Gastrulation in the cnidarian Nematostella vectensis occurs via invagination not ingress

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Abstract

Gastrulation is a central event in metazoan development, involving many cellular behaviors including invagination, delamination, and ingress. 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 ingress 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 ingress (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
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 mesodasoon 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 ingestion (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 ingestion 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 metazoa. 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
expression domains appear, in the absence of a clear morphological marker at these early stages, to define the endoderm/ectoderm boundary (arrows in Figs. 1B′, C′, D′).

An overview of Nematostella development from gastrulation through early planula formation

In order to examine in detail the cellular movements and shape changes that occur during gastrulation in Nematostella, embryos were stained with propidium iodide (PI, to label nuclei), and phalloidin (to label filamentous, or f-actin) and examined by confocal microscopy. Since f-actin is enriched in the cortices of individual cells, it is a useful tool for visualizing cellular morphologies.

Early development in Nematostella consists of a chaotic cleavage pattern that is highly variable from embryo to embryo, leading to the formation of a spherical blastula. Gastrulation begins at approximately 24 h post-fertilization (hpf) at 16 °C with the apical constriction of the presumptive endodermal cells at one pole of the embryo (Figs. 2A, A′; constricting cells indicated by the bracket in Fig. 2A′). This apical constriction results in the buckling of the presumptive endoderm and the subsequent initiation of invagination at the future oral pole of the embryo (Figs. 2B, B′). During this process, the archenteron forms a mushroom-like shape, in which the lateral edges are pulled towards the side of the internal lateral surface of the embryo (Figs. 2B–C′; arrows in B′). By 30 hpf the internalization of the endoderm is complete, and the endodermal cells lie beneath the overlying ectoderm (Figs. 2C, C′). Throughout this process, the endoderm remains a monolayer of cells, even during the final stages. At no time is the blastocoel filled with a mass of mesenchymal, endodermal cells, as would be expected if EMT were occurring. Once the endoderm is fully internalized, mitotic cells can be seen (e.g. arrows in Fig. 2C′) associated with the apical edge of the endoderm (the edge facing the archenteron). Interestingly, this is the same cell surface that dividing cells in the ectoderm remain associated with during mitosis (see Figs. 4A–I, below), perhaps indicating the continued presence of apical adhesive contacts in both germ layers.

By 36 hpf the endodermal cells flatten against the overlying ectoderm (Figs. 2D, D′). Endodermal flattening results in the opening up of the coelenteron, allowing space for the internalization of the pharyngeal ectoderm (Figs. 2D–E′). This movement is distinct from the initial invagination event in that it is an involution of the pharyngeal ectoderm through the open blastopore rather than the invagination of a contiguous sheet of cells, but may share some similarities in the behavior of the pharyngeal endodermal cells. Prospective pharyngeal endodermal cells must flatten against the body wall endodermal cells much the same way the endoderm flattens against the overlying ectoderm during the initial invagination, and may involve similar crawling mechanisms (Figs. 2D′, E′). The involution of the pharyngeal ectoderm continues through 48 hpf (Figs. 2F, F′). By 60 hpf, the embryo has elongated into a planula larva (Figs. 2G, G′). In contrast to some cnidarians in which the endoderm consists of a mass of mesenchymal cells (e.g. Phialidium gregarium; Byrum, 2001), the endoderm in a Nematostella planula consistently retains morphologically distinct regions, with the pharyngeal endoderm associated with the pharyngeal...
ectoderm and distinct from the body-wall endoderm (Figs. 2G, G', 6). The elongation of the planula continues through 72 hpf, and will be followed by the formation of tentacle buds during polyp formation (Figs. 2H, H' and data not shown).

Gastrulation in Nematostella occurs through invagination, not ingression or delamination

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). We have examined this process in detail using a combination of microinjection techniques and confocal microscopy. We do not, however, observe any evidence of ingression (n = 50 embryos examined). At no time are loose mesenchymal cells observed within the blastocoel. Our initial investigations involved staining gastrula-stage embryos with phalloidin, to outline cell boundaries, and propidium iodide, to highlight nuclei. We then collected confocal z-stacks and reconstructed the three-dimensional morphologies of gastrulating endodermal
cells. The results of this analysis reveal that as gastrulation begins, the invaginating endodermal cells form bottle-like morphologies due to the constriction of their apices (e.g. false-colored cells in Fig. 3A′). Cells at the edges of the blastopore lip adopt particularly extreme bottle cell morphologies, while cells in the center of the endodermal mass form less dramatic bottle-like shapes. The internal surface of cells lining the edges of the blastopore lip form actin-rich protrusions and reach out for the overlying ectoderm (Figs. 3B, B′). The endoderm and ectoderm then zip together as invagination proceeds, resulting in the blas-

Fig. 3. Gastrulation in Nematostella occurs by invagination. (A–E) Gastrulating Nematostella embryos (24–36 h of development at 16 °C) stained with phalloidin (green) and PI (red). The images shown are single optical slices from a confocal microscope. (A′–C′) Images from panels A, B, and C, respectively, with two blastopore lip cells in each image false-colored to highlight their morphologies. Note that at no time do the invaginating endodermal cells detach from the endodermal mass and migrate into the blastocoel as individuals. They maintain projections back to the archenteron throughout (seen in the most extreme fashion in panels B′, C′), and remain a monolayer even after the endoderm has been fully internalized and lies beneath the overlying ectoderm (D). Following gastrulation, the endodermal cells assume a more squamous morphology (E), and cell division can be observed (arrow in panel E). (F–I′) Gastrulation stage embryos in which one blastomere was injected during cleavage stages with texas-red dextran. In these cases, the resulting clones are endodermal, and the shapes of individual bottle cells can be seen. Note the projections back to the archenteron (arrows in panels F′–I′), present in all cells despite their contact with overlying ectoderm. Images are representative examples from n=50 embryos examined. Asterisks in all images indicate the position of the archenteron.
tocoel being obscured by the invaginating endodermal plate. Throughout this process the endodermal cells maintain projections back to the archenteron (the archenteron is indicated by the asterisks in all panels in Fig. 3), which contacts the apical surface of the endoderm. These projections could be important in pulling the archenteron into the mushroom-like shape it exhibits, which would provide a mechanism to widen the coelenteron and allow space for the involution of the pharyngeal ectoderm (Figs. 2D–F).

At the completion of the invagination process, the endoderm remains a monolayer of cells (Fig. 3D; see false-colored cells). Cell division within the endoderm begins at this stage however (e.g. Fig. 2C′, arrow in Fig. 3E), and both body wall and pharyngeal endodermal cells flatten and adopt a more squamous morphology (false-colored cells in Fig. 3E).

In order to verify our confocal reconstruction data, we performed cell-labeling experiments in which we injected fluorescently labeled dextrans into individual blastomeres of cleavage-stage embryos to generate labeled clones in gastrula-stage embryos. We then fixed and counterstained these embryos with phalloidin to visualize the morphology of individual cells in the intact embryo. Clones that happened to fall within the endoderm allowed us to clearly visualize individual cells at the edges of the clone (Figs. 3F–I). In agreement with our confocal data, we did not observe migrating, mesenchymal cells in 32 clones examined. Instead, all cells maintained their epithelial character and retained projections back to the archenteron throughout gastrulation (arrows in Figs. 3F′, G′, H′, I′).

Delamination does not occur during gastrulation in Nematostella

Another potential mechanism for internalizing cells during gastrulation is delamination. To verify that delamination is not a contributing mechanism during Nematostella gastrulation, we stained embryos with antibodies to Tubulin to label mitotic spindles and PI to label nuclei (Figs. 4A–I). If the mitotic spindles are oriented parallel to the surface of the embryo, cytokinesis would result in two daughter cells both within the plane of the blastoderm. If the mitotic spindles are oriented perpendicular to the surface of the embryo, however, this would result in one daughter cell remaining in the plane of the blastoderm, and the internalization of the other (Table 1). Cells undergoing mitosis adopted a spherical morphology and were invariably located at the apical, or outer, surface of the ectoderm (Figs. 4C, F, I). Greater than 96% of mitotic spindles observed (178/185 from 5 embryos; Fig. 4J) were oriented parallel to the plane of the blastoderm (e.g. arrows in Figs. 4B–C, E–F, H–I), with the remainder (7/185) slightly oblique to the surface of the embryo. These data indicate that delamination is not an internalization mechanism utilized during Nematostella gastrulation.

Ectodermal cells secrete material into the blastocoel during gastrulation that is internalized into endodermal cells

While performing the dextran-labeling experiments to visualize cellular morphologies during gastrulation, we noticed that when the labeled clone was present in the aboral region of the ectoderm they were often accompanied by labeled material in the blastocoel (Figs. 5A–B′; arrows in Figs. 5A, B′). In contrast, clones that labeled the lateral ectoderm and/or the endoderm did not deposit labeled, anucleate material into the blastocoel, and labeled dextran was not observed in cells outside the labeled clone (Figs. 5C, C′). Once secreted into the blastocoel, the endodermal cells take up the labeled material. This is most clearly demonstrated by early planula stage embryos possessing aboral ectodermal clones (Figs. 5D, D′), as the labeled material can be seen within the unlabeled endodermal cells (arrow in Fig. 5D′). The composition and function of this material are currently unknown, though secretion of yolk into the blastocoel has been described for a number of anthozoans and may serve a nutritive role (reviewed in Siewing, 1969).

Ultrastructural analysis of Nematostella gastrulation

One of the major cellular functions that have to be regulated properly during gastrulation is that of cell–cell adhesion. To gain more insight into the role of cell–cell adhesion during gastrulation in Nematostella, we examined the morphology of cell–cell junctions in the gastrula-stage embryo using transmission electron microscopy (TEM). Ectodermal cells exhibit an apical junctional complex at their apical-most edge (Figs. 6A, A′). The cells of the ectoderm are columnar, regularly arranged, approximately equal in size, and similar in shape throughout the gastrula. Each is ciliated at its apex, has small electron dense vesicles concentrated towards the apex and larger, round, membrane-bound inclusions of at least 2 types basally (based on their electron density). The nuclei are large, irregularly shaped, and typically in the apical one-third to one-quarter of the cell. Cells contain numerous mitochondria at both their apices and bases, though relatively few in the middle, and are full of ribosomes.

The ectodermal cells (including the lip cells) are joined by adherens junctions. The apical-most of these occurs as a belt in approximately the same place in all of the cells (arrows in Figs. 6A, A′). In addition to the apical adherens junction, previous studies have also reported the presence of more basally located septate junctions (Kraus and Technau, 2006). We do not, however, observe septae between any junctional complexes, even on very high magnification, suggesting that in contrast to what has been previously reported, Nematostella embryos lack septate junctions. The ectodermal cell junctions occur at regular intervals in the apical half of the cell; towards the basal end of the cell they are shorter and less closely spaced. The apical-most junction seems to be the strongest, as it results in the greatest “pulling” between cells, and involves more filaments than the others. There is no narrowing at either end of the adherens junction, and the distance between the cell membranes is constant.

In the endoderm, junctions between the cells are similar to those present in the ectoderm, although they are fewer in number and tend to be shorter in length. We observe only a few adherens junctions between adjacent cells in the center of the endodermal plate; in cells at the edge of the plate there is one or none. The junctions are at the apical, constricted end of the cells (arrows in Fig. 6C). There is more space between cells than in...
the ectoderm (Fig. 6B), and in some places the cells are only tangentially in contact. At least some endodermal cells retain their cilia (Fig. 6C).

Once gastrulation is complete, the endodermal cells exhibit adherens junctions at their apical end, closest to the coelenteron. The junctions are about the same size as those of the ectoderm, and longer than those of the endodermal plate, though still lack septae (Fig. 6B′; arrowheads in Fig. 6D). These ultrastructural observations confirm our data from the phalloidin-stained preparations, in which the early endodermal epithelium is not as

Fig. 4. Delamination does not occur during gastrulation in Nematostella. (A–I) Gastrula-stage Nematostella embryos stained with antibodies to Tubulin (green) and PI (red). Boxed regions in panels A, D, and G are shown in close-up in panels B, E, and H, respectively. Images in panels C, F, and I are cross-sections taken from other regions of the embryos in panels A, D, and G, respectively. Greater than 96% of mitotic spindles are oriented parallel to the surface of the embryo (arrows in panels B–C, E–F, H–I). The remaining spindles are oriented somewhat oblique to the embryo surface, but none is oriented perpendicular, indicating that cells are not internalized through delamination. (J) Quantitation of spindle orientation data. $n=185$ total mitotic spindles from 5 gastrula-stage embryos.
organized as the ectodermal epithelium and the cells are not as columnar. In some instances we observe exocytosis occurring from the endodermal cells (Fig. 6D), suggesting that these cells are undergoing a great deal of remodeling.

Our analysis has also revealed filopodia on the basal surface of the ectoderm and on the surface of the leading edge of the endodermal plate cells, which may act to facilitate the attachment of the two cell layers (arrows in Fig. 6E). These appear to be actin-rich outgrowths of the cell surface, as we observe these protrusions in the phalloidin-stained embryos as well (e.g. arrows in Figs. 5A′, B′). They are reminiscent of the so-called adhesion zippers in mammalian cells that are important in the formation and maturation of adherens junctions (Vasioukhin et al., 2000), suggesting that a similar mechanism may be involved in Nematostella during the adhesion of the endoderm to the overlying ectoderm.

Assessing snail function

The Nematostella snailA and snailB genes are zygotically expressed in the presumptive endoderm before invagination begins. In order to directly assess the role of Snail during gastrulation in Nematostella, we attempted both a knockdown of Snail function to generate a loss-of-function phenotype, and overexpression of a Snail-GFP fusion protein. For the loss-of-function experiments, we utilized splice-blocking morpholinos, since they allow for easy determination of their effectiveness via RT-PCR (Draper et al., 2001). Both snailA and snailB possess one intron and two exons. Splice blocking MOs should affect both genes by causing intron insertions, resulting in a frameshift for snailA and a premature stop codon for snailB (Fig. 7A). Through microinjection of morpholinos targeting both SnailA and SnailB, we are able to reduce wild-type transcript levels of both genes to approximately 13% and 29%, respectively, of the total transcript amount (Fig. 7A). This does not, however, result in a detectable phenotype (Figs. 7B–D′). Embryos injected with snail morpholinos (either snailA alone, snailB alone, or a combination of snailA and snailB) form bottle cells indistinguishable from wild-type embryos (compare Figs. 7B′, C′ with images in Fig. 3) and go on to form swimming planulae.

In order to determine whether Snail is sufficient to cause the cellular behaviors observed among endodermal cells during gastrulation, we overexpressed a SnailA-GFP fusion protein by injecting cleavage-stage blastomeres with SnailA-GFP mRNA, along with rhodamine-dextran as a tracer (Figs. 7B–E). Doing

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Table 1
Quantitation of mitotic spindle orientations

Fig. 5. Ectodermal cells secrete material into the blastocoel, which is taken up by the endoderm. (A–C′) Gastrulating Nematostella embryos in which single blastomeres were injected with texas-red dextran during the early cleavage stages (2–32 cell). The resulting labeled clones are indicated in red, with embryos counterstained with phalloidin (green). Embryos in panels A–B′ are also counterstained with propidium iodide in the red channel to identify nuclei. Close-ups of the embryos in panels A–C are shown in panels A′–C′, respectively. Note the labeled material present within the blastocoel (arrows in panels A, B′) when the labeled clone is restricted to the ectoderm. This material is not seen when the labeled clone is within the endoderm and lateral ectoderm (C, C′). Note the sometimes-long projections extending from the endodermal cells to the overlying ectoderm (e.g. arrow in panel A′). (C, C′) The labeled material is taken up by the endodermal, but not ectodermal, cells (e.g. arrow in panel C′). The embryo in panels C, C′ was not stained with propidium iodide.
so resulted in the expression and nuclear localization of SnailA-GFP within the marked clone of cells. In no case, however, did we observe these cells forming ectopic invaginations or losing their epithelial organization. Cells in ectodermal clones remained on the surface of the embryo (Figs. 7B–E), and endodermal cells expressing SnailA-GFP invaginated normally (e.g. Fig. 7E). These data indicate that SnailA is not sufficient to drive the process of invagination.

Discussion

Gastrulation in Nematostella occurs via invagination

Nematostella gastrulation has been characterized as a combination of invagination and ingestion (Byrum and Martindale, 2004; Fritztenwanker et al., 2004; Kraus and Technau, 2006), though Hand and Uhlinger (1992) described Nematostella as a strictly invagination gastrula. The initiation of gastrulation in Nematostella involves cell shape changes very reminiscent of invagination processes observed in other organisms (Keller et al., 2003), namely apical constriction of the endodermal cells and the formation of bottle cells at the endodermal margin. This apical constriction results in the buckling of the endodermal plate and the beginning of invagination (Fig. 8). Prior to this study, our preliminary observations of Nematostella gastrulae under DIC microscopy suggested that some cells were detaching from the endodermal plate and migrating inward as individuals (Byrum and Martindale, 2004). In addition, there have been recent reports purporting to show immigrating cells during gastrulation in Nematostella (Fritzenwanker et al., 2004; Kraus and Technau,
In this study, however, our analyses using confocal microscopy, in situ expression analysis and microinjection have revealed that although the lateral endodermal cells do adopt extreme bottle-like morphologies and form basal protrusions (filopodia and lamellipodia), these cells do not detach from the rest of the endodermal cells. Rather, they maintain projections back to the archenteron that may be important in the formation of the archenteron’s characteristic mushroom-like shape.

The question of what does and does not constitute EMT is an important one. The adoption of this extreme bottle-like morphology has been termed an “incomplete” or “partial” EMT by some (Barrallo-Gimeno and Nieto, 2005; Kraus and Technau, 2006). If one defines EMT as the process through which an epithelial cell loses epithelial characters, detaches from an epithelial layer, and subsequently migrates away as mesenchyme, the failure of the endodermal cells in Nematostella to ultimately detach from the endodermal plate would argue that EMT does not occur. While these cells do adopt some mesenchymal characters (e.g. form basal actin-rich protrusions), they do not ingress and become mesenchyme, a crucial aspect of EMT (Shook and Keller, 2003). Minimally, it cannot be said that these cells undergo “complete” EMT. The blastocoel is never simply filled with a mass of mesenchymal cells.

The discrepancies between our results and those of others may have to do with the method of analysis. Byrum and Martindale (2004) described gastrulation in Nematostella as a combination of invagination and unipolar ingression on the basis of DIC microscopy and the position of Hoechst-labeled nuclei, techniques which do not have the resolution required to observe the long, narrow projections of the cells at the endodermal margin. Fritzenwanker et al. (2004) also claimed to observe EMT in Nematostella embryos examined using light micro-
scopy, in this case on whole-mount in situ preparations. Kraus and Technau (2006) utilized SEM to examine the morphologies of the endodermal cells during gastrulation in fractured embryos, and reported cells immigrating as individuals. Given that the projections extending back to the archenteron are very thin, however, they may have been damaged during processing, leading to the impression that cells were detaching completely from their neighbors. Our study has examined cellular morphology in intact embryos, which were prepared differently and analyzed via confocal microscopy. In addition, our technique of labeling clones of cells through microinjection allows us to examine individual cellular morphologies in the endogenous context, and we do not observe immigrating cells. It is possible that a very small number of cells do detach from the endodermal margin, though our failure to yet observe any cells doing this would suggest at least that this does not occur in all embryos and is not a major factor in Nematostella gastrulation.

**Biomechanics of gastrulation in Nematostella**

Studies of morphogenesis in a variety of organisms have revealed some general principles of the cell biology underlying invagination processes. One common feature is that of coordinated apical constriction, resulting in the formation of a group of wedge-shaped cells and the resulting buckling of the region of tissue they inhabit, due to the presence of apical junctions that transmit forces from cell to cell (Keller et al., 2003). In Drosophila and C. elegans, apical constriction is accompanied by flattening of the apical surface of these cells due to the presence of an apical contractile actin network in each cell (Costa et al., 1993; Nance and Priess, 2002). The endodermal cells in Nematostella, by contrast, do not undergo apical flattening. Instead, the apical region of each endodermal cell bulges outward and flattening does not occur despite the presence of a high concentration of actin (see Fig. 2 and data not

Fig. 8. Schematic representation of Nematostella gastrulation. (A) Invagination begins with the apical constriction of the endodermal cells, resulting in the buckling of the endodermal plate. (B) The endodermal cells adopt bottle-like morphologies as invagination proceeds, with the cells at the lateral edges of the endodermal plate adopting the most extreme bottle shapes. They form actin-rich protrusions and reach out for the overlying ectoderm, but maintain projections back to the archenteron (see inset). (C) The endoderm continues to zip up against the overlying ectoderm beginning with the lateral-most endodermal cells. The zippering proceeds medially, with the endoderm progressively obscuring the blastocoel. The projections of the bottle cells at the margins keep the archenteron in a mushroom-like shape (see inset). (D) Once invagination is complete, the endoderm flattens against the ectoderm, opening the gastrocoel and allowing space for the internalization of the pharynx. (E) The pharynx rolls inward, resulting in the internalization of its ectodermal component. (F) Following the internalization of the pharynx, the embryo elongates into a free-swimming planula larva (G).
shown), suggesting that a simple purse-string mechanism may be acting to constrict each cell. We and others have also observed endo- and exocytic activity at the apical margins of the endodermal cells, suggesting that the need for cytoskeletal elements to coordinate this process could preclude flattening (Kraus and Technau, 2006).

Once invagination is initiated, the progression of gastrulation is likely the result of a combination of forces, including the continued apical constriction of the bottle cells and the involution of the ectodermal epithelium. Our data also suggest that the “zipping” of the lateral endodermal cells to the inner surface of the blastocoel could also be involved in pulling the endodermal plate into the blastocoel. The cells at the lateral edges of the endodermal plate form basal filopodia and lamellipodia and reach out for the overlying ectoderm (see Figs. 4A′–C′). Additionally, we observe filopodia reaching from the ectodermal to the endodermal cells as the latter progressively zips up against the ectoderm. The fact that both cell layers exhibit filopodial projections is highly reminiscent of the interdigitating filopodia involved in adherens junction formation in mammalian cells (Vasioukhin et al., 2000), and suggests that a similar mechanism may be operating in the formation of adhesive contacts between the endoderm and ectoderm in *Nematostella*. Future identification of the molecular components required for this adhesion will shed light on the cell-biological details of this process.

The fact that the bottle cells maintain projections back to the archenteron, and their concomitant maintenance of some apical cell–cell adhesive contacts, may be important in the opening of the gastrocoel to allow proper pharyngeal morphogenesis. If many cells were detaching from the endodermal layer and immigrating as individuals, it is possible that the lack of apical adhesive contacts would not allow the archenteron to be pulled into its characteristic mushroom shape or allow the bottle cells at the margins to pull the rest of the endodermal plate inward. Additionally, the endoderm does maintain a very definite structural organization throughout gastrulation, in contrast to what has been observed in some other cnidarian planulae (e.g. Byrum, 2001), and the blastocoel is never simply filled by a mass of mesenchymal cells. Instead, the endoderm maintains a monolayer configuration until invagination is complete, at which time the endodermal cells adopt more squamous morphologies, flatten against the ectoderm and begin proliferating (see mitotic cells in Fig. 2C′). This endodermal organization also argues against immigration of single mesenchymal cells as being a major factor in *Nematostella* gastrulation.

Pharyngeal morphogenesis occurs as a continuation of the initial internalization of the endoderm. The oral-most ectoderm involutes as the endodermal cells flatten, resulting in the formation of an epithelial tube of ectodermal origin (pharyngeal ectoderm) surrounded by squamous, endodermal cells (pharyngeal endoderm; see Figs. 2D–F). The entire structure extends into the gastrocoel and is ringed by body-wall endoderm. The forces driving this process are likely largely the result of the involution of the pharyngeal ectoderm, though adherence of the pharyngeal endodermal cells to the body wall endoderm could also be involved (similar to the crawling of the endodermal cells along the ectoderm described above).

Following gastrulation, the embryo elongates into a swimming planula. The forces driving this elongation are not clear, but may involve proliferation as we observe a large number of mitotic cells, particularly in the endoderm, once gastrulation is complete. Interestingly, although it does form a swimming planula, *Nematostella* is best characterized as a direct developer. The internal structure of the polyp is formed during gastrulation with the formation of the body wall endoderm, which will line the gastrocoel, and the pharynx. Polyp “metamorphosis” involves a continuation of the elongation begun in the planula stage, followed by the development of tentacle buds at the oral pole and elaboration of the internal mesenteries, but not a whole scale remodeling of the type seen in other cnidarians (such as the hydrozoan *Hydractinia*; Frank et al., 2001). A detailed description of this process awaits future studies.

**Yolk secretion into the blastocoel**

The presence of yolk in the blastocoel has been documented in a number of anthozoans (reviewed in Mergner, 1971; Siewing, 1969). Indeed, the secretion of anucleate yolk particles into the blastocoel is a character common to anthozoans, as opposed to the invasion of the blastocoel by whole cells as is found in some scyphozoans (Mergner, 1971). While our data do not demonstrate conclusively that the material secreted into the blastocoel is yolk, this seems likely given its uptake by endodermal cells, which may be indicative of a nutritive role. Although in some anthozoan cnidarians the presence of yolk in the blastocoel can result in deformations of the invaginating endoderm (Mergner, 1971), in *Nematostella* it does not appear to dramatically affect cellular morphologies. Future experiments will be necessary to clarify the role of this material on subsequent development.

**Regulation of gastrulation in Nematostella**

A number of genes involved in gastrulation in other organisms are expressed in the proper time and place to play a role during gastrulation in *Nematostella*. Relatives of the DNA-binding zinc-finger protein Snail have been shown to be important regulators of gastrulation and EMT in a variety of organisms, primarily through direct repression of the transcription of cell–cell adhesion genes (Barral-Gimeno and Nieto, 2005). In *Nematostella*, there are 2 *snail* genes, both of which are expressed throughout the presumptive endoderm (see Fig. 1 and Fritzenwanker et al., 2004; Martindale et al., 2004), consistent with a role in the regulation of gastrulation movements. Forkhead proteins have also been shown to be involved in a diverse array of morphogenetic processes, including epithelial organization, gastrulation, and axial patterning (Carlsson and Mahlapuu, 2002). *NvFoxA* is expressed in the pharyngeal ectoderm (Fig. 1), suggesting a role in the patterning or morphogenesis of the pharynx during gastrulation (Fritzenwanker et al., 2004; Martindale et al., 2004). The expression domains of the *snail* genes and *NvFoxA* never overlap (Fig. 1), and their boundary can be viewed as the boundary between the endoderm and ectoderm. The function of...
NvFoxA in this context is likely to involve regulating the cellular movements required for the involution and morphogenesis of the pharyngeal endoderm, and keeping these cells morphologically distinct from the invaginating endodermal cells (Kraus and Technau, 2006). These hypothesized functions are consistent with prior studies documenting roles for Forkhead proteins in epithelial morphogenesis, for example during development of the lung epithelium in the mouse (Costa et al., 2001) and gut development in Drosophila (Weigel et al., 1989). Future work will be required to determine the precise role of NvFoxA during gastrulation in Nematostella.

Previous reports have advanced the hypothesis that snail genes in Nematostella are involved in regulating EMT, and that this represents the ancestral function of this family of proteins (Fritzenwanker et al., 2004). However, given our observation that EMT is not occurring during Nematostella gastrulation the role of Snail in this organism is best characterized differently. Additionally, snail expression is found in all endodermal cells, even those of the mid-endodermal plate that do not form extreme bottle-like morphologies (and have not been described as undergoing EMT), also arguing for a more complex role for Snail than simply regulating EMT. Significantly, Snail has been shown in other organisms to have effects on cellular morphogenesis and movement when cells migrate as groups without undergoing true EMT, such as during axial mesendoderm migration in zebrafish (Montero et al., 2005; Yamashita et al., 2004) and the re-epithelialization of cutaneous wounds in mice (Savagner et al., 2005), suggesting a more general role in adhesion or other cellular behavior.

Our observation that overexpression of SnailA-GFP is not sufficient to drive invagination or the adoption of bottle cell morphologies by ectodermal cells argues against a role for SnailA as an organizer of this process, though does not rule out a necessary role. We do not, however, observe a phenotype associated with injection of splice-blocking morpholinos targeting the Nematostella Snail genes. It could be argued that the approximately 15% of wild-type snailA transcript still remaining in these experiments is sufficient to generate enough functional protein to get the embryo through the early stages of development, and we have not reduced transcript levels dramatically enough to uncover a loss-of-function phenotype. Alternatively, it is possible that we do not recover a phenotype because the function of Snail in Nematostella is different from what would be predicted based on other systems that undergo true EMT. For example, perhaps Snail must act synergistically with other genes, rather than playing an organizing role itself. Further refinements of gene knockdown strategies in Nematostella will be crucial to resolve this issue.

Conclusions

In this study we have utilized a variety of techniques to investigate the cellular basis of gastrulation in Nematostella. We find that in contrast to prior reports, gastrulation in Nematostella occurs through invagination, not ingestion. The Nematostella embryo exhibits true epithelial organization, with the invaginating endoderm and pharynx forming via epithelial folding. The endoderm remains structured throughout the process, and mesenchymal cells are never seen in the blastocoel. Future studies into the molecular regulation of this process will be instrumental in providing insight into the evolutionary history of the cell biology underlying gastrulation in this species.

Methods

Double-label in situ hybridizations

Embryos from various stages were fixed in fresh ice-cold 3.7% formaldehyde with 0.2% glutaraldehyde in 1/3× seawater for 90 s and then post-fixed in 3.7% formaldehyde in 1/3× seawater at 4 °C for 1 h. Fixed embryos were rinsed 5× in PTw (PBS buffer plus 0.1% Tween-20) and once in deionized water, and transferred to 100% methanol for storage at ~20 °C. Early embryos were removed from the jelly of the egg mass by treating with freshly made 2% cysteine in 1/3× seawater (pH 7.4–7.6) for 10–15 min. In situ hybridization using 1–2 kb digoxigenin-(snailA) or fluorescein-(NvFoxA) labeled riboprobes were performed as previously described (Finnerty et al., 2003). Probe concentration was 1.0 ng/ml and hybridizations were performed at 60 °C for 24–48 h. Alkaline phosphatase reaction products were visualized with NBT-BCIP (snail) and FastRed (Sigma; NvFoxA). Specimens were photographed on a Zeiss Axioplan with a Nikon Coolpix 990 digital camera.

Immunofluorescence

Phalloidin staining

Embryos from various stages were fixed in fresh 4% paraformaldehyde with 0.2% glutaraldehyde in 1/3× seawater for 90 s and then post-fixed in 4% paraformaldehyde in 1/3× seawater at 4 °C for 1 h. Fixed embryos were rinsed 5× in PTw. Embryos were then incubated in 10 μl Alexa 488-conjugated phalloidin (200 U/ml; Molecular Probes) in 1 ml PTw for 1 h at room temperature. Embryos were dehydrated into isopropanol (3 washes over 5 min) and mounted in Murray’s Mounting Media (MM; 1:2 benzyl benzoate: benzyl alcohol), then visualized on a Zeiss LSM 510 confocal microscope. Determination of cellular morphologies during gastrulation was accomplished by generating confocal z-stacks from phalloidin-stained embryos, then tracing cell boundaries from slice to slice to reconstruct the three-dimensional shape of individual gastrulating cells. Image processing was done using ImageJ (NIH).

Tubulin staining

Embryos were fixed in fresh 4% paraformaldehyde in MEMPfa buffer (0.1M MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO4) for 1 h at room temperature. The fix buffer was then replaced with Dent’s buffer (80% methanol, 20% DMSO) for 15 min. Embryos were bleached by treatment in 2 parts Dent’s: 1 part 30% H2O2 for 30 min and washed 3× in PBS. Following bleaching, embryos were incubated overnight in 1° antibody (1:5 anti-α-Tubulin, DSHB) at PTw at 4 °C. They were then washed 3× in PTw, blocked 1 h at room temperature in PTw+2% normal goat serum, and incubated overnight in 2° antibody (1:500 Alexa 594-conjugated α-mouse, Molecular Probes). Embryos were dehydrated into methanol and mounted in MOM, then visualized on a Zeiss LSM 510 confocal microscope, and analyzed using ImageJ to determine spindle orientations.

Propidium iodide

PI staining was done by incubating embryos in 1 μg/ml propidium iodide (Sigma) and 50 μg/ml RNase in PTw for 1 h, then washing 5× in PTw. For phalloidin staining, this was done concurrently. To label embryos stained with antibodies, the propidium iodide treatment was done following the antibody treatment.

Dextran injections

Uncleaved or early cleavage stage embryos were injected with glass microelectrodes by pressure with 0.2 mg/ml Texas red-dextran (Molecular Probes) in 0.2M KCl, diluted 3:2 dextran:100% glycerol (for 40% final glycerol concentration). Injected embryos were cultured at 16 °C in 1/3× 0.22 mm
filtered sea water (1/3× FSW) for 24–60 h. They were then fixed and stained with phalloidin as described above and visualized on a Zeiss LSM 510 confocal microscope. Image processing was done using Velocity (Improvision) and ImageJ.

Electron microscopy

Embryos 12 h old or younger were fixed for 1 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer containing 0.1M sucrose. After fixation, embryos were rinsed three times in the fixation buffer, and post-fixed for 1 h in 1% osmium tetroxide, buffered as in fixation and rinsing. Embryos were then dehydrated through a graded series of ethanol and propylene oxide, and then infiltrated and embedded in Spurr resin. Sections 70 nm thick were cut using a diamond knife on a Reichert Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and observed on a Philips CM 12 TEM at 80 kV.

Morpholinos

Antisense morpholinos were designed to target the splice acceptor site of snailA and the splice donor site of snailB (Gene Tools, LLC). The sequences are as follows:

5′-CTTGCTCCTCATCAAGTGAATAAA-3′ (snailA acceptor)
5′-GAGCAATGAACTCACGGTGTCGGG-3′ (snailB donor)
5′-CCTCTACCTCAGTTACAAATTATA-3′ (standard control oligo).

Morpholinos were resuspended in dH2O. Control morpholino (1 mM) and mixed snail morpholinos (500 μM each) were injected in 40% glycerol with texas-red dextran, as described above. Twenty-four to forty-eight hours after injection, embryos were fixed and stained with phalloidin (also as described above), to assess phenotype.

SnailA-GFP overexpression

The SnailA-GFP fusion protein was constructed by cloning the snailA coding sequence into a variant of the pCS2 vector containing the GFP coding sequence inserted in frame as a snailA fragment to generate a C-terminal fusion with GFP and verified by sequencing. mRNA was transcribed in vitro using the SP6 mMessage mMachine kit (Ambion) and injected into cleavage stage blastomeres as described above at a final concentration of 2 μg/μl. Injected embryos were visualized live on a Zeiss Axioplan with an Orca camera (Hamamatsu), and images captured using Volocity acquisition software.

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