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Tube formation by complex cellular processes in *Ciona intestinalis* notochord

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ABSTRACT

In the course of embryogenesis multicellular structures and organs are assembled from constituent cells. One structural component common to many organs is the tube, which consists most simply of a luminal space surrounded by a single layer of epithelial cells. The notochord of ascidian *Ciona* forms a tube consisting of only 40 cells, and serves as a hydrostatic "skeleton" essential for swimming. While the early processes of convergent extension in ascidian notochord development have been extensively studied, the later phases of development, which include lumen formation, have not been well characterized. Here we used molecular markers and confocal imaging to describe tubulogenesis in the developing *Ciona* notochord. We found that during tubulogenesis each notochord cell established *de novo* apical domains, and underwent a mesenchymal–epithelial transition to become an unusual epithelial cell with two opposing apical domains. Concomitantly, extracellular luminal matrix was produced and deposited between notochord cells. Subsequently, each notochord cell simultaneously executed two types of crawling movements bi-directionally along the anterior/posterior axis on the inner surface of notochordal sheath. Lamellipodia-like protrusions resulted in cell lengthening along the anterior/posterior axis, while the retraction of trailing edges of the same cell led to the merging of the two apical domains. As a result, the notochord cells acquired endothelial-like shape and formed the wall of the central lumen. Inhibition of actin polymerization prevented the cell movement and tube formation. *Ciona* notochord tube formation utilized an assortment of common and fundamental cellular processes including cell shape change, apical membrane biogenesis, cell/cell adhesion remodeling, dynamic cell crawling, and lumen matrix secretion.

Introduction

The precise integration of individual cell behaviors, such as directed migration, cytoskeletal modification, membrane biogenesis, extracellular matrix production, and adhesion remodeling that results in the assembly of functional multicellular structures and organs is not well understood. The formation of multicellular tubes is a particularly interesting question for morphogenesis. Tubes are a fundamental unit of biological design and are found throughout metazoan bodies in such organs as the digestive system, lung, and kidney (Lubarsky and Krasnow, 2003). During development and in the adult, tubes serve diverse functions including transport of nutrients, waste, and gases, and structural support. The shape, size, and arrangement of tubes are very diverse. Several common themes have been recognized in their development (Bryant and Mostov, 2008; Chung and Andrew, 2008; Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003). Three cellular activities appear to be essential for the construction of tubes. The first is the establishment of apical/basal (A/B) polarity and acquisition of epithelial identity. The second is the formation of a lumen. The third is a set of dynamic cell movements that drive the cell shape changes and rearrangements in order to make a tube. These processes of tubulogenesis have been extensively studied in cultured Madin–Darby canine kidney (MDCK) cells (O'Brien et al., 2002). Addition of hepatocyte growth factor stimulates a cluster of MDCK cells to migrate and form a solid cord 2 to 3 cells in diameter. Subsequently, at various positions along the cord cells begin to establish A/B polarity. Apical membranes are produced at interior cell/cell interfaces, where cells then separate and pockets of lumen appear. Molecular details underlying the cellular events in this and other tube formation processes have been described (Bryant and Mostov, 2008).

The notochord of the larval stage ascidian *Ciona intestinalis* is a straight tube closed at both ends (Jiang and Smith, 2007). It consists of 40 endothelial-like cells enclosing a single large lumen. The cells are in turn bounded by a basal lamina and a sheath of connective tissue

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fibrils (Cloney, 1964). The notochord is located in the central position of the larval tail, flanked on both sides by muscles. It functions presumably as a hydrostatic "skeleton" essential for the locomotion of the swimming larva (Keller, 2006; McHenry, 2005). The tension-resisting fibrils of the notochordal sheath allow the notochord to become rigid as the lumen inflates. To aid in swimming, this stiff yet flexible rod recoils the trunk and tail after each alternating stroke of the lateral muscles.

The development of *Ciona* notochord can be divided into two distinct phases. In the first phase, the notochord cells are induced at the blastula stage (Kumano and Nishida, 2007) and subsequently undergo a morphogenetic process during gastrula, neurula and early tailbud stages called convergent extension (C/E) that results in the formation of a columnar notochord of 40 cells in length and a single cell in diameter (Miyamoto and Crowther, 1985). This process has been studied in detail in another ascidian species *Boltenia villosa* (Munro and Odell, 2002). In the second phase, the morphogenesis of the notochord undergoes a series of dynamic changes which culminate in the formation of a central lumen by the swimming larval stage. While previous investigations have described cell shape changes during tubulogenesis (Cloney, 1964), conflicting results have been presented on whether lumen formation in ascidians is extracellular or intracellular (Burighel and Cloney, 1997; Miyamoto and Crowther, 1985). In this report we characterized the tubulogenesis of *C. intestinalis* notochord and presented it as a promising model system for the study of tubulogenesis. We first documented cell shape changes by confocal imaging and 3-D reconstruction. We then examined the emergence of apical domains as notochord cells underwent mesenchymal–epithelial transition. In addition, we performed biometric analysis to quantify the transformation of individual cells and of the entire notochord. Finally, we characterized cell junction remodeling that occurred as notochord cells rearranged into a hollow tube. In summary, our report presented a novel model system for tube formation that incorporated such cellular processes as cell shape changes, apical membrane biogenesis, cell–cell junction remodeling, dynamic cell motility, and lumen matrix secretion.

Materials and methods

Ascidians and embryos

Adult *C. intestinalis* were collected from a fjord in Bergen, Norway. The animals were kept in the Sars Centre Ascidian Culture Facility, in running filtered seawater for seven days under constant light to accumulate gametes. Eggs were then dissected and mixed in seawater with sperm from other individuals. Five minutes after fertilization, the eggs were washed with seawater through a nylon filter to remove sperm and debris.

Plasmid construction

A membrane GFP notochord expression vector was constructed by modification of Ci-Bra::GFP (Corbo et al., 1997; Deschet et al., 2003). The GFP fragment was amplified by PCR (primers: memGFP5, 5′-AAAAGATCTGGCACCATGCTTTGTTGTATGAGAAGAACAAAACAAGTTGAAAAAAACGATGAAGATCAAAAAATTAGTAAAGGAGAAGAACTTTTCACT-3′ and memGFP3, 5′-TTTGCGGCCGCTTATTTGTATAGTTCATCCATGCCATGTGT-3′) with the first 20 amino acids of *Ciona* GAP-43 included in the 5′ primer to create a GAP-43-GFP fusion. This fragment was inserted back into Ci-Bra-GFP at Bgl II and Not I sites to obtain Bra::memGFP.

To create plasmids expressing actin and E-cadherin fluorescent fusion proteins in the notochord, we first modified pSP1.72BSSPE-R3-*ccd*B/CmR-R5::RfA (Roure et al., 2007) to generate two destination constructs, *Minos*-B3-eBra-bpFOG-B5::Kozak-turboGFP-R1-*ccd*B/CmR-R2 and *Minos*-B3-eBra-bpFOG-B5::R1-*ccd*B/CmR-R2-mCherry. Full length human actin was amplified with primers hAct5 (5′-ATGGATGATGATATCGCCGCG-3′) and hAct3 (5′-CTAGAAGCATTTGCGGTGGAC-3′) and cloned into the pCR®8/GW/TOPO® vector (Invitrogen) to obtain an entry clone. This was recombined into the destination vector to create *Minos*-B3-eBra-bpFOG-B5::Kozak-turboGFP-B1-hActin-B2 using the Gateway LR reaction (Invitrogen). The Dm-E-cadherin entry clone (a gift from Agnès Roure and Patrick Lemaire) was recombined into the destination vector to create *Minos*-B3-eBra-bpFOG-B5::B1-Dm-E-cadherin-B2-mCherry.

Electroporation

Electroporation was performed as described previously with some modifications (Corbo et al., 1997). Eight-hundred μl of dechorionated fertilized eggs was mixed with 50–100 μg of plasmid DNA and electroporated in 4 mm cuvettes with a Gene Pulser Xcell System (BIO-RAD), using a square pulse protocol (50 V and 15 ms per pulse). After electroporation, embryos were allowed to develop at 16 °C to the desired stages for confocal observation.

Antibody production and immunohistochemistry

For preparation of an antibody to recognize the *C. intestinalis* homologue of SLC26-2 (Ci-SLC26-2), a cDNA fragment encoding the carboxyl-terminal cytoplasmic region of Ci-SLC26-2 (D658-N762) was amplified by PCR from the original cDNA clone ciht022e16 using a pair of primers, 5′-AGAgatCtTCGATGGCAGAGCGCCT-3′ and 5′-TTaaGCtTCATTCACGTCGTTAGCC-3′ (the small letters in the primer sequences indicate mismatched nucleotides introduced to generate restriction sites), and cloned into the pQE40 vector (Qiagen GmBH). The plasmid construct was introduced into the *E. coli* strain XL1-Blue (Stratagene). The carboxyl-terminal region of Ci-SLC26-2 was expressed as a fusion protein with dihydrofolate reductase and a histidine tag, then isolated and used to immunize mice. Ascidian embryos were fixed with 10% formalin in artificial seawater for 3 h at 4 °C. After fixation, the embryos were washed with PBS containing 0.1% Triton X-100 (PBST), and treated with 10% goat serum in PBST for 3 h. The embryos were then incubated overnight with the primary antiserum diluted 1000-fold with the blocking buffer and washed with PBST for 8 h at 4 °C. The specimens were then incubated with an Alexa 594-conjugated anti-mouse IgG goat antibody (Molecular Probes). The embryos were counter-stained with BODIPY phalloidin (488 nm excitation; Molecular Probes) for filamental actin. After rinsing several times with PBST, the specimens were mounted in Murray Clear for confocal observation (Horie et al., 2008).

Actin localization in notochord cells from embryos electroporated with the turboGFP-actin construct was visualized using anti-turboGFP antibody (Evrogen) and Alexa-488-conjugated secondary antibody (Invitrogen).

Actin inhibitor treatment

10 μM Latrunculin B was applied to embryos at 23 hpf for one hour then washed away. The embryos were cultured for two more hours before observation. Latrunculin B was substituted by DMSO in the control embryos.

Laser scanning confocal microscopy and 3D reconstruction

Confocal images were taken with a Leica TCS SP5 confocal laser scanning microscope (CLSM) equipped with 40× oil and 63× water immersion objectives (numerical aperture 1.25 and 1.40, respectively). Z-series were taken at intervals of 1–1.2 μm, resulting in stacks of 20–40 images. Image analysis and 3D reconstruction were performed with Leica TCS SP5 systems LAS AF software packages. Adobe PhotoShop was used to pseudocolor the images.