Spring 5-15-2016

Mechanics of Early Retina and Lens Development in the Embryo

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Mechanics of Early Retina and Lens Development in the Embryo

by

Alina Oltean

A dissertation presented to the Graduate School of Arts & Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2016
Saint Louis, Missouri
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Acknowledgments

The completion of this dissertation would not have been possible without the support and guidance from advisers, mentors, friends, and family.

I would like to thank my committee members for their contributions and sharing their knowledge and expertise at lab meetings, conferences, in classrooms or individual discussions: Dr. Larry Taber, Dr. Steven Bassnett, Dr. Philip Bayly, Dr. Spencer Lake, Dr. Ruth Okamoto, and Dr. Jin-Yu Shao. I thank my thesis adviser, Dr. Larry Taber, for countless scientific discussions and feedback on this dissertation. I would like to also thank Dr. David Beebe, a great scientist, mentor, and collaborator who passed away before the completion of this dissertation. The collaborative and interdisciplinary atmosphere at Washington University has provided a great learning environment throughout my doctoral work.

I would like to thank past and current members in the Taber lab for great scientific and non-scientific discussions and support: Ben Filas, Victor Varner, Yunfei Shi, Matt Wyczalkowski, Gang Xu, Kara Garcia, Gaby Espinosa, and Hadi Hosseini. I also wish to thank my undergraduate mentors at the University of Minnesota for inspiring my initial interest in biomedical research.
I thank my parents, sister, and extended family for support throughout my education. I also wish to thank my amazing and kind friends across the country.

This work was supported by NIH grant R01 NS070918 and a fellowship through the Imaging Sciences Pathway at Washington University (NIH T32 EB014855).

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August 2016
Mechanical forces play an essential role in morphogenesis, the shaping of embryonic structures. This research focuses mainly on eye development, a problem that has been studied for decades using a variety of approaches. However, the mechanics of the early stages of eye formation remain incompletely understood.

The embryonic eyes begin as bilateral protrusions called optic vesicles (OVs) that grow outward from the anterior end of the brain tube. The optic vesicles contact and adhere to the overlying surface ectoderm (SE) via extracellular matrix (ECM). Then, both layers thicken in the region of contact to form the retinal and lens placodes, which bend inward (invaginate) to form the roughly spherical optic cup (OC, primitive retina) and lens pit, respectively. These two structures then separate, and the lens pit continues to fold until it closes to create the lens vesicle (LV). The primary aim of this research is to determine the biophysical mechanisms that create the OC and LV in the chick embryo. Throughout, we used a combination of experiments and computational modeling.

First, we explored the hypothesis that the ECM locally constrains the OV as it grows, forcing it to thicken and invaginate. The integrity of the matrix was disrupted by removing its major source,
the SE, and degrading the matrix chemically at different stages of development. At relatively early stages of invagination, removing the SE caused the curvature of the OV to reverse as it ‘popped out’ and became convex, but the OV remained concave at later stages and invaginated further during subsequent culture. Disrupting the ECM had a similar effect, with the OV popping out at early to mid-stages of invagination. These results suggest that the ECM is required for the early stages but not the late stages of OV invagination. A finite-element model consisting of a growing spherical OV attached to a relatively stiff layer of ECM reproduced the observed behavior, as well as measured changes in OV curvature, wall thickness, and invagination depth reasonably well. These results support the matrix constraint hypothesis for retinal placode formation and invagination.

Next, we studied the forces that create the LV. Studies have shown that, like the retinal placode, the lens placode is produced by growth of the SE constrained locally by ECM, while actomyosin contraction at the apical surface of the lens placode plays a major role in its subsequent invagination. Our experiments and modeling support this view but also suggest that apical contraction alone does not generate sufficient force to close the LV. We propose that programmed cell death, or apoptosis, which has been observed near the opening in the lens pit, supplies the required force for closure by causing tissue surrounding the opening to shorten circumferentially. Finite-element modeling and experiments involving apoptosis inhibitors support this idea.

Finally, we investigated the possible role of mechanical feedback in regulating the contractile behavior of neuroepithelia such as the OC and primitive brain tube. Using a novel loading technique, we subjected isolated brain tubes to compressive loads during culture. Normally, cell nuclei in the wall of the brain tube are elongated in the radial direction, and a previous study has shown that compression of the tube triggers a decrease in contractile force with the nuclei becoming rounder. We found that exposure to a gap junction blocker prevents this response, suggesting that compression without the blocker releases a molecular signal that diffuses through the wall to inhibit
contraction. Our results also show that nuclear shape changes are likely caused by changes in wall stress. These results lay the groundwork for future studies of mechanotransduction in the embryonic brain.

Our experimental and computational findings expand our knowledge of early eye development and demonstrate key roles for extracellular matrix, actomyosin contraction, and apoptosis in morphogenesis. We also have shown that chemomechanical feedback can play a role in regulating contractility of neuroepithelial tissues such as the brain and retina. Future investigations of developmental mechanics may reveal similar mechanisms in the formation of other organs as well.
Chapter 1

Introduction

1.1 Summary

The embryo initially consists of three primary germ layers that form through rapid cell division and undergo gastrulation (Solnica-Krezel, 2005). Soon after, cell growth, division, movement, and shape changes coordinate formation of the primitive heart, brain, and eye. Biochemical, genetic, and mechanical processes are critical to the complex shape changes, or morphogenesis, of the embryo. Here we focus on the mechanical forces in development to determine the mechanisms involved in early eye formation.

1.2 Mechanical Forces in Development

Mechanical forces have been investigated in morphogenetic processes such as early heart development (Voronov and Taber, 2002; Shi et al, 2014), neurulation (Benko and Brodland, 2007), early gut looping (Savin et al, 2011), and primary brain vesicle formation (Filas et al, 2012). The role of mechanics can be evaluated with experiments such as measures of stress (Xu et al, 2010), strain
(Alford and Taber, 2003), stiffness (Zamir et al, 2003), and distributions of contractile proteins (Zolessi and Arruti, 2001). Computational modeling based on the theory of elasticity including volumetric growth (Rodriguez et al, 1994; Taber, 2001) allows testing of hypotheses using parameters determined from experiments.

Several cellular components are critical in morphomechanics. The cytoskeleton contributes to cell structure, stiffness, and shape changes. Microtubules, intermediate filaments, and actin filaments form a dynamic network of proteins connecting intracellular components and the cell membrane to the local environment (Fletcher and Mullins, 2010). Non-muscle myosin II in the cell allows actin filaments to contract and generate forces necessary for tissue shape changes. Contraction of apical actin, for example, drives apical constriction to create wedge-shaped cells, as observed during lens invagination (Plageman et al, 2011).

Extracellular matrix facilitates cell adhesion, cell migration, and can bind signaling molecules (Rozario and DeSimone, 2010; Kwan, 2014). Its role in development has been studied in lung morphogenesis (Wessells and Cohen, 1968), sea urchin gastrulation (Lane et al, 1993), and early eye development as it drives and maintains tissue shape changes (Zwaan and Hendrix, 1973; Huang et al, 2011).

Cells can adapt and respond to mechanical forces in the embryo (Ramasubramanian and Taber, 2008; Guilluy and Burridge, 2015). Through mechanotransduction, mechanical signals activate biochemical and genetic responses in the cell and nucleus (Guilluy and Burridge, 2015). In the Drosophila embryo, for example, apical localization of myosin is required for mesoderm invagination, but this process is absent in snail-mutant embryos. Mechanical indentation can rescue myosin localization and trigger invagination in these mutants, dependent on a Fog signaling pathway (Pouille et al, 2009).
Mechanical forces also impact nuclei directly. In the early chick brain, mechanical loading has been shown to change nuclear shape and tissue stiffness (Filas et al, 2011). Mechanical forces can induce nuclear shape changes, remodeling of the nuclear lamina, and changes in gene expression (Salbreux et al, 2012; Guilluy and Burridge, 2015).

1.3 Early Eye and Brain Development

The primitive, vertebrate brain originates from a columnar epithelium called the neural plate which bends to form a tube through the process of neurulation (Lowery and Sive, 2009). In vertebrates, the anterior brain tube develops expansions and boundaries forming distinct regions called the forebrain, midbrain, and hindbrain (Fig. 1.1A). The posterior brain eventually becomes the spinal cord (Lowery and Sive, 2009). The early eyes expand bilaterally from the neuroepithelium of the forebrain as optic vesicles. Each optic vesicle grows and contacts the outer surface ectoderm, becoming tightly adhered through an extracellular matrix composed of several proteins such as fibronectin, collagen, and glycosaminoglycans (Hendrix and Zwaan, 1975; Hilfer and Randolph, 1993).

The optic vesicle and surface ectoderm thicken in the contact region to form the retinal placode and lens placode, respectively. The two placodes then bend inward, or invaginate, developing the bilayered optic cup and the lens pit. Apical actin is located on the shortening side of the lens pit where it drives lens invagination, but it is found on the opposite side of the optic cup (Martinez-Morales and Wittbrodt, 2009). Actomyosin contraction has therefore been studied in lens pit formation (Plageman et al, 2011). The inner layer of the optic cup forms the neural retina and the outer layer forms the retinal pigmented epithelium (Fuhrmann, 2010). As the optic cup continues to develop, it remains connected to the brain tube through the optic stalk, which precedes the optic nerve (Wang et al, 2015). The lens pit begins to separate from the optic cup and closes to
Figure 1.1: Early brain and eye development visualized with (A) brightfield microscopy and (B, C) scanning electron microscopy in the chick embryo. (A) Stage HH12 chick (embryonic day 2) with brain tube and optic vesicles (OVs) outlined. The primary brain vesicles are the forebrain (FB), midbrain (MB), and hindbrain (HB). The OV invaginates to form the (B) optic cup (OC), and the adjacent surface ectoderm tissue forms the lens vesicle (LV) at HH17- (day 2.3). (C) LV lumen is filled as primary lens fiber cells elongate at stage HH20 (day 3). (B, C) ©Schuetze and Goodenough (1982); originally published in the Journal of Cell Biology 92(3):694-705. Scale bars: (A) 1 mm, (B, C) 100 µm.
form a fully closed sphere called the lens vesicle; the lumen then fills as the posterior cells elongate (Fig. 1.1B–C) (Cvekl and Ashery-Padan, 2014). Apoptosis in the anterior lens has been suggested to separate the lens vesicle from the outer surface ectoderm (Francisco-Morcillo et al, 2014).

Since the early retina and lens develop concurrently, scientists have wondered for over a century whether one organ predominantly causes formation of the other (Swindell et al, 2008). Hans Spemann and Hilde Mangold’s discovery of the organizer that can induce development of the neural plate in transplanted amphibian experiments pioneered research in induction (Spemann and Mangold, 1924). In 1901, prior to this Nobel prize-winning organizer paper, Spemann suggested the retina induces the lens since excising the optic vesicle from frog embryos prevented lens development (Spemann, 1901). For decades, researchers found conflicting results for the importance of contact between the optic vesicle and surface ectoderm (Hendrix and Zwaan, 1975; Swindell et al, 2008). Placing a cellophane strip between the two cell layers prevented lens formation in the chick embryo (Langman, 1956); however, agar strips and Millipore filters did not prevent lens formation in the chick and mouse, respectively (McKeehan, 1958; Muthukkaruppan, 1965).

More recently, Hyer et al (2003) discovered that ablating the lens after placode formation did not prevent optic cup formation, but the lens needed to be present before placode formation. While many researchers have shown that signals crossing the extracellular matrix may induce the lens or retina (Swindell et al, 2008; Hyer et al, 2003), the mechanical restraint provided by the extracellular matrix may be sufficient to explain placode formation and early invagination (Zwaan and Hendrix, 1973; Huang et al, 2011). Evaluating the mechanics of early eye development can help determine which mechanisms are biologically feasible and likely to drive optic cup and lens vesicle formation.
1.4 Research Significance

Abnormalities during development can cause serious birth defects in the brain and eye. Defects in the early brain are known to cause serious neurological disorders such as anencephaly, hydrocephalus, autism, and schizophrenia (Lowery and Sive, 2009). Malformations of the eye are related to coloboma (missing part of the eye), microphthalmia (abnormally small eye), or anophthalmia (missing eye) (Mic et al, 2004; Slavotinek, 2011). Abnormal lens development in particular can cause congenital cataracts (opaque lens), microphakia (small lens), and aphakia (missing lens) (Khong, 2015).

Connections between embryonic development and cancer development make the chick embryo a valuable learning tool for cancer research as well. Studying developmental processes, such as epithelial to mesenchymal transitions, can improve understanding of cancer metastasis, angiogenesis, and tumorigenic growth (Kain et al, 2014). Cancer cell lines can also be directly injected in the chick embryo to study cell invasion (Busch et al, 2013).

A better understanding of embryonic development can also contribute to the field of tissue engineering. Creating constructs for regenerative medicine that replicate native tissue requires knowledge of the signals, microenvironment, and mechanical forces that are necessary during embryonic development (Ghosh and Ingber, 2007). Researchers have created self-assembling optic cups using human embryonic stem cells suspended in a matrix-rich media (Nakano et al, 2012). While several obstacles remain in culturing and innervating these optic cups, future studies considering both biochemical and mechanical factors may one day lead to their use in retinal degeneration treatment.
1.5 Summary of this Dissertation

In this research, we investigated the mechanical forces driving early retina and lens development. Additionally, we studied nuclear shape changes during mechanical loading of the early brain.

In Chapter 2, we consider the role of extracellular matrix in optic cup (OC) formation due to its importance for mouse lens placode formation (Huang et al, 2011). Our computational and experimental results show a stage-dependent need for the matrix that is secreted between the early retina and lens to constrain the optic vesicle (OV) as it grows. At early stages of OV invagination, removing the surface ectoderm, which helps secrete matrix, caused the OV to revert from a concave to a convex shape. At mid-stages of invagination, the OV remained concave without the surface ectoderm; however, this concavity often reversed when the matrix was degraded with collagenase. During late stages of OC formation, the OC remained concave without the adjacent lens tissue or matrix. These stage-dependent effects were reproducible in a computational model. We also predict a stiffness gradient across the matrix with higher stiffness at the center of the OC.

After investigating OC formation, in Chapter 3 we consider the mechanics of early lens development. Our computational models suggest critical roles for extracellular matrix, apical contraction, and apoptosis to thicken, invaginate, and then close the lens vesicle. Inhibiting apoptosis with caspase inhibitors in the chick embryo reduced lens vesicle closure after overnight culture. Our results indicate a novel mechanical need for apoptosis in development.

Finally, in Chapter 4, we find an important role for gap junction communication in nuclear shape changes seen in response to loading the embryonic brain. We discuss a new technique to locally or globally compress the embryonic brain during culture. Quantifying nuclear shape changes in the brain indicated that nuclear shape depends on apical-basal positioning in the brain and gap junction communication. Inhibiting gap junction communication in the chick prevented most of
the observed nuclear shape changes. Therefore, our results suggest that a small mechanosensitive molecule drives changes in contractility that cause the changes in nuclear shape in the loaded brain.

In Chapter 5, we summarize our conclusions and discuss future directions for investigating eye and brain development.
Chapter 2

Tissue Growth Constrained by Extracellular Matrix Drives Invagination during Optic Cup Morphogenesis

2.1 Summary

In the early embryo, the eyes form initially as relatively spherical optic vesicles (OVs) that protrude from both sides of the brain tube. Each OV grows until it contacts and adheres to the overlying surface ectoderm (SE) via an extracellular matrix (ECM) that is secreted by the SE and OV. The OV and SE then thicken and bend inward (invaginate) to create the optic cup (OC) and lens vesicle, respectively. While constriction of cell apices likely plays a role in SE invagination, the mechanisms that drive OV invagination are poorly understood. Here, we used experiments and computational modeling to explore the hypothesis that the ECM locally constrains the growing OV, forcing it to invaginate. In chick embryos, we examined the need for the ECM by (1) removing SE at different developmental stages and (2) exposing the embryo to collagenase. At relatively early stages of invagination (Hamburger-Hamilton stage HH14-), removing the SE caused the curvature of the
OV to reverse as it ‘popped out’ and became convex, but the OV remained concave at later stages (HH15) and invaginated further during subsequent culture. Disrupting the ECM had a similar effect, with the OV popping out at early to mid-stages of invagination (HH14- to HH14+). These results suggest that the ECM is required for the early stages but not the late stages of OV invagination. Microindentation tests indicate that the matrix is considerably stiffer than the cellular OV, and a finite-element model consisting of a growing spherical OV attached to a relatively stiff layer of ECM reproduced the observed behavior, as well as measured temporal changes in OV curvature, wall thickness, and invagination depth reasonably well. Results from our study also suggest that the OV grows relatively uniformly, while the ECM is stiffer towards the center of the optic vesicle. These results are consistent with our matrix constraint hypothesis, providing new insight into the mechanics of OC (early retina) morphogenesis.

2.2 Introduction

In the embryo, the eyes begin to develop as bilateral spherical protrusions called optic vesicles (OVs) that extend outward from the forebrain and soon contact the overlying surface ectoderm (SE). Within the region of contact, the OV and SE epithelia adhere and thicken to form the retinal and lens placodes, which then invaginate to create the bilayered optic cup (OC; future retina) and the lens vesicle, respectively (Fig. 2.1) (Hendrix and Zwaan, 1974). Morphogenetic abnormalities during eye development can cause ocular defects such as coloboma (missing part of eye) and microphthalmia (abnormally small eye) (Mic et al, 2004). Studies suggest that SE invagination to create the lens is driven primarily by apical constriction (Plageman et al, 2011), but the biophysical mechanisms that drive OV invagination remain poorly understood.

\[^1\]This Chapter was originally published in Biomechanics and modeling in mechanobiology (Oltean et al, 2016). Reprinted with permission of Springer.
Previous experimental studies have provided valuable information needed to understand the mechanics of OC formation. First, it is important to note that actin is located primarily on the apical sides of both the SE and OV in chick and mouse, corresponding to the initial outer and inner surface, respectively, of these layers (Martinez-Morales and Wittbrodt, 2009; Borges et al, 2011). The location in the SE is consistent with an apical constriction mechanism for lens placode invagination, but it is on the wrong side of the OV to drive retinal placode invagination. This process has been studied for over a century, with Spemann (1901) finding that contact between the OV and SE is required for lens invagination. More recently, Hyer et al (2003) found that contact with the SE is needed only until the placode stage for invagination to occur in chick embryos, as ablating the lens after this time does not prevent OC formation. These findings indicate that the developing lens (SE) is not the main driver of OV (retinal placode) invagination and that actomyosin contraction does not play a major role in this process.

Another important mechanical consideration comes from the observation that the contact area between the OV and SE remains essentially constant during placode formation in chicken and mouse embryos (Zwaan and Hendrix, 1973; Huang et al, 2011). Zwaan and Hendrix (1973) attributed this observation to the constraining effects of extracellular matrix (ECM) between these layers (Fig. 2.1). These authors also speculated that cell proliferation in the constrained region creates cell crowding, which causes the presumptive lens tissue of the SE to thicken into a placode that eventually buckles and invaginates. More recently, evidence obtained by Huang et al (2011) indicates that the ECM constrains the spreading of both the OV and SE in their mutual region of contact as they grow, causing them to thicken locally and form placodes.

The present study extends these ideas to the process of OV invagination. In particular, we explore the hypothesis that OV growth, constrained by a relatively stiff extracellular matrix causes formation of the retinal placode, as well as subsequent invagination to create the OC. Consequently, the
SE is involved only indirectly by secreting and inducing the OV to also secrete the necessary matrix. Rather than being a type of buckling, this represents a differential growth mechanism similar to that which bends a bimetallic strip, whereby one layer expands more than the other in response to an increase in temperature. Differential growth apparently plays a major role in the development of other organs, including the heart, brain, and gut (Taber, 2014).

To test the physical plausibility of this hypothesis, we integrated experimental measurements of morphogenetic changes in geometry, as well as cell and matrix stiffness, with finite-element modeling of the invagination process. The model was tested experimentally using perturbations whereby the SE was removed from the OV and the matrix was degraded at various times during invagination. Consistent with our experimental results, the model indicates that the matrix is needed to initiate invagination but is no longer necessary for invagination to proceed after reaching a critical developmental stage. In addition, comparing experimental and numerical results suggests that the growth rate in the invaginating region of the SE is relatively uniform, while the ECM is stiffer near the center of the retinal placode. These details have subtle but potentially significant effects on OC (retinal) morphology.

2.3 Materials and methods

2.3.1 Embryo Preparation and Culture

Fertilized white Leghorn chicken eggs were incubated at 38°C for 45-55 hours to reach Hamburger-Hamilton stages 12–15 (HH12–HH15) (Hamburger and Hamilton, 1951). Embryos were removed from the egg using a method in which the vitelline membrane adheres to a ring of filter paper (Whatman #2) to lift the embryo from the yolk. A second filter paper ring was placed on the ventral side of the embryo to keep the membranes from slipping. Embryos were cultured in approximately
1 ml chick media (Dulbecco’s modified Eagle’s medium with 10% chick serum and 1% antibiotics) in 35 mm Petri dishes placed in a small plastic bag with a mixture of 95% oxygen and 5% carbon dioxide (Voronov and Taber, 2002). To image further developed control embryos, additional eggs were incubated up to 80 hours (HH21), and right eyes were imaged while in the yolk.

2.3.2 Removal of Surface Ectoderm and Matrix

After embryos were removed from the egg, the SE was dissected from the right eye with the left eye serving as a control. During dissection, embryos were submerged in thin albumen collected from eggs. A small region of vitelline membrane was removed near the right eye with fine glass needles. Then <2 μl of 2% Nile Blue sulfate was applied to the SE on and near the right eye with a glass capillary tube as described previously (Hyer et al, 2003; Yang and Niswander, 1995). After a short time (<1 min), the SE blistered and was removed with a glass needle and forceps. The Nile Blue sulfate tended to have a stronger effect on SE blistering when embryos were submerged in thin albumen than in PBS (Dulbecco’s phosphate-buffered saline). After dissection, the albumen was replaced with PBS. After removal of the SE, a group of embryos was treated locally with 4 μl crude collagenase (1 mg/ml, C5138; Sigma-Aldrich, St Louis, MO) to degrade the ECM on the basal surface of the OV.

2.3.3 Stiffness Measurements

Tissue stiffness was measured in the eye using a custom-made microindentation device, consisting of a calibrated glass cantilever beam with a cylindrical tip (approximately 20 μm in diameter) at the end (Zamir et al, 2003; Xu et al, 2010). Motion of the cantilever was controlled by a piezoelectric motor, which moved the tip 200 μm in 5 s. The membranes surrounding the brain were removed for easier manipulation, and the heart was removed to eliminate motion caused by the heartbeat.
With one OV held by suction onto a glass micropipette, the opposite eye was indented. Video recording of the indentation was used to quantify beam and tissue deflection as described in Zamir et al (2003). Tissue stiffness before and after collagenase treatment was calculated as the slope of an average force-displacement curve at multiple indentation depths (10 µm, 20 µm, and 30 µm).

2.3.4 Imaging and Measuring Tissue Morphology

Brightfield images and video recordings were captured with a Nikon EOS T3 camera on a Leica DMLB MZ 8 microscope. Optical coherence tomography (OCT) images were acquired with a Thorlabs (Newton, NJ) OCT system (approximately 10 µm resolution).

Measurements of central curvature, invagination depth, and tissue thickness were made from OCT sections using ImageJ (National Institutes of Health, Bethesda, MD). A circle was fit through approximately five markers near the center of the OV along the basal surface, and curvature was calculated as \( k = 1/r \) where \( r \) is the radius of the circle fit. Curvature was normalized by dividing values by the average, initial curvature at HH12. Normalized invagination depth, measured in the plane orthogonal to the optic stalk, is defined as the vertical distance between the top of the OV to the center of the neural retina \( (D) \) divided by the radius of the OV \( (R) \) defined by the distance from the center of the OV to the outer edge (see Fig. 2.11E inset). To quantify thickness of the retinal placode, six measurements were made in each eye near the center of the OV (three measurements in two orthogonal planes). The thickness of the edge of the retinal placode region was measured by averaging four measurements at the dorsal edge in the plane through the optic fissure. The orthogonal plane did not have sufficient contrast at the placode edge to measure in this second plane. Cross-sectional area of the retinal placode was measured in two orthogonal planes for each eye in OCT images. Thickness and area were normalized by corresponding values at HH12.
To better visualize the 3D morphology of the developing eye, the geometry of some OVs and OCs were reconstructed from OCT image stacks by tracing the surface of the OV in ImageJ. The segmented image volumes were then imported into the software package Caret (Van Essen et al, 2001) to create a smoothed surface from the outlines. The reconstructed volumes were then visualized in MATLAB (MathWorks, Natick, MA).

2.3.5 Actin and Matrix Visualization

Embryos were stained after overnight fixation in 3.7% formaldehyde. To visualize filamentous actin, embryos were first blocked in 0.1% Triton X-100 (Sigma) and 1% bovine serum albumin (BSA) in PBS and then stained with phalloidin (1:100, Alexa Fluor 488 phalloidin or Alexa Fluor 633 phalloidin, Molecular Probes) in PBS with 0.1% Triton X-100 and 1% BSA.

To visualize ECM, two separate fibronectin antibodies were used. Fixed chick embryos were permeabilized in 1% Triton X-100 (Sigma) in PBS for 30 minutes and blocked in 5% BSA in PBS for 3 hours (Rifes and Thorsteinsdóttir, 2012). Embryos were incubated with a primary antibody of mouse monoclonal anti-chick fibronectin (DSHB, clone B3/D6, 1:50) in 1% BSA and 0.1% Triton X-100 in PBS for 48 hours at 4°C. Embryos were rinsed in PBS and then incubated with the secondary antibody Alexa Fluor(R) 488 F(ab’)2 Fragment of Goat Anti-Mouse IgG (1:250, Invitrogen) in 1% BSA and 0.1% Triton X-100 in PBS for 48 hours at 4°C. Additional fixed embryos were rinsed in PBS, then soaked in blocking buffer (2% goat serum with 0.1% Triton X-100 in PBS) for 3 hours. Samples were incubated in primary antibody in blocking buffer (Sigma F3648, 1:100) for 24 hours at 4°C, rinsed with PBS three times, and incubated with a secondary antibody of Alexa Fluor(R) 488 Goat anti-Rabbit IgG (H+L) (Invitrogen A11034, 1:100) in blocking buffer overnight at 4°C. Samples were then rinsed with 0.1% Tween 20 (Sigma) and DAPI (Sigma) in PBS three times. The B3/D6 fibronectin antibody was developed by D.M. Fambrough and was
obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Actin-stained embryos were rinsed in PBS three times and then imaged using a fluorescence Leica DMLB microscope (x5, x20 objectives). Before imaging fibronectin distributions with confocal microscopy, tissues were embedded in 4% agarose in PBS and cut into thick sections (120 µm) using a tissue slicer (Electron Microscopy Sciences, Hatfield, PA). Confocal images were captured with a Zeiss LSM 510 or 710 confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY).

Fibronectin distribution was analyzed by tracing the ECM between the SE and OV in ImageJ with a wide, segmented line. The line was then smoothed with the Fit Spline tool and the image straightened with the Straighten tool (see Fig. 2.15). The fluorescence intensity was calculated across the thickness of the ECM with respect to arc length with the Plot Profile tool, and data were exported to plot in Matlab. Arc length was normalized from 0 (the dorsal edge of the placode) to 1 (the center of the placode). Fluorescence intensity was normalized by dividing the raw data by the average intensity along the ECM for each sample.

2.3.6 Cell Proliferation

Cell proliferation was quantified for the mouse OV from image data generously provided by the authors of Huang et al (2011). As described in Huang et al (2011), embryonic mice were exposed for 1 h to BrdU which incorporates into cells in S-phase. Fixed embryos were then sectioned and counterstained with hematoxylin. Using their images, we quantified cell proliferation in the OV in three equally sized regions of the retinal placode and in the outer OV at multiple stages of eye development. BrdU positive and negative cells were counted to calculate the percent of BrdU incorporation in each region of the mouse OV. Measurements were analyzed across stages and
placode regions using two-way repeated measure ANOVA and post-hoc Tukey’s test in SigmaPlot (v12.5, Systat Software Inc.).

### 2.3.7 Finite-Element Models for Morphogenesis

To simulate OV morphogenesis, we created axisymmetric finite-element models using COMSOL Multiphysics (Version 4.2a, COMSOL AB, Providence, RI, USA). The OV is modeled as a spherical shell with a normalized outer radius of 1 and wall thickness of 0.2. With OV and ECM geometries estimated from OCT images and fibronectin staining, respectively (e.g. Fig. 2.1, Fig. 2.2C), the model is divided into three regions representing the inner OV (placode region), the outer OV, and the ECM (Fig. 2.8B). The ECM is represented by a relatively thin layer of the outer wall spanning 1/10 the OV thickness and 80 deg of the circumference (future placode region) (see Fig. 2.9A). To prevent rigid-body motion, a point at the bottom of the OV is fixed. In some models, the invaginating region contacted the bottom of the OV. In these cases, the Contact Pair feature of COMSOL was used with a contact penalty factor of $2/h$ where $h$ is mesh element size.

With all layers treated as pseudoelastic, growth is simulated by modifying the governing equations within COMSOL based on the theory of Rodriguez et al (1994). Accordingly, the total deformation gradient tensor is defined as $F = F^* \cdot G$, where $F^*$ is the elastic deformation gradient tensor and $G$ is the growth tensor. The Cauchy stress tensor is given by (Taber, 2004)

$$\sigma = \frac{2}{J^*} F^* \cdot \frac{\partial W}{\partial C^*} \cdot F^{\ast T}, \quad (2.1)$$

where $W$ is the strain-energy density function, $C^* = F^{\ast T} \cdot F^*$ is the right Cauchy-Green elastic deformation tensor, and $J^* = \det F^*$ is the elastic volume ratio. Details for incorporating growth in COMSOL 4.2a are provided in Hosseini et al (2014).
Assuming the material properties for the OV are essentially the same as those for the rest of the brain tube during these stages, the strain-energy density function for each region is taken in the modified Blatz-Ko form (Xu et al, 2010; Taber, 2004; Blatz and Ko, 1962)

\[
W = \frac{1}{2} \mu \left[ I^* - 3 + \frac{1}{\alpha} (J^* - 2\alpha - 1) \right]
\]  

(2.2)

for an isotropic material, where \( \mu \) is the shear modulus and \( \alpha = \nu / (1 - 2\nu) \), with \( \nu \) being Poisson’s ratio in the limit of small strain. In addition, \( I^* = \text{tr} \mathbf{C}^* \) is the first strain invariant. For the OV, we set \( \mu = 220 \text{ Pa} \) and \( \nu = 0.45 \) (Xu et al, 2010). Poisson’s ratio is taken the same for the ECM, but the shear modulus is larger as detailed later for each model.

In each region, the growth tensor is taken in the form

\[
\mathbf{G} = G_R \mathbf{e}_R \mathbf{e}_R + G_\Theta \mathbf{e}_\Theta \mathbf{e}_\Theta + G_\Phi \mathbf{e}_\Phi \mathbf{e}_\Phi
\]  

(2.3)

where \( \mathbf{e}_R, \mathbf{e}_\Theta, \) and \( \mathbf{e}_\Phi \) are undeformed unit vectors in spherical polar coordinates (Fig. 2.9A), and \( G_R, G_\Theta, \) and \( G_\Phi \) are specified functions of space and time. In all models, the ECM layer does not grow \( (G_R = G_\Theta = G_\Phi = 1) \), while, for axisymmetric deformation, the OV grows equally in the circumferential and meridional directions as defined by

\[
G_\Theta = G_\Phi \equiv G, \quad G_R = 1
\]  

(2.4)

\[
G = 1 + \dot{G}(\Theta) t
\]  

(2.5)

where \( t \) is dimensionless time. The spatial distributions of the growth rate \( \dot{G} \) for each model are given in the Results section.
2.4 Results

2.4.1 OCT Imaging of Eye Morphology

At HH12 to HH13, the SE and OV are visible in OCT images as distinct cell layers of ectodermal origin (Fig. 2.1, bottom row). The lens placode and retinal placode form as these respective layers thicken locally in their mutual region of contact during stage HH13+. These placodes then invaginate to create the lens pit and bi-layered OC. The lens pit eventually closes to create the lens vesicle, the inner and outer regions of the OC become the neural retina and retinal pigment epithelium, respectively, and the part of the SE outside the lens fuses into a continuous sheet in contact with the lens (Fig. 2.1). The resolution of our OCT system (about 10 µm) is not fine enough to visualize the ECM between the OV and SE. The cell layers and ECM are depicted schematically in the top row of Fig. 2.1.

As embryonic structures acquire different shapes and functions, their names often change. During development, for example, a circular region of the SE becomes the lens placode, the lens pit, and the lens vesicle before becoming the lens itself. For simplicity, since this can be a source of confusion, we refer to the two main layers as the OV and SE at all stages. Each of these layers is composed of two regions. Regardless of the amount of invagination, we consider the OV as divided into the inner OV (iOV; retinal placode composed of neural ectoderm) and outer OV (oOV; retinal pigment epithelium), while the SE is divided into the inner SE (iSE; lens placode, lens pit, lens vesicle) and the outer SE (Fig. 2.1, top row).

In addition, we define the following developmental phases for the OV (Fig. 2.1):

Phase 0: pre-placode (HH12)
Phase 1: placode (HH13+)
Phase 2: early invagination (HH14-)
Phase 3: mid invagination (HH14)
Phase 4: late invagination, optic cup (≥HH15)

Finally, it is important to note that the apical side of the SE becomes the concave side of this membrane in the invaginating region, while the basal side becomes convex. The opposite is true for the OV.

2.4.2 Actin and ECM Staining

Tissues stained for F-actin were visualized with fluorescence microscopy in whole embryos (Figure 2.2A, A’) and with confocal microscopy in sectioned embryos (Figure 2.2B). Actin was concentrated primarily along the apical (initially convex then concave) side of the iSE, as has been previously reported in chick and mice (Zolessi and Arruti, 2001; Smith et al, 2005; Chauhan et al, 2009). In the iOV, actin intensity was considerably weaker than in the iSE but was stronger on the apical (convex) side than the basal (concave) side.

Previous work in mice has shown that fibronectin is required for the lens placode to form, as well as for subsequent lens invagination. In mouse embryos, fibronectin is visible as a dual-layered ECM located between the SE and OV (Huang et al, 2011). Here, in chick embryos, the ECM was also typically visible as two layers between the SE and OV as shown by fibronectin staining (Fig. 2.2C).
Figure 2.1: Morphology of optic cup in five phases. Top row: Schematic diagram of phases of optic cup formation (OV = optic vesicle, blue; oOV = outer OV, blue; iOV = inner OV, red; SE = surface ectoderm, purple; iSE = inner SE, purple; RP = retinal placode, red; LP = lens placode, purple; ECM = extracellular matrix, orange). Bottom row: Representative OCT images for each phase. Dashed red lines indicate boundary between the SE and OC. **Phase 0, pre-placode:** The OV and SE are in contact but have not thickened. **Phase 1, placode:** The two cell layers become tightly adhered via ECM and thicken to form the RP and LP. **Phase 2, early invagination:** The OV and iSE begin to invaginate. **Phase 3, mid-invagination:** Significant curvatures are apparent. **Phase 4, late invagination:** The OC and LV have formed. Scale bars: 100 µm.
Figure 2.2: Distributions of actin and ECM in the eye. (A, HH14) Whole-mount stain of F-actin. Panel (A’) is an enlarged view of yellow box in (A) showing concentrated actin along the apical surface of the SE (yellow arrow) and OC (white arrow) but not the basal surface of the OC (blue arrow). (B, HH13+) Section during early invagination also showing F-actin concentrated mainly along the apical (initially convex) surface of the SE (yellow arrow). The basal (concave) side of the OV lacked substantial F-actin (blue arrow). (C, HH13+) Section with fibronectin staining shows a bilayered ECM (blue arrow) between the invaginating OV and SE. (D, HH14-) ECM remained after SE removal (blue arrow). Scale bars: 100µm.
2.4.3 Effects of SE and ECM Removal

To determine whether the mechanics of OV invagination in the chick embryo are similar to those in mice, we first repeated the SE removal experiments of Hyer et al (2003), who ablated the SE using a small amount of Nile Blue sulfate solution applied locally in ovo (see Materials and Methods section). We removed the iSE at multiple stages of eye development before, during, and after placode formation. The SE generally grew back as a thin layer, but this occurred a few hours after dissection or collagenase observations were made (longer if the ablated region was relatively large).

Consistent with the results of Hyer et al (2003), removing the SE at Phase 0 (before placode formation) inhibited OC formation (Fig. 2.3A, A’, A”), while dissecting the SE after this time usually led to OVs invaginating into OCs without lens vesicles (Fig. 2.3B, B’, B”). In some embryos, the SE healed after several hours of culture and grew a small lens placode or lens vesicle from the newly created SE tissue.

As shown later, although these results are crucial, the tissue behavior immediately after dissection yields important clues concerning the underlying morphogenetic mechanisms. Interestingly, the short-time behavior of dissected samples also depended on the stage of development. At Phase 0 and 1, the OVs maintained approximately the same shape just after SE removal (Fig. 2.3A, A’). At Phase 2, the concavity of OVs usually reversed, i.e., the invaginated region ‘popped out’ when the inner SE was ablated (24/34) (Fig. 2.3B, B’). At later phases of invagination (Phases 3 and 4), the OV typically remained invaginated after SE removal (15/40 popped out at Phase 3 and 0/15 at Phase 4) (e.g., see Fig. 2.4B, B’). Notably, some of the OVs that popped out after shallow invagination later resumed the invagination process to form relatively normal-looking OCs (Fig. 2.3B”).
Figure 2.3: OCT images of OV after SE removal and continued culture. Dashed red lines indicate the boundary between the SE and OV; dashed yellow lines indicate OV shape after overnight culture. (A, A’, A”) SE removal before placode formation had little effect on OV shape immediately after dissection (A, A’, Phase 0, HH12+) but inhibited subsequent invagination (A”). (B, B’, B”) Removing the SE after placode formation (B, B’, Phase 2, HH14-) caused immediate loss of concavity in most embryos (n = 24/34), but invagination still occurred in some embryos that were cultured overnight (n = 4/6) (B”), as reported by Hyer et al (2003). Scale bar: 100µm.

Taken together, these results suggest that OV invagination generally requires the presence of the SE through the period of placode formation (Phase 1), but not afterwards. Evidence from a previous study suggests that the matrix secreted by the SE and OV (triggered by the contact between the two cell layers) rather than the SE itself, supplies a mechanical constraint needed for OV invagination (Huang et al, 2011). To explore this idea further, we disrupted the matrix at various stages of invagination (Phases 2–4).

First, to determine how SE removal affects the ECM, embryos were fixed and stained for fibronectin after SE ablation. A bilayered ECM was still visible in dissected embryos, indicating that the matrix was not destroyed in the process of removing the SE (Fig. 2.2D).

Next, we applied collagenase locally to degrade the ECM in eyes that remained invaginated after SE removal and observed changes in morphology. The matrix between the OV and SE in the chick contains several components including collagen, fibronectin, laminin, and glycosaminoglycans (GAGs) (Hendrix and Zwaan, 1974, 1975; Webster et al, 1983; Hilfer and Randolph, 1993).
Crude collagenase disrupts both collagen and fibronectin, which are major determinants of material properties in ECM (Oberhauser et al, 2002).

Within a few minutes of collagenase exposure, many of the Phase 2 OVs slowly reversed their concavity and became convex (5/6) (Fig. 2.4A, A’, A”). One became less invaginated but remained concave (1/6). Several eyes (9/14) at Phase 3 remained concave after collagenase treatment and showed relatively little change in morphology (Fig. 2.4B, B’, B”). To verify the effects of collagenase, in a few embryos both eyes were dissected, and only the right eye was treated with collagenase. The embryos were fixed and stained for fibronectin. As shown in Fig. 2.5, the crude collagenase decreased fibronectin staining and therefore degraded the ECM without affecting actin (see Fig. 2.6).

Figure 2.7 summarizes the main results of our SE and ECM perturbation studies. During early placode formation (Phase 1), OVs remained fairly unaltered following SE removal (Fig. 2.7A).
Figure 2.5: Effects of collagenase on matrix. (A) Confocal images of fibronectin staining (red) of two eyes from a representative embryo (Phase 3, HH14+). The basal surface of the OV was stained after SE removal (A, A’) and after SE removal followed by local collagenase treatment (B, B’) with the same confocal laser settings. (A, B) Stacks of 8 images of the ECM, combined in ImageJ according to maximum intensity of each image, show brighter staining in the eye without collagenase treatment. (A, B) Cross-sections of the ECM also show weaker fibronectin staining in the collagenase case (cyan = cell nuclei (dapi)). (C) Ratio of fibronectin intensity for three embryos (Phase 2–3). Ratio was computed as sum of intensity in B (collagenase treated) / sum of intensity in A (control). Collagenase reduced fibronectin staining in all embryos with an average fluorescence ratio of 0.39. Scale bars: 100µm.
Figure 2.6: Effect of collagenase on F-actin. The right eyes of four chick embryos were stained for F-actin after SE removal and collagenase treatment (C, D, C’, D’). Left eyes served as controls (A, B, A’, B’). Eyes were stained, then sectioned in agarose and imaged with a confocal microscope. Four representative eyes are shown with actin in yellow (A–D, A’–D’) and nuclei (DAPI) in cyan (A’–D’). Actin remained intact after SE removal and collagenase treatment. Scale bars: 50μm.
At early to mid-stages of invagination (Phases 2–3), many OVs became convex after SE removal, while others did not pop out until the ECM also was degraded (Fig. 2.7B). At Phase 2, 71% of the OVs became convex after SE ablation (24/34), while this fraction dropped to 38% at Phase 3 (15/40). For those Phase 2 OVs that remained concave after SE ablation, collagenase exposure caused 83% (5/6) to become convex (Fig. 2.7D). By Phase 3, this fraction dropped to 64% (9/14), and by Phase 4 all of the more developed OCs remained concave following SE removal ($n = 15$) and collagenase treatment ($n = 4$) (Fig. 2.7C, D).

To determine whether the effects of SE removal and ECM degradation on OV shape were statistically significant, results were compared using Fisher’s exact test. After SE removal, a significantly higher fraction of OVs popped out at Phase 2 compared to Phase 3 ($P < 0.001$), indicating that the SE is more essential for invagination at the early phases. Although a higher percentage of Phase 3 OVs became convex after subsequent collagenase exposure, the difference relative to Phase 2 was not significant ($P = 0.613$), suggesting that the ECM is required for continued invagination at Phase 3.

Taken together, these results suggest that the presence of the SE is required to initiate OV invagination, but only the ECM is needed to continue the process at later stages. It would be interesting to conduct similar experiments in which the ECM is degraded without removing the SE, but our attempts to do so encountered technical difficulties.

### 2.4.4 Estimation of ECM Stiffness

To explore the morphogenetic function of the ECM during OC formation, we measured mechanical properties and changes in geometry during invagination. These data were used to create and test finite-element models to assess the physical plausibility of the matrix-constraint hypothesis for OV invagination.
Figure 2.7: Summary of shape changes in OV after SE removal and ECM degradation. (A-C) Schematics of observed shape changes (purple = SE, blue = OV, orange = ECM). Arrow size reflects the likelihood of each shape change as plotted in (D). (A, Phase 1) Removing the SE at the placode stage prior to invagination caused relatively little immediate shape change. (B, Phase 2 & 3) During OV invagination (HH14- to HH14+), SE (lens placode) removal sometimes caused the OV to lose its concave shape and become convex. Exposing the remaining concave OVs to collagenase caused most to pop out and become convex. (C, Phase 4) At later stages of invagination (about HH15 onward), lens removal and subsequent collagenase treatment had relatively little effect on the shape of the OC. (D) Fraction of OVs that became convex after treatment. As invagination deepened, optic cups were more likely to remain concave after SE removal (P<0.001, Fisher’s exact test comparing Phases 2 and 3). However, collagenase had a similar effect at these two phases (P<0.613, Fisher’s exact test). Numbers above bars indicate sample size. All OVs remained concave at Phase 4 after both SE removal and collagenase treatment.
As shown later (see Fig. 2.13), results from our model suggest that the proposed mechanism requires the ECM to be considerably stiffer than the cells comprising the OV. Since it is not practical to measure the properties of the matrix directly, we estimated its modulus relative to that of the OV by conducting microindentation tests on partially invaginated OVs (Phases 2–3) with and without ECM (after removing the SE).

After indenting the center of the OV three times and averaging the results (see Fig. 2.8A), we repeated the tests on the same sample after several minutes of local collagenase exposure. Force-displacement curves for three embryos were averaged and fit to a quadratic polynomial for both conditions (with and without collagenase), and the slope of the curves at multiple indentation depths provided incremental stiffnesses. The measured force-displacement curves after SE removal and collagenase treatment were relatively linear (Fig. 2.8C). Degrading the ECM with collagenase decreased the average stiffness of the OV. At 20% indentation, the stiffness decreased about 38% from 11.3 to 7.0 nN/µm, while at 40% indentation the stiffness decreased about 42% from 12.6 to 7.3 nN/µm.

To test for possible side effects of collagenase on the cells, Phase 0 embryos with intact SE tissue were indented before and after treatment. Collagenase had little effect on the stiffness of these OVs, presumably due to the lack of ECM between the OV and SE at these stages (data not shown). These results suggest that the softening effect of collagenase seen in our data was caused by degradation of the ECM and not by weakening of the cells.

To estimate the value of the ECM shear modulus relative to that of the OV, we created a finite-element model for the indentation test. As discussed in the Materials and Methods section, the OV was modeled as a spherical shell with the wall divided into three regions representing the iOV, the oOV, and the ECM (Figs. 2.8B). The shear modulus $\mu_{OV}$ is taken as uniform and equal in both regions of the OV. The force of a cylindrical indenter, with a radius 25% of the OV thickness, was
Figure 2.8: Microindentation testing to determine relative ECM stiffness. (A) Image of OV being indented (BT = brain tube). Black mark on the indenter tip was tracked to determine displacement. (B) Finite-element model for OV indented at top. (C) Comparison of nondimensional force-displacement curves between experiment and model ($F =$ indenter force; $\mu =$ shear modulus of OV; $H =$ undeformed OV thickness). Model results are shown for various values of $\bar{\mu} = \mu_{\text{ECM}}/\mu_{\text{OV}}$. Experimental curves represent the averages of three OVs without the SE before (red dots) and after (blue dots) collagenase treatment; error bars represent standard deviation. Indentation stiffness (slope) decreases after collagenase exposure.
applied normal to the center of the ECM. Nondimensional force-displacement curves were computed for various values of $\bar{\mu} = \mu_{\text{ECM}}/\mu_{\text{OV}}$ and compared with experimental results (Fig. 2.8C).

These results indicate that the value $\bar{\mu} = 50$ is reasonable for the intact OV with ECM but not SE (Fig. 2.8A). Moreover, the curve for $\bar{\mu} = 1$ falls relatively close to the experimental curve for collagenase treatment, consistent with the assumption that collagenase effectively eliminates the ECM as a factor in the mechanics.\(^2\)

### 2.4.5 Computational Models for OV Invagination

Finite-element models were used to help answer the following questions: (1) Can the ECM constrain growth-driven OV expansion enough to cause invagination without the SE? (2) What distributions of placode growth and ECM stiffness produce the most realistic morphology during invagination?

To address these questions, the model from the indentation analysis was modified to include regional tissue growth (Fig. 2.9A). The shear modulus is taken as 220 Pa in both regions of the OV (Xu et al, 2010). We also assume that the ECM does not grow, while the OV grows only in the $e_\Theta$ and $e_\Phi$ directions (see Eqs. (3) and (4)). To give a realistic change in OV radius ($R$ in Fig. 2.11E), the oOV is assumed to grow uniformly at the rate $\dot{G} = 0.15$ (see Eq. (5)). This leaves ECM stiffness and iOV growth as free variables.

Here, we consider three models: (1) **uniform model**: uniform ECM modulus and uniform iOV growth; (2) **growth gradient model**: uniform ECM modulus and growth gradient in iOV (with higher growth rate at the center); and (3) **stiffness gradient model**: ECM stiffness gradient (with

\(^2\)For simplicity, since our model does not include tissue remodeling, which occurs in vivo to partially maintain the relatively thicker OV after ECM degradation, the indentation tests were simulated for the OV in its initial state. As a check, we also ran these simulations for a partially invaginated OV with and without ECM. Although the computed stiffnesses were lower, the value of $\bar{\mu}$ for the intact OV is approximately the same.
Figure 2.9: Three models for OV invagination. The models differ only in the distributions of growth and stiffness in the placode and ECM regions, respectively: (1) uniform growth and stiffness; (2) growth gradient with uniform stiffness; and (3) stiffness gradient with uniform growth. (A) Initial OV geometry is a spherical shell divided into three regions representing the iOV (light blue), oOV (dark blue), and ECM (red). (B, C) Spatial distributions of shear modulus ratio ($\bar{\mu} = \mu_{ECM}/\mu_{OV}$) and growth rate in each model. (D) ECM arc length remains almost constant (1–2% change) in each model as the placode flattens and begins to invaginate ($t < 0.5$), and then increases significantly with time. The uniform model is shown during early invagination ($t = 0.55$, right). (E-G) Invaginated shapes for each model at $t = 6$. (E’-G’) Outlines of models at various times. (E”-G”’) 3D representations of each model.
higher ECM stiffness at the center) and uniform growth in iOV. Values of the specified growth rate, \( \dot{G} \), and the ratio \( \bar{\mu} = \mu_{\text{ECM}}/\mu_{\text{OV}} \) for each model are given in the following paragraphs. (For a given geometry, the behavior of the model depends on the ratio of the shear moduli, rather than their individual values.)

For the first two models, we take \( \bar{\mu} = 50 \) from our indentation data. In the third model, the distribution for the ECM was taken in the form (see Fig. 2.9B)

\[
\bar{\mu} = 96e^{-\beta(\Theta/\Theta_{\text{max}})^2}
\]  

(2.6)

where \( \beta = 2.75 \) and \( \Theta \) is the meridional angle from the axis of symmetry with \( \Theta_{\text{max}} = 40 \) deg defining the margin of iOV (see Fig. 2.9A). This expression corresponds to an average ECM shear modulus of 50 \( \mu_{\text{OV}} \). The value for \( \beta \) was chosen to give a reasonably realistic thickness gradient in the iOV (see Fig. 2.11C and Fig. 2.10).

Cell proliferation in mouse eyes was higher in the iOV than the oOV during Phases 1 and 4 (see Fig. 2.14A). However, changes in cell size and cell death also can contribute to tissue growth. Hence, as discussed in the next subsection, the growth rate in the iOV was determined by matching experimental and numerical results for the temporal change in iOV cross-sectional area (see Fig. 2.11A). This procedure yielded \( \dot{G} = 0.5 \) for the (uniform) nondimensional growth rate in the uniform and stiffness gradient models. For the growth gradient model, we take

\[
\dot{G} = 0.84e^{-\gamma(\Theta/\Theta_{\text{max}})^2}
\]  

(2.7)

in the placode region with \( \gamma = 2 \) and the average growth rate maintained at \( \dot{G} = 0.5 \) (Fig. 2.9C). The value for \( \gamma \) was chosen based on morphology and curvature (see Fig. 2.12). All three models were run until the dimensionless time \( t = 10 \) at which point the growth \( G \) in the iOV reached 6 (or an average of 6 in the growth gradient model).
Figure 2.10: Effect of changing stiffness gradient parameter $\beta$ (see Eq. (6)) in OV model while preserving the average shear modulus ratio ($\bar{\mu} = 50$). Baseline stiffness gradient model is underlined. (A) Lower values of $\beta$ decrease the gradient in stiffness across the ECM and produce similar morphology as the uniform model. (B) Increasing $\beta$ leads to a lower shear modulus near the edge of the iOV and less constraint on iOV expansion. For this reason, large values of $\beta$ increase the invagination depth to levels considerably greater than those measured experimentally. Color bar shows values for growth ($G$).
All three models invaginate (Fig. 2.9E-G), suggesting that the ECM is stiff enough to produce an invagination without the SE. Moreover, there are some notable similarities as well as distinct differences in the morphogenetic behavior of these models.

In all three models, the iOV thickens into a relatively flat placode just prior to the start of invagination (Fig. 2.9E’–G’, green outlines). During this early phase, the placode thickness varies among the models (see below), but the radial arc length of the ECM-OV boundary remains relatively constant in all models (Fig. 2.9D). Near t = 0.5, the arc length reaches a local minimum in the growth gradient and uniform models and changes by only +2% in the stiffness gradient model and -1% in the other models. This behavior is consistent with measurements in chick and mouse embryos, where the contact area between the OV and SE remains relatively constant during placode formation (Zwaan and Hendrix, 1973; Huang et al, 2011). As the invagination grows deeper, the ECM is stretched significantly by the growing iOV (Fig. 2.9D) as the oOV expands outward (Fig. 2.9E’-G’).

At the end of the simulation (t = 6), the center of the iOV is closest to the bottom of the oOV in the stiffness gradient model, while the uniform model has the largest gap (see Fig. 2.9E-G, E’-G’). In addition, the matrix inhibits tangential expansion of the growing placode region, with the added tissue volume causing the placode to thicken in all models. The wall thickness at the center of the OV increases most in the stiffness gradient model and least in the uniform model (Fig. 2.9E-G). As discussed below, quantitative comparisons of experimental and model geometries were used to determine the most realistic of these three models.

Cut-out 3D representations of the final shapes of the OC are shown in Fig. 2.9E”-G”. In addition, we conducted a parameter study to determine how changes in OV growth and relative ECM modulus affect invagination morphology. Decreasing $\bar{\mu}$ from 50 to 16 has relatively little effect on shape, but for $\bar{\mu} < 8$ the iOV evaginates (see Fig. 2.13A). Between the values of 8 and 16, the model did not converge past early placode formation, possibly due to structural instability in the transition
from convex to concave phenotype (Ramasubramanian and Taber, 2008). Finally, increasing the
growth rate in the oOV relative to that in the iOV does not change the shape of the iOV signifi-
cantly, but the edge of the invaginating region within the oOV becomes less sharp (Fig. 2.13C).
Modifying the parameters $\beta$ in the stiffness gradient model and $\gamma$ in the growth gradient model
affects iOV curvature and invagination depth (see Fig. 2.10 and Fig. 2.12).

### 2.4.6 Stiffness Gradient Model Best Simulates Optic Cup Formation

To determine which of the three models produces the most realistic behavior, we compared numerical results to experimental data acquired from chick embryos. Our evaluation is based on temporal changes in curvature, invagination depth, and wall thickness at the center of the iOV.

First, we correlated time scales using changes in cross-sectional area of the iOV. When normalized by initial area at Phase 0, the area obtained from OCT images increased approximately linearly with incubation time based on Hamburger-Hamilton staging (Fig. 2.11A) (Hamburger and Hamilton, 1951). As mentioned above, when the average growth rate in the iOV is taken to be the constant value $\hat{G} = 0.5$ (see Eq. (5)), the models yield area vs. time curves that agree reasonably well with the data (Fig. 2.11A).

Meridional curvature at the iOV surface (normalized by initial curvature), wall thickness (normalized by initial thickness), and invagination depth (normalized by OV radius) were measured over time (see Fig. 2.11B-E). Curvature was determined at $t=2$ and $t=6$ in the models and at comparable stages in the chick (HH14 and HH17). The growth gradient model produces the greatest curvature at both time points, while the uniform and stiffness gradient models more closely match the flatter curvature of the chick embryo (Fig. 2.11B).

Wall thickness and invagination depth at the center of the iOV change rapidly at first and then more slowly as the OV invaginates. Of the three models, the stiffness gradient model yields the
Figure 2.11: Comparison of experimental and model-predicted optic cup morphology. (A) Cross-sectional area of the iOV (normalized by average initial area), averaged from OCT images in two orthogonal planes, versus hours of incubation. Gray region shows the range of iOV area in the three models versus nondimensional time. A linear regression ($R^2=0.996$) through the model data was correlated with experimental area to determine corresponding times for the models. (B) Experimental curvature at center of iOV (normalized by initial curvature) decreased between $t=2$ (Phase 3, HH14) and $t=6$ (Phase 4, HH17). For all models, the curvature decreased a similar amount to the measurements, while the magnitude of the measured curvature was similar to that given by the uniform and stiffness gradient models but considerably lower than that of the growth gradient model. (C) Measured wall thickness at center and outer edge of the iOV. (D) Comparison of experimental and model wall thickness (normalized by initial thickness at Phase 0) at center of iOV as function of time. Thickness reached a local maximum early in each model as the placode flattened and increased afterwards. Stiffness gradient model yields the best qualitative agreement with the data. (E) Comparison of experimental and model invagination depth versus time. Normalized invagination depth is defined as pit depth $D$ divided by maximum OV radius $R$ (see inset). In panels D and E, outlines of the stiffness gradient model mark each time point on the time axes. Stiffness and growth gradient models agree reasonably well with qualitative trends in the data.
Figure 2.12: Effect of changing growth gradient parameter $\gamma$ (see Eq. (7)) in stiffness gradient model while preserving average iOV growth rate ($\dot{G} = 0.5$). The baseline growth gradient model is underlined. As $\gamma$ increases, the growth gradient increases. Increasing $\gamma$ creates less realistic curvature in the iOV. Color bar shows values for growth ($G$). Models are shown at $t = 6$ except for $\gamma = 5$ due to convergence issues near the edge of the iOV.

best agreement with the thickness vs. time data (Fig. 2.11D). In addition, although the thickness at the outer edge of the iOV was relatively difficult to measure accurately due to OCT resolution, the wall thickness near the center became higher than that near the edge as invagination proceeded (Fig. 2.11C). Only the stiffness gradient model captured this trend (see Fig. 2.9E–G).

Finally, the invagination depth vs. time curves given by the growth gradient and stiffness gradient models are similar to each other but are quite different from the curve given by the uniform model at later stages (Fig. 2.11E). The trends provided by the two gradient models are a better match to experimental data (Fig. 2.11E). Note, however, that the early blip in the curves predicted by these models was not seen consistently in our data. This behavior is caused by a snap-through type of instability that occurs as the curvature reverses from convex to concave (Ramasubramanian and Taber, 2008). Geometric imperfections in the actual OV would be expected to produce a smoother transition.

In summary, the growth gradient model is the only one of our three models that yields results in reasonably good agreement with all of our data. Taken together, therefore, the present results support the hypothesis that OV invagination is driven by uniform growth in the iOV constrained by a relatively stiff attached ECM. Our results also suggest that the stiffness of the ECM decreases
Figure 2.13: Effect of changing parameters in the uniform model (baseline model is underlined in each row). (A, B) Evagination occurs for low values of shear modulus ratio ($\bar{\mu} \leq 8$), while higher values lead to invagination and greater placode thickening. (C) Increasing the growth rate of the oOV had little effect on placode morphology but increased the distance between the iOV and oOV. Color bar shows values for $G$. 
with distance from the center of the OV. Although it is possible that the actual mechanism involves a combination of growth and stiffness gradients, our data indicate that the gradient in stiffness dominates the fundamental behavior.

We used other data to test the model predictions of uniform growth in the iOV and a stiffness gradient in the ECM. To examine the growth question, we used data from another study (Huang et al, 2011) to quantify cell proliferation in three regions of the iOV of the mouse embryo. No significant differences were found between regions at Phases 0 and 1 (two-way repeated measures ANOVA and post-hoc Tukey’s test) (Fig. 2.14A). There was a significant difference between the dorsal third and ventral third of the iOV by Phase 4 ($P < 0.05$), but these regions were not significantly different from the middle third. Although growth may not be caused by cell division alone and some differences exist in eye development between mouse and chick, these results suggest that a strong growth gradient in the iOV is unlikely. In addition, at Phases 1 and 4, proliferation in the oOV was significantly lower than the iOV, similar to published findings in the chick and at later stages of human eye development (Tsukiji et al, 2009; Božanić and Saraga-Babić, 2004). These data are consistent with the different growth rates specified in the iOV and oOV of our model.

Using microindentation, we tried to measure regional stiffness variations in the iOV, but the relatively small size and concave curvature of this region made these measurements unreliable. Since fibronectin content has been found to correlate with tissue stiffness in arteries (Kakou et al, 2009), we used the intensity of fibronectin staining as an indicator of qualitative trends in ECM stiffness (see Fig. 2.15 for more details). Normalized intensity for six chick embryos is plotted as a function of normalized arc length from the center to the dorsal edge of the iOV (Fig. 2.14A’,B). Data for the ventral half of the iOV were quite irregular, possibly due to the developing optic stalk, and were ignored. Intensity was significantly higher near the center than near the dorsal edge (38% higher in the cubic regression fit; $P < 0.001$, t-test comparing data within 10% of the total arc length from
Figure 2.14: Cell proliferation and ECM distribution in embryonic eyes. (A) Cell proliferation at Phase 0 (E8.5), Phase 1 (E9.5), and Phase 4 (E10.5) was measured in the mouse embryo by counting the percent of BrdU positive cells in cross-sections in the oOV and three equal regions of the iOV (see A’). Horizontal black bars show significant differences between regions at a given phase, and gray bars between phases within a given region (two-way repeated measure ANOVA with Tukey’s test, *P < 0.05, # P < 0.001). Differences were significant between certain regions and stages with significant interaction between stage and region (P < 0.001). At Phase 0 and Phase 1, proliferation in the three iOV regions was not significantly different suggesting uniform proliferation in the iOV. At Phase 4, proliferation of the dorsal third of the iOV was significantly lower than in the ventral third (P < 0.05). Proliferation significantly decreased in the oOV between each stage. (A’) Diagram shows the oOV and three regions of the iOV. The normalized arc length plotted in (B) is outlined in black from 0 to 1 (0 = dorsal contact point between OV and SE; 1 = center of the ECM, where the OV was most invaginated). (B) Fibronectin distribution between the OV and SE in the chick embryo at Phases 2–3. Fluorescence intensity was greater in the center than near the edge of the iOV (P < 0.001, t-test comparing data between regions where arc length ≤ 0.1 and arc length ≥ 0.9), suggesting that the ECM is denser near the center of the iOV. Fluorescence intensity was normalized by average intensity in the ECM of each sample. A cubic polynomial was fit to the raw data ($R^2 = 0.34$, $y = -0.71x^3 + 1.05x^2 - 0.013x + 0.85$).
the center and edge). These results are consistent with the ECM being stiffer at the center than at the edge of the iOV.

Figure 2.15: Matrix visualization in OV. (A) Confocal image of the invaginating OV and SE. Nuclei are cyan, fibronectin is yellow. Red asterisks (*) mark the start and end of the contact between the two cell layers. Orange arrows point to a few mitotic cells along the apical surfaces of the SE and OV during interkinetic nuclear migration. (B) The ECM from A was traced and the image straightened in ImageJ. (C) Fluorescence intensity was calculated and plotted by summing the columns along the dorsal half of the ECM arc length. Intensity and arc length were normalized for multiple samples to obtain the results shown in Fig. 9B.

Taken together, the cell proliferation and ECM staining data seem to support the stiffness gradient model for OV invagination. To compare morphology, the stiffness gradient model is superimposed over OCT images of the chick OV in Fig. 2.16.

2.4.7 A Stage Dependent Need for the ECM

As discussed above, our experiments have shown that degrading the ECM with collagenase during the early phases of invagination often caused the OV to pop out, converting the invagination into an evagination. At later phases, however, the invagination was more likely to remain concave after treatment (Fig. 2.7). We used this behavior to test the predictive ability of the stiffness gradient model. (Notably, the behavior of our other models is similar for this perturbation.)
Figure 2.16: Overlay of chick OCT images at different stages and corresponding time points in the stiffness gradient model (yellow). Chick OVs (outlined in dashed red lines) are shown in the plane orthogonal to the optic stalk for greater symmetry. Model images were each scaled for the respective OCT image. Scale bar: 100\(\mu\)m.

To simulate degradation of the ECM, growth was paused at a specified level of invagination, the shear modulus of the ECM was decreased to match that of the OV, and the value of \(G\) in the ECM layer was increased to match the value of the iOV (Fig. 2.17A, A’, B, B’). This procedure effectively eliminated the ECM from the model. Results from this procedure are shown for early invagination (Phase 2) and mid invagination (Phase 3). Consistent with our experiments, the OV reverted to a convex shape after ECM removal during early invagination (Fig. 2.17A, A’), but it remained concave after the deeper invagination (Fig. 2.17B, B’). Hence, the model captures this unexpected behavior reasonably well. Interestingly, removing the ECM at Phase 3 allowed the iOV to expand, decreasing its thickness and center curvature (Fig. 2.17B, B). In our collagenase experiments, thickness and curvature did not appear to change significantly, possibly due to cell remodeling. We attempted to find the critical invagination depth for popping out, but convergence issues near the critical point hindered this aspect of our study. However, the model results indicate that the critical point occurs between about HH13+ and HH14 (\(t = 0.9\) to 2.1 in the model).
Figure 2.17: Simulation of ECM degradation (stiffness gradient model). To simulate matrix degradation, growth is paused at a specified invagination depth (A, B) and the ECM is converted to iOV tissue by decreasing its shear modulus and increasing its growth to match those of the iOV (A’, B’). (A, A’) Phase 2 (G = 1.4 in iOV); ECM degradation causes the OV to pop out, switching from concave to convex. (B, B’) Phase 3 (G = 2.25 in iOV); the OV remains invaginated. (B”) To simulate further invagination after the lens and optic cup have detached, growth is continued after ECM degradation of B’. (B’’) 3D representation of the model in B”. (C) Phase 4 (HH17) chick optic cup (OCT cross-section) shows the model qualitatively captures the flattening at the center of the iOV. (D) Reconstruction of optic cup from OCT images shows 3D shape with a groove along the optic stalk (OS). Scale bar: 100µm.
Figure 2.18: Intermediate steps for matrix degradation simulations. (A) Model evaginates (pops out) at Phase 2 (see Fig. 10A, A’). As ECM degrades, bending stresses at the edge of the invagination invert the iOV. Since the iOV has grown slightly larger than the opening created by the invagination, the iOV pushes the oOV outwards as it passes through the opening. To aid visualization, all arrows are the same length in each row and represent distance from the center of the OV to the edge of the invaginating region before degradation begins (left image). (B) Model remains invaginated at Phase 3 (see Fig. 10B, B’). The iOV has grown too large to overcome the constraint of the oOV as it tries to pass through the opening.
Hendrix and Zwaan (1974) found that the density of the matrix increases through early OV invagination and then decreases as the lens pit begins to separate from the OC to close and form the lens vesicle. To simulate the later stages of invagination without the ECM, we restarted growth in the Phase 3 model without ECM (shown in Fig. 2.17B’). As invagination deepened, the iOV eventually contacted the oOV (Fig. 2.17B”,B”’) to form a bilayered OC with a shape similar to that seen in the chick at about HH17 (Fig. 2.17C,D).

2.5 Discussion

The results of the present study support the hypothesis that ECM-provided constraint on lateral expansion of the growing iOV is sufficient to drive the invagination that creates the OC. The behavior of our model indicates that invagination requires that the matrix be considerably stiffer than the cells in the OV (see Fig. 2.13), consistent with our stiffness measurements. Our results also suggest that the shape of the invaginated iOV (neural retina) is regulated, in part, by a stiffness gradient in the ECM, which is stiffer near the center than near the margin of the iOV. This gradient affects the local curvature and thickness of the retina.

As mentioned in the Introduction, Zwaan and Hendrix (Zwaan and Hendrix, 1973; Hendrix and Zwaan, 1974) suggested a similar invagination mechanism for the iSE (lens), and they speculated that constrained growth may also be involved in OC development. However, although the ECM has been studied as a potential source of morphogenetic signals to the OV (Wride, 1996), how the matrix might influence OV morphogenesis has remained poorly understood.

3Notably, the extensional stiffness of the ECM is most important for constraining cellular expansion in the OV, rather than the bending stiffness. The matrix does not need to be stiffer than the cells to cause a flat epithelium to bend. However, for a spherical shell the curvature stiffens the structure and complicates the mechanics. According to our model, a relatively large ECM stiffness is required to force a reversal of curvature (see Fig. 2.13).
A mechanical function for ECM has been found in other problems in morphogenesis. In branching morphogenesis, for example, matrix is more abundant in cleft (concave) regions than in expanding (convex) regions (Daley and Yamada, 2013). Treatment with collagenase can reverse cleft formation in salivary gland (Grobstein and Cohen, 1965) and the early lung (Wessells and Cohen, 1968), similar to the popping out effect we observed in the OV. During gastrulation in the sea urchin embryo, Lane et al (1993) suggested that differential swelling of the ECM drives invagination, a mechanism supported by computer modeling (Davidson et al, 1995, 1999). Hence, accumulating evidence suggests that ECM plays a major biomechanical role in shaping the embryo.

### 2.5.1 Evidence Supporting the Constrained Growth Hypothesis for OV Invagination

Most previous studies of early eye morphogenesis have focused on the lens, but some offer important clues for solving the OV problem. In \textit{Pax6}\textsuperscript{CKO} embryos, for example, lens placode formation is absent, the ECM between the OV and SE is less abundant, and cell-matrix adhesion is reduced. As a result, the dividing cells spread laterally instead of maintaining constant contact area, and neither the lens nor the OV invaginate (Huang et al, 2011). Fibronectin knockout mice (\textit{Fn1}\textsuperscript{CKO}) yield similar results (Huang et al, 2011). Fibronectin is a primary matrix constituent during eye development (Kwan, 2014). These results suggest that placode formation is a prerequisite for invagination in the eye, as has been found in other morphogenetic problems (Huang et al, 2011; Jidigam and Gunhaga, 2013), and that retinal and lens placode formation requires the ECM. However, they do not prove that the ECM is necessary for the invagination process itself.

The experiments of Hyer et al (2003) provide further clues to our problem. As discussed in the Introduction, removing the SE prior to lens placode formation (Phase 1) blocks OV invagination, although the neuroepithelium of the iOV still differentiates, whereas OV invagination generally
proceeds unimpeded when the lens placode is removed after Phase 1. We repeated these experiments and obtained similar results. To explain these findings, Hyer et al (2003) speculated that the iSE must be present long enough to send a biochemical signal to the iOV that induces invagination. Once the signal is received, the iSE (lens) is no longer needed.

Here, we offer an alternative possibility. We found that dissecting the SE using the method of Hyer et al (2003) leaves the matrix relatively intact in a considerable number of embryos (Fig 2.2D; Fig. 2.5), and our computational model suggests that the ECM without the iSE provides sufficient constraint on OV expansion to cause invagination (Fig 2.9). Moreover, once the invagination has reached a critical depth, invagination can proceed without the ECM (Fig 2.17B–B”). Accordingly, we speculate that the iSE is required only to generate sufficient matrix to reach the critical depth. Dissecting the iSE prior to the placode stage (Phase 1) removes the source of ECM before enough has accumulated to cause sufficient invagination, and the process does not complete. Later dissection, however, removes the source but leaves enough ECM intact to reach the critical stage. After this time, invagination can proceed even if the matrix degrades further.

This mechanism is also supported by our ‘popping out’ experiments. Removing the SE or disrupting the ECM during Phase 2 causes most OVs to immediately become convex (Fig 2.7), as the mechanical constraint is lost. However, the fraction of OVs that remain concave after dissection or collagenase exposure increases at later times (Phases 3 and 4). By Phase 4, removing the iSE or the ECM no longer induces any OVs to pop out. The results given by our model are consistent with this behavior (Fig. 2.17).

The reason that the ECM is required to maintain invagination only until a certain depth can be understood by considering the behavior of the model as ECM degradation is simulated (see Fig. 2.18).

4Natural variations in ECM and OV geometry or material properties could account for the different behaviors of some OVs at intermediate stages of invagination, especially near the critical invagination depth, which is especially sensitive to imperfections.
When the constraint provided by the matrix is eliminated, the relatively large bending stresses generated near the edge of the invaginated region force the iOV toward reversing its curvature and pop out as these stresses are relieved. Since the iOV grows faster than the oOV, the increased surface area of the iOV must fit through a relatively smaller opening in the oOV during the transition from concave to convex curvature. Hence, the iOV pushes the edge of the spherical oOV radially outward while stretching it in the circumferential (hoop) direction. At Phase 2, the bending stresses can overcome these additional hoop stresses. By Phase 3, however, the growth disparity and corresponding hoop stresses are too large to overcome, and the iOV remains invaginated. Notably, this constraint is a consequence of the spherical geometry of the model; a cylindrical model would not provide such strong resistance to evagination.

Finally, it is important to note the following. While our study indicates that constrained growth likely drives OC formation on a global scale, the spatial distributions of growth and matrix stiffness can affect the more detailed shape of the invaginated iOV, i.e., the neural retina (Figs. 2.9E–G, 2.10, 2.12, and 2.13). While these gradients are not necessary for invagination to occur, a sharper gradient in ECM stiffness, for example, would produce an increased gradient in iOV thickness as well as a change in retinal curvature. Since the shape of the retina significantly affects vision, these morphogenetic factors should not be overlooked.

2.5.2 Possible Alternative Mechanism from Stem Cell Studies

Recently, researchers have devised ways to generate self-assembling OCs in vitro using either mouse or human embryonic stem cells (Eiraku et al, 2011; Nakano et al, 2012). Notably, these structures invaginate without SE tissue, but laminin staining showed that ECM was present along the basal (outer) surface of the OV. Moreover, disrupting cell-matrix adhesion with anti-integrin-β1 antibody prevented OC formation in the mouse stem cell culture (Eiraku et al, 2011).
Although these results do not contradict the constrained growth hypothesis, Eiraku et al (2011) proposed an alternative mechanism for OC formation based on growth and regulated actomyosin contraction along the apical surface of the OV. This mechanism, which does not require ECM, consists of four phases: (1) uniform contraction produces a spherical OV; (2) relaxation in the iOV creates a placode; (3) apical constriction at the iOV margin causes local cell wedging and a small invagination; and (4) growth of the iOV deepens the invagination. These investigators used staining of phosphorylated myosin, stiffness measurements, inhibitors of apical contraction, and a computer model to support their hypothesis (Eiraku et al, 2011, 2012).

While the elegant experimental and numerical results of Eiraku et al (2011) offer strong support for this alternative hypothesis, it does not seem consistent with our ECM degradation experiments which suggest an important role for the matrix. We offer three possibilities. First, as discussed by Sasai et al (2012), the mechanism of OV invagination may be fundamentally different in vitro. Second, a back-up mechanism may be triggered in vitro when the SE is not available to generate enough matrix to cause invagination. Third, the researchers may have uncovered a parallel mechanism that normally works in concert with constrained growth. In fact, other researchers have obtained evidence supporting a role for contraction in this process (Brady and Hilfer, 1982; Plageman et al, 2011). Future studies are needed to evaluate these possibilities.

### 2.5.3 Limitations

With the focus here being mechanics, we did not consider the genetic and molecular signals involved in OV invagination. Reviews have explored the role of signals such as BMPs and FGF during eye development (Adler and Canto-Soler, 2007; Fuhrmann, 2010). Researchers have studied specification of the retina by signals from nearby tissues such as the mesenchyme and SE (Fuhrmann, 2010). Knocking out BMP7, Six3, or Pax6 can cause various eye defects in mice (Fuhrmann, 2010). Mutated genes such as SIX3, SHH, PAX6, and SMOC1, a BMP antagonist,
have been associated with anophthalmia (missing eye) and microphthalmia (small eye) in humans (Slavotinek, 2011). Future work could elucidate how these signals may disrupt the mechanical properties of the ECM or cell-matrix adhesion in the eye. For example, in an anophthalmic mouse strain ZRDCT-Ch, researchers have found decreased sulfated glycosaminoglycan staining on the basal side of the OV (Webster et al, 1984).

Mechanisms other than those considered here also may contribute to OC formation. For example, in those embryos that developed fully invaginated OCs without the iSE, the final morphology of the cultured lens-less OC sometimes was not completely normal. In some embryos, the iOV and oOV were not pressed together as seen in controls; this separation is also visible in the images of Hyer et al (2003). This observation suggests that the invaginating lens may impart some secondary stresses that affect the final shape of the OC, e.g., by pushing against the OV. In addition, interkinetic nuclear migration may contribute to OV invagination by placing more cell nuclei near the apical side of the OV, causing it to expand (Zwaan et al, 1969; Norden et al, 2009) (see Fig. 2.15).

Further studies are needed to verify the distributions of growth and ECM stiffness suggested by our data. Our results indicate that the growth rate in the iOV is relatively uniform, but subtle differences in growth rate may exist. More precise estimates of growth distributions would require measuring local changes in tissue volume. In addition, the matrix and epithelial stiffnesses depend on their composition (material properties) and geometry (thickness). The ECM thickness could not be measured consistently due to sectioning difficulties. Moreover, the estimated shear moduli represent macroscopic averages of microstructural properties, but this is consistent with our tissue-level model. Although our microindentation tests indicate that the ECM shear modulus is high enough to provide the constraint on iOV expansion needed to drive invagination, technical challenges prevented us from using indentation to determine regional differences in ECM stiffness.

For the constrained growth mechanism to be feasible, the ECM must be tightly bound to the iOV. We did not examine the distribution of integrins connecting these layers, but we did notice that the
center of the SE was more difficult to remove from the iOV than the outer region, especially at later stages, suggesting a stronger binding between the SE and OV near the center. Such a gradient in binding strength could also increase the effective ECM stiffness near the center of the iOV, thereby influencing retinal shape.

Finally, it is important to note that the mechanisms of eye morphogenesis may be considerably different in some species. In fish, for example, cells migrate over the rim from the oOV to the iOV during invagination (Li et al, 2000).

In conclusion, our results support the hypothesis that the ECM between the SE and OV plays a crucial role in OC formation. Our experiments and modeling suggest that the matrix constrains lateral expansion of the inner region of the growing OV, causing it to thicken and invaginate to create the primitive retina, while a gradient in ECM stiffness refines the final shape of the retina. Our study provides new understanding as well as poses new questions related to the mechanics involved in early eye development.
Chapter 3

Apoptosis Generates Mechanical Forces that Close the Lens Vesicle in the Chick Embryo

3.1 Summary

The embryonic eye begins to develop when the optic vesicle (OV) grows outward on each side of the forebrain and contacts the surface ectoderm (SE). In the region of contact, the OV and SE secrete extracellular matrix (ECM) and thicken locally to create the retinal placode and lens placode, respectively. These layers then invaginate to create the optic cup (primitive retina) and lens vesicle (LV). Studies have shown that the ECM constrains expansion of the growing SE and OV, leading to placode formation and SE invagination, while actomyosin causes the lens to invaginate. Here, we use computational modeling and experiments on the chick embryo to show that these mechanisms are plausible, but our study shows that contraction likely does not cause the invaginating lens placode to close to create the LV. Here, we suggest a novel role for apoptosis to close the LV. Estimates of the proliferation and apoptosis in the early lens show that apoptosis occurs
at a sufficient rate to close the LV by generating circumferential tension. Apoptosis inhibition with caspase inhibitors Q-VD-OPh and Z-VAD-fmk significantly reduced LP closure in the chick embryo. A finite-element model based on this mechanism suggests that this idea is feasible, as it provides results that agree reasonably well with experimental data. Our results suggest a significant mechanical role for apoptosis in lens development.

3.2 Introduction

During the early stages of eye development, the lens forms from a region of surface ectoderm (SE) that contacts the optic vesicle (OV) as it grows laterally outward from either side of the forebrain. Within the region of contact, the OV and SE become tightly adhered by extracellular matrix (ECM), and these epithelia thicken to form the retinal placode and lens placode, respectively (Zwaan and Hendrix, 1973). These placodes then invaginate together to form the optic cup (future retina) and lens pit (LP). Eventually, the nascent lens separates from the optic cup and surrounding SE and becomes the fluid-filled lens vesicle (LV). Later, the cells in the LV wall elongate and fill the lumen to create the lens (Cvekl and Ashery-Padan, 2014). Malformations of the lens can cause birth defects such as congenital cataracts (opaque lens), microphakia (small lens), ectopia lentis (abnormal lens position), and aphakia (absence of the lens) (Khong, 2015).

Published reports have explored the biophysical mechanisms that create the lens placode and drive subsequent invagination. These studies suggest that the matrix constrains lateral expansion of the growing SE, causing the membrane to thicken locally to form the relatively circular placode (Huang et al, 2011; Plageman et al, 2010). Then, contraction of actomyosin fibers located on the apical side of the lens placode causes it to invaginate (Medina-Martinez and Jamrich, 2007). Here, we use new data from chick embryos and computational modeling to confirm that these ideas are consistent with physical law. However, our model also reveals that apical constriction alone is
not sufficient to close the LV into a complete fluid-filled shell. The main objective of our work, therefore, is to determine the mechanism that closes the opening in the LP to create the LV. To our knowledge, this important aspect of LV morphogenesis has been almost completely overlooked in the literature.

Closing the LV is similar to closing a circular wound in an epithelium. From a mechanics perspective, the problem is more complicated than it may appear, and, depending on the circumstances, nature has devised multiple mechanisms to accomplish this task. In the adult, cells near the wound proliferate and crawl into the wound, create matrix to strengthen the patch, contract to reduce the size of the wound, and remodel to complete the healing process, but often leaving a scar (Martin, 1997; Redd et al, 2004). In contrast, wounding of an embryonic epithelium typically triggers the formation of an actomyosin cable at the wound margin that contracts and closes the hole by a “purse string” mechanism. Filopodia-mediated fusion then seals the wound without scarring (Redd et al, 2004). Notably, this mechanism requires the circumference of the hole to shorten from some finite value to zero, causing significant tissue stresses that are partially alleviated by cell remodeling and rearrangement (Wyczalkowski et al, 2013). As will be shown, results from our computational model indicate that an actomyosin purse string can close the hole in the LV, but actin staining does not support such a mechanism.

Here, we suggest an alternative mechanism that operates similarly to a contractile ring, but the required circumferential tension is generated by apoptosis, or programmed cell death, near the hole. Researchers have known for decades that apoptosis occurs in the developing lens, especially near the opening in the LV at later stages of invagination, and some have associated it with several morphogenetic processes, including LP invagination, LV shaping, and detaching the LV from the SE (Francisco-Morcillo et al, 2014). Some investigators have speculated that apoptosis also may play a role in LV closure (Božanić et al, 2003; Yan et al, 2006), but how this may occur has not been studied.
Results from our model suggest that an apoptosis-driven mechanism for LV closure is feasible from a mechanics viewpoint. Such a mechanism also is supported by experiments showing that inhibiting apoptosis reduces lens closure in overnight culture. These results reveal an important mechanical function for apoptosis in lens development that may also play a role in other morphogenetic processes.

### 3.3 Materials and methods

#### 3.3.1 Embryo Culture and Pharmacological Treatment

Fertilized white Leghorn chicken eggs were incubated at 38°C to reach Hamburger-Hamilton stages 11–17 (HH11–17, 40–64 hours) (Hamburger and Hamilton, 1951). Embryos were removed from eggs using a ring of filter paper adhered to the vitelline membrane and placed ventral side up into 35 mm Petri dishes, as described previously (Voronov and Taber, 2002). To eliminate artifacts due to surface tension, a second filter paper ring was placed on the embryo to keep it submerged under approximately 1 mL of culture media. Embryos were cultured in a plastic bag with supplemented oxygen (Voronov and Taber, 2002).

To inhibit apoptosis, the irreversible caspase inhibitor Q-VD-OPh (ApexBio, A1901) or Z-VAD-fmk (ApexBio, A1902) was added to the media at stages HH11–HH14-, before significant invagination occurs, for a concentration of 100, 150, or 200 µM. Embryos were cultured overnight (16 to 23 hours), then imaged as described below.
3.3.2 Imaging of Tissue Morphology

Brightfield images were captured with a camera (Nikon EOS T3) mounted on a dissecting microscope (Leica DMLB MZ 8). Sections of living embryos were acquired using optical coherence tomography (OCT) with 10 µm resolution (Thorlabs, Newton, NJ).

To analyze lens morphology, stacks of OCT images were imported into ImageJ (National Institutes of Health, Bethesda, MD) and oriented through the dorsal-ventral plane of the lens and optic cup. Three to four values of wall thickness were measured and averaged at the center of the developing lens. Apical arc length, basal arc length, and area of the SE or lens pit were also measured without including the outer SE. To quantify the gap before LV closure, the distance between both sides of the LP was measured within the plane oriented through the optic stalk in control and treated embryos. The lens pit gap distance was statistically analyzed between different treatment groups after overnight culture with one-way ANOVA.

3.3.3 Visualization of Cells and Matrix

Various staining protocols were used to visualize tissue constituents. Unless stated otherwise, images of stained samples were captured with a Zeiss LSM 710 confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Prior to staining, all embryos were fixed for 1–24 h in PBS containing 3.7% formaldehyde.

Fibronectin stain was used to visualize ECM. Fixed embryos were rinsed in PBS, then soaked in blocking buffer (2% goat serum with 0.1% Triton X-100 in PBS) for 3 hours as described previously (Oltean et al, 2016). Embryos were incubated in primary antibody (Sigma F3648, 1:100) for 24 hours at 4°C, rinsed with PBS three times, and then incubated with a secondary antibody (Alexa Fluor(R) 488 Goat anti-Rabbit IgG (H+L), Invitrogen A11034, 1:100) overnight.
at 4°C. Samples were rinsed with 0.1% Tween 20 (Sigma) and DAPI (Sigma, 1:1000) in PBS three times before sectioning in agar and imaging with confocal microscopy as described above.

To observe F-actin, embryos were blocked in 0.1% Triton X-100 (Sigma) and 1% bovine serum albumin (BSA) in PBS and then stained with Alexa Fluor 488 phalloidin (1:100, Molecular Probes) with 0.1% Triton X-100 and 1% BSA (Oltean et al, 2016). To stain cell nuclei, embryos were incubated at room temperature with DAPI (Sigma, 1:1000) in PBS for 10 minutes. Samples were imaged as intact embryos or sectioned in agar.

To visualize cell death, 2 µl of 2% Nile Blue sulfate was added per mL of media. Embryos were incubated at room temperature on a shaker and then imaged using brightfield microscopy. Nile Blue sulfate shows approximate regions of cell death, including lysosomal and phagocytotic activity (Elmore, 2007). For more specific staining of dying cells, activated caspases associated with apoptosis were visualized with CellEvent(R) Caspase 3/7 Detection Reagent (ThermoFisher Scientific C10423, 1:1000). Live embryos were incubated with CellEvent(R) in media for 30 minutes. Some embryos were fixed as described above, then incubated with DAPI for 10 minutes. Fixed embryos were sectioned and imaged with confocal microscopy. Additional, live embryos were imaged at multiple time points between incubation with a fluorescence Leica DMLB microscope (10x, 20x, and 40x objectives).

### 3.3.4 Probing Tissue Stress

A microsurgical device (Gastromaster, Xenotek Engineering, Belleville, IL; white tip), was used to test for compression or tension in the SE surrounding the lens. The membranes over the eye were removed with a pulled glass capillary, and embryos were submerged in sufficient PBS (Dulbecco’s phosphate-buffered saline) to completely cover the cutting tip. Shallow cuts were made in different directions around the LP (perpendicular and tangential). Images were captured within a
few minutes after dissection using brightfield microscopy and OCT to observe if the cut opened, indicating tension, or remained closed, indicating compression or negligible tension.

### 3.3.5 Quantification of Cell Proliferation and Apoptosis

In mouse embryos, cell proliferation in the SE was quantified from images (n=9 embryos) provided by the authors of Huang et al (2011). As described in their paper, embryos were exposed for 1 h to BrdU which incorporates into cells during S-phase. Fixed embryos were then sectioned and counterstained with hematoxylin. Using their images, we quantified cell proliferation in five equally sized regions of the primitive lens and in two regions of the surrounding SE on the lateral dorsal and ventral side at multiple stages of development: embryonic day 8.5 (pre-placode stage), 9.5 (placode stage), and 10.5 (lens pit stage). BrdU positive and negative cells were counted to calculate the percent of BrdU incorporation in each region (the proliferation index), as done previously in the mouse optic cup (Oltean et al, 2016). Measurements were analyzed across stages and spatial regions using two-way ANOVA and post-hoc Tukey’s test as needed in SigmaPlot (v12.5, Systat Software Inc.). To calculate the proliferation rate, P, the proliferation index and cell cycle length must both be known. Zwaan and Pearce (1971) calculated the cell cycle in the early chick lens as 9 hours. Here, the proliferation rate was calculated by dividing the average proliferation index by the cell cycle length of 9 hours.

To calculate the apoptotic rate, A, in the lens pit, analogous calculations to proliferation were made. The apoptotic index has been previously measured in the mouse embryonic lens with TUNEL staining (Yoshimoto et al, 2005; Le et al, 2012). Here we evaluated apoptosis distribution in the chick embryo using CellEvent(R) and DAPI staining in sectioned eyes as described above. The time it takes for apoptotic cells to degrade has not been well characterized (Jacobson et al, 1997). To calculate this clearance time, we imaged activated caspases 3 and 7 in real-time as described above using fluorescence microscopy. At each time point, newly fluorescing cells were noted as
beginning to die. These cells were tracked across multiple time points until they fragmented and were no longer part of the epithelium. The times from beginning to end were averaged from multiple embryos (n=4) to estimate the apoptosis time period in or near the chick eye. The apoptotic rate was calculated by dividing the apoptotic index by the average clearance time.

3.3.6 Computational Modeling

To simulate formation of the LV, we created axisymmetric finite-element models using COMSOL Multiphysics (Version 4.2a, COMSOL AB, Providence, RI, USA). Model geometry in the initial configuration (HH12) was determined from OCT and confocal images. The pre-lens SE is modeled as a shallow spherical shell consisting of three layers representing a passive epithelium (normalized outer radius = 1.09 and inner radius = 1) sandwiched between a relatively thin apical contractile layer and passive layer of matrix (thickness = 0.01 for both). To simulate placode formation, another spherical shell (thickness = 0.2) representing the optic vesicle is attached to the matrix layer (Oltean et al, 2016). To prevent rigid-body motion, the top point of the model is fixed.

With all layers treated as pseudoelastic, growth is simulated by modifying the governing equations within COMSOL based on the theory of Rodriguez et al (1994). Accordingly, the total deformation gradient tensor is defined as \( F = F^* \cdot G \), where \( F^* \) is the elastic deformation gradient tensor (relative to the zero-stress state) and \( G \) is the growth tensor. The Cauchy stress tensor is given by (Taber, 2004)

\[
\sigma = \frac{2}{J^*} F^* \cdot \frac{\partial W}{\partial C^*} \cdot F^{*T},
\]

(3.1)

where \( W \) is the strain-energy density function, \( C^* = F^{*T} \cdot F^* \) is the right Cauchy-Green elastic deformation tensor, and \( J^* = \det F^* \) is the elastic volume ratio. Details for incorporating growth in COMSOL 4.2a are provided in Hosseini et al (2014).
As in Xu et al (2010); Taber (2004); Blatz and Ko (1962), all layers are assumed to be isotropic with the strain-energy density function defined as

\[
W = \frac{1}{2} \mu \left[ I^* - 3 + \frac{1}{\alpha} (J^* - 2\alpha - 1) \right],
\]  

(3.2)

where \( \mu \) is the shear modulus, \( \alpha = \nu/(1 - 2\nu) \), and \( \nu \) is Poisson’s ratio in the limit of small strain. In addition, \( I^* = \text{tr}C^* \) is the first strain invariant. For the SE (and OV), we set \( \mu = 220 \text{ Pa} \) and \( \nu = 0.45 \) (Xu et al, 2010). Poisson’s ratio is taken the same for the ECM and actin layers, but the shear modulus is varied. The ECM begins with a shear modulus 50x stiffer than the shear modulus of the cell layers, \( \mu_{SE} \), as determined previously (Oltean et al, 2016). Then, from \( t=4.5 \) to \( t=5.5 \), the ECM shear modulus is decreased to become only 10x stiffer than the SE to simulate separation of the SE and OV near HH14 (Hendrix and Zwaan, 1974). The actin layer begins with a shear modulus equal to \( \mu_{SE} \) then increases to \( 50\mu_{SE} \), over the time period \( t=3 \) (lens placode stage) to \( t=5 \). For continued thickening of the LP as observed in the chick embryo, the shear modulus of actin continued to increase throughout the model, reaching a maximum value of \( \mu_{max} \) as described below (see Eq. 3.7).

In each region, the growth tensor is taken in the orthotropic form

\[
G = G_R e_R e_R + G_\Theta e_\Theta e_\Theta + G_\Phi e_\Phi e_\Phi
\]  

(3.3)

where \( e_R, e_\Theta, \) and \( e_\Phi \) are undeformed unit vectors in spherical polar coordinates (Fig. 3.2), and \( G_R, G_\Theta, \) and \( G_\Phi \) are specified functions of space and time. The SE and OV are modeled as 80 deg of a spherical shell.

In all models, the ECM layer does not grow \( (G_R = G_\Theta = G_\Phi = 1) \), while, for axisymmetric deformation, the SE and OV grow only in the circumferential and meridional directions. For the SE
(and OV), growth was approximated to increase linearly with time by

\[ G_R = 1 \] (3.4)

\[ G_\Theta = 1 + \dot{G}_\Theta t \] (3.5)

\[ G_\Phi = 1 + \dot{G}_\Phi t \] (3.6)

where \( \dot{G}_\Theta \) and \( \dot{G}_\Phi \) are growth rates for the meridional and circumferential directions. The actin layer contracts from \( t=3 \) to \( t=5 \) by increasing the shear modulus as described above and by linearly decreasing \( G_\Theta \) from 1 to 0.1. \( G_\Phi \) decreases as well with greater contraction at the edge of the LP to create a more circular lens shape. The minimum of \( G_\Phi \) varies spatially and linearly from 0.04 to 0.1 as shown in Fig. 3.5B, B’. The final, maximum shear modulus in the actin layer is described by

\[ \mu_{\text{max}} = \frac{\mu_{\text{SE}}}{(G_\Theta G_\Phi)^\beta} \] (3.7)

where \( \beta = 1.2 \). A previous model of contraction in the embryonic brain used a similar equation with \( \beta = 1.5 \) (Filas et al, 2012).

Net growth is a combination of proliferation and apoptosis. Therefore, the growth rates were estimated as \( \dot{G}_\Theta = P_\Theta \) and \( \dot{G}_\Phi = P_\Phi - A_\Phi \) where \( P_\Theta \) and \( P_\Phi \) are the proliferation components in each direction and \( A_\Phi \) is apoptotic rate in the circumferential direction in the region of the SE with significant cell death. We set \( A_\Theta = 0 \) (see Fig. 3.11). We assume that proliferation causes equal growth in both directions and define

\[ P_c \equiv P_\Theta = P_\Phi. \] (3.8)

The proliferation rate, \( P \), and apoptotic rate, \( A \), were calculated for the early lens as described above and tested in the computational model. Since apoptosis was only specified in the circumferential direction, \( A_\Phi = A \). Overall proliferation can be approximated as the average proliferation in the
circumferential and meridional directions with \( P = 1/2(P_\Phi + P_\Theta) \). Therefore \( P \) and \( P_c \) should be equal.

Eqs. 3.4–3.6 can be redefined as:

\[
G_R = 1
\]

\[
G_\Theta = 1 + P_c t
\]

\[
G_\Phi = 1 + (P_c - A)t
\]

### 3.4 Results

#### 3.4.1 Morphology of Early Lens Development

In the chick embryo, the OV grows outward and contacts the SE by HH10. At HH12, more ECM begins to be deposited between the OV and SE setting the stage for coordinated placode formation and invagination (Hendrix and Zwaan, 1974). With OCT imaging, both cell layers are visible with the SE much thinner than the OV (Fig. 3.1A). After the two layers form placodes and start bending inward, the lens pit continues to thicken and separate from the optic cup (OC) (Fig. 3.1B–F).

As shown previously (Zwaan and Hendrix, 1973; Ettensohn, 1985), actin is abundant on the apical surface of the SE before and during invagination (Fig. 3.1G,I). Bilayered ECM is present on the basal surface of the lens placode and lens pit (Fig. 3.1H). The ECM has been proven to be essential for lens placode formation in the mouse embryo (Huang et al, 2011) and important for OC formation in the chick (Oltean et al, 2016). The ECM density partially decreases mid-invagination to assist LP detachment from the OC (Hendrix and Zwaan, 1974). The abundant ECM between the OC and LP is reduced, but each cell layer still maintains a thin matrix layer.
Figure 3.1: Lens development in the chick embryo. (A) OCT cross-sections of developing eye. The optic vesicle (OV) and surface ectoderm (SE) are tightly adhered (HH13) by ECM (red dashed line) on the basal side of the lens. (B) Both layers thicken forming the retinal placode (in the OV) and lens placode (Plac). (C) Layers invaginate to form the lens pit (LP) and optic cup (OC). (D–F) The LP continues to bend and closes to form the ellipsoidal lens vesicle (LV) by HH17. Apical side of the primitive lens is outlined in yellow. (G) Confocal sections of F-actin (red) and cell nuclei (cyan) shows actin concentrated on apical sides of LP and OV. (H) Fibronectin (yellow) marks location of ECM between layers. (I) Actin staining remains strong on the apical side of the LP as it invaginates. Scale bar: 100μm.
3.4.2 Mechanics of Placode Formation and Early Invagination

The effects of ECM and actin on early lens development were studied in computational models with a growing SE and OV. BrdU measurements in the mouse embryonic lens indicated no statistically significant differences across different regions or stages (Fig. 3.10). $P_c$ was varied in a parameter study to optimize LP area and thickness over time as seen in the chick embryo (see Fig. 3.7C, D). This led to a value of $P_c = 0.17$ for $t = 0$ to $t = 5$.

Placode formation was simulated using the axisymmetric model. At $t = 5$, the SE and OV thicken due to the ECM constraint (Fig. 3.2A,A’). Although the ECM is sufficient to cause invagination of the OV (Oltean et al, 2016), the early lens requires apical actin to invert and become concave. The ECM is necessary, however, to first thicken the SE otherwise the model is unable to overcome the inversion step and cannot converge past the flat SE shape (model not shown). With the addition of apical contraction that begins to stiffen and constrict at $t = 3$, the SE still forms a thickened placode and becomes concave by $t = 5$ (Fig. 3.2B,B’).

To test the effects of simulating the ECM without the OV, the SE was first modeled with just a basal ECM (Fig. 3.2C,C’). The ECM causes the SE to thicken, but without the OV restraint, the SE becomes more convex. With the addition of actin but no OV, the SE is able to become concave and the final morphology is relatively unaffected without the OV (Fig. 3.2D,D’). Therefore, subsequent models for lens invagination include the ECM but not OV for simplicity.

3.4.3 Role and Limitations of Contraction in Lens Vesicle Formation

While apical constriction has been studied in lens pit invagination (Borges et al, 2011; Plageman et al, 2010), the role of actomyosin contraction specifically in lens pit closure has not been evaluated in literature, to our knowledge, especially from a mechanical viewpoint. Contraction has
a significant role in invagination as inhibition of apical contraction with blebbistatin, a myosin II inhibitor, prevents lens pit formation in the chick embryo (Borges et al, 2011).

The axisymmetric model of the growing SE with basal ECM and apical contraction was modified to include ECM degradation, but the LP could not close to form the LV (Fig. 3.3C, t = 11, HH17). The ECM stiffness was decreased, from $\mu_{ECM} = 50\mu_{SE}$ to $10\mu_{SE}$, at $t=4.5$ (HH14) to simulate the decrease in matrix density that allows separation of the LP and OC. After $t = 5$, the average growth in the SE was doubled to increase the LP’s size sufficiently, and actin continued to stiffen to reach a maximum shear modulus ($\mu_{max}$) at $t = 11$ (see Eq. 3.7). After increasing $P_c$ to 0.34 after $t = 5$, a slight growth gradient was added to the SE to modify the overall shape (uniform growth causes too circular of a shape) as described by

$$P_{grad} = 0.4 e^{-0.5(1-\Theta/40)^2}$$

(3.12)

where the average value across the SE is 0.34. A portion of the outer SE was added as a 10 deg extension of the spherical shell. The tissue was pulled outward until $t = 5$ to create tension (as seen in stress cutting experiments, Fig. 3.9A-A”). The outer SE then grew in the $\Theta$ and $\Phi$ directions with $P_c = 0.1$ to prevent tissue thinning while maintaining tension. Adding the outer SE did not have a large effect on LP closure distance but helped prevent the edge of the LP from curling inwards.

Closing the spherical model requires the generation of high forces to overcome large circumferential stresses in the tissue. For comparison, a similar cylindrical model was created (Fig. 3.3D). The growth tensor for the cylinder model is defined as

$$G = e_R e_R + G_{\Theta} e_\Theta e_\Theta + e_Z e_Z$$

(3.13)
Figure 3.2: Initially spherical models of lens placode formation illustrating effects of various layers. Cross-sections are shown with deformed shapes at (A’–D’) t = 5. (A, A’) Growth of surface ectoderm (SE) constrained by a relatively stiff ECM on basal side and growing OV. SE thickens and becomes flatter. (B,B’) Contraction of apical actomyosin layer (actin) causes additional thickening and invagination. (C,C’) Without the OV, the SE still thickens from the ECM constraint but becomes more convex. (D,D’) Apical actin helps thicken the SE and reduce the evagination seen in (C’). Dashed line denotes axis of symmetry.

where the SE grows in $G_\Theta$, and the apical layer contracts with a decreasing $G_\Theta$. The growth rate was set to $P_c = 0.17$, and the ECM shear modulus was defined as in the axisymmetric model. In the cylinder model, contraction quickly constricts the apical surface of the SE and closes the lumen. Therefore contraction was decreased (final $G_\Theta = 0.3$), and the SE completely closed while maintaining a lumen (Fig. 3.3F, t = 5.4). To close the spherical model, additional mechanisms must be involved.

3.4.4 Apoptosis is Sufficient and Necessary to Close the Lens Vesicle

Cell death has been observed in the developing eye such as in the ventral OC possibly contributing to choroid fissure formation (Morcillo et al, 2006). In the developing lens, apoptosis has been
suggested to play a role in separating the LV from the outer SE (Francisco-Morcillo et al, 2014). Here, we investigated the possibility that apoptosis drives LV closure.

Nile Blue sulfate staining in the chick embryo indicates cell death along the anterior LP (Fig. 3.4A,B) beginning with strong dorsal staining at HH14, then staining in a complete ring around the LP at HH15. Since Nile Blue sulfate is not a specific stain for apoptosis (Elmore, 2007), we also incubated embryos with CellEvent(R) Caspase 3/7 to visualize signals associated with apoptosis. Fixed embryos were stained with DAPI, and then sectioned eyes were imaged with confocal microscopy. Caspase-positive cells were concentrated along the anterior edge of the LP and through the center of the OC (Fig. 3.4C). Apoptotic bodies were also apparent with DAPI staining as small, bright, segmented nuclei in the anterior LP and OC.

To simulate apoptosis in a computational model, we first estimated the apoptotic rate from the apoptotic index and clearance time. The apoptotic index has been measured previously by two groups studying the embryonic mouse lens. The apoptotic index was measured as 7.5% or 17% at E10.5 in the anterior lens pit then decreased dramatically at E11.5 and E12.5 (Yoshimoto et al, 2005; Le et al, 2012). Here, we used the average of these values, 12%, as the apoptotic index. Clearance time for apoptotic cells has rarely been measured but has estimated to be in the range of minutes to several hours depending on the cell type and system (Teng and Toyama, 2011), such as 40 minutes in the neonatal rat retina (Cellerino et al, 2000). Chick embryos were incubated with CellEvent(R) and imaged live with fluorescence microscopy to watch cells enter and complete apoptosis at multiple time points for several hours. Newly fluorescent cells were tracked to measure clearance time in or near the LP. Average clearance time was 35 minutes (standard deviation ±16 minutes, n=4 embryos).

Due to choosing the growth rate with a parameter study as described above, the experimental apoptotic rate was not implemented in the model directly. However, it was calculated to verify if apoptosis is sufficiently greater than proliferation to close the LP. The experimental apoptotic rate
Figure 3.3: Effects of geometry on invagination caused by apical contraction. Behavior of spherical model (top row) and cylindrical model (bottom row) are compared. (A) Spherical model in initial configuration with small segment of outer SE. The ECM modulus in the model of Fig. 3.2 is reduced at $t = 4.5$ to simulate separation of the OV and SE during later stages. (B) Although contractile strength continues to increase as the SE grows, the model fails to close to create a lens vesicle at $t = 11$. (C) 3D representation of model in (B). (B,C) Final spherical model shown at half the scale used in (A). (D) Cylindrical model in initial configuration. (E) Even with less growth and less contraction than the spherical model (minimum $G_0 \approx 0.3$), the cylindrical model closes completely by $t = 5.4$. (F) 3D representation of model in (E). Color legend indicates growth in meridional direction.
was calculated as 0.21, almost 4x the measured proliferation rate of 0.056. The model apoptotic rate, $A$, was chosen by calculating the necessary value for $G_{\Phi}$ to approach 0 at $t = 11$ (HH17). Apoptosis was implemented in the outer edge of the LP (outer 5 deg) beginning at $t = 5$ (HH14+). The growth rate in the growing and apoptotic region of the SE is plotted in Fig. 3.5D, D’. At $\Theta = 40$ deg, for example, $P_c = 0.4$ and $A = -0.71$.

The apoptotic region constricted the region near the LP opening to form the LV by $t=10.93$ (HH17). Once $G_{\Phi}$ in the apoptotic region began decreasing below 1, the adjacent ECM layer with no growth distorted the basal surface of this region. To simulate removing the ECM of each apoptotic cell and prevent excessive accumulation of ECM in the apoptotic region, the ECM growth was also decreased locally in the circumferential direction ($G_{\Phi} < 1$).

After the computational model showed apoptosis can potentially close the LV, we inhibited apoptosis in the chick embryo to determine if this mechanism was biologically plausible. Apoptosis is mediated by caspases, and Q-VD-OPh is a pan-caspase inhibitor known to strongly inhibit caspases with very little toxicity to cells (Caserta et al, 2003). Z-VAD-fmk is also a broad caspase inhibitor that preferentially inhibits caspase 3 at low concentrations (Caserta et al, 2003). Embryos were incubated with either caspase inhibitor beginning at stage HH11 to HH14- and cultured overnight (see Fig. 3.6). The LP gap distance was measured, and one-way ANOVA showed statistically significant differences between gap size in control and all treatment groups ($P < 0.001$). There was no significant difference between 100$\mu$M Q-VD-OPh and 100$\mu$M Z-VAD-fmk ($P=0.2$).

Apoptosis inhibited eyes had reduced lens closure and reduced Nile Blue sulfate staining under OCT imaging and brightfield microscopy respectively. Embryos appeared healthy overall (see Fig. 3.6F), and confocal imaging revealed a dramatic reduction of caspase staining with only a few dots in the LP or OC (Fig. 3.6E). DAPI staining also showed intact nuclei without apoptotic bodies in treated embryos.
Figure 3.4: Effects of apoptosis on lens vesicle formation. (A, B) Brightfield image of Nile Blue staining shows a ring of cell death surrounding the opening to the lens pit (LP) at stage HH14 (A) and HH15 (B). (C) Confocal section with caspase 3 and 7 staining (yellow) confirming apoptotic cell death in the optic cup (OC) and anterior LP at HH15. Large dots show nuclei in early apoptosis, while very small dots show cells in late apoptosis. Image shows combination of multiple slices in a stack using the maximum brightness function in ImageJ. (C’) Nuclear staining shows early lens morphology, with small dots indicating apoptotic bodies. (D) Spherical model of Fig. 3.3A without the outer SE. Beginning at $t = 5$, apoptosis is simulated by negative growth ($G_\Phi < 1$) in indicated region. (E) At $t = 7$, the edge of the LP begins to pull inward. (F) The model closes to produce a lens vesicle ($t = 10.93$). Compare with model at $t = 11$ in Fig. 3.3B,C. (G) 3D representation of model in (F). Legend indicates growth in the circumferential direction ($G_\Phi$). Scale bars: 100µm.
Figure 3.5: Spatial and temporal values of model parameters in actin layer (B, B’, C), SE (D, D’), and ECM (E). (A) Model of early lens formation with three layers and an apoptotic region as shown in Fig. 3.4. (B, B’) Plots of growth in the contractile layer over time (B) and as a function of $\Theta$ (B’). The layer contracts by decreasing growth between $t = 3$ and $t = 5$. (C) The actin layer’s shear modulus increases with time reaching a maximum value at $t = 11$ dependent on the final growth values (see Eq. 3.7). (D) The surface ectoderm (SE) grows at a rate of 0.17 until $t = 5$ when the growth rate averages 0.34 and varies as a function of $\Theta$ (see Eq. 3.12). (D’) Spatial variation of the SE growth rate after $t = 5$. The growth rate is negative ($\dot{G}_\Phi < 0$) in the apoptotic region. (E) The relative shear modulus of ECM begins at 50 then decreases to 10, simulating detachment of the SE and optic cup. (F) Table shows corresponding model time points for stages HH12–HH17.
3.4.5 Comparison of Experimental and Model Results

To evaluate the biological accuracy of the apoptosis model, we measured several morphology characteristics temporally in the apoptosis model and chick embryo. We compared the area of the overall lens, average cell wedging (apical / basal length), LP gap distance, and thickness of the center of the SE or LP.

Outlines of the model with apoptosis and without apoptosis were overlaid on representative OCT images of a control chick embryo and an embryo treated with Q-VD-OPh (Fig. 3.7A',B'). The model with apoptosis, although not as thick as the chick lens, had a similar overall elliptical shape with flatter curvature near the top of the LV. The chick LP treated with apoptosis inhibitor did not grow as large as the model without apoptosis.

Time in the model was correlated to incubation times in the chick embryo based on Hamburger-Hamilton stages 12–17 (see Fig. 3.5F) (Hamburger and Hamilton, 1951). The growth rate, \( P_c \), was selected from a parameter study to optimize area of the lens while still allowing closure by \( t = 11 \). The amount of actin contraction and maximum actin stiffness, \( \mu_{max} \), were then chosen with a second parameter study to increase thickness without drastically reducing lens size. The ECM and apical constriction increased the wall thickness in the model rather linearly although only by a factor of 2.8, and not 4 as found in the chick (Fig. 3.7D).

In the model, the SE initially grows, constricted by a relatively stiff ECM until \( t = 3 \) (HH13), then contraction begins. The ECM begins to relax at \( t = 4.5 \) (HH14), up to which point the ECM maintains almost a constant basal arc length in the SE as a placode formed (Fig. 3.7E). This agrees well with previous experimental data in the chick and mouse showing constant area on the basal side of the lens before and after placode formation (Hendrix and Zwaan, 1974; Huang et al, 2011). The apical arc length, however, varies and reflects the timing of contraction and stiffening. Overall cell wedging was computed as the ratio of apical to basal arc length (Fig. 3.7F). The model and
Figure 3.6: Effects of inhibiting apoptosis on lens vesicle formation. Embryos beginning at stage HH11–HH14 were cultured overnight in control media or in media treated with Q-VD-OPh and imaged the next day with (A, B) OCT imaging and (C, D) Nile Blue staining under a brightfield microscope. (A) Most control eyes formed a lens vesicle (LV) the next day; however, many treated embryos (B) did not have a closed LV. (C, D) Considerably less cell death was evident in treated eyes than in controls when stained with Nile Blue sulfate (dark blue). (E, E’) Caspase and nuclear staining revealed little to no apoptosis in treated eyes. Image shows overlay of multiple slices in a stack with the maximum brightness function. (F) Representative embryo treated with 150µM Q-VD-OPh imaged with brightfield microscopy after overnight culture. Apoptosis inhibition did not cause adverse effects in overall embryo health or heartbeat compared to controls. (G) To verify the effects of inhibiting caspase, a second caspase inhibitor (Z-VAD-fmk) was also used. Plot shows the average gap size (0 indicates a closed LV) for each group after overnight culture. One-way ANOVA showed significant differences between each treatment group ($P < 0.001$) except for the pair indicated by NS ($P = 0.2$). Scale bars: 100µm.
chick data agree reasonably well, beginning with an increase as the placode forms, then a sudden decrease in cell wedging that approaches a steady value of approximately 0.4.

Measurements of the lens pit gap indicate the LP closes fairly linearly with time in the chick but with varying rates in the model (Fig. 3.7G). The model includes apoptosis as a linear decrease in growth; however, the actual apoptotic rates may vary temporally in the chick. Quantifying the apoptotic index at different stages may elucidate this process in more detail.

While some parameters of the model could be further optimized to more consistently match experimental data, the model does suggest crucial roles for ECM, apical contraction, and apoptosis in lens development. The diagram in Figure 3.8 summarizes our hypotheses for early eye development. First the ECM constrains the OV and SE to create the retinal and lens placodes. Next, apical constriction of actin inverts the SE to invaginate and form the LP, while the ECM causes the OV to bend inward and form the OC. Then apoptosis in the anterior LP significantly shrinks the tissue to close the LV while the OC continues to grow.

### 3.4.6 Possible Alternate Mechanisms for Lens Morphogenesis

Additional mechanisms were considered for early lens development. First, the stress was probed in the outer SE to determine if this tissue could help close the LP. Small cuts were made in the outer SE in tangential or perpendicular directions around the LP using the Gastromaster tool (Fig. 3.9A-A”). OCT imaging revealed that cuts opened, indicating tension and not compression in the tissue. Tension in the outer SE would pull the LP open further rather than contribute to LV closure.

Actomyosin purse strings have been implicated in embryonic wound healing and morphogenetic events such as dorsal closure in Drosophila (Redd et al, 2004; Martin et al, 2009). To simulate a purse string in the LP, a small region of contraction was added at the edge of the SE in the model. This region begins to contract at $t = 5$ by decreasing growth in the circumferential direction.
Figure 3.7: Comparison of model and experimental results. (A) Closed lens model (including apoptosis) at $t = 10.93$ and (B) model without apoptosis at $t = 11$. (A’, B’) Representative OCT images of a control lens vesicle (A’, HH17) and a LP exposed to apoptosis inhibitor (B’, 150µM Q-VD-OPh) overlaid with outlines of the models shown in (A, B) in red. (A’) The model with apoptosis (red) is slightly thinner than the chick LV but has a similar overall shape. (B’) The model without apoptosis (red) grows larger than the treated chick LP (yellow dashed line) but is scaled here to compare overall shape. (C–G) Comparison of experimental and apoptosis model results for cross-sectional area of the early lens, center thickness, apical and basal arc lengths, overall cell wedging, and lens pit gap size after invagination begins. In the model, apoptosis begins at $t = 5$. 
Figure 3.8: Schematic of hypothesis for lens vesicle formation. (A) The optic vesicle (OV, purple) and surface ectoderm (SE, orange) are initially bonded together by relatively stiff extracellular matrix (ECM, dashed black line). (B) The ECM constrains expansion of both layers as they grow, causing the layers to thicken and form the retinal placode (RP) and lens placode (Plac) in the region of contact. (C) Matrix-constrained growth eventually causes the OV to invaginate and form the optic cup (OC), while actomyosin contraction (actin, dashed red line) causes the SE to invaginate and form the lens pit (LP). Although occurring simultaneously, the mechanisms that drive these invaginations are largely independent of each other. (D) Cell death, or apoptosis (blue dots), occurs in a ring near the opening in the LP and generates circumferential tension that closes the LP to form the lens vesicle (LV). (E) The completed LV separates from the OC and outer SE.
(\(G_\Phi < 1\)) and increasing the shear modulus to a maximum of \(478\mu_{SE}\) by \(t = 10.96\). The model did close, but actin staining revealed no concentrated actin along or near the LP border in HH14 or HH15 embryos (Fig. 3.9C,D).

Another mechanism that may affect LV formation is active cell wedging caused by interkinetic nuclear migration (Zwaan and Hendrix, 1973). During the cell cycle, nuclei shift back and forth between the apical and basal surface, dividing only at the apical surface in the early lens or retina. To simulate active cell wedging in the model, the growth rate was varied linearly across the SE from 0.17 to 0.47 (apical to basal; average growth rate of 0.3). This did not significantly affect the thickness or overall morphology of the LP (Fig. 3.7G).

### 3.5 Discussion

The results from this study support the hypothesis that ECM constraint, apical contraction, and apoptosis are each necessary in successive steps for lens development (Figure 3.8). The ECM has been implicated in mouse lens placode formation and chick OC formation (Huang et al, 2011; Oltean et al, 2016). Here, computational modeling suggests a mechanical need for the ECM to help thicken the SE. Although actomyosin contraction likely drives LP invagination, our computational model and experimental data suggest it is not sufficient to close the LP. We hypothesize that apoptosis, localized to the anterior SE, is necessary for LV formation. Our computational and experimental results suggest apoptosis is sufficient and necessary to shorten the edge of the LP opening and cause lens closure.
Figure 3.9: Other possible mechanisms involved in lens vesicle formation. (A–A’’) To examine the possibility that the outer surface ectoderm (SE) pushes the edges of the lens pit (LP) closed, small circumferential incisions were made in the SE around the LP. (A, A’) OCT cross sections before and after cutting. Cuts open (arrows), indicating tension directed normal to the LP, rather than compression that would be associated with pushing the LP closed. (A’’) The stack of OCT images from (A’) were visualized in 3D to show the complete cuts. (A’’’) Additional cuts to completely isolate the LP did not cause the LP to open further. (B) To determine whether a contractile ring could contribute to LP closure, a region of circumferential contraction (orange) was added at the edge of the model without apoptosis to simulate an actomyosin purse string. (B’) The model closes to create an LV. Shown at half the scale used in (A). (C, D) To examine whether such a purse string exists, stacks of confocal images were combined to visualize multiple layers of actin near the LP opening. Top-down views of the LP show a network of actin but no distinct, concentrated ring near the opening at stage HH14 (C) or HH15 (D). (E–G) To determine whether cell wedging, possibly caused by interkinetic nuclear migration toward the basal surface, could play a significant role in shaping the LV, a linear growth gradient (higher growth rate toward basal side) was included in the model without apoptosis. (G, t = 10) Cell wedging caused more bending at the free end of the SE but did not have strong global effects compared to a model with uniform growth. (F, G) Final models shown at half the scale used in (E). Color legend indicates circumferential growth $G_\Phi$. Scale bar: 100 µm.
3.5.1 Effects of Matrix and Contraction in Early Lens Development

Hendrix and Zwaan studied the ECM in early eye development and suggested its role in restricting expansion of the SE and OV (Zwaan and Hendrix, 1973; Hendrix and Zwaan, 1974). They noticed constant contact area between the two cell layers during placode formation, presumably from the ECM deposited during this stage. Huang et al (2011) confirmed this result before and after lens placode formation in mice, and showed that the conditional knockout of fibronectin prevents lens placode formation and invagination. Hendrix et al (1993) presented a geometric analysis of the forming LV from ECM and growth alone, but this model does not account for mechanical forces. While the ECM can cause invagination of the OV (Oltean et al, 2016), our mechanical model shows that the SE requires contraction to invaginate (Fig. 3.2, Fig. 3.3).
Invagination in processes such as Drosophila gastrulation or inner ear development (the otic cup) requires contraction-driven apical constriction (Martin et al, 2009; Sai and Ladher, 2015). The role of apical constriction has been studied in the early lens for several decades, supported by the localization of actin and myosin II on the apical (shortening) side of the LP (Zwaan and Hendrix, 1973; Ettensohn, 1985; Plageman et al, 2010). Blebbistatin prevents lens invagination in the chick embryo, supporting the requirement of apical constriction in this process (Borges et al, 2011).

While our cylindrical model demonstrates that apical contraction is sufficient to close a cylinder, it is not sufficient to close the LV (Fig. 3.3). As described in the Introduction, closing a spherical shell is more similar to wound healing than formation of a tube, since the edge of a wound must decrease from a finite circumference to zero. In embryonic wound healing, an actomyosin cable forms around the wound and helps close the hole with a purse-string contractile mechanism (Redd et al, 2004). While localized actin in a purse string can close our computational model of the lens (Fig. 3.9B,B’), we did not find evidence of concentrated actin in the chick LP. However, other
proteins important for actin contraction or stabilization, such as tropomyosin (Nicholson-Flynn et al, 1996), may be localized in this region. We also considered the effect of the outer SE on LV closure; however, cutting experiments indicated tension and not compression in the outer SE which contradicts the idea that the SE pushes the LP closed (Fig 3.9A–A”). Active cell wedging with greater basal growth was also considered but did not have a significant effect on the model (Fig 3.9G). We hypothesize that another mechanism, specifically apoptosis, is required to close the LP.

3.5.2 A Novel Role for Apoptosis in Lens Vesicle Formation

While ECM and actomyosin contraction have been studied and strongly implicated in lens development (Zwaan and Hendrix, 1973; Huang et al, 2011; Chauhan et al, 2009; Plageman et al, 2010), apoptosis has not been evaluated as a mechanism to close the LV. Apoptosis has been observed in the early lens and OV primarily with TUNEL staining, caspase staining, or examination of nuclei ultrastructure in the mouse, rat, chick, and human embryos (Arya et al, 2015; Yoshimoto et al, 2005; Le et al, 2012; Trousse et al, 2001; Mohamed and Amemiya, 2003; Božanić et al, 2003). Macrophages have also been imaged near the lens tissues containing debris from dead cells (Mohamed and Amemiya, 2003; Francisco-Morcillo et al, 2014). Here, we verified the presence of apoptosis with Nile Blue sulfate and activated caspase 3 and 7 staining (Fig. 3.4). Caspases are cysteine proteases central to apoptosis that are initially inactive and must be activated by initiator caspases or other proteins (McIlwain et al, 2013).

Once cells begin to apoptose, they can be fragmented and extruded from the epithelium within minutes or hours (Teng and Toyama, 2011). Due to the fast clearance time (35 minutes in our study), studying the individual cell behavior of apoptotic and neighboring cells is challenging. Our estimates from literature values and experimental data of the proliferation rate (0.056) and apoptotic rate (0.21) suggest that apoptosis occurs at a fast enough rate to close the LP. Here, we assumed
cells quickly fill the space left by apoptotic cells since no holes are visible in the epithelium (see tightly packed nuclei in Fig. 3.1H,I). As demonstrated in the schematic in Figure 3.11, removing 1/3 of cells in concentric rings of cells can cause the circumferential direction to shrink by more than 1/3 as neighboring cells squeeze together and rearrange to fill the gaps. This pattern would hold for any fraction of cells undergoing apoptosis (1/3 was chosen arbitrarily to demonstrate the cell behavior). In our computational model with apoptosis in the perimeter of the LP, the SE closed by negative growth in the circumferential direction (Fig. 3.4F).

Apoptosis in the LV has been primarily suggested as a driver for separation between the LV and remaining SE (Ozeki et al, 2001). Inhibition of apoptosis has been shown to prevent LV detachment, causing an eye defect called Peters’ anomaly (Ozeki et al, 2001; Cvekl and Ashery-Padan, 2014). Inhibition of apoptosis with caspase inhibitors in our study significantly reduced LV closure in chick embryos (Fig. 3.6). The inhibitors were validated with caspase staining which showed almost complete inhibition of activated caspases 3 and 7 in the LP and OC. Adding the inhibitor during LV formation reduced separation of the anterior LV from the SE and separation of the LV from the OC in the very center of the OC (data not shown), as has been studied previously (Cvekl and Ashery-Padan, 2014). These data suggest multiple roles for apoptosis in LV development.

Apoptosis has important mechanical roles in several processes. Cell death can help define cell boundaries, remove unwanted tissues, or aid in various shape changes necessary for morphogenesis. For example, apoptosis causes regression of the tail in tadpoles, removes the interdigital webs in limb development, and aids drosophila dorsal closure or fold formation in the leg disc (Penaloza et al, 2006; Pérez-Gario and Steller, 2015). As shown in previous studies (Francisco-Morcillo et al, 2014) and confirmed in our study with caspase staining, apoptotic cells are abundant in the OC where the choroid fissure begins to form. Bmp7 mutant mouse embryos do not form the choroid fissure and have reduced cell death and proliferation in the ventral OC (Morcillo et al,
Apoptosis may play a role in fissure formation although another mutant with reduced cell death has been shown to still form the choroid fissure (Silver and Robb, 1979).

Apoptosis may not be a simple passive process, as it can also induce local actomyosin contraction at the site of extruded cells. In the chick embryonic leg epithelium, neighboring cells form rosette shapes and an actin-myosin ring as dying cells are extruded from the epithelium (Rosenblatt et al, 2001). This cell-sized purse string was also seen in 2D cultures of MDCK cells where inhibition of ROCK (an important signal for apical constriction) prevented extrusion of apoptotic cells (Rosenblatt et al, 2001). Recently, Monier et al (2015) found that apoptotic cells in the early Drosophila embryo exert forces on the leg epithelium through dynamic myosin II cables along the apico-basal length of the cell. Localized cell death causes an actin-dependent shortening and bending of the epithelium. Inhibiting apoptosis with Q-VD-OPh prevented the apical shortening and formation of local myosin II cables. Interestingly, ROCK-I can be directly activated by caspases, and inhibiting myosin II with blebbistatin was shown to decrease cell fragmentation (specifically blebbing) of apoptotic cells (Leverrier and Ridley, 2001; Orlando et al, 2006). This interplay between contraction and apoptosis may complicate elucidating the effects of contraction alone in lens development. While we did not examine actin or myosin in individual apoptotic cells, actomyosin contraction may help strengthen the mechanical effects of apoptosis in the early lens.

3.5.3 Limitations and Future Work

Results from our computational model were compared to multiple characteristics of the chick lens during development at analogous time points (Fig. 3.7). Lens placode formation, initial invagination, and LP closure were simulated within reasonable time periods in the model. A near constant basal arc length in the model indicates that the ECM is able to constrict expansion of the SE as the placode forms. Although the center of the SE becomes thicker over time in the model and chick, the model thickens by a factor of 2.8 rather than 4 as in the chick eye. Future work investigating
possible variations in the proposed mechanisms, e.g., more proliferation, may improve the model. Since extrusion of an apoptotic cell can cause a cell-sized purse string in other systems (Rosenblatt et al., 2001), future studies could investigate actin and myosin localization in the LP in more detail.

While this study included estimations of the proliferation rate and apoptotic rate, we assumed these rates were fairly constant (once apoptosis is triggered, \( t = 5 \) to \( t = 11 \)). Quantification of caspase staining at several stages of lens development would help detail the temporal and spatial distribution of apoptosis in the chick LP and possibly improve the behavior of the model. Researchers have shown that apoptosis in the mouse lens varies greatly between stages E10.5, E11.5, and E12.5 (Yoshimoto et al., 2005; Le et al., 2012).

To estimate the proliferation rate, BrdU-positive cells were counted in multiple regions of the mouse embryonic lens (Fig. 3.10). Cell proliferation was consistent spatially across the SE and averaged approximately 0.5 in the pre-placode and placode stages. The proliferation index decreased to 0.41 in the LP stage, but one-way ANOVA showed no significant differences caused by variations in the region or stage of the early lens. Based on a cell cycle of 9 hours in the chick lens (Zwaan and Pearce, 1971), the proliferation rate, \( P \), was calculated to be 0.056 (i.e. 5.6% of existing cells divide each hour). This can be approximated in the model with \( P = 0.056 \) for the growth equations; however, this was too low to provide adequate expansion of the LP over time. Instead, the growth rate in the model was determined by a parameter study to try to replicate tissue level morphology (area and thickness of the lens pit over time, Fig. 3.7C, D).

While the proliferation index does not significantly vary across the stages of lens development (Fig. 3.10) and agrees well with other proliferation measurements in the chick and mouse eye (Huang et al., 2011; Tsukiji et al., 2009), the cell cycle has not been studied as extensively and may vary with time. In chick neural crest cells, the cell cycle can vary from 4.5 hours to 16 hours depending on location and stage (Ridenour et al., 2014). In the mouse retina, the cell cycle decreases from 7 hours at E11.5 to approximately 5 hours at E13 (Klimova and Kozmik, 2014).
Future studies could confirm the cell cycle length in the chick lens during invagination and LV closure.

This study investigated the mechanical role of ECM, contraction, and apoptosis in early lens development. Other than caspases, we did not investigate genetic or molecular signals in the SE. Researchers have studied the signals involved in apical constriction such as Shroom3 and ROCK (Lang et al, 2014). Mutations in Shroom3 and p120 prevented separation of the SE and LV in mice (Lang et al, 2014), which may suggest interactions between these signals and apoptosis. The signals Pax6, Six3, and Sox2 have been studied extensively in lens development (Cvekl and Ashery-Padan, 2014). Pax6 conditionally deleted in the SE decreases ECM deposition and prevents placode formation in the mouse (Huang et al, 2011). Mutations in Pax6, Foxe3 and several other genes are associated with Peters’ anomaly where the LV fails to detach from the SE (Cvekl and Ashery-Padan, 2014). Future studies could investigate how caspase inhibition in the chick embryo affects these cell signals.

In conclusion, our study supports the hypothesis that apoptosis is required for LP closure. Our computational model suggests apical constriction is required for invagination but is not sufficient to close the LP. Apoptosis inhibition with caspase inhibitors prevented LV formation in the chick embryo, and our model showed that apoptosis at the edge of the LP was sufficient to close the lens. This work suggests a critical role for apoptosis in lens development that may be important in other morphogenetic processes as well.
Chapter 4

Contractile Response of the Embryonic Brain to Mechanical Loads

4.1 Summary

The embryonic chick brain has been shown to respond to mechanical loading with changes in nuclear shapes caused by changes in contractility. Here, we examine nuclear remodeling and changes in contraction using a novel method for compressing regions of the brain. A simple cylindrical finite-element model illustrated inhomogeneous stress distribution in the wall of the compressed brain tube. The immediate shape of nuclei after compression in the chick embryo appears to reflect the local stress-state in the brain wall. After compressed brains were cultured for three hours, the nuclei remodeled and became rounder. This response was prevented with a gap junction inhibitor. Our results suggest that stress triggers a small molecular signal that diffuses through the brain tube and alters contractility, causing nuclei to remodel into a relatively uniform shape throughout the wall. Future work is needed to investigate which signals are essential for this process.
4.2 Introduction

The role of mechanics during embryonic development has been investigated in several processes such as neural tube formation (Yamaguchi and Miura, 2013), primary brain vesicle formation (Filas et al, 2012), heart looping (Shi et al, 2014), and gastrulation (Rauzi et al, 2015). In addition to morphogenesis, mechanics is important for cell adhesion, division, differentiation, and motility (Campas et al, 2014; Lovett et al, 2013). How cells respond to mechanical signals has been increasingly studied on multiple scales in the field of mechanotransduction (Hamada, 2015).

Cells can detect their mechanical environment and communicate with neighboring cells through gap junctions and biochemical signals (Fedorchak et al, 2014). Within a cell, mechanical signals such as tension can be transmitted to the nucleus and affect nuclear shape (Guilluy and Burridge, 2015). In the early chick brain, different loading conditions cause nuclear shape and tissue stiffness changes in an actomyosin-dependent process (Filas et al, 2011). Actin filaments affect cell shape, tissue stiffness, and are directly connected to the nucleus through a protein complex (Salbreux et al, 2012; Guilluy and Burridge, 2015).

The chick embryo provides a useful model to study how cells respond to mechanical stress. As shown previously, nuclei in the brain tube become rounder in response to compression during culture, possibly to restore a homeostatic stress state (Filas et al, 2011). These shape changes are accompanied by a reduction in tissue contractility. In this study, we developed a new method to locally or globally compress the embryonic brain. Culturing in the presence of a gap junction blocker prevented the observed nuclear rounding, as nuclei in brains cultured for several hours resembled those of brains examined immediately after compression. This suggests the diffusion of a mechanical signal through the neuroepithelium induces changes in contractility that alter nuclear shape.
Additionally, flattening a cylinder can cause an uneven stress distribution across the wall as the cross-section bends. This leads to spatial variations in nuclear shape, especially in regions of strongest bending, that later become more homogeneous. Future studies are needed to reveal which molecular signals are responsible for inducing these changes in contractility and their possible impact on gene expression in the developing brain.

4.3 Materials and methods

4.3.1 Embryo Preparation and Compression during Culture

Fertilized white Leghorn chicken eggs were incubated at 38°C to reach Hamburger-Hamilton stages 10–12 (HH10–12, 36–48 hours) (Hamburger and Hamilton, 1951). Eggs were cracked over a dish to remove embryos from the surface of the yolk using a ring of filter paper (Voronov and Taber, 2002). A second filter paper ring was placed on the ventral side of the embryo, and embryos were submerged under approximately 1 mL growth media in 35 mm Petri dishes. Some embryos were dissected using pulled glass micropipettes and microdissection scissors to isolate the brain tube as described previously (Filas et al, 2012). The mesenchyme, heart, and other tissues surrounding the brain vesicles were removed while preserving attachment to posterior tissues adhered to the filter paper.

To apply a localized force to the chick embryo, small iron-agar bars were made to position over the brain tube. First, iron particles were sterilized with 70% ethanol and allowed to dry. Next, 3 mL of melted 5% agar in PBS (phosphate buffered saline) was poured into a 35 mm petri dish. Then, approximately 0.07g of iron particles was mixed into the agar (2.3% iron concentration, w/v). The amount of iron can be increased or decreased to vary the force on the embryo. Once the agar cooled and solidified, a thin bar was cut from the agar with a razor blade and forceps
under a brightfield microscope (Leica DMLB MZ 8). Dimensions of the bars were approximately 5 mm across, 200-300 µm deep, and 200 µm–1 mm wide. Width was customized to cover either a portion of the brain such as the hindbrain or the whole, anterior portion of the embryo. The bar was positioned over an isolated brain under a brightfield microscope. A small disc magnet (MagCraft Rare Earth Magnet, NSN0802, 0.5 in diameter) was attached to the inside of a second, empty 35 mm petri dish. Then the magnetic dish was taped beneath the embryo’s culture dish to lower the iron-agar bar and compress the brain. Embryos were cultured with control media or media with 200µM carbenoxolone (Cbx, a gap junction inhibitor), and dishes were placed in a plastic bag with supplemented oxygen (Voronov and Taber, 2002).

4.3.2 Imaging of Tissue Morphology, Nuclei, and Actin

Brightfield images were captured with a Leica DMLB MZ 8 microscope and a Nikon EOS T3 camera. Stacks of optical coherence tomography (OCT) images were acquired with a Thorlabs (Newton, NJ) OCT system (approximately 10 µm resolution).

Embryos in control and loaded conditions were fixed in 3.7% formaldehyde for 1–24 hours. To visualize filamentous actin, some embryos were first blocked in 0.1% Triton X-100 (Sigma) and 1% bovine serum albumin (BSA) in PBS and then stained with phalloidin (1:100, Alexa Fluor 488 phalloidin) in PBS with 0.1% Triton X-100 and 1% BSA. Nuclei were stained with DAPI (Sigma, 1:1000) in PBS for at least 10 mins. Embryos were then sectioned in agar and imaged with confocal microscopy (Zeiss LSM 710, Carl Zeiss Microimaging, Inc., Thornwood, NY).
4.3.3 Quantifying Nuclear Changes in the Brain

Image stacks of nuclei from confocal microscopy were viewed and traced in ImageJ (National Institutes of Health, Bethesda, MD). The position and elliptical fit were measured to calculate the circularity of each nucleus (short axis/long axis). Measurements were analyzed in Matlab R2015a (MathWorks, Natick, MA) for the different loading conditions. For consistency and to avoid regional variations, this study focused on nuclei in the hindbrain. Nuclei were also compared at the apical (inner) and basal (outer) wall in the corners of compressed brains. The mean and standard deviation were calculated for each experimental group. Circularity was plotted in histograms and fit with a lognormal PDF (probability distribution function). The normal PDF did not fit circularity data as well as the lognormal PDF as determined by the probplot function in Matlab comparing data to each distribution function. Statistical significance was tested with ANOVA on ranks, which does not require data to have a normal distribution, followed by Dunn’s test in SigmaPlot (v12.5, Systat Software Inc.). The metric Cohen’s d, to determine effect size between groups, was also calculated in data sets with large sample sizes by

\[
d = \frac{M_1 - M_2}{\sqrt{(\sigma_1^2 + \sigma_2^2)/2}} \tag{4.1}
\]

where \(M_1\) and \(M_2\) are the means in two groups, and \(\sigma_1\) and \(\sigma_2\) are the respective standard deviations (Damania et al., 2010; Sullivan and Feinn, 2012).

While nuclei are elongated in the radial direction of the early brain tube (Filas et al., 2011), 2D circularity measurements were compared to 3D nuclear morphology with fractional anisotropy (FA), a metric typically used for measuring diffusivity (Tang et al., 2013). We analyzed nuclear geometry in two test cases previously shown to cause more elliptical nuclei (40 nM calyculin A) and rounded nuclei (60 µM blebbistatin) (Filas et al., 2011). High-resolution stacks of DAPI staining were traced in ImageJ to visualize the 3D shape of nuclei and perform an ellipsoidal fit.
The 3 axes from the ellipsoid fit were defined as $\lambda_1$, $\lambda_2$, and $\lambda_3$ (listed in decreasing size). The fractional anisotropy was calculated as a shape metric to measure how spherical or elongated a nucleus was with the equation

$$FA = \frac{\sqrt{\left(\lambda_1 - \lambda_2\right)^2 + \left(\lambda_2 - \lambda_3\right)^2 + \left(\lambda_1 - \lambda_3\right)^2}}{\sqrt{2\left(\lambda_1^2 + \lambda_2^2 + \lambda_3^2\right)}}.$$  \hspace{1cm} (4.2)

Fractional anisotropy was then compared to 2D circularity measurements.

### 4.3.4 Observing Diffusion in the Brain Tube

Several methods have been established to observe gap junctional intercellular communication such as microinjection, electroporation, patch clamping and scrape loading (Abbaci et al, 2008). For scrape loading, 2D cultures of cells are scraped, using a tool such as a scalpel, in the presence of a gap junction permeable tracer. The tracer can then enter cells along the cut due to the mechanical break in cell membranes. The dye can then be visualized with fluorescence microscopy to see if it is transported to neighboring cells through gap junctions under different conditions (Abbaci et al, 2008).

The scrape loading technique was modified for our 3D brain geometry. Chick embryos were submerged in control media or media treated with 200$\mu$M Cbx. Lucifer yellow (LY, VWR IC15526710), a fluorescent, gap junction permeable dye (molecular weight 457 Da), was added to the media at a concentration of 0.025%. Microdissection scissors were used to make a few cuts in the brain and anterior embryo in the presence of this dye. Each embryo was then rinsed, and new control or Cbx treated media was added to the dish. Embryos were imaged live at different time points with fluorescence microscopy (Leica DMLB microscope).
4.3.5 Computational Modeling

As mentioned above, sharp bending at the corners of flattened brain tubes likely causes both compression and tension. The inner curvature of the wall experiences compression while the outer curvature experiences tension. We created a cylinder model for a cross-section of the passive brain tube using COMSOL Multiphysics (Version 4.2a, COMSOL AB, Providence, RI, USA). As described in Chapters 2 and 3, the brain tube was modeled as isotropic with the strain-energy density function taken as

\[ W = \frac{1}{2} \mu \left[ I^* - 3 + \frac{1}{\alpha} (J^* - 2\alpha - 1) \right], \tag{4.3} \]

where \( \mu \) is the shear modulus, \( \alpha = \nu / (1 - 2\nu) \), and \( \nu \) is Poisson’s ratio in the limit of small strain (Blatz and Ko, 1962). In addition, \( I^* = \text{tr} C^* \) is the first strain invariant. The shear modulus was set to \( \mu = 220 \) Pa, and we set the Poisson ratio to \( \nu = 0.45 \) (Xu et al, 2010). For simplicity, a force was applied to the top of the brain with no growth in the model.

The geometry of the brain tube cross-section was initially defined as the right half of a circle with a normalized outer radius of 1 and inner radius of 0.6. Rollers were applied at the boundaries through the center of the circle. A downward pressure was applied to a small boundary region at the top of the brain (10 degrees of the outer circle), and a small segment of the bottom edge of the brain was fixed. Four ellipses (circularity of 0.5) with the same properties as the brain were placed inside the brain wall to simulate nuclei and observe their shape changes. The nucleus in an undifferentiated, embryonic cell has the same stiffness as the remainder of the cell (Pajerowski et al, 2007). Stress was plotted qualitatively to view compression and tension in the brain after loading.
4.4 Results

4.4.1 Imaging of Compressed Chick Brains

In control embryos, the lumen of the brain tube was visible in OCT cross-sections of the forebrain (FB), midbrain (MB), and hindbrain (HB), as shown for stages HH10–H12 (Fig. 4.1A–C”). In previous work, Filas et al (2011) used surface tension at the fluid-brain interface to compress the fully isolated brain until the lumen was completely closed. With the iron-agar bar method of compression presented in this study, a mechanical load can be applied locally or globally by placing the agar bar in specified locations, with a magnet beneath the dish pulling the iron particles embedded in the agar downwards. Since the iron particles are scattered within the agar bar, the embryo is still visible under brightfield microscopy without the magnet (Fig. 4.1D,D’,E).

With OCT imaging, the agar was fairly transparent while iron particles appeared as bright, white dots. OCT imaging also revealed the lumen only collapsed where the agar bar was placed, whether it spanned the whole brain or only the hindbrain (Fig. 4.1F,G). The magnet held the agar bars firmly in place, allowing embryos to be moved between microscope and incubator without significant movement of the bar.

4.4.2 Nuclear Shape Changes in Response to Compressing the Hindbrain

Previous work showed that compressing the whole brain tube induces nuclear rounding (i.e., an increase in circularity) (Filas et al, 2011), as the contractility of the brain decreased. In contrast, unloading the brain leads to enhanced contraction as nuclei become more elongated (Filas et al, 2011). Here, we confirmed that compressing the brain tube with an agar bar also induced nuclear shape rounding. Hindbrain cross-sections were examined with DAPI staining for several
Figure 4.1: Brain tube morphology and compressive loading. (A-C’’) Early brain tube geometry (HH10, HH11, and HH12) and transverse cross-sections through the forebrain (FB, red dashed line), midbrain (MB, orange dashed line), and hindbrain (HB, blue-dashed line) (A, B, C) Images from brightfield microscopy with dashed lines corresponding to (A’-A’’, B’-B’’, C’-C’’) OCT cross-sections. (D) Brains were isolated by removing surrounding tissues while leaving posterior tissues intact. (D’) HH10 embryo seen through large iron-agar bar used for compressive loading. (E) Different HH10 embryo with narrow agar bar. (F) Longitudinal OCT cross-section showing complete compression of embryo in (D’) when a magnet is placed below the dish. (G) Longitudinal OCT cross-section of embryo in (E) locally compressed in the HB. Scale bars: 100µm.
conditions: control non-dissected embryos, dissected brains immediately after compression, and dissected brains after 3 hours of compression in control media or media containing Cbx (Fig. 4.2).

With confocal microscopy, images of the brain cross-section showed nuclei elongated radially through the wall with actin strongly localized to the apical (inner) surface (Fig. 4.2A).

Nuclear circularity was measured in at least three embryos per loading condition, and data was plotted in histograms, each with a lognormal PDF fit. In Figure 4.2(A’, B’, C’, D’), the PDF fits were scaled by the area under each histogram to overlay the plots. The circularity data did not fit a normal distribution as well as a lognormal distribution due to the wider tail on the right side of the histograms. The circularity fits are plotted together in Figure 4.3A, and the data are summarized in a table in Figure 4.3B.

In control embryos, the nuclei had an average circularity of 0.46 with the smallest standard deviation across the groups (as seen by the narrower PDF in Figure 4.3A). Immediately after compression (t0), nuclei were slightly more elliptical, and the circularity fit became shifted towards the left (towards lower circularity, average of 0.42). Culturing compressed brains for 3 hours (t3) produced more rounded nuclei with greater variance in shape. The average circularity, 0.51, was 21% higher than embryos fixed immediately after compression. One-way ANOVA on ranks followed by Dunn’s test suggested statistically significant differences between almost all test cases in Figure 4.3A (P<0.001) except for the compressed t0 and Cbx t3 groups. Due to the large sample sizes in each group (N>400 nuclei), these tests for significance tend to overpredict meaningful differences. Effect size (d), which is independent of sample size, was also calculated (Fig. 4.3C, eq. 4.1) and suggests small differences between controls and all other culture conditions (d < 0.40). Nuclear circularity in initially compressed embryos was similar to nuclear circularity in compressed brains cultured with Cbx for 3 hours (d = 0.13). There were moderate effects (d = 0.66) between compressed embryos at t0 and t3 with nuclei becoming rounder after culturing. Cbx was able to
Figure 4.2: Changes in nuclear shape in brains subjected to compressive loading. (A, B, C, D) Representative cross-sections of embryos in control and different loading conditions: non-dissected control case, compressed and immediately fixed, compressed and cultured for 3 hours, and compressed embryos exposed to 200\(\mu\)M carbenoxolone (Cbx, gap junction inhibitor) for 3 hours. Nuclei (cyan) were imaged with confocal microscopy. (A) F-actin (red) is localized to the apical lumen as shown in a control HH12 embryo. (A', B', C', D') Histograms and lognormal curve fits for nuclear circularity (short axis / long axis). At least 3 embryos were analyzed in each test condition. Scale bars: 100\(\mu\)m.
prevent this rounding with a moderate effect size \( (d = 0.53, \) a difference in means of approximately half the pooled standard deviation).

### 4.4.3 Stress-Dependent Variations in Nuclear Shape across the Brain Wall

We also evaluated nuclei based on position in the brain wall. Compression of the brain tube would cause uneven stress distributions across the wall, especially at the two sharp corners that form. We demonstrated this with a simple cylinder model where a circular brain tube was compressed with a boundary load (Fig. 4.4A–C). The circumferential stress plot (Fig. 4.4C) shows compression along the apical half and tension along the basal half of the walls near the corners. We quantified nuclei circularity in HB sections where staining in the corner regions was clearly visible across the wall; this decreased sample size in these data sets considerably (Fig. 4.4G, \( N \leq 70 \) nuclei per group). Figure 4.4(D,E) shows a closer view of nuclei staining in two brains immediately after compression and 3 hours after compression during culture, with the apical and basal regions of the wall marked in the corner of the HB.

Circularity values were fit to lognormal PDFs as in Figure 4.2 for different test conditions and for apical (inner wall) versus basal (outer wall) positions (Fig. 4.4F). Data are summarized in a table in Fig. 4.4G. In embryos immediately fixed after compression (t0), the basal nuclei were rounder than apical nuclei with 11% higher circularity. This corresponds to stress differences expected across the wall, since apical nuclei would experience circumferential compression along the short axis and become less circular while basal nuclei would have an expanded short axis due to tension. However, this was not statistically significant as tested by Dunn’s test after ANOVA on ranks. Culturing compressed embryos for 3 hours eliminated these stress-dependent differences. The nuclei remodeled to become rounder in the reverse directions; apical nuclei were 6.5% rounder than basal nuclei (not significant) and 40% rounder than apical nuclei in the t0 case \( (P < 0.001) \).
Figure 4.3: Summary of nuclear shape data. (A) Comparison of circularity curve fits for different media conditions (as plotted in histograms in Fig. 4.2). (B) Table summarizing nuclear shape data shown in (A). One-way ANOVA on ranks followed by Dunn’s test determined statistically significant differences between all groups \((P < 0.001)\) except for Compressed \(t_0\) versus Cbx \(t_3\) (not significant). However, large sample sizes lead to overestimated differences in these statistical tests, so (C) effect size was also calculated (see eq. 4.1). Values over 0.5 (bolded in table) indicate a difference in means over half the pooled standard deviation and suggest moderate differences between the groups. Effect sizes below 0.5 indicate small effects; Compressed \(t_0\) versus Cbx \(t_3\) had the smallest difference.
Figure 4.4: Stress effects through the brain tube wall. (A) Cylinder model of undeformed cross-section with randomly placed nuclei. (B) Compression of the brain tube by local pressure load (red arrow). (C) Circumferential stress showing compression in the inner wall of the flattened brain corners (blue) and tension in the outer wall of the brain (yellow and red). (D) Nuclei in HB fixed immediately after compression and (E) in HB fixed 3 hours after compression and culturing. (F) Nuclear shapes were analyzed in the left and right corners of compressed brains (HB). Regions are indicated with white lines in (D, E). (G) Table summarizing data in (F). Statistical significance between groups was tested with ANOVA on ranks followed by Dunn’s test (* indicates $P < 0.001$). Mean circularity increased with time in untreated brains but remained nearly constant in brains exposed to gap junction blocker (Cbx). Scale bar: 50µm.

Treatment with Cbx was able to prevent most of the observed nuclear rounding and prevented apical nuclei from becoming rounder than basal nuclei (basal nuclei were approximately 2% rounder than apical nuclei; no statistically significant difference).
4.4.4 Validating Gap Junction Inhibition and 2D Analysis of 3D Nuclear Geometry

The inhibition of gap junctions with Cbx was tested with the fluorescent dye LY in cutting experiments similar to the scrape loading technique. Control and Cbx treated embryos were cut in the presence of LY to incorporate the small molecule dye in cells next to the cut. The dye was imaged under fluorescence microscopy at multiple incubation time points. In control embryos, the dye diffused away from the cut as seen with reduced staining (Fig. 4.5). In Cbx treated embryos, LY remained near the cuts much longer than in controls suggesting the successful inhibition of gap junctions needed to transport the dye between cells. Future work could quantify this transport with confocal microscopy.

Our 2D circularity measurements were validated by comparing 2D metrics of circularity with 3D measures of ’sphericity’. Although nuclei in controls are known to be oriented radially, outward from the lumen (Filas et al, 2011), we measured 3D nuclear shape in treated embryos to determine if 2D measurements were excluding important out-of-plane effects. Stacks of high-resolution confocal images were analyzed in two extreme cases: an isolated brain cultured with 40nM calyculin A (a myosin II enhancer), and an isolated brain treated with 60µM blebbistatin (a myosin II inhibitor). Both embryos were cultured for 3 hours. As shown in 2D in Filas et al (2011), calyculin A caused more elongated nuclei while blebbistatin induced rounding of nuclei. This trend was apparent in 3D visualizations of the nuclei as well (Fig. 4.6A,B).

Fractional anisotropy (FA) was measured for individual nuclei by fitting each shape to an ellipsoid in ImageJ, which returned three axes for each ellipsoid (λ₁, λ₂, and λ₃). The 3D metric, FA, was compared to 2D circularity in two ways: using the ellipsoid fit values (λ₃/λ₁) (to test if 2 axes can capture most of the 3D variation in shape) or manually measuring circularity in 2D images of the nuclei (to test if confocal images reflect 3D geometry). FA and circularity were inversely
Figure 4.5: Effects of blocking gap junctions on diffusion in the brain tube. (A) Embryos were immersed in control media with 0.025% Lucifer yellow (LY) or (D) media with LY and 200µM carbenoxolone (Cbx), then cut (red dashed lines, arrows) with microdissection scissors similar to a 3D scrape loading test (cuts indicated by dashed lines or arrows). LY is incorporated into cut cells and begins to diffuse to neighboring cells (brightfield images). (B, E) About 2 min after cutting, LY was visible under fluorescence microscopy near the cuts, and faintly in the brain lumen and mesenchyme. (C) After 30 min, LY was only faintly visible near the cuts in control embryos due to diffusion from cells near the cut. (F) In embryos treated with Cbx, LY remained near the cuts even after 45 min, suggesting successful blocking of gap junctions.

related, since a perfect sphere would have values of 0 and 1 with these metrics respectively. Using the ellipsoid fit for both the FA and circularity measurements correlated very well, especially for the calyculin A case (Fig. 4.6A’, $R^2 = 0.97$) where nuclei were strongly elongated in the radial direction. 2D measurements of circularity correlated to FA values in the blebbistatin treated brain although with a lower $R^2$ value (0.56). These measurements indicate that 2D measurements generally reflect 3D nuclear geometry.

### 4.4.5 Extending Loading Technique to Other Model Organisms

The utility of our iron-agar loading technique for other model organisms was considered by attempting to compress zebrafish. Zebrafish embryos (1 dpf) were provided by the Solnica-Krezel
Figure 4.6: 2D circularity correlates well with 3D nuclear geometry. To verify if 2D circularity measurements reflect 3D nuclear geometry well, stacks of confocal images were analyzed for two extreme nuclei geometries: (A) HH12- isolated brain cultured with 40nM calyculin A (myosin enhancer) for 3 hours, and (B) HH11 isolated brain cultured with 60µM blebbistatin (myosin inhibitor) for 3 hours. (A) 3D view of DAPI staining in ImageJ 3D Viewer before manual tracing. (B) 3D view of nuclei after manually tracing from DAPI staining in blebbistatin case. Individual nuclei were fit to an ellipsoid to calculate the 3 axes $\lambda_1$, $\lambda_2$, and $\lambda_3$. All 3 axes were used to calculate fractional anisotropy, a measure of how elongated or spherical (value of 0) the ellipsoid is. (A’) Calyculin A nuclei were more elongated than (B’) blebbistatin nuclei. (A’) Measuring circularity from the ellipsoid fit parameters ($\lambda_3/\lambda_1$) correlated very well (inversely) with fractional anisotropy in calyculin A treated nuclei ($R^2 = 0.97$). This suggests $\lambda_1$ and $\lambda_3$ can be used to describe most of the ellipsoid geometry, and $\lambda_2$ has minor effects on fractional anisotropy. (B’) For the blebbistatin test case, circularity was measured manually from 2D confocal images to determine if 2D cross-sections produce similar trends as 3D data. Circularity correlated fairly well to fractional anisotropy with a decreasing linear fit ($R^2 = 0.56$). These test cases suggest 2D circularity accurately reflects 3D nuclear geometry.
Figure 4.7: Compressive loading technique applied to zebrafish. (A) Brightfield image of 4 zebrafish embryos (1 dpf). The left two have the outer membrane (chorion) removed. (B) Top-down OCT image of zebrafish embryo with thin agar bar over the posterior region. (B’) Longitudinal cross-section through red line shown in (B) showing local compression of the zebrafish with an agar bar and magnet below the dish. (C) Brightfield image of embryo in (B, B’) after bar was removed. Yolk sac extension near the notochord remained compressed (red arrow points to small indent). Scale bar: 200µm.

The laboratory at the Washington University School of Medicine. Embryos were immobilized with tricaine, had the outer membrane removed with forceps, and then imaged with brightfield microscopy and the OCT system (Fig. 4.7). The zebrafish embryos were clearly visible with OCT imaging. Iron-agar bars were placed over the posterior portion of the embryo and lowered with a magnet as in the chick embryo experiments. The agar bars were able to compress the zebrafish, although this caused some irreversible compression to the yolk sac extension in one embryo (Fig. 4.7C).

4.5 Discussion

Our results indicate that a new technique for compressing regions of the chick embryo can induce nuclear shape changes and remodeling in the brain. A previous study by Filas et al (2011) showed that compression with high surface tension causes nuclear rounding and tissue softening as contractility decreased. Inversely, reducing loads with no surface tension caused nuclear elongation.
and tissue stiffening with increased contraction. Here, we showed that some of these contractility effects rely on a small molecular signal that is transported between cells through gap junctions. Additionally, nuclear shape changes immediately after compression partially reflected the uneven stress distribution in the brain wall; this effect was reduced with gap junction inhibition. Future work may elucidate which molecular signal (or signals) is crucial to these responses.

Compressing the brain locally in the chick embryo with an iron-agar bar flattened the lumen directly beneath the agar as seen with OCT imaging (Fig. 4.1G). This provides a method to study regional differences across the brain vesicles in future studies. Here, the hindbrain was compressed in multiple test conditions, and nuclear shape was analyzed. The distribution of nuclear circularity was plotted for controls, embryos compressed and immediately fixed, and embryos cultured while under compression in control media or media containing the gap junction blocker Cbx. Isolated brains that were compressed and immediately examined for nuclear morphology had more elliptical nuclei than controls (Fig. 4.3A, B). However, when cultured for 3 hours, the nuclei became rounder than controls.

Lognormal probability distribution functions were fit to the circularity data to visualize variance in the nuclei population. Control embryos had more narrow curves (smaller standard deviation) than embryos cultured for 3 hours with wider lognormal curves. This suggests that while nuclei on average became rounder, the remodeling did not occur uniformly; otherwise, the curve would have simply shifted to the right in Figure 4.3A. Treatment with the gap junction inhibitor Cbx inhibited this remodeling during embryo culture. The circularity distribution curves for immediate compression and 3 hour compression with Cbx were almost identical (Fig. 4.3A).

Compressing the brain causes sharp bending at the corners of the flattened brain (Fig. 4.2B, C, D). To simulate the immediate effects of this flattening and visualize stress in the wall, a cylinder model of the brain was compressed (Fig. 4.4A, B, C). The brain was modeled as an isotropic tissue with equivalent elliptical shapes dispersed through the wall to simulate a few nuclei. After
flattening, strain in the brain tube was variable but mostly compressive (data not shown). Stress in the corners of the bent wall varied dramatically from compressive along the inner (apical) half and tensile along the outer (basal) half of the wall (Fig. 4.4C).

Nuclear shapes in the model partially reflected this uneven stress distribution. For example, the maroon ellipse in the outer edge of the corner (Fig. 4.4C) became slightly rounder than the more apical (pink) ellipse due to tension along the outer wall. This small difference was also observed in the chick brain immediately after compression with more elongated nuclei positioned along the apical surface. After three hours of culturing compressed brains, apical nuclei were 40% rounder than those in immediately compressed brains (Fig. 4.4G). Basal nuclei became rounder but not as dramatically (18% increase in circularity). This discrepancy in remodeling across the wall could suggest that nuclei respond more dramatically to compression than tension; however, more detailed studies where stress could be quantified across the brain would be needed. Interkinetic nuclear migration may also affect comparisons of apical and basal nuclei as nuclei move apico-basally based on the cell cycle (Guthrie et al, 1991). In actin staining, round dividing cells were located along the apical surface of the brain wall. Inhibiting proliferation with a chemical such as aphidicolin would help separate the effects of nuclear rounding caused by stress and not mitosis (Davidson et al, 1995).

The ability of Cbx to block small molecular transfer through gap junctions was tested with a technique similar to scrape loading. Cutting the brain with small scissors trapped the fluorescent dye LY in cells near the cuts. In controls, the dye faded away from these cells whereas Cbx treated embryos maintained LY fluorescence near the cuts (Fig. 4.5). This suggests Cbx inhibited intercellular transport of small molecules. Future work could quantify the speed of gap junction transport in the chick brain and quantify the effectiveness of Cbx. Which molecule triggers nuclear remodeling in the chick brain, however, remains unknown.
Our present study provides interesting results but raises several questions about mechanotransduction in the chick brain. For example, why are gap junctions essential to nuclear remodeling? Intercellular communication may be used to bring the tissue toward an average homeostatic stress state (Filas et al, 2011; Chien, 2007) instead of each nucleus responding to its individual, local environment. Gap junctions provide direct communication between neighboring cells by allowing direct passage of small molecules between the cells. Small molecules less than 1000 Da such as ATP, glucose, calcium, or triphosphate inositol can pass through gap junctions (Abbaci et al, 2008). Gap junctions are regulated by voltage, phosphorylation, ECM, and other signals. These channels can also be inhibited with licorice plant derivatives or carbenoxolone (Cbx) used in this study; Cbx has been shown to block up to 80% of gap junction function (Rozental et al, 2001).

Future work could evaluate calcium signaling as a possible regulator of nuclei remodeling in the compressed chick brain. Intracellular calcium concentrations can vary rapidly from transport through the cytoplasmic membrane or release from intracellular reservoirs (Liu and Lee, 2014). Calcium signaling has been studied in response to compression, tension, and shear stress (Liu and Lee, 2014). Chemicals such as the calcium chelator BAPTA (Liu et al, 2010) could be used to block calcium signaling in the chick brain. Live imaging of calcium waves is also possible with fluorescent chemicals such as Fluo-4 AM (Liu et al, 2010). Cbx in our study may have also affected ATP, another signal associated with contraction. Adding ATP to permeabilized chick brains has been shown to induce rapid contraction of the brain (Filas et al, 2012). Compressing control chick brains, however, reduced contractility and stiffness (Filas et al, 2011); therefore we predict the mechanotransduction signal to be able to directly inhibit contraction and not enhance contraction as ATP does.

In conclusion, our study demonstrated the importance of gap junctions for nuclei to respond to stress perturbations in the chick brain. We also validated our 2D measurements with comparisons to 3D nuclear shape metrics (Fig. 4.6). Our iron-agar bar method was able to compress both chick
and zebrafish embryos (Fig. 4.7). Therefore, our experiments may be repeatable in a model organism more suited for genetic manipulations such as the mouse or zebrafish. For example, repeating the loading experiments in mice with knockouts of nuclear envelope proteins, such as lamin a/c (Fedorchak et al., 2014), could provide clues for how the nucleus changes shape in response to mechanical loading.
Chapter 5

Conclusion

In this work, we investigated the biophysical mechanisms involved in early eye development in the chick embryo using experimental and computational techniques. We also studied mechanical feedback in the early brain using a novel loading technique. Here we summarize our main results and consider future directions for research.

5.1 Early retina morphogenesis

In Chapter 2, we studied the role of extracellular matrix (ECM) in optic vesicle (OV) invagination. Removing the surface ectoderm (SE) at early stages of invagination caused the concave OV to ‘pop out’ and become convex. At later stages of invagination, the OV remained concave without the adjacent SE tissue. Degrading the ECM caused the earlier stages of the remaining concave OVs to pop out as well. Our computational models suggest that the ECM restricts the OV as it grows, causing the OV to bend inward and form the optic cup. Our model reproduced these popping out effects. These results support the hypothesis that the ECM restricts the OV to drive retinal placode and optic cup (OC) formation.
Our results suggest the ECM between the OV and SE has a gradient in stiffness, with greater stiffness at the center of the OV. The spatial properties of the ECM could be characterized in detail in future studies with techniques such as atomic force microscopy (Ludwig et al, 2008). Greater ECM stiffness at the center of the OV would restrict expansion of the center cells more than outer cells of the OV. Therefore, strain experiments could be compared to model results to elucidate the regional effects of the ECM. Here, we did not investigate the possible effects of contraction on OC formation, since actin is localized on the outer curvature of the bending OV. However, apical contraction may help refine the shape of the early retina such as the sharp bend or hinge between the inner and outer layer of the optic cup.

5.2 Early lens morphogenesis

In Chapter 3, we studied the role of ECM and actomyosin contraction in early lens development. As in the OV, the ECM helps restrict the SE to form the lens placode. Apical contraction then drives invagination of the SE; however, our experiments and models indicate that apical constriction is not sufficient to close the lens pit to form the lens vesicle (LV). We hypothesize that apoptosis at the edge of the lens pit provides the mechanical force to close the LV. Computational modeling and experiments with apoptotic inhibition support this hypothesis.

Our model of lens development assumes a multi-step process of ECM restriction, actomyosin contraction, and apoptosis as the lens grows. Our results show that apoptosis occurs at a faster rate than proliferation; however, the temporal and spatial distribution of cell death remains to be characterized. Additionally, variations in cell cycle across stages of eye development could affect the shaping of the LV. Therefore, future work could investigate apoptosis and proliferation in greater temporal and spatial detail. Apoptosis may also trigger local, cell-sized purse strings as
cells are extruded from the epithelium (Rosenblatt et al, 2001). Future studies will be needed to determine the interactions between contraction and apoptosis in the closing LV.

5.3 Mechanical feedback in the brain tube

Finally, in Chapter 4, we investigated the role of mechanical feedback in regulating contractility during compression of the early brain tube. We described a novel technique to locally or globally load the brain. Compressing the brain caused normally elongated nuclei to become rounder, which previous work suggests corresponds to reduced contraction (Filas et al, 2011). Our results with a gap junction inhibitor suggest that this nuclear shape change depends on a molecular signal that diffuses through the wall to inhibit contraction. Additional research will be needed to determine which molecular signal is crucial to this process.

Our novel loading technique was applied to the forebrain (including the OVs), midbrain, and hindbrain. Here, we focused on compression of the hindbrain; however, future studies could investigate the regional effects of compressing other parts of the brain tube. Our measurements suggest the immediate changes in nuclear shape depend on local stress distributions. Quantifying the local stress in loaded brains could be used to directly compare stress and mechanical feedback in the brain. Future work could also investigate the effects of compressing the brain on gene expression and morphogenesis. Our results lay the groundwork for further investigations of mechanotransduction and morphogenesis in the brain and eye.


McKeehan MS (1958) Induction of portions of the chick lens without contact with the optic cup. The Anatomical Record 132(3):297–305


Spemann H (1901) Über korrelationen in der entwicklung des auges. Verhandlungen der Anatomischen Gesellschaft 15:61–79


Appendix: Numerical Convergence for Shell Buckling

As described in Chapters 2 and 3, to simulate optic cup formation and lens development we created axisymmetric finite-element models using COMSOL Multiphysics (Version 4.2a, COMSOL AB, Providence, RI, USA). While the mechanical properties of the optic vesicle (OV) and surface ectoderm (SE) were detailed in previous chapters, the solver settings in COMSOL were also important to reach final time points in the models. Snap-through buckling in the transition from convex to concave curvature was particularly challenging. Here, these model settings are detailed to indicate which settings led to greater convergence in our models.

Both the OV and SE were modeled as portions of spherical shells (see Fig. 2.9A and 3.4D). The OV model geometry was discretized into triangular mesh elements, while the SE model was discretized into mapped elements. The choice of mesh elements did not appear to have a significant effect on convergence.

For the OV model, the study time and time stepping settings had large effects on the final time point reached for a converged solution. The initial invagination or buckling of the model was often a stopping point for the model where convergence could not be reached. COMSOL includes two time-dependent solvers for approximating solutions to a system of equations: the BDF and generalized-alpha solvers. With the BDF time stepping method and strict or intermediate time stepping, the final OV time point of $t = 6$ was reached (Fig. 5.1A’). The generalized alpha solver
Figure 5.1: Computational models of (A, A’) OV formation and (B) LV formation. (A) The OV model with uniform extracellular matrix (ECM) stiffness with various study settings often did not converge past $t = 0.5$. At $t = 0.5$, the inner OV is a flat placode before becoming concave. (A’) Final time point, $t = 6$, for OV invagination model. (B) Final time point, $t = 10.93$, for the early lens model.

was not able to converge past $t = 0.5$ at which point the retinal placode is flat and about to become concave (Fig. 5.1A). According to COMSOL documentation, the BDF solver uses backward differentiation formulas with strong damping effects. The generalized alpha method includes less damping but is considered less stable.

For the lens model, the BDF solver with strict steps provided the greatest convergence (Fig. 5.1B, $t = 10.93$), but the model required the Jacobian matrix to be updated on every iteration instead of minimally as in the OV model. Updating the Jacobian more frequently in the time-dependent solver adds computation time but can improve convergence. With minimal updates to the Jacobian, the SE model does not converge past $t = 3.55$. 

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The study ‘Times’ setting allows choosing the range of output times for the model and affects convergence in the OV model. With steps of 0.025 between output times, the model only reached $t = 0.5$. Steps of 0.01 allowed the model to reach $t = 5.48$, and with Jacobian updates at every iteration, the model reached $t = 6$. The ‘Times’ setting with a change in step size over time (0.01 until $t = 0.4$, then 0.025 after) was also able to converge at $t = 6$ with minimal Jacobian updates.

Other techniques to improve convergence in the finite-element models could have also been used. For example, the sudden drop in stiffness ($\mu$, shear modulus) across the ECM to OV or ECM to SE boundary could be changed to a continuous decline to possibly reduce extreme deformation of local, boundary elements. Our computational models were able to simulate both OV and SE invagination while corresponding fairly well to experimental data (Fig. 2.11 and 3.7).
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August 2016