkAT-1 mRNA (1.85 μg/μl), and DsRed-tagged AnkAT-1 mRNA (3.1 μg/μl).

Microarray

Sample preparation, microarray and data processing were described previously (Wei et al., 2006). The double-strand cDNAs synthesized from control and SpFoxQ2-MO1-injected embryos were labeled, hybridized and scanned by Nimblegen microarray services.

Quantitative PCR

Quantitative PCR (QPCR) was performed as described previously (Ransick, 2004) with some modifications. Total RNA from 200 to 300 embryos of *H. pulcherrimus* was isolated using TRI reagent (Sigma) and reverse transcription was performed using Superscript III (Life Technologies). iQ SYBR Green mix (Bio-Rad) was used for PCR reactions carried out with an Thermal Cycler Dice Real Time system (Takara). Primer pairs used for PCR reactions were following:

AnkAT-1-qF1; 5′-TTTGCAGAACACGACTGTCC-3′,
AnkAT-1-qR1; 5′-CTGGTCCGGTTGTAATTGCT-3′,
MT12S-rRNA-qF1; 5′-GGCCCACAACACTAAGCAAT-3′,
MT12S-rRNA-qR1; 5′-GGCACGTATTTTACCCCCTT-3′.