Video Article

Probing the Roles of Physical Forces in Early Chick Embryonic Morphogenesis

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Abstract

Embryonic development is traditionally studied from the perspective of biomolecular genetics, but the fundamental importance of mechanics in morphogenesis is becoming increasingly recognized. In particular, the embryonic chick heart and brain tube, which undergo drastic morphological changes as they develop, are among the prime candidates to study the role of physical forces in morphogenesis. Progressive ventral bending and rightward torsion of the tubular embryonic chick brain happen at the earliest stage of organ-level left-right asymmetry in chick embryonic development. The vitelline membrane (VM) constrains the dorsal side of the embryo and has been implicated in providing the force necessary to induce torsion of the developing brain. Here we present a combination of new *ex-ovo* experiments and physical modeling to identify the mechanics of brain torsion. At Hamburger-Hamilton stage 11, embryos are harvested and cultured *ex ovo (*in media). The VM is subsequently removed using a pulled capillary tube. By controlling the level of the fluid and subjecting the embryo to a fluid-air interface, the fluid surface tension of the media can be used to replace the mechanical role of the VM. Microsurgery experiments were also performed to alter the position of the heart to find the resultant change in the chirality of brain torsion. Results from this protocol illustrate the fundamental roles of mechanics in driving morphogenesis.

Video Link

The video component of this article can be found at https://www.jove.com/video/57150/

Introduction

Modern developmental biology research largely focuses on understanding development from the perspective of molecular genetics[1,2,3,4,5,6,7,8,9,10,11,12,13]. It is known that physical phenomena play a central role in morphogenesis, or the generation of biological form[14,15,16,17]; however, specific mechanical mechanisms of development remain largely unstudied. Ventral flexure and rightward torsion of the primitive brain tube after Hamburger-Hamilton stage 11 (HH 11)[18] are the two main processes that contribute to embryonic shape change[19,20]. In particular, the physical mechanism underlying the torsional development in the embryonic brain remains incompletely understood.

The embryonic torsion in chick embryo is among the earliest morphogenetic events of left-right (L-R) asymmetry in development. When the process of L-R asymmetry is perturbed, birth defects such as *situs inversus*, *isomerism*, or *heterotaxia* will occur[21].
Here we present a protocol which combines *ex-ovo* experiments[22,23] with physical modeling to characterize mechanical forces during early embryonic brain development. The goal of the method presented is to identify the mechanical forces responsible for brain torsion and the factors that affect the degree of torsion during early development[12]. Based on the experimental observation that the vitelline membrane (VM) constrains the dorsal side of the embryo, we hypothesized that the VM provides the force necessary to induce torsion of the developing brain. Therefore, in this method, we removed the part of the VM that covers the brain area to find out the effects on brain torsion. Furthermore, the method of applying fluid surface tension was used to confirm the mechanical role of the VM and provide an estimate of the force needed for brain torsion, which had not been done previously. Measuring the forces during embryonic morphogenesis is a challenging task. Notably, in a pioneering study, Campàs and co-workers[24] developed a novel method to quantify the cellular stresses using injected microdroplets. Nevertheless, this method was limited to measure forces at the cellular level, hence not applicable to probe forces at tissue- or organism-level. The protocol presented in this paper was developed to partially fill this gap.

Protocol

1. Preparation of Tissue Culture Media

1. Use a 0.5 L bottle of Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, sodium bicarbonate, and L-glutamine as the base for the culture media.
2. In a sterile laminar flow hood, add 10 mL of antibiotics to the 0.5 L of DMEM.
3. Using a sterile pipette, transfer 50 mL of the DMEM antibiotics solution to a sterile 50 mL conical tube.
4. Add 50 mL of chick serum to the remaining DMEM antibiotics solution in the 0.5 L bottle in the sterile hood.
5. Store the final solution (referred to hereafter as chick culture media [CCM]) in 50 mL conical tube aliquots at -20 °C.

2. Egg Incubation

1. Use delicate wipes with 70% ethanol to clean fertilized specific pathogen-free White Leghorn chicken eggs. Arrange eggs in a longitudinal orientation on holders.
2. Turn on an egg incubator to set a target temperature of 37.5 °C and maintain the humidity at 48 - 55%. The humidity is controlled by adding an appropriate amount of water to the incubator.
3. Incubate the eggs to HH11-13, approximately 40 - 44 h.
4. Let the eggs cool down at room temperature for approximately 15 - 30 min prior to spraying/cleaning with 70% ethanol.

3. Pull Glass Capillaries

1. Mount a 10 cm-long glass capillary tubes with an outer diameter of 1.0 mm and an inner diameter of 0.5 mm on a micropipette puller.
2. Set the heat and pull parameters to 750 and 400, respectively. Press the pull button to pull the capillary tube into thin needles.

4. Filter Paper Carrier Method

1. Cut circles of approximately 3 cm in diameter from filter paper.
2. Cut a rectangle roughly 1 cm by 2 cm from the circle using a hole puncher. Make sure to remove any protruding or sharp corners.

5. Embryo Harvesting and Preparation

1. Crack eggs from the bottom, pull apart the shell gently and carefully deposit contents into a 150 mm x 15 mm petri dish. To ensure the embryo side is up, keep the eggs in the same orientation they were incubated while cracking them.
2. Remove the thin albumin using a disposable Pasteur pipette.
3. Separate the thick albumin from the yolk using blunt ended forceps. Ensure that the thick albumin has been removed by lightly scraping the top of the yolk with the blunt ended forceps.
4. Use fine-tipped forceps to center and place a filter paper ring over the embryo, matching the long axis of the ring with the long axis of the embryo.
5. Cut the yolk surrounding the filter paper ring with scissors.
6. Pull the ring and embryo off the yolk in an oblique direction towards the site where the yolk was first cut.
7. Rinse the embryos in two sequential 100-mm dishes with room temperature 1x phosphate buffered saline (PBS).
8. Place a filter paper ring into a 35-mm petri dish first. Then place the embryo dorsal side up onto the filter paper already in 35-mm petri dish.
9. Place a stainless-steel ring on top of the filter paper sandwich. Make sure not to damage the embryo.
10. Add 3 mL of the previously prepared CCM to each petri dish.
11. Remove the VM of each embryo by lightly skimming the pulled capillary needle across the top of the embryo and peeling the VM away, starting from the anterior end (right above the forebrain) and proceeding to the notochord (**Figure 1A**).
12. Place eight 35-mm Petri dishes into one 150 mm dish that was lined with water saturated delicate task wipes (to maintain humidity).
13. Place the 150-mm petri dish into a sealable plastic bag then fill the bag with a gas mixture comprised of 95% O_2 and 5% CO_2.
14. Seal the bag and place it in a 37.5 °C incubator.
15. Incubate the embryos for an additional 27 h until HH15-HH16 (**Figure 1B**).

6. Inducing Surface Tension

1. Remove the embryos from the incubator and use an optical coherence tomography (OCT) system to image them. Use OCT to determine the torsional angle of the neural tube (NT) (**Figure 2**).
2. Transfer the embryos to a light microscope and visualize at 10X magnification. Use a 200 microliter pipette to incrementally remove 0.2 mL of media from the petri dish.
3. Take brightfield images at each interval to observe the effects of the media-air interface on the embryo.
4. Remove media until the surface tension across the embryo induces torsion (**Figure 1C**).
5. Image the embryo using the OCT system once again to establish a final torsional angle for comparison to control embryos. NOTE: The Bright-field images were acquired by using a dissecting microscope. An optical coherence tomography system with an attached microscope

was used to acquire cross-sectional image stacks of live embryos. Images were obtained in a 3 x 10 mm x 3 mm^3 scanning domain, then processed in an imaging software. Lastly, the physical model images were taken with a digital single-lens reflex camera.

7. Physical Modeling of Surface Tension/VM Forces

1. Develop a simplified 3D geometry that resembles an embryo between HH14-17 in commercial modeling software (**Figure 3A**).
2. Design the negative mold of the 3D geometry in commercial 3D computer graphics software.
3. Use a 3D printer loaded with 1.75 mm acrylonitrile butadiene styrene filament to 3D print the designed mold, in stereolithographic (.stl) format.
4. To cast the mold, mix silicone rubber elastomer components A and B in equal parts and pour the mix into the mold promptly; set the casting mold to cure at room temperature for 12 h (**Figure 3B**).
5. Mark the physical model of the embryo along the NT on the dorsal side to visualize torsion.
6. Use a coverslip to replicate the force applied to the 3D physical model which mimics that of the VM or surface tension (**Figure 3D**).
7. Insert a series of rigid wires of equal length to the side of the physical model. After the coverslip exerts an external force on the brain model, the wires become tilted at an angle that depends on the location. Determine the rotational angle by arctan of the projected length over the original length of each wire (**Figure 3E**).

8. Altering the Direction of the Heart Loop

1. Follow the steps in 3.1 and 3.2 to obtain a pulled capillary tube.
2. Follow the steps in 5.1 through 5.10 to prepare the embryo.
3. Use a pair of forceps to flip the filter paper so that the embryo becomes ventral-side-up.
4. Use the pulled capillary tube to cut open a slit in the splanchnopleure (SPL) membrane.
5. Use the capillary tube to exert a mechanical force to push the heart from the right-hand side to the left-hand side.
6. Follow the steps in 5.12 through 5.15 to observe the change in torsion.

Representative Results

In this study, the VM of the embryo at HH11 was removed from the anterior end to the thoracic flexure. The embryos were imaged by an OCT system. At this stage, the torsion of brain tube has not started (**Figure 1A**). After being incubated to HH15-16, embryos with their VM removed exhibited reduced brain tube torsion, approximately 35 degrees (**Figure 1B**) compared to control embryos, which exhibit torsion of around 90 degrees. When the media level was lowered to induce surface tension on the dorsal end of embryos with their VMs removed, the brains twisted to levels comparable to those in control embryos (**Figure 1C**). **Figure 2** shows a representative OCT image of an HH 13 embryo with its thoracic orientation angle and cranial orientation angle marked (**Figure 2A**). The angles are measured from a vertical position of the cross-section of the NT (**Figure 2B, C**). Results from our experiments suggested that normal brain tube torsion is driven by external loading on the dorsal end of the embryo and that this essential load is supplied by the VM[20,25]. Moreover, in a normal embryo the brain turns rightward as the heart loop goes to the right-hand side whereas in an embryo with the heart loop pushed to the left-hand side at an early stage (*i.e.*, before HH stage 12), the brain also turns leftward following another 20 h of incubation (Figure 3c in Ref. [12]), suggesting that the position of the heart resulted in the asymmetry in brain torsion.

Data gathered in experiments enabled us to reconstruct a simplified geometry of the chick embryo without the VM from HH14-17 (**Figure 3A**). In this computational model, the brain and right looped heart were modeled as curved rods. A block representing the splanchnopleure (SPL) membrane was contact with the heart rod. By designing a negative mold from this computational model, 3D printing this mold, and casting the mold with a silicone elastomer, we fabricated a physical model from the simplified computational geometry (**Figure 3B-D**). A coverslip pressed downward on the dorsal end of the physical model replicated the mechanical load provided by the VM or surface tension from our experiments (**Figure 3D**). The model exhibits comparable geometry and brain torsion with an actual embryo, cultured *ex-ovo* to HH14-17 (**Figure 3E**).

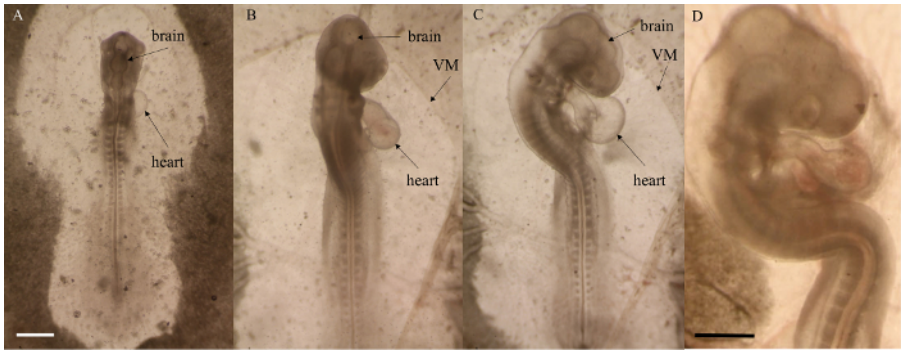

Figure 1: Morphology of embryo with VM removed and effects of external forces on rightward brain tube torsion. (A) Harvested embryo with VM removed at HH11. **(B)** The same embryo incubated for 27 h post VM removal showing reduced torsion. **(C)** The same embryo underwent brain torsion, upon application of fluid surface tension. **(D)** Control embryo with normal brain torsion at a comparable stage. Scale bars, (A-C) 1 mm, (D) 1 mm. Images were captured at 10X magnification. Adapted from Ref. [12] with permission. Please click here to view a larger version of this figure.

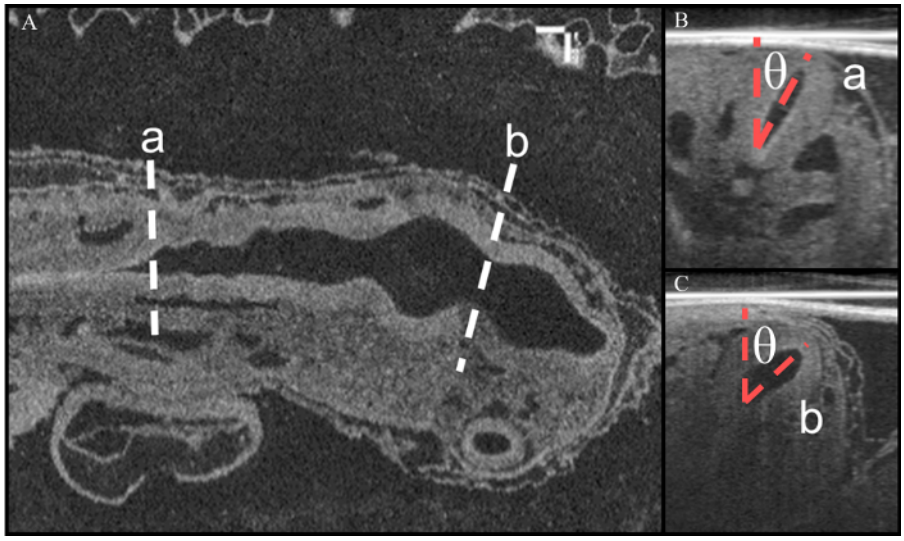

Figure 2: OCT image of an HH13 embryo. (A) The figure has its thoracic orientation angle marked at (a) and its cranial orientation angle measured at (b). **(B), (C)** A cross-section of the NT at positions (a) and (b). Angles are measured from a vertical position. Please click here to view a larger version of this figure.

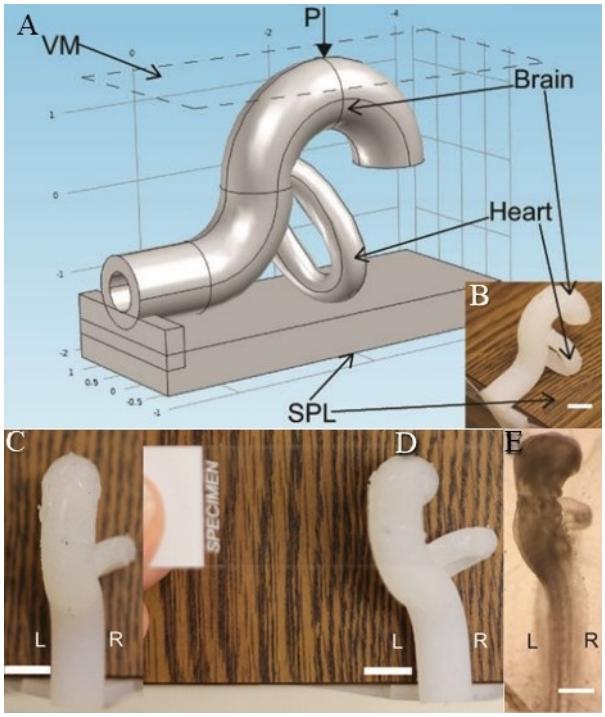

Figure 3: A physical model of the embryo demonstrating brain torsion. (A) A simplified geometry of a chick embryo without VM at HH14-17 **(B)** Silicone elastomer physical model of a chick embryo. **(C)** Dorsal view of model with the heart on the right side. **(D)** Dorsal view of the model under an external force applied by a coverslip, starting to show rightward brain torsion. **(E)** Chick embryo cultured *ex-ovo* beginning to twist rightwards at HH14 for comparison. Scale Bars, (B-D) 1 cm, (E) 1 mm. Adapted from Ref. [12] with permission. Please click here to view a larger version of this figure.

Discussion

While physical phenomena play an integral role in morphogenesis[26,27,28,29,30], the specific mechanical mechanisms, along with the coordination of mechanical and molecular mechanisms, remain largely unexplored. It is known that the ventral flexure and rightward torsion of the primitive brain are two central processes that contribute to early embryonic morphogenesis[18,31,32,33,34], but no prior studies addressed the mechanical origins of brain torsion, one of the earliest organ-level left-right asymmetry event.

The key steps of the protocol include the removal of the VM to identify the mechanical driving force for brain torsion and the application of fluid surface tension to further confirm the findings. The troubleshooting of this technique took place to identify the initial stage at which the VM should be removed to induce significant changes in torsion.

The downward passive force of the VM was shown to be a fundamental mechanical boundary for the growing embryonic brain tube. When the VM of the embryo was removed, the brain no longer twists to a normal degree but could be made to twist to the control level through the subsequent application of surface tension by lowering the fluid level. The known surface tension of water at ambient temperature is 72.01 ± 0.1 mN/m, and the contact length is of the order of millimeters, the force can then be calculated. Thereby we estimated the VM exerted a force of approximately 10 mN on the HH 14-17 embryo[12].

Using this protocol, we were able to determine that VM plays the key mechanical role in embryonic brain torsion. The results obtained using this new protocol suggests that the VM is a critical structure for the progression of normal brain torsion during embryonic morphogenesis, as it supplies the geometric constraints and mechanical load necessary for the twisting of the brain[35]. The results also indicated that the position of the heart determines the direction of brain torsion. L-R asymmetry of the embryo during development leads to a right-looped shape of the heart, which in turn drives rightward twisting of the brain[36,37,38,39]. It is worth mentioning that the mechanical method of displacing the heart's position differs from the chemical method developed by other researchers[13] and is better for delineating the role of mechanics in morphogenesis. Altogether, our results illustrate the fundamental role of mechanics in driving torsional brain morphogenesis in the chick embryo.

In the future, the protocol may be applied to identify how genetic and mechanical factors together regular embryonic torsion and unveil how these different factors work in concert to ensure appropriate morphogenesis.

Disclosures

The authors declare no conflicts of interest.

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