Mechanics of blastopore closure during amphibian gastrulation

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ABSTRACT

Blastopore closure in the amphibian embryo involves large scale tissue reorganization driven by physical forces. These forces are tuned to generate sustained blastopore closure throughout the course of gastrulation. We describe the mechanics of blastopore closure at multiple scales and in different regions around the blastopore by characterizing large scale tissue deformations, cell level shape change and subcellular F-actin organization and by measuring tissue force production and structural stiffness of the blastopore during gastrulation. We find that the embryo generates a ramping magnitude of force until it reaches a peak force on the order of 0.5 μN. During this time course, the embryo also stiffens 1.5 fold. Strain rate mapping of the dorsal, ventral and lateral epithelial cells proximal to the blastopore reveals changing patterns of strain rate throughout closure. Cells dorsal to the blastopore, which are fated to become neural plate ectoderm, are polarized and have straight boundaries. In contrast, cells lateral and ventral to the blastopore are less polarized and have tortuous cell boundaries. The F-actin network is organized differently in each region with the highest percentage of alignment occurring in the lateral region. Interestingly F-actin was consistently oriented toward the blastopore lip in dorsal and lateral cells, but oriented parallel to the lip in ventral regions. Cell shape and F-actin alignment analyses reveal different local mechanical environments in regions around the blastopore, which was reflected by the strain rate maps.

Introduction

Morphogenesis relies on cell- and tissue-level control of mechanical properties and highly regulated, spatio-temporally controlled forces to dynamically reshape tissues (Heisenberg and Bellaiche, 2013; Holtfreter, 1943, 1944; Koehl, 1990; Lecuit et al., 2011). By tailoring force production to material properties the embryo is able to facilitate large scale tissue movements necessary to organize the three prospective germ layers during gastrulation (Davidson, 2011; Moore et al., 1995). Developmental programs regulate mechanical processes by regulating cell motility, cell shape, and cell adhesion through direct genetic control as well as through biochemical and mechanical signaling pathways (Belousov et al., 1988; Discher et al., 2005; Orr et al., 2006). These programs coordinate cell behaviors and drive morphogenetic movements, but also encode cell responses to variations in both the local microenvironment as well as to larger scale environmental cues.

Tissue-scale mechanics are especially important during the large scale rearrangements of the germ layers during amphibian gastrulation (Keller et al., 2000). During gastrulation, large regions on the surface of the embryo involute and pass into the embryo while the remaining surface tissues expand to enclose the embryo. Gastrulation begins when a patch of epithelial cells known as bottle cells apically constrict and lengthen along their apical basal axis to create a local invagination that ultimately forms the anterior-most end of the future archenteron. Bottle cell constriction encircles the large yolky cells of the vegetal endoderm. Involution starts when dorsal marginal zone cells adjacent to the bottle cells and deepening groove move inward. The bending of the dorsal marginal zone as involution progresses forms the blastopore lip. Rather than being a static group of cells, the blastopore lip is a dynamic annular mass of cells, composed of both superficial epithelia and deep mesenchymal cells that transiently reside in the lip as they move into the embryo. The processes of bottle cell formation, blastopore lip formation, and involution all begin dorsally and spread laterally, and...
posterior-ventrally, to encircle the yolk plug formed from the large cells of the vegetal endoderm. As large scale tissue reorganization occurs during gastrulation, the blastopore progressively decreases in diameter and closes (blastopore closure; BC) at a constant rate (Fig. S1).

A number of cellular behaviors are thought to drive closure but the precise biomechanics of these processes and their relative contributions to the integrated tissue mechanics that drives closure remain unknown (Keller and Shook, 2008). On the dorso-anterior face of the embryo, presumptive notochordal and somitic mesoderm cells involute and undergo convergent extension. Mesoderm involution and convergent extension are thought to be driven by active mediolateral intercalation, which elongates the tissue and is proposed to generate an arc of tension that contributes to BC (Keller et al., 2003; Keller and Shook, 2008). In the posterior-ventral region, involuting marginal zone cells undergo convergent thickening which produces a thicker tissue further contributing to BC (Keller and Shook, 2008). The completion of blastopore closure signifies the end of gastrulation and is an early milestone of normal embryonic development. Defects during gastrulation, including delays or failure in BC, can lead to incorrect organization of the primary germ layers, lesions in organogenesis, and serious developmental defects.

Cell and tissue movements accompanying Xenopus gastrulation have been described previously (Ewald et al., 2002; Keller et al., 2003; Keller and Shook, 2008; Moosmann et al., 2013; Tyszka et al., 2005) but to understand the physical mechanics of BC requires quantitative measurement of spatial and temporal changes in cell and tissue rearrangement, cellular force generation, and tissue mechanical properties. Such quantitative studies require tools with which strain or strain rates can be measured after application of known forces or loads (Davidson and Keller, 2007).

To understand how the strain rate patterns relate to the global mechanics of blastopore closure we have developed a method to measure force production and material properties of the tissues surrounding the blastopore in vivo and used quantitative image analysis to map mechanical strain rates of dorsal and ventral tissues surrounding the blastopore during gastrulation. To complement our tissue-scale analysis of biomechanics, we collected high resolution confocal images to characterize shape and cytoskeletal orientation of cells surrounding the blastopore lip, and use latrunculin B to evaluate the role of F-actin in the mechanics of blastopore closure. By combining a biomechanical analysis of gastrulation including strain rates, tissue force production and stiffness with descriptions of cell shapes and apical F-actin cytoskeleton we aim to separate the contribution of active from passive tissue shape changes to blastopore closure.

Results

Changing patterns of radial strain rate from mid- to late gastrulation

To understand the location and direction in which cellular forces are being generated, we analyzed mechanical strain rates in tissues surrounding the blastopore using digital image correlation on time-lapse sequences (Fig. 1A, G, M; Supplementary Video S1). Strain rate is a scale- and geometry-free measure of tissue deformation over time that can be used to identify potential sources of force production or regions where mechanical properties change (see Methods for a definition of strain (Blanchard et al., 2009; Davidson et al., 2009)). In contrast to simple deformation or trajectory maps, strain rate maps can indicate where tissues are expanding or contracting in radial and circumferential directions by comparing the displacement of multiple pixels together and calculating whether the distance between them is larger or smaller than in previous time frames. To calculate strain rate we estimate a displacement field or mathematical transform needed to align the two sequential images (Arganda-Carreras et al., 2006). The displacement field produced from this analysis consists of an array of two-dimensional (2D) vectors that bring each pixel in the first image into alignment with the second image. Displacement fields can be visualized by superimposing a subset of these vectors onto the original time lapse images (Fig. 1B, H and N). Spatial gradients of these displacement vectors produce strain rate tensors which can be displayed as maps that reveal local variations in strain rate (Fig. 1C–F, I–L, O–R). In principle, displacement and strain measured between images collected at different times represent the near-instantaneous velocity and the strain rate over a time interval. To recast the strain rates from image-coordinates onto embryonic axes we used a geometric transformation to calculate strains perpendicular to the blastopore (e.g. radial strain) and strain parallel to the blastopore lip (e.g. circumferential strain) for each stage (see Methods). During early gastrulation, after dorsal lip formation, nearly all tissues surrounding the blastopore are expanding with the greatest expansive strain rate appearing dorsally (Fig. 1E). As gastrulation progresses, that radial strain rate at the dorsal lip becomes contractile at Stage 11 (Fig. 1K) then expansive again by Stage 12.5 (Fig. 1Q). Fig. 1 represents strain and displacement results of a single embryo to clearly illustrate our analyses. Typical magnitudes and directions of changes in median strains around the embryo at Stages 10, 11 and 12.5 are consistent amongst 4 other embryos and are summarized in Fig. S2.

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During mid-gastrulation involution spreads from the dorso-anterior lip progressively reaching the ventral–posterior region of the blastopore by late-gastrulation and the surface contractility we observe correlates with these large scale involution movements. For instance, radial strain dips in posterior-ventral regions of the embryo as prospective posterior mesoderm and endoderm initiate involvment (Fig. 1Q).

Force of blastopore closure

Since strain is a product of the application of force against a mechanical structure, we measured both the force of blastopore closure and the mechanical resistance of the blastopore lip within the intact embryo. To quantify tissue force production we used flexible cantilever beams constructed from aramid-polymer fibers. Aramid fibers allow the construction of 5–30 μm diameter cantilevers that can be fashioned into force transducers that are sensitive to nano-Newton (10−9 kg m/s2) scale forces. To measure the force produced during blastopore closure we fabricated a dual-cantilever device consisting of two fibers mounted on a single manipulator. The cantilevers were mounted so their tips were initially separated by ~300 μm, approximately the diameter of the blastopore. We inserted the cantilever tips into opposite sides of the blastopore at Stage 10.5 (Fig. 2A and B). As time progressed, the cantilevers deflected under the force of the closing blastopore and we recorded a “ramp-like” linear increase in force leading to a plateau phase approximately 4 h into BC (mid- to late-gastrula, Stage 12; Fig. 2C; n=3). At the plateau phase, the mechanical resistance of the cantilever stalls progress of blastopore closure between Stages 12 and 12.5, suggesting the force reported by the cantilever at the plateau phase is the peak force generated during closure. The peak force ranged from 300 nN to 500 nN for the embryos tested. We also measured force production along the mediolateral axis of the blastopore. Neither the ramp-like increase nor the peak-force differed significantly from forces measured from probes placed parallel to the dorso-ventral axis (peak forces of 330, 380 and 440 nN in 3 embryos; Fig. 2C) suggesting closure forces are integrated from multiple cellular sources and distributed equally around the
blastopore. Peak force reported by the cantilever as the blastopore closes could reflect changing levels of force production at the cellular level, how these forces are integrated, or changing levels of mechanical resistance of the blastopore.

Blastopore structural stiffness increases during gastrulation

To assess the integrated mechanics of blastopore closure we needed to understand how force production might deform the blastopore. For instance, the magnitude of strain production and tissue deformation depends on both the force generating capacity of the component cells as well as the mechanical resistance of the tissue to those forces, e.g. the force needed to deform a stiff tissue is greater than the amount of force needed to deform a compliant tissue to the same degree. Thus, the increasing force production we observed could be a response to tissue stiffening over the course of BC. Since the rate of blastopore closure is nearly constant (Supplementary Fig. 1) we suspected that tissue stiffness also increases with stage, as previously shown in tissue explants (Moore et al., 1995; Zhou et al., 2009) and intact embryos (Levental et al., 2007; Majkut et al., 2013; Varner et al., 2010; von Dassow and Davidson, 2009). To measure stiffness we replaced the dual cantilever device used for force measurement with two single cantilevers allowing each cantilever to be positioned independently. To immobilize the blastopore we fashioned a single short, high-stiffness cantilever to a fixed base allowing the cantilever to be inserted on one side of the blastopore lip. To apply a defined force to the blastopore we fashioned a second, more compliant cantilever and mounted it to a computer controlled stage; this single

![Fig. 1. Strain mapping of blastopore closure. Strain and displacement analysis was done on Stage 10 (A–F), Stage 11 (G–L) and Stage 12.5 (M–R) embryos. This figure shows analysis of a single embryo; however results from 4 other embryos are generally consistent and are summarized in Fig. S2. Tissue net displacements (B,H,N) and strains were mapped using UnwarpJ during BC (A,G,M). Radial and circumferential tissue strains are represented in polar (C,I,Q) and Cartesian (E,K,Q & F,L,R) coordinates during the beginning (A–F), middle (G–L) and end (M–R) of BC. The Cartesian plots (E,K,Q & F,L,R) represent the median (magenta line) and 75% and 25% quartiles of pixel-by-pixel strain at specific angles around the blastopore, binned every 1°. Cartesian plots were calculated within the ‘flat region’ to avoid distorted strain measurements from the embryo curvature. Displacement vectors are scaled 20 μm. Regions outside the embryo and within the yolk plug are manually shaded gray. Involution is occurring between ~45° and 45° at Stage 10; between ~90° and 90° at Stage 11; and all around the blastopore circumference at Stage 12.5. Scale bar in A represents 200 μm.](image-url)
cantilever was used to apply force to the opposite side of the blastopore lip (Fig. 2D). This apparatus allowed single measurements in different embryos as well as multiple measurements of stiffness over the course of blastopore closure in a single embryo. The mechanical resistance measured by this device is a complex function of the component materials and how they are organized in tissue architecture and is best characterized by the term structural stiffness rather than modulus (Vincent, 2012). We first measured blastopore structural stiffness of a single embryo from the earliest indication of the dorsoanterior blastopore lip (early gastrulation; Stages 10.5; Supplementary Video S2) to stage 4 hours later when the blastopore was nearly closed (mid to late gastrulation; Stage 12; Fig. 2E; Supplementary Video S3). To limit the potential influence of mecanochemical feedback and potential viscoelastic effects of repeated testing, we next collected single measurements from single embryos over the same range of stages (Fig. 2F). In both cases we found blastopore structural stiffness increased 1.5-fold over these stages ($p$-value < 0.001; $n = 13$ embryos per group, 3 clutches, Mann–Whitney $U$-test; Fig. 2F).

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**F-actin depolymerization reduces blastopore structural stiffness**

To test the contribution of the cytoskeleton on blastopore structural stiffness, we treated Stage 11.5–12 embryos with two cytoskeletal inhibitors prior to stiffness measurements (Table 1, Supplementary Table 1). We chose these as stages to evaluate cytoskeletal inhibitors due to the large size of the blastopore and repeatability of probe insertion. Embryos at this stage have a well formed blastopore lip surrounding the yolk plug allowing stable insertion of the force probes at any location. Probes inserted at earlier stages frequently puncture the ventral endoderm. Probes can easily be inserted at later stages but the progressive stiffening of the blastopore and smaller size reduces the resolution of our technique. Previous work by our group has shown that depolymerizing F-actin with latrunculin B (LATB) and inhibiting Rho Kinase with the inhibitor Y-27632 reduces the modulus of neural plate stage dorsal axial tissues (Zhou et al., 2009) so we applied these same inhibitors to the whole embryo to determine the contribution of actomyosin and microtubules to the structural mechanics of the embryo during blastopore closure. Treatment of embryos with 0.3 μM LATB for 20 min resulted in a 22% decrease.
in stiffness \((p \text{-value} = 0.001; n = 15); \text{Kruskal–Wallis test}\) whereas embryos incubated with 80 \(\mu M\) Y-27632 for 60–90 min at 21 °C showed no significant change in stiffness. Incubation with higher doses of LATB caused cell dissociation. The effect of Y-27632 on the structural stiffness of the blastopore differs from prior measurements of the time-dependent Young’s modulus of dorsal tissue isolates (Zhou et al., 2009, 2010) but these previous values are based on longer duration compression tests than our 15 s tests. Structural stiffness depends on both the material modulus (e.g. Young’s modulus) of the component parts as well as the anatomical architecture of the blastopore.

**Cell shapes differ in cells around blastopore**

To understand how cells near the blastopore lip may be contributing to forces of blastopore closure or passively responding to those forces we characterized epithelial cell shapes around the blastopore. We characterized cells in three regions (Fig. 3A); dorsal (Fig. 3C), lateral (Fig. 3C), and ventral (Fig. 3C) to the blastopore. Preliminary experiments revealed surprisingly irregular cell–cell boundaries in ventral and lateral regions of the embryo (Supplementary Video S4) with conventional hexagonally shaped polarized cells found in the dorsal region. To compare cell shapes in these different cell populations around the blastopore we used cell outlines to calculate cell tortuosity. Cell tortuosity is a measure of the complexity of the cell’s morphology (see Methods; see example yellow cell outline and blue convex hull in Fig. 3A); a tortuosity of 0 indicates perfectly straight boundaries whereas a tortuosity greater than 1 indicates irregular cell–cell boundaries (Fig. 3B–B’). Cells are least tortuous in the dorsal region and most tortuous in ventral \((p \text{-value} = 0.001); \text{Mann Whitney U-test}\) and lateral regions \((p \text{-value} < 0.001); \text{Mann Whitney U-test}; \text{Fig. 3D}\). We found no significant difference in tortuosity between ventral and lateral regions \((p \text{-value} = 0.78); \text{Mann Whitney U-test}\) and no dependence on distance from the blastopore (within 120 \(\mu m\); Fig. 3D).

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Cells around the blastopore differ in their degree of elongation and whether their long axis is aligned towards or parallel to the blastopore lip. We find many elongated cells with their major axes directed towards the blastopore lip at all positions around the blastopore (Fig. 3E). However, elongation is weakest in the ventral region and is most evident in the dorsal region (Fig. 3E). To evaluate PA distributions we calculated angular deviations (as described in Methods) in each region. The dorsal region had the lowest angular standard deviation \((26.4°); \text{indicating greatest concentration around 0°}\) followed by the lateral region \((31.6°); \text{verifying our previous observation of relatively more polarized cells at the dorsal blastopore lip}\). In summary, we observe cells are strongly polarized towards the blastopore in dorsal cells and more weakly polarized in lateral and ventral regions.

To determine how cell morphology changes with distance from the blastopore lip, we further analyzed polarity angle in cells as they approached the blastopore lip. We observed that fewer cells were oriented toward the blastopore lip with increasing distance from the dorsal lip (Fig. 3F). The cells in the dorsal region were divided into two groups depending on the distance from the blastopore lip: near (0–80 \(\mu m\); approximately two cell rows from the lip) and far (80–160 \(\mu m\)). Cells at all distances have similar mean polarity angles but exhibit considerable changes in variance with angular standard deviation values decreasing as cells approached the blastopore lip; near and far groups had angular standard deviations of 23.9° and 39.3° respectively. Cells show less variance in PA as they approach the blastopore in the dorsal region. No relationship between cell alignment and distance from blastopore lip is evident in the lateral and ventral regions. However, cells further than 2 rows from the blastopore in the dorsal region exhibit less polarity than cells proximal to the blastopore.

Since we observed highly polarized cells with highly tortuous boundaries in ventral and lateral regions, we then wanted to examine the relationship between polarity and cell boundary tortuosity. Cells having low PA (between –30° and 30°) and a high PI (greater than 2) represent cells that are elongated and highly polarized towards the blastopore lip. Highly polarized cells were found in dorsal and lateral regions. Cells oriented towards the blastopore lip (PA between –30° and 30°) in the dorsal region have a high PI indicating a large degree of polarization towards the blastopore lip (Fig. 3G). As mentioned earlier, cells in the dorsal region exhibit significantly less tortuous boundaries. The largest PI we observed was found in a single sample of six cells in the lateral region (Fig. 3G, see labels 1–6) which, unlike the highly polarized dorsal cells, have highly tortuous boundaries (Fig. 3G top inset) and are relatively close (within 50 \(\mu m\)) to the blastopore lip (Fig. 3G, bottom inset). The strong orientation of dorsal and lateral cells toward the blastopore was accompanied by very different shapes and suggests different local mechanical processes that can produce the same degree of polarity on dorsal and lateral regions.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Treated</th>
<th>Kruskal–Wallis sig.</th>
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<tbody>
<tr>
<td></td>
<td>(n \text{ (Embryos; clutches)})</td>
<td>(\text{Normalized stiffness})</td>
<td>(n \text{ (Embryos; clutches)})</td>
</tr>
<tr>
<td>Latrunculin B</td>
<td>13;5(^c)</td>
<td>1.00 ± 0.10</td>
<td>15;5(^f)</td>
</tr>
<tr>
<td>Y27632</td>
<td>7;3(^c)</td>
<td>1.00 ± 0.23</td>
<td>8;3(^f)</td>
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\(^a\) See Supplementary Table for detailed breakdown by clutch and treatment.

\(^b\) Control embryo stiffness measurements normalized to mean of controls within each clutch.

\(^c\) Treated embryo stiffness measurements normalized to mean of controls within each clutch.

\(^d\) Tested normalized control means vs. normalized treated means using treatment as the independent factor and normalized stiffness as the dependent factor, for each treatment.

\(^e\) Measurements collected from 3 days with 3, 2, and 3 measures per clutch.

\(^f\) Measurements collected from 5 days with 2, 3, 4, 3, and 3 measures per clutch.
Apical F-actin arrays are strongly oriented in cells surrounding the blastopore

Preliminary analysis revealed that polarized cells and cells with tortuous boundaries contained organized arrays of F-actin within their apical cortex. Live confocal time-lapse sequences were collected from mid- to late-gastrula stage embryos (Stage 11.5–12.5) expressing GFP-utrophin (Burkel et al., 2007; Kim and Davidson, 2011) and cells were identified containing (i) disorganized actin, (ii) partially organized F-actin arrays where filament microdomains aligned in two or more different directions, and (iii) strongly oriented F-actin arrays where most filaments are oriented in the same direction (Fig. 4A). F-actin arrays were also observed in fixed, phalloidin-stained samples confirming F-actin organization observed in live samples was not an artifact of GFP-utrophin expression (Supplementary Fig. 4). All three types of F-actin organization were found in cells on dorsal, ventral, and lateral regions but the percentage of cells with highly oriented F-actin arrays varied with their position around the blastopore. The majority of cells surrounding the blastopore contain highly organized F-actin arrays but a significantly greater proportion of cells in ventral and lateral regions contained highly organized actin compared to the dorsal region (Fig. 4B) with the ventral region containing the largest fraction of organized actin. Furthermore, the F-actin arrays are frequently oriented toward the blastopore. Highly organized F-actin arrays in dorsal and lateral regions are aligned toward the blastopore (within a range of 45°); in contrast, highly organized actin arrays in the ventral region align parallel to the blastopore.

Discussion

We have carried out a multilevel biomechanical description of blastopore closure. This analysis involved a range of in vivo analyses: measuring patterns of strain rate surrounding the closing blastopore, measuring structural stiffness and tissue force production of the blastopore, describing cell level shape changes during Xenopus laevis blastopore closure, and conclude with a description of a highly organized apical F-actin array within these cells. Using digital image correlation, we mapped the radial and circumferential strain rate patterns in tissues surrounding the blastopore over time and observed changing patterns of radial strain rate as development progressed. Local patterns of strain rate indicate stress is being generated in the embryo; such stresses may be autonomous to the stained cells, e.g. through apical contraction, or may reflect exogenously generated forces. In particular, high rates of radial expansion are found in the dorsal region during early stages of closure and in the ventral region during later stages. High rates of radial expansion suggest that specialized epithelial cell behaviors in these regions may be contributing to forces of blastopore closure or that epithelial cells are being passively deformed by forces produced by cells deeper in the embryo. The radial strain rate becomes progressively contractile from dorsal to ventral regions as closure proceeds. These local epithelial cell behaviors could augment the role of convergent extension in the dorsal region and convergent thickening in the ventral region as driving forces of blastopore closure (Keller and Shook, 2008).

Peak forces measured by the cantilever during blastopore closure are in the range of 0.5 μN. These peak forces are similar in magnitude to forces of neural tube closure measured in the newt Ichthyosaura alpestris (formerly Triturus alpestris) (Waddington, 1939) and the salamander Ambystoma mexicanum (Selman, 1958). The forces we report, like these earlier studies, include force production of multiple tissues and the dissipation of these forces by viscoelastic processes. These peak forces are likely the sum of forces generated from various cellular movements including radial and mediolateral intercalation in the mesoderm, mesendoderm migration, and thickening of mesoderm at the posterior-ventral end of the blastopore. To estimate the relative contribution of these distinct morphogenetic machines on the cellular scale will require further biomechanical studies with tissue explants and detailed computer simulations.

Stage dependent changes in blastopore structural stiffness parallels changes we have observed previously in converging and extending dorsal tissue isolates (Zhou et al., 2009) and within patches of dorsal tissues within the dorsal marginal zone (von Dassow and Davidson, 2009). Blastopore structural stiffening occurs at a time when both the rates of closure and radial strain are relatively constant. Maintaining levels of force production throughout the course of blastopore closure suggests several possibilities: (i) a mechanical feedback model in which cells near the blastopore lip balance their force production to match local increases in stiffness in order to sustain blastopore closure, (ii) a feed-forward model that couples actomyosin contractility and stiffness, and (iii) structural feedback in which strain aligns contractile apical actomyosin filaments producing anisotropic stiffness in tissues that move over the blastopore lip. There may be differences between observed changes in mechanics due to cytoskeletal inhibitors obtained with previous analyses and our current methods. The mechanical changes in modulus observed after cytoskeletal perturbation of dorsal isolates (Zhou et al., 2009, 2010) used an unconfined uniaxial compression test to evaluate the axial modulus of dorsal isolates. Dorsal isolates are organized into a “brick-like” structure which allowed Zhou and coworkers to relate the effects of cytoskeleton modifiers to the material properties of embryonic tissues. By contrast, our measurement of structural stiffness does not allow us to directly relate stiffness measurements to material properties and may reflect changes in the connectivity or overall mechanical structure of tissues that form the blastopore.

Fig. 3. Cell shape around the blastopore. (A) Schematic of gastrulating embryo at Stage 11 indicating regions of imaging (scale bar represents 200 μm). Cell shape imaging was carried out in Stage 10.5–12 embryos. (A’) Example image demonstrating methods used to make tortuosity and polarity measurements. Tortuosity=1 (contour area(yellow dashed)/convex hull area(green solid)); polarity index (PI)=major axis (blue dashed)/minor Axis (red solid); polarity angle (PA)=acute angle between major axis of enclosed ellipse and line from blastopore centroid to cell centroid. A PA of 0° indicates the cell’s major axis is perpendicular to the blastopore lip. (B) Original and (B’) skeletonized image in the lateral region with associated cell boundary tortuosity measurements as a reference. Scale bar = 20 μm. Representative (C) dorsal, (C’) lateral, and (C”) ventral cells. Asterisks indicate large yolky cells in the blastopore. Scale bar = 50 μm. (D) Tortuosity measurements for dorsal, ventral, and lateral regions with lowest tortuosity in the dorsal region (p-value < 0.001 for dorsal vs. ventral and dorsal vs. lateral; Mann-Whitney U-test). (D’) Tortuosity as a function of distance from the blastopore lip (r² values obtained from linear regression show insignificant correlation). (E) Distributions of polarity angle (PA) on dorsal, ventral, and lateral regions; angular deviation shows highest concentration of points around the mean in the dorsal region followed by the lateral region; angular deviation was lowest in dorsal region (26.4°) followed by lateral (31.6°) and ventral regions (38.5°). (F) PA of dorsal cells as a function of distance from the blastopore lip showing decreasing polarity towards the blastopore with increasing distance (pink vs. blue). Data was divided into two groups: close (0–80 μm) and far (80–160 μm) from blastopore lip. Box plots indicate the same mean but much greater standard deviations for the far group. (G) PI as a function of PA indicates high polarity indices for cells polarized towards the blastopore lip in dorsal and lateral regions. All cells with a PI of less than 2 are excluded to only observe highly polarized cells. Cells having low PA (between ~30 and 30°) and a high PI (greater than 2) represent cells that are elongated and highly polarized towards the blastopore lip. Highly polarized cells in the dorsal region do not have tortuous boundaries. In contrast, highly polarized cells (labeled 1–6) in the lateral region do have highly tortuous boundaries (G top inset). These cells are also close to the lateral blastopore lip (within 50 μm of the lip; G bottom inset). 82 cells from 7 embryos, 58 cells from 3 embryos, and 44 cells from 3 embryos for dorsal, lateral and ventral regions, respectively, were used in cell shape analyses in D–G.
Estimating the true blastopore closure force requires accounting for the unknown force required to deform the embryo, in addition to the force on the cantilever. By considering the elastic and viscous limiting cases for embryo deformation behavior (Supplementary Text), we find that the blastopore closure force closely follows the timing of cantilever force (Fig. S3), although the blastopore closure force is twice the cantilever force at the elastic limit. The observed ramped forces of blastopore closure are consistent with predictions of our earlier study on the temperature dependence of blastopore closure (von Dassow et al., 2014). These limiting cases suggest that the blastopore closure force displays a ramp and plateau of a similar magnitude and time course to the measured cantilever force. Viscoelastic deformation behavior would fall between these limits.
To investigate the local mechanical environment in cells within a few cell diameters from the blastopore, we characterized apical cell shape changes and altered dynamics of apical actomyosin as blastopore closure progresses (Fig. 5A). Epithelial cells dorsal to the blastopore are polarized towards the blastopore lip, have non-tortuous cell boundaries, and organize their F-actin fibers in the same direction in which they are polarized. Cells lateral and ventral to the blastopore are less polarized with tortuous cell boundaries. Many of these same cells exhibit highly organized arrays of F-actin but are increasingly less oriented. This suggests that cells dorsal to the blastopore lip experience a different mechanical environment than cells lateral and ventral to the blastopore. Polarized cells with tortuous boundaries are observed in lateral and ventral regions of the blastopore suggest these cells are compressed along their apical surface.

The tortuous cell boundaries we observe surrounding the blastopore lip differ from cell shapes seen in other embryonic epithelia which show nearly ideal polygonal boundaries (Gibson et al., 2006) but are similar to convoluted cells observed transiently in the amnioserosa during dorsal closure in Drosophila (Mateus et al., 2011; Solon et al., 2009; Toyama et al., 2008). Mateus et al. (2011) propose these irregularities are due to reduced levels of endocytosis and membrane trafficking on apical membrane content and conclude that Rab5, which is a marker of early endosomes, is required in the amnioserosa to remove membrane. However, the processes that produce irregular cell–cell borders in Xenopus may be much different from those that shape amnioserosa cells since Xenopus cells are much more static in their shapes (Supplementary Video S5) and do not oscillate as amniosera cells. Since tensile apical actomyosin arrays are likely responsible for the maintenance of ideal polygonal shapes it remains unclear how tortuous cell–cell boundaries might arise and remain stable during blastopore closure.

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The presence of highly organized, apical F-actin around the blastopore is unusual in epithelial cells. Further perturbation studies are needed to understand the role of apical F-actin arrays during gastrulation. Oriented arrays of F-actin stress fibers are frequently found at the basal surface of epithelial cells (Nusrat et al., 1995). In some cases, these F-actin arrays are highly sensitive to the local mechanical microenvironment, aligning either parallel or transverse to fluid-flow induced shear stress (Barbee et al., 1994). Another instance of highly organized cytoskeleton can be found in the developing Drosophila embryo. Microtubules (MT) can form highly organized arrays in the apical cortex during squamous epithelial morphogenesis in Drosophila and have been hypothesized to play mechanical roles in the reshaping of cells by extending apically then bending perpendicularly to increase apical area (Pope and Harris, 2008). The high degree of actin organization we observe in the ventral region resembles that of the MT network in Drosophila and may reflect underlying tissue thickening processes described in previous studies (Keller and Shook, 2008). F-actin aligned towards the blastopore in the dorsal region along with the high degree of cell polarity may indicate active cell behaviors coordinating mechanical processes of blastopore closure, strengthening tissues in the direction of highest strain rate, perhaps integrating stresses generated by convergent extension movements with those produced by the blastopore. Conversely, F-actin aligned perpendicular to the direction

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**Fig. 4.** Actin orientation in cells around the blastopore. (A) Representative confocal single section images of single cells from Stage 11.5 to 12.5 embryos categorized with organized (O), intermediate (I), and disorganized (D) apical F-actin arrays. (B) Stacked bar graph representing relative frequencies of each type of F-actin organization in each of the 3 regions around the blastopore. Total number of cells analyzed per group below each bar.

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**Fig. 5.** Cell shapes and strain patterns around the blastopore. (A) Schematic depicting conclusions on cell morphology (cell boundary tortuosity, cell polarity and F-actin organization) in dorsal, lateral and ventral regions of the embryo. White, gray, and black cells represent cells with organized, intermediate and disorganized apical F-actin arrays. (B) Strain patterns around the blastopore during gastrulation. Regions of positive radial strain and positive circumferential strain (+R, +C; yellow) is suggestive of epiboly-like tissue movements. Regions with positive radial and negative circumferential strains suggest converging and extending tissues and regions with negative radial and negative circumferential may indicate tissues undergoing convergent thickening.
of closure in the ventral region may indicate that these cells are passively involuting or actively contracting laterally to close the blastopore.

Our multilevel description of blastopore closure reports complex patterns of strain rate (Fig. 5B), the integrative force of closure and structural stiffness of the blastopore, the irregular shapes of epithelial cells and the presence of aligned F-actin at their apical surface and suggests novel roles for the epithelial cells during amphibian gastrulation. At the level of the tissue we see complex patterns of contraction and expansion. At early stages we observe large regions surrounding the blastopore that exhibit strain rate patterns indicative of epiboly (e.g. radial and circumferential expansion). At late stages strain rates appear to reflect underlying processes of convergence and extension (e.g. radial expansion and circumferential contraction) and convergent thickening (e.g. radial and circumferential contraction). Interestingly, epithelial cells do not exhibit common tiling patterns seen in passive arrays of cells but instead exhibit complex, often torturous shapes, with strongly aligned apical arrays of F-actin. We propose these arrays develop in response to complex patterns of strain. Compressive strains in both direction may cause apical cell junctions to buckle. Anisotropic strain patterns may initiate the formation of aligned F-actin as is found at the basal surface of cells exposed to shear flows. These observations suggest epithelial cells are adapting to strain in the epithelium in unexpected ways and may either contribute to force production of blastopore closure or serve to guide movements of blastopore closure or later morphogenetic movements by offering anisotropic resistance.

Methods

Embryos

*X. laevis* embryos are obtained by standard methods (Kay, 1991), fertilized in vitro, dejellied in 2% cysteine and cultured in 1/3 × MBS (Sive et al., 2000) at 14.5–21°C to Stage 10 (Nieuwkoop, 1967). Before running mechanical testing, vitelline membranes of embryos were removed with forceps (Fine Science Tools Inc., Foster City, CA). For BC imaging and during mechanical testing, embryos were held in place using modeling clay (Van Aken, Dick Blick Art Materials) in Petri dishes. The dishes were completely filled with 1/3 × MBS. All force and stiffness experiments were done at room temperature, 21°C. The two cantilevers were inserted approximately 45° from the axis perpendicular to the blastopore, not more than 10 μm deep just within the blastopore lip on either end. Images and time-lapse sequences of the embryo and embedded cantilevers were collected using a CCD camera (Scion Imaging Corp.) attached to the video port of a stereomicroscope (Stemi 2000C; Carl Zeiss Microscopy). A small glass cover-slip was placed across the rim of the Petri dish, directly above the embryo without interfering with the cantilevers, which partially sealed the dish and prevented disruptive convective flow, to optimize imaging.

Strain rate mapping

To characterize the strain rate in the embryo, image sequences were collected at 26°C and analyzed using an open source image analysis software (ImageJ; (Rueden and Eliceiri, 2007); available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) and a custom plugin (bUnwarpyJ) (Arganda-Carreras et al., 2006). This plugin allows us to calculate strain by using image registration of two images based on elastic deformations represented by B-splines. Custom macros were created to sequentially obtain strain maps throughout an image sequence. By tailoring our time interval for image registration we were able to uncover strain rates over short (< 2 min) and long time scales (> 30 min) throughout development. A colormap for the strain rate at each pixel was calculated and plotted, depicting blue as contraction and red as expansion. A custom ImageJ plugin was used to display the displacement as vectors superimposed on the original data. Radial and circumferential strain rates were calculated by transforming the strain rate tensor, \(\varepsilon = [\varepsilon_{rr} \varepsilon_{\theta r} \varepsilon_{r\theta} \varepsilon_{\phi r} \varepsilon_{\theta\phi} \varepsilon_{r\phi}]\), to polar coordinates (Korukonda and Dooley, 2012). Radial and circumferential sweep boxplots were calculated for regions within 5–10 cell diameters of the blastopore to avoid potential distortions from surface curvature (MATLAB, R2013; Mathworks Inc., Natick, MA).

Force of closure test

To measure the force of blastopore closure, we constructed a device holding two aramide-fiber cantilevers. These cantilevers were mounted in glass capillary tubes which could be easily exchanged and mounted on a micro-positioner (M33 Märzhäuser). The aramide-fibers were calibrated by applying various weights at the tip (data not shown) and the spring stiffness calculated from the measured deflections. Aramide-fiber cantilevers exhibited linear spring stiffness over the range of deflection values used in these studies. The spring stiffness ranged from 0.002 to 0.004 N/m. To begin mechanical testing, Stage 10 embryos were oriented with the blastopore facing upwards using non-toxic modeling clay (Van Aken, Dick Blick Art Materials) in Petri dishes. The dishes were completely filled with 1/3 × MBS. All force and stiffness experiments were done at room temperature, 21°C. The two cantilevers were inserted approximately 45° from the axis perpendicular to the blastopore, not more than 10 μm deep just within the blastopore lip on either end. Images and time-lapse sequences of the embryo and embedded cantilevers were collected using a CCD camera (Scion Imaging Corp.) attached to the video port of a stereomicroscope (Stemi 2000C; Carl Zeiss Microscopy). A small glass cover-slip was placed across the rim of the Petri dish, directly above the embryo without interfering with the cantilevers, which partially sealed the dish and prevented disruptive convective flow, to optimize imaging.

Stiffness test

Two cantilevers, each mounted on a micro-positioner were used. Embryos were immobilized using clay in dishes as described above. One of the cantilevers was stiff and used to fix the position of the dorsal lip of the blastopore. The other cantilever was compliant and its stiffness was calibrated as described above. The compliant cantilever was mounted on a moveable micro-positioner fitted with a computer controlled DC servo motor (Thorlabs Inc., Newton, New Jersey). The stiff and the compliant cantilevers were inserted perpendicular to the plane of the blastopore less than 10 μm below the blastopore lip with the stiff cantilever in the dorsal region and the compliant cantilever in the ventral region. The servo motor controlling the compliant cantilever was programmed to produce a series of 50–100 μm step-displacements with a 15 s period between displacements. The force applied by the cantilever on the blastopore lip was calculated by using the observed compliant fiber flexure and the compliant cantilever stiffness. Linear force–displacement profiles were obtained and the slope used to calculate the spring stiffness of the blastopore lip.

mRNA injection and confocal microscopy

To visualize F-actin, we injected 1–4 cell stage embryos with mRNA encoding the eGFP-utrophin as used in previous studies (Burkel et al., 2007; Kim and Davidson, 2011). mRNA was synthesized and purified using an in vitro transcription kit (Epitrend, Madison WI). A 20 × objective allows the visualization of cell outlines whereas a high n.a. 63 × objective was required to visualize intracellular F-actin morphology. Single sections and time-lapse sequences were collected using a confocal laser scanner (SP5, Leica Microsystems, Inc., Wetzelar, Germany) mounted on an inverted compound microscope (DMI6000, Leica Microsystems, Inc.) using automated acquisition software (LASAF, Leica Microsystems, Inc.). Laser power, scanline frequency, pinhole size, and PMT gains were adjusted in order to
optimize image quality and minimize phototoxicity (Joshí et al., 2012; Kim and Davidson, 2013).

Cell shape characterization

Quantitative morphology was used to characterize cell shapes including cell tortuosity (morphology of cell–cell boundaries), polarity angle (cell orientation), and polarity index (cell elongation). Cell boundary tortuosity was measured using semi-automated image analysis tools (ImageJ; Rueden and Eliceiri, 2007). Tortuosity was measured in ImageJ using the index [1-solidity]. Briefly, solidity is measured by manually outlining a cell boundary and then automatically fitting a convex hull around the manually traced perimeter. Solidity is the ratio between the traced perimeter and convex hull area. The polarity index (PI) is the length to width ratio automatically calculated from the manually traced cell boundary using image analysis software. The polarity angle (PA) is calculated by measuring the acute angle between the major axis of the manually traced cell boundary and a line from the blastopore centroid to cell centroid. Angular standard deviation, s, was measured using the equation: \[ s = \sqrt{2(1 – R)} \], where \( R \) is the resultant vector length (RVL). RVL is a measurement of circular spread in directional statistics, ranging from 0 to 1, with high values indicating a greater concentration of the distribution around the mean polarity angle (Zar, 1999) and is the magnitude of the vector sum of n polarity angles divided by \( n \) (all vectors have a magnitude of 1). The blastopore centroid was approximated from the location and position of the nearest blastopore lip observed using low magnification confocal images of the regions of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.11.011.

References


