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Gastrulation in the cnidarian *Nematostella vectensis* occurs via invagination not ingression

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Abstract

Gastrulation is a central event in metazoan development, involving many cellular behaviors including invagination, delamination, and ingression. Understanding the cell biology underlying gastrulation in many different taxa will help clarify the evolution of gastrulation mechanisms. Gastrulation in the anthozoan cnidarian *Nematostella vectensis* has been described as a combination of invagination and unipolar ingression through epithelial to mesenchymal transitions (EMT), possibly controlled by *snail* genes, important regulators of EMT in other organisms. Our examination, however, fails to reveal evidence of ingressing cells. Rather, we observe that endodermal cells constrict their apices, adopting bottle-like morphologies especially pronounced adjacent to the blastopore lip. They retain apical projections extending to the archenteron throughout gastrulation. Basally, they form actin-rich protrusions, including interdigitating filopodia that may be important in pulling the ectodermal and endodermal cells together. Endodermal cells retain cell–cell junctions while invaginating, and are organized throughout development. Never is the blastocoel filled by a mass of mesenchyme. Additionally, injection of splice-blocking morpholinos to *Nematostella snail* genes does not result in a phenotype despite dramatically reducing wild-type transcript, and overexpression of Snail-GFP in different clonal domains has no effect on cell behavior. These data indicate that EMT is not a major factor during gastrulation in *Nematostella*.

Keywords: Snail; Forkhead; *Nematostella*; Gastrulation; EMT

Introduction

Gastrulation, the process through which an embryo internalizes and re-organizes the cells that will form the various structures of the adult animal, is the primary morphogenetic event during early development. Metazoans consist of multiple "layers": an inner gut derived from endoderm, an outer surface derived from ectoderm, and, in triploblastic animals, a middle layer of mesoderm. There are many cellular strategies for accomplishing the formation of these germ layers, including invagination (the internalization of cells through epithelial folding), involution (the coordinated movement of sheets of cells into the interior of the embryo), epiboly (the spreading of one group of cells over the surface of another group), delamination (mitoses in which the spindle is oriented perpendicular to the embryo surface, resulting in one daughter remaining on the surface, and the other entering the blastocoel) and ingression (the migration of individual cells to the interior of the embryo) (reviewed in Keller et al., 2003). These are complicated morphogenetic processes that require the coordination of a number of cellular behaviors. An understanding of the evolution of gastrulation mechanisms, therefore, promises to provide insight into the evolution of the cell biology underlying them as well as such outstanding questions as the origin of distinct germ layer-specific cell fates.

All the cellular behaviors described above require the coordinated regulation of cell biological processes such as adhesion, changes in cell shape, contractile activity, and the regulation of the cytoskeleton. Examination of these processes across a wide range of taxa is crucial to an understanding of the ancestral states of these processes. As the likely sister group to the Bilateria (Collins, 1998; Medina et al., 2001), the phylum Cnidaria (which includes sea anemones, jellyfish and corals) is ideally placed to provide insight into the evolution of gastrulation. Cnidarians are diploblastic (i.e. have only 2 germ

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layers rather than the 3 present in bilaterian metazoans) and lack much of the anatomical complexity of most bilaterians. In addition to their phylogenetic position, cnidarians are interesting due to the extreme diversity of gastrulation mechanisms they exhibit. All gastrulation mechanisms observed in bilaterians can be found in the Cnidaria, raising the potential for powerful comparative studies between members of this phylum (Byrum and Martindale, 2004; Tardent, 1978). There are four major clades of cnidarians: the Anthozoa (sea anemones and corals) and the 3 medusazoan clades, Scyphozoa, Cubozoa, and Hydrozoa, which are distinguished by the presence of a pelagic medusoid stage in their life cycle (e.g. jellyfish). Among the Cnidaria, anthozoans appear to be the most relevant for comparison to bilaterian taxa because of their sister-group relationship to the medusazoans and simple life history. Anthozoans also show less diversity in gastrulation mechanisms than the other classes, perhaps indicating a closer relationship to the ancestral form of cnidarian gastrulation.

The starlet sea anemone, *Nematostella vectensis*, has recently emerged as an important cnidarian developmental model system for use in studies aimed at inferring character states ancestral to the evolution of the Bilateria (Fritzenwanker and Technau, 2002; Hand and Uhlinger, 1992). Gastrulation in *Nematostella* has been characterized as a combination of invagination and unipolar ingression (Byrum and Martindale, 2004; Fritzenwanker et al., 2004; Kraus and Technau, 2006). In this mode of gastrulation, cells begin to invaginate on one side of the embryo, and as gastrulation proceeds it is accompanied by the ingression of a subset of individual, presumptive endodermal cells undergoing an epithelial-to-mesenchymal transition (EMT) from the same region of the embryo. If *Nematostella* does gastrulate in this way, it would represent an excellent opportunity to examine the evolutionary history of the cell biology underlying EMT and its regulation. Regardless, an examination of *Nematostella* gastrulation promises to contribute to our understanding of the cell-biological basis of gastrulation in non-bilaterian metazoans.

Based on studies in other organisms, regulation of cell-cell adhesion is a crucial aspect of the control of EMT. This is accomplished, at least in part, by repressing transcription of adhesive proteins present in the apical junctional complex (AJC; Shook and Keller, 2003). The DNA-binding zinc-finger protein Snail can bind directly to the promoter of the adherens junction (AJ) protein E-cadherin (the primary epithelial cadherin) and repress its transcription (Batlle et al., 2000). Snail can also directly repress transcription of the tight junction (TJ) proteins claudin and occludin in cultured mouse epithelial cells (Ikenouchi et al., 2003), indicating a general role for Snail in the regulation of the AJC. *snail* was initially identified as a gene involved in mesoderm formation and gastrulation in *Drosophila melanogaster* (Boulay et al., 1987). In *Drosophila snail* mutant embryos, mesodermal precursors on the ventral surface fail to invaginate, and E-cadherin levels in those cells remain high (Oda et al., 1998). *snail* family members have subsequently been shown to be important regulators of EMT in both tissue culture systems and *in vivo*. Ectopic expression of Snail in cultured mammalian epithelial cells results in their adoption of an invasive phenotype (Cano et al., 2000), and Snail expression is inversely correlated with E-cadherin in some epithelial tumors (Blanco et al., 2002; Rosivatz et al., 2002). Mouse embryos homozygous for null mutations of *snail* have gastrulation defects (Carver et al., 2001), and although they form a mesodermal layer, cells in this layer fail to adopt the mesenchymal characteristics seen in wild-type embryos and instead retain an epithelial morphology. All these data indicate a central role for *snail* in the regulation of EMT. It remains to be determined whether this is an ancestral or derived function, and whether EMT in "primitive" metazoans requires *snail*. In *Nematostella*, *snail* is expressed at the future site of gastrulation and is maintained in the endoderm throughout that process. This makes it an excellent candidate to regulate EMT during gastrulation (Fritzenwanker et al., 2004; Martindale et al., 2004), as we would expect if that is its ancestral role.

In this study we examine the process of gastrulation in *Nematostella* using a diversity of techniques, including expression analysis, cell labeling, confocal microscopy, and TEM, to elucidate the details of this process in a non-bilaterian metazoan. We find that in contrast to what has been reported previously, gastrulating endodermal cells in *Nematostella* do not undergo EMT, but instead gastrulate through invagination alone. This provides an opportunity to examine the regulation of gastrulation in an anthozoan cnidarian relative to other organisms, and investigate the role of *snail* genes in an organism that does not undergo EMT to gain insight into the ancestral role of these genes.

Results

snailA and NvFoxA expression domains are complimentary

A definitive characterization of genes involved in regulating the cellular behaviors required for gastrulation awaits functional studies. We can, however, gain some insight into likely candidates based on the expression of genes involved in similar processes in other organisms. As previously reported, genes of the Snail and Forkhead families of transcription factors are expressed in domains suggestive of a role in regulating gastrulation in *Nematostella* (Fritzenwanker et al., 2004; Martindale et al., 2004). Two-color in situ expression analysis confirms that their expression domains are complimentary, with *snailA* expressed in the invaginating endoderm (as is *snailB*, though less robustly; Martindale et al., 2004) and *NvFoxA* (one of the *Nematostella forkhead* genes) expressed in the ectoderm surrounding the presumptive endoderm (Fig. 1). *NvFoxA* is initially expressed in patches of cells surrounding the *snailA* expression domain (Figs. 1A, A′). As the endodermal cells invaginate, the patches of *NvFoxA* expression begin to connect into a ring of expression (Figs. 1B, B′). The endoderm and ectoderm progressively zip together as invagination proceeds (the zippering front is indicated by arrowheads in Fig. 1C′). Expression of *NvFoxA* reaches its highest levels when the blastopore closes and the pharyngeal ectoderm has involuted (Figs. 1C–D′). *snailA* is expressed in all endodermal cells throughout gastrulation and is maintained during polyp formation (Fig. 1). The boundary between the *snailA* and *NvFoxA*