Biophysical Mechanisms of Early Heart Morphogenesis

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Biophysical Mechanisms of Early Heart Morphogenesis

by

Yunfei Shi

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

August 2014
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Contents

List of Figures ................................................................. v
Acknowledgments .............................................................. vii
Abstract ............................................................................ x

1 Introduction ...................................................................... 1
  1.1 Formation of the heart tube ............................................ 4
  1.2 Cardiac c-looping ...................................................... 7
  1.3 Synopsis of dissertation .............................................. 12

2 Why Is Cytoskeletal Contraction Required for Cardiac Fusion Before but not
   After Looping Begins? ..................................................... 19
  2.1 Summary ................................................................... 19
  2.2 Introduction ............................................................ 20
  2.3 Experimental methods .............................................. 23
    2.3.1 Embryo preparation and culture ............................. 23
    2.3.2 Optical coherence tomography .............................. 23
    2.3.3 Stiffness measurements ..................................... 24
    2.3.4 Visualizing microindentation with OCT ................. 24
    2.3.5 Strain measurements ......................................... 25
    2.3.6 Stress measurements ......................................... 26
    2.3.7 Statistical analysis ........................................... 26
  2.4 Computational methods ............................................. 27
    2.4.1 Theory for modeling morphogenesis ..................... 27
    2.4.2 Models for microindentation ................................ 27
    2.4.3 OV model with endodermal contraction ................. 30
  2.5 Experimental results ................................................ 32
    2.5.1 Stiffness around AIP .......................................... 33
    2.5.2 Tissue movements and deformation around AIP .......... 36
    2.5.3 Tissue stress around AIP ...................................... 38
  2.6 Computational results .............................................. 41
    2.6.1 Models for microindentation around AIP ............... 41
    2.6.2 OV model with endodermal contraction ................. 43
  2.7 Discussion ............................................................. 45
    2.7.1 Tissue mechanics around AIP .............................. 46
### 3 Bending of the Looping Heart: Differential Growth Revisited

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Summary</td>
<td>57</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>58</td>
</tr>
<tr>
<td>3.3 Background</td>
<td>60</td>
</tr>
<tr>
<td>3.3.1 Structure of the early heart tube</td>
<td>60</td>
</tr>
<tr>
<td>3.3.2 Possible bending mechanisms during c-looping</td>
<td>60</td>
</tr>
<tr>
<td>3.4 Experimental methods</td>
<td>64</td>
</tr>
<tr>
<td>3.4.1 Preparation and culture of the embryonic heart</td>
<td>64</td>
</tr>
<tr>
<td>3.4.2 Optical coherence tomography</td>
<td>65</td>
</tr>
<tr>
<td>3.4.3 Strain measurements</td>
<td>65</td>
</tr>
<tr>
<td>3.4.4 Stress measurements</td>
<td>66</td>
</tr>
<tr>
<td>3.4.5 Statistical analysis</td>
<td>66</td>
</tr>
<tr>
<td>3.5 Computational methods</td>
<td>67</td>
</tr>
<tr>
<td>3.5.1 Modeling of morphogenesis</td>
<td>67</td>
</tr>
<tr>
<td>3.5.2 Cylindrical model for heart bending</td>
<td>68</td>
</tr>
<tr>
<td>3.5.3 Material properties</td>
<td>70</td>
</tr>
<tr>
<td>3.5.4 Looping simulation</td>
<td>71</td>
</tr>
<tr>
<td>3.5.5 Model based on realistic heart geometry</td>
<td>77</td>
</tr>
<tr>
<td>3.6 Experimental results</td>
<td>78</td>
</tr>
<tr>
<td>3.6.1 Perturbations of HT growth and bending</td>
<td>78</td>
</tr>
<tr>
<td>3.6.2 Morphogenetic strains</td>
<td>82</td>
</tr>
<tr>
<td>3.6.3 Myocardial stresses</td>
<td>83</td>
</tr>
<tr>
<td>3.7 Computational results</td>
<td>86</td>
</tr>
<tr>
<td>3.7.1 Single-mechanism models</td>
<td>86</td>
</tr>
<tr>
<td>3.7.2 Baseline model</td>
<td>90</td>
</tr>
<tr>
<td>3.7.3 Model based on realistic heart geometry</td>
<td>93</td>
</tr>
<tr>
<td>3.8 Discussion</td>
<td>94</td>
</tr>
<tr>
<td>3.8.1 Hypertrophic myocardial growth correlates with bending</td>
<td>95</td>
</tr>
<tr>
<td>3.8.2 Differential myocardial growth is the primary bending mechanism</td>
<td>96</td>
</tr>
<tr>
<td>3.8.3 Other mechanisms contribute to the bending process</td>
<td>97</td>
</tr>
<tr>
<td>3.8.4 Limitations</td>
<td>99</td>
</tr>
</tbody>
</table>

### 4 Bending and Twisting the Embryonic Heart: A Computational Model for C-Looping Based on Realistic Geometry

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Summary</td>
<td>106</td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>107</td>
</tr>
<tr>
<td>4.3 Background</td>
<td>108</td>
</tr>
<tr>
<td>4.4 Material and methods</td>
<td>111</td>
</tr>
<tr>
<td>4.4.1 Preparation and culture of the embryonic heart</td>
<td>111</td>
</tr>
<tr>
<td>4.4.2 Measurement of HT rotation</td>
<td>111</td>
</tr>
<tr>
<td>4.4.3 Computational model geometry</td>
<td>112</td>
</tr>
</tbody>
</table>
# List of Figures

1.1 Schematic of embryogenesis shown in *Xenopus* embryo. .................. 2
1.2 Mechanical forces play an important role during morphogenesis. .......... 3
1.3 Heart fields during early cardiac development of chick embryo. .......... 4
1.4 Heart tube (HT) formation shown in transverse cross section. .......... 5
1.5 Tissue movements along anterior intestinal portal (AIP) before mesodermal fusion begins. ................................................. 6
1.6 Cardiac looping in the chick embryo. ........................................ 8
1.7 Myocardial growth during c-looping. ........................................ 11
1.8 Biophysical mechanism for twisting of the HT during c-looping. ........ 11

2.1 Endodermal contraction and cardiac field fusion around AIP. ............ 21
2.2 Finite-element models for microindentation around AIP. .................. 29
2.3 Torus model for omphalomesenteric veins (OVs) with endodermal contraction. ................................................................. 31
2.4 Stiffness measurements around AIP. .......................................... 34
2.5 Cross sections around AIP obtained via optical coherence tomography (OCT). 35
2.6 Tissue movement and deformation around AIP. ................................. 37
2.7 Tissue stress around AIP. ....................................................... 39
2.8 Results from finite-element models for microindentation around AIP. .... 42
2.9 Results from finite-element model for OV with endodermal contraction. 44
2.10 Mechanical regulation around AIP. ........................................... 49

3.1 Cardiac c-looping in chick embryo. ............................................ 61
3.2 Schematic of configurations in computational modeling. .................... 67
3.3 Finite-element models for bending of isolated heart. ......................... 69
3.4 Bending of isolated hearts in different culture conditions. ................ 79
3.5 Short-term exposure to blebbistatin (Bleb) reveals effects of myocardial contraction in isolated hearts. ................................................. 79
3.6 Morphogenetic strains measured during bending of isolated heart. ........ 81
3.7 Changes in myocardial cross-sectional area and outer radius of isolated hearts cultured for 24 hr. .................................................. 82
3.8 Residual stresses in the myocardium as revealed by microsurgical cuts. 84
3.9 Immediate effects of heart isolation on myocardial stress. ................. 85
3.10 Single-mechanism cylinder models for bending of Bleb-treated heart. ... 87
3.11 Differential growth model for bending of control hearts. .................. 89
3.12 Baseline cylinder model for bending of isolated heart. ..................... 91
3.13 Model for bending of control heart based on realistic geometry.  

4.1 Primary mechanisms in model for c-looping.  
4.2 Finite-element model for embryonic heart.  
4.3 Stress distributions in baseline model for c-looping.  
4.4 Rotation of HT during c-looping.  
4.5 Time history of regional longitudinal strains in baseline model.  
4.6 Time history of regional circumferential strains in baseline model.  
4.7 Effects of inhibiting contraction or vein fusion.  
4.8 Effects of removing external constraints on HT.  
4.9 Effects of removing OVs or HT.  

A.1 Characterizing tissue deformation during microindentation using OCT.  
A.2 Effects of material moduli and endodermal contraction on strain and stress distributions from OV model.  
A.3 Effects of geometric dimensions on strain and stress distributions from OV model.  
A.4 Cell shapes and orientations in prelooped and c-looped chick heart.  
A.5 Effects of altering myocardial growth parameters in differential growth model for Bleb-treated heart.  
A.6 Parameter study for baseline cylinder model for control heart.  
A.7 Exponential form for the strain-energy density function does not significantly change the results of baseline model.  
A.8 Effects of inhibiting contraction before looping begins.  
A.9 Effects of elastic modulus of splanchnopleure on torsion of HT.  
A.10 Effects of growth in OVs on torsion of HT.
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Yunfei Shi

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This dissertation is dedicated to my beloved parents and mentors, who teach and inspire me every day to be a better man.
The heart is the first functioning organ in the developing embryo. Initially, the heart is a relatively straight tube created by folding and fusion of the cardiogenic fields, which lie bilaterally within the blastoderm. Shortly after formation, the primitive heart tube (HT) undergoes the morphogenetic process of c-looping as it bends and twists into a c-shaped tube. All these transformations require physical forces, which remain poorly understood.

The aim of this dissertation is to elucidate some of the biophysical mechanisms that create and shape the early HT. Our work involves a combination of ex ovo experiments and computational modeling. Experiments were performed on embryonic chicken hearts, which are morphologically similar to human hearts during development.

First, we explored a somewhat puzzling aspect of early heart development. Previous studies have shown that myosin-II-based cytoskeletal contraction is required for fusion of the heart fields before looping begins, but not as these tissues continue to fuse and extend the length of the HT during subsequent c-looping. To investigate this fundamental change in behavior, we focused on the tissues around the anterior intestinal portal (AIP), where fusion
takes place. Our results indicate that stiffness and tangential tension decreased bilaterally with distance from the embryonic midline along the AIP. The stiffness and tension gradients increased to peaks at Hamburger-Hamilton (HH) stage 9 and decreased immediately afterward. Along with experimental results of contraction inhibition, finite-element models indicate that the measured mechanical gradients are consistent with a relatively uniform contraction of the endoderm along the AIP. Taken together, these results suggest that, before looping begins at HH10, cytoskeletal contraction pulls the bilateral cardiogenic fields toward the midline where they begin to fuse to create the HT. By HH10, however, the fusion process is far enough along to enable apposing cardiac progenitor cells to subsequently undergo filopodia-mediated “zippering” without the continuing need for contraction.

Next, in light of recently published data, we examined the possible role of differential hypertrophic growth in driving the bending component of c-looping. Using cultured isolated hearts, which bend without the complicating effects of external loads, we found that myocardial growth patterns correlate with bending. We also developed finite-element models that include previously measured regional changes in myocardial growth during c-looping. The simulations show that differential growth alone can produce results that agree reasonably well with trends in our experimental data, including changes in HT morphology and tissue strains and stresses. Incorporating other mechanisms into the model, such as active changes in myocardial cell shape, provides closer agreement. These results suggest that regional difference in hypertrophic myocardial growth is the primary cause of the bending component of c-looping, with other mechanisms playing lesser roles.

Finally, we extended the model of the previous study to explore the physical plausibility of a hypothesis for the entire process of c-looping. According to our hypothesis, bending is driven primarily by differential hypertrophic growth in the myocardium, torsion is mainly caused by compressive loads exerted by the overlying splanchnopleuric membrane, and
looping direction is determined by asymmetric regional growth in the omphalomesenteric veins at the caudal end of the HT. Our model includes both bending and torsion of the HT, realistic 3D geometry, and loads exerted by neighboring tissues. The behavior of the model is in reasonable agreement with available experimental data from control and mechanically perturbed embryos, offering support for our hypothesis. The results also suggest, however, that several other mechanisms contribute secondarily to normal looping, and we speculate that these mechanisms play backup roles when looping is perturbed.

In summary, studies of this dissertation address several important questions during early cardiac development. The results should enrich our understanding of the underlying biophysical mechanisms.
Chapter 1

Introduction

In animals, embryonic development begins with a single zygote (Fig. 1.1). First, the zygote divides to give rise to a blastula (or blastocyst in mammals), which consists of the cellular blastoderm and an acellular cavity, called the blastocoel. The blastoderm then enters gastrulation, during which it is reorganized to form a gastrula containing (in most cases) three layers – ectoderm, mesoderm, and endoderm. The gastrula undergoes a series of morphogenetic events to create specialized tissues and organs. Ever since the era of Aristotle, we (as humans) have never ceased to wonder why nature creates life in this way (Aristotle, 1965; Varner, 2011).

Despite the rapidly growing knowledge of the genetic and molecular regulation of embryogenesis in the past decades, relatively little is known about the biophysical aspects. Obviously, the transformations during every phase of embryonic development require action of physical forces. As one of the most fundamental building blocks of cytoskeletal structures, actin provides many force-generating mechanisms for shaping the developing embryo, primarily through polymerization of its filamentous form and interactions with myosins (Fig. 1.2).

This dissertation focuses on the biomechanics of the early heart development. Here, after giving a brief background of early cardiac morphogenesis, we outline the studies in this dissertation.
Figure 1.1: **Schematic of embryogenesis shown in *Xenopus* embryo.** (Reprinted from Wozniak and Chen (2009).) Key developmental processes include proliferation of zygote, gastrulation, and tissue morphogenesis. Zygotic cell proliferation gives rise to a blastula. Through gastrulation, the blastula is transformed into a gastrula containing (in most organisms) three germ layers – mesoderm, ectoderm and endoderm. After gastrulation, the gastrula undergoes several morphogenetic events that give rise to specialized tissues and organs of the embryo.
Figure 1.2: **Mechanical forces play an important role during morphogenesis.** (A1–A3) Apical constriction (white arrows) in *Xenopus* bottle cells. (Reprinted from Lee and Harland (2007).) (A4) Schematic of the ratchet model of apical constriction. (Reprinted from Martin et al. (2009).) (B1,B2) Filopodia-mediated zippering during *Drosophila* dorsal closure. (Reprinted from Millard and Martin (2008).) (C1–C3) Increasing regional acto-myosin contraction (asterisks) using calyculin significantly changes the brain morphology of the chick embryo (F=forebrain, M=midbrain, H=hindbrain). As shown in cross sections, the original circular lumen of the stage 12- chick brain changes into diamond, triangular, and tear-drop shapes, which appear in the normal zebrafish embryo. (Reprinted from Filas et al. (2012).)
Figure 1.3: **Heart fields during early cardiac development of chick embryo.** (Modified from Abu-Issa and Kirby (2007).) (A) At Hamburger and Hamilton (HH) stage 5, the bilateral heart fields reside on either side of the embryonic midline. (A’) Enlarged left cardiogenic area in (A). The primary heart field (blue) gives rise to left ventricle (lv) and atria (a), whereas the anterior heart field (red) gives rise to conus (c), truncus (t), and right ventricle (rv). (B) During development, the bilateral heart fields move along the anterior intestinal portal (AIP) toward the embryonic midline, where they fuse to create the heart tube (HT). (C) Fused heart fields shown in HH11 heart, which has entered c-looping. The HT continues to lengthen by fusion of the omphalomesenteric veins (OVs).

### 1.1 Formation of the heart tube

In vertebrate embryos, the heart is the first mechanically functioning organ. For many reasons, the chick embryo has been a well established model organism to study cardiac development. Compared to other animal models, the chick embryo is relatively easy to harvest and incubate, and most importantly, development of the avian heart parallels that in human (DeHaan, 1967).

During the first day of development, the chick embryo is initially organized as a flat sheet of blastoderm (Patten, 1922; Hamburger and Hamilton, 1951; DeHaan, 1967). Among the three germ layers, the mesoderm gives rise to the embryonic heart. At Hamburger and Hamilton (HH) stage 5 (∼20 hr) (Hamburger and Hamilton, 1951), myocardial progenitor cells are organized as two separate heart fields that reside on bilateral sides of the primitive streak (Fig. 1.3A,A’) (Abu-Issa and Kirby, 2007).

During approximately the next 10 hr, the lateral mesoderm gradually splits into splanchnic and somatic layers, which are separated by the enlarging coelomic cavity (see phase 1 in 1.4) (Stalsberg and DeHaan, 1969; Linask, 1992; Abu-Issa and Kirby, 2007). Meanwhile,
Figure 1.4: **Heart tube formation shown in transverse cross section.** (Modified from van den Berg et al. (2009).) Between HH6 and HH8, the endoderm (green) contracts to bring the bilateral separate fields of cardiogenic mesoderm (red and blue) toward the embryonic midline (arrows in 2 and 3 indicate tissue motions). At about HH9, cardiogenic mesoderm begins to fuse to create the heart tube (HT) at the midline (arrowhead in 4 indicates the initial fusion site). Meanwhile, the splanchnopleure (SPL) and foregut (FG) are also forming. By HH10, a relatively straight HT has formed along the midline and is about to undergo subsequent c-looping. At HH11, the HT twists normally rightward, and the dorsal mesocardium (DM) ruptures (arrowhead in 5). Note that cell proliferation primarily occurs in the somatic mesoderm and DM (red), whereas relatively little proliferation occurs in the HT (blue). (CJ = cardiac jelly)
the bilateral heart fields, which are restricted to the splanchnic mesoderm, fold ventrally and medially from their respective sides and fuse at the ventral midline to create the heart tube (HT) (Figs. 1.3B,C and 1.4) (Abu-Issa and Kirby, 2007).

By HH10- (~33 hr), a relatively straight HT has formed (see phase 4 in Fig. 1.4). As soon as the primitive myocardium moves into its proper position, its component cells begin to secrete cardiac jelly (CJ, extracellular matrix), which is speculated to inflate the HT (De Jong et al., 1990). At this stage, the dorsal side of the HT remains open, and as a result, the cross section of the HT is U-shaped (Taber, 2006).

In addition, during the process of mesodermal fusion, part of the splanchnic mesoderm remains contacting the endoderm, and together, they form the splanchnopleuric membrane (SPL), which covers the ventral side of the HT (see phase 4 in Fig. 1.4) (Taber, 2006). Meanwhile, the primitive foregut forms underneath the somatic mesoderm. This process
is achieved by descending of an arch-like structure, called anterior intestinal portal (AIP), which serves as the entrance to the foregut (see Fig. 1.3B,C).

It has been well established that the endoderm is required for early cardiogenesis, as removal or dissection of the endoderm can cause abnormal heart development, such as cardia bifida (DeHaan, 1959; Nadal-Ginard and García, 1972). The endoderm is generally considered as an inductive substrate for the cardiogenic mesoderm (Nascone and Mercola, 1995; Schultheiss et al., 1995, 1997; Lough and Sugi, 2000; Alsan and Schultheiss, 2002). Recently, Varner and Taber (2012) found that the endoderm also actively contracts along the AIP to bring the bilateral heart fields toward the embryonic midline, where they fuse to form the HT (Fig. 1.5).

1.2 Cardiac c-looping

At HH10 (33–38 hr), the heart is a relatively straight tube located inside the pericardial cavity. The HT consists of three layers – a two-cell-thick outer layer of myocardium, a one-cell-thick inner layer of endocardium, and a relatively thick middle layer of CJ (Fig. 1.6A,B). The HT is connected cranially to the outflow tract (conotruncus), caudally to the omphalomesenteric veins (OVs, primitive atria), and dorsally to the dorsal mesocardium (DM), which attaches the entire length of the HT to the foregut wall. In addition, the SPL presses against the ventral side of the HT and wraps around the caudal sides of the OVs at the AIP (Männer, 2000; Taber, 2006; Männer, 2009). Shortly after formation of the HT, the first contraction occurs, and looping begins (Patten, 1922; Hiruma and Hirakow, 1985; Romanoff, 1960; Taber, 2006).

The entire looping process consists of two main phases – c-looping and s-looping (Männer, 2000; Taber, 2006; Männer, 2009).\(^1\) During c-looping (up to HH12), the initially straight HT gradually bends ventrally and twists normally rightward, transforming into a c-shaped tube (Fig. 1.6C). By HH12 (45–49 hr), the original ventral and dorsal sides of the straight HT have become the outer curvature (OC) and inner curvature (IC) of the curved tube.

\(^1\)In fact, researchers have different definitions for the division of cardiac looping, and the meaning of looping may differ from one literature to another. See Männer (2000) for detailed discussion. In this dissertation, since we focus on c-looping (from HH10 to HH12), the term looping is used synonymously with c-looping, unless stated otherwise.
Figure 1.6: **Cardiac looping in the chick embryo.** (A) Schematic of transverse cross-section of heart tube (HT) at HH10. (CJ = cardiac jelly, DM = dorsal mesocardium, EN = endocardium, FG = foregut, MY = myocardium, SPL = splanchnopleure; modified from Ramasubramanian et al. (2008).) (B,C) SEM images of c-looping heart. During c-looping, the relatively straight HT at HH10 (B) bends ventrally and twists normally rightward, as its original ventral and dorsal sides become the outer curvature (OC) and inner curvature (IC) of the curved tube, respectively, at HH12 (C). Torsion of the HT is indicated by artificial labels (yellow dots) placed along the ventral midline of the straight HT at HH10. (Arrows indicate edges of the removed SPL.) (AIP = anterior intestinal portal, AT = atrium, CT = conotruncus, OT = outflow tract, OV = omphalomesenteric vein, VE = ventricle) (D) Schematics of embryonic heart development. (Panels (B–D) are modified from Männer (2009).)
respectively (Männer, 2000; Taber, 2006). In addition, the DM begins to rupture after HH11, and the conotruncus begins to bulge near the end of c-looping (Taber et al., 1995; Männer, 2000; Taber, 2006). During s-looping (HH14 to HH18, 52–68 hr), the atrium moves cranially and becomes superior to the ventricle (Fig. 1.6D) (Taber, 2006). After the atrium and ventricle move to their final positions, the tubular heart is divided into four chambers through septation (Taber, 2006).

As cardiac looping represents the first major morphological sign of left-right asymmetry in the vertebrate embryo (Srivastava and Olson, 1997; Harvey, 1998) and abnormalities during this process can lead to congenital heart defects (Ramsdell, 2005; Männer, 2009), it has intrigued generations of developmental biologists (Patten, 1922; Stalsberg, 1969b; Levin et al., 1995; Harvey, 1998; Linask et al., 2002; Männer, 2004; Taber, 2006; Schulte et al., 2007). Despite decades of study, however, the biophysical aspect of looping has been overshadowed by the relatively rich understanding of the genetic and molecular mechanisms and remained poorly characterized (Patten, 1922; Stalsberg, 1970; Männer, 2000; Taber, 2006). One of the causes of the current situation is that many researchers did not appreciate the fact that c-looping consists of both bending and torsional components (Butler, 1952; Männer, 2000; Taber, 2006).

In a pioneering study, Patten (1922) attributed bending of the HT to buckling as the HT outgrows the allotted distance between its ends. This hypothesis, however, was contradicted later by the experimental finding that the HT cultured in isolation bends without constraints (Butler, 1952; Manning and McLachlan, 1990). This result indicates that bending is intrinsic to the HT. Other prominent bending mechanisms include differential growth of the myocardium (Stalsberg, 1969a), active changes in myocardial cell shape (Manasek et al., 1972; Latacha et al., 2005; Auman et al., 2007), differential contraction (Manasek et al., 1984a; Itasaki et al., 1991; Taber et al., 1995), dorsally constrained stretching as CJ swells and inflates the HT (Manasek et al., 1984b), as well as bending forces exerted on the HT by remnants of the DM after it ruptures (Taber et al., 1995). However, few of these hypotheses have survived the test of time. (See Section 3.3.2 for detailed discussion.)

It was generally thought that the embryonic heart grows primarily by cell hyperplasia (increase in cell number) before birth and by cell hypertrophy (increase in cell volume) after birth (Grossman, 1980). Differential myocardial growth was thus ruled out as a possible bending mechanism, when no clear mitotic patterns were found in the HT (Sissman, 1966;
Relatively recently, Soufan et al. (2006) found that during c-looping, while myocardial cells in the HT stay at a low proliferative state, the average cell volume increases significantly at the OC but remains the same at the IC (Fig. 1.7; see also HT in Fig. 1.4). This spatial pattern of hypertrophic growth (increase in cell volume) is qualitatively consistent with the observed bending deformation, suggesting that differential myocardial hypertrophic growth may play a role in causing the bending component of c-looping.

Although bending and twisting of the HT are coupled to some extent, studies have shown that the torsional component of c-looping is mainly caused by external forces exerted on the HT (Fig. 1.8). Over 60 years ago, Butler (1952) speculated that the left OV grows noticeably larger than the right OV, exerting a torque on the HT that causes normal rightward torsion (Fig. 1.8B,B′). Recent studies have shown that abnormal leftward looping can be produced by reducing the size of the left OV or increasing the size of the right OV (Voronov et al., 2004; Kidokoro et al., 2008; Ramasubramanian et al., 2008), providing support for the hypothesis of Butler (1952). Moreover, it has been shown that the SPL also plays an important role in causing torsion, as removal of the SPL significantly reduces or delays twisting of the HT (Voronov and Taber, 2002; Voronov et al., 2004; Ramasubramanian et al., 2008). These results suggest that looping direction is primarily determined by the vein forces exerted on the caudal end of the HT (Fig. 1.8B,B′) and torsion is enhanced by the compressive loads exerted by the SPL on the ventral side of the HT (Fig. 1.8C,C′). In addition, differential hyperplastic growth on the left and right sides of the DM (Linask et al., 2005), as well as differential cytoskeletal contraction on the caudal sides of the OVs (Voronov et al., 2004) may also contribute to torsion.

Taken together, the bending and torsional components of c-looping are caused by physical forces that are intrinsic and external to the HT, respectively. It is likely that multiple redundant mechanisms are involved in reducing the incidence of abnormal looping (Stalsberg, 1970; Taber, 2006).
Figure 1.7: **Myocardial growth during c-looping.** (Reprinted from Soufan et al. (2006).) Cell hypertrophy (increase in cell size) and hyperplasia (increase in cell number) of the myocardium are shown in reconstructed 3D geometry of c-looping hearts.

Figure 1.8: **Biophysical mechanism for twisting of the heart tube (HT) during c-looping.** (Reprinted from Ramasubramanian et al. (2008).) (A–C) C-looping heart shown in ventral view. (A′–C′) Transverse cross sections of embryonic heart. (A,A′) Relatively straight HT at HH10-. (B,B′) During c-looping, the left omphalomesenteric vein (OV) normally grows relatively larger than the right OV, pushing the HT slightly rightward at the caudal end. (C,C′) Then, the splanchnopleuric membrane (SPL) presses against the HT on the ventral side, enhancing the rightward torsion of the HT. (AIP = anterior intestinal portal, CJ = cardiac jelly, EN = endocardium, FG = foregut, MY = myocardium)
1.3 Synopsis of dissertation

Studying early heart development is very important. Cardiac looping represents the first major morphological sign of left-right asymmetry in the vertebrate embryo (Harvey, 1998; Männer, 2009). Abnormal development during these critical stages may underlie some of the congenital heart defects that occur in as many as 1% liveborn and 10% stillborn of human births (Srivastava and Olson, 1997; Ramsdell, 2005). In addition, understanding how tissues and organs are created during normal development is crucial to tissue engineering, which aims to mimic the physiological conditions for tissue constructs. Recent studies have shown that physical forces can regulate local gene expression during *Drosophila* development (Farge, 2003; Pouille et al., 2009).

The goal of this dissertation is to elucidate some of the biophysical mechanisms that create and shape the early HT.

In Chapter 2, we explore why myosin-II-based cytoskeletal contraction is required before but not after looping begins. Our results suggest that, before HH10, endodermal contraction is required for pulling the bilateral heart fields toward the midline where they begin to fuse to create the HT (see Fig. 1.5). By HH10, however, the fusion has proceeded far enough along to allow apposing cells to undergo filopodia-mediated “zippering” (see Fig. 1.2B1,B2) without the need for continued contraction.

In Chapter 3, we examine the role of differential hypertrophic growth in driving the bending component of c-looping. Our experimental data indicate that growth patterns in the myocardium correlate with bending. Results of our finite-element models suggest that differential myocardial hypertrophic growth is the primary driver of the bending component of c-looping, with other mechanisms playing secondary roles.

In Chapter 4, we present a 3D finite-element model for the c-looping HT that includes both bending and torsion, as well as realistic heart geometry. Results from this model support our newly proposed looping hypothesis, i.e., bending of the HT is driven primarily by differential myocardial hypertrophic growth, whereas torsion is mainly caused by external loads exerted by the SPL and OVs. Our results also suggest that other mechanisms may play backup roles when looping is perturbed.
Last, we summarize the conclusions of these studies and discuss future directions in Chapter 5.

In summary, the studies in this dissertation shed light on some of the important problems during early cardiac development. The results of these studies add new knowledge to the underlying biophysical mechanisms, fertilizing the field of research.
References


Chapter 2

Why Is Cytoskeletal Contraction Required for Cardiac Fusion Before but not After Looping Begins?²

2.1 Summary

Cytoskeletal contraction is crucial to many morphogenetic processes, but its role in early heart development is poorly understood. For example, studies in chick embryos have shown that inhibiting myosin-II-based contraction prior to Hamburger-Hamilton (HH) stage 10 impedes the fusion of cardiogenic mesoderm (heart fields) that creates the heart tube (HT), as well as the ensuing process of cardiac looping. If contraction is inhibited at or after HH10, however, fusion and looping proceed relatively normally. Here, we combine experiments and computational modeling to explore the mechanical reasons behind this seemingly fundamental change in behavior. First, we measured spatiotemporal distributions of tissue stiffness, strain, and stress around the anterior intestinal portal (AIP), where endodermal contraction and mesodermal fusion occur. The results indicate that stiffness and tangential tension decreased bilaterally with distance from the embryonic midline along the AIP. During development, the AIP stiffness gradient, tension gradient, and strain rates increased to peaks at HH9 and decreased afterward. Exposure to the myosin II inhibitor

²The OCT experiment and computer simulation of microindentation tests in Sections 2.3.4 and 2.4.2 were conducted by Victor Varner. Experimental data for stage HH8+/9- were included from Varner and Taber (2012).
blebbistatin reduced these effects, suggesting that they are mainly generated by active cytoskeletal contraction. Results from finite-element models indicate that the measured mechanical gradients are consistent with a relatively uniform contraction of the endoderm in conjunction with a gradient in passive mechanical properties and constraints imposed by the attached mesoderm. Taken together, our results suggest that, before HH10, endodermal contraction pulls the bilateral cardiogenic fields toward the midline where they begin to fuse to create the HT. By HH10, however, the fusion process is far enough along to enable apposing cardiac progenitor cells to subsequently undergo filopodia-mediated “zippering” without the continuing need for high contractile forces. These findings should shed new light on a perplexing question in early heart development.

2.2 Introduction

The heart is the first mechanically functioning organ in the vertebrate embryo. Similar to other organ primordia (Lubarsky and Krasnow, 2003), the embryonic heart emerges from a relatively simple cell sheet and forms as a 3D tube situated along the ventral side of the embryo (Stalsberg and DeHaan, 1969; Taber, 2006; Abu-Issa and Kirby, 2007). Shortly after being created, the initially straight heart tube (HT) undergoes cardiac looping, which transforms the HT into a curved tube, laying out the blueprint for the future four-chambered heart (Männer, 2000, 2004; Taber, 2006). Understanding how the HT is created and undergoes looping is of fundamental importance, as abnormalities during these vital processes can lead to congenital heart defects and spontaneous abortions during the first trimester (Srivastava and Olson, 1997; Ramsdell, 2005).

Given that avian heart development parallels that in humans (DeHaan, 1967), the chick embryo is well suited to studies of early cardiogenesis (Taber, 2006). During gastrulation, the early chick embryo is organized as a flat sheet of cells, consisting of three primary germ layers – endoderm, mesoderm, and ectoderm. The cardiac progenitor cells reside in the lateral mesodermal plate, where they form a pair of epithelia, called heart fields, situated on the bilateral sides of the embryonic midline (Fig. 2.1A,A’) (Stalsberg and DeHaan, 1969; Abu-Issa and Kirby, 2008). After the head fold forms at about Hamburger-Hamilton (HH) stage 6 (Hamburger and Hamilton, 1951), the initial planar geometry of the embryo is broken, and the cardiogenic mesoderm folds out of plane and moves toward the embryonic
midline. Then, the bilateral heart fields begin to fuse behind the anterior intestinal portal (AIP) at HH9 and gradually form the primitive HT and omphalomesenteric veins (OVs, primitive atria) (Fig. 2.1B,B′) (Moreno-Rodriguez et al., 2006; Cui et al., 2009; Varner and Taber, 2012).

Over the last few decades, researchers have identified many of the important genetic factors and biochemical signaling pathways involved in cardiac specification and differentiation (Brand, 2003). The mechanical forces that physically shape the HT, however, have remained poorly understood. Our recent experimental study shows that the endoderm that remains in close contact with the cardiogenic mesoderm plays an important mechanical role during formation of the HT (Varner and Taber, 2012). In addition to being inductive (Linask and Lash, 1986; Schultheiss et al., 1995), the endoderm actively contracts around
the AIP and pulls the fields of cardiogenic mesoderm toward the midline before HH9, enabling them to fuse and create the HT (Varner and Taber, 2012). Blocking endodermal contraction before HH9 by exposing the embryo to blebbistatin impedes the mesodermal movements and fusion (Varner and Taber, 2012). Moreover, stiffness and tension of the AIP tissue were found to decrease with distance from the embryonic midline, and we postulated that these mechanical gradients are likely responsible for driving the movements of the cardiogenic mesoderm (Varner and Taber, 2012).

After HH10, the HT loops as it continues to lengthen through fusion of what now are OVs at its caudal end. During the first phase of looping (c-looping), the HT bends ventrally and normally twists rightward (Männer, 2000; Voronov et al., 2004). Although differential contraction has long been speculated as one of the biophysical mechanisms that drive c-looping (Itasaki et al., 1991; Taber et al., 1995), our recent studies have shown that non-muscle myosin-II-based cytoskeletal contraction is not necessary for either c-looping or continued fusion of the OVs (Rémond et al., 2006; Rémond, 2006).

These recent findings prompted us to ask why contraction of the endoderm is required for normal cardiac morphogenesis before but not after HH10. Here, we explore this question by studying the biomechanics of the tissues around the AIP from HH8+ to HH11. Measurements revealed spatiotemporal variations in AIP stiffness, strain, and stress. Interestingly, AIP stiffness and tension gradients, as well as the endodermal shortening rate, peaked at HH9 and decreased afterward. Finite-element models showed that these results are consistent with a relatively uniform distribution of endodermal contraction around the AIP that peaks at HH9. From these data, we speculate that endodermal contraction pulls the cardiogenic mesoderm toward the midline prior to HH9, when fusion begins. After this time, however, fusion can continue through filopodial mediated “zippering” without the need for continued contraction.

The present work offers new insight into the spatiotemporal patterning of the heart fields during the HT formation and early-stage looping. It also raises new questions about the cellular mechanisms and signaling networks that regulate these morphogenetic events.
2.3 Experimental methods

2.3.1 Embryo preparation and culture

The method for preparation and culture of the embryo is adopted from Voronov and Taber (2002). Fertile white Leghorn chicken eggs (Sunrise Farms, Catskill, NY) were incubated in a humidified atmosphere at 38°C for 27–45 hr to yield embryos at HH stages 8 to 11 (Hamburger and Hamilton, 1951). Embryos were extracted from eggs using filter paper rings (Waterman) and rinsed in PBS. The sandwich structure of embryo and filter paper rings was transferred to a sterile 35 mm culture dish (Fisher Scientific, Foster City, CA), and a stainless steel ring was placed on top to hold it in place. The sandwich structure was then covered with a thin layer of liquid culture medium consisting of 89% Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO), 10% chick serum (Sigma), and 1% antibiotics to eliminate surface tension artifacts (Voronov and Taber, 2002). Finally, culture dishes were sealed in plastic bags filled with a humidified mixture of 95% O₂ and 5% CO₂, and put into an incubator for continued culture.

To specifically inhibit myosin-II-based cytoskeletal contraction, embryos were cultured in medium containing 50 µM (−)-blebbistatin (Bleb, Sigma). A stock solution of 20 mM Bleb in dimethylsulfoxide (DMSO, Sigma) was diluted 1:400 in the culture medium to give the final concentration. For controls, the same amount of DMSO was added. To prevent photoinactivation of Bleb during culture and manipulation, culture dishes were covered with aluminum foil whenever possible, and exposure to light was limited. Effectiveness of drug treatments was verified by diminished but not abolished heartbeat and reduced tissue tension.

2.3.2 Optical coherence tomography

Optical coherence tomography (OCT) provides sub-surface structural information of living tissues with high spatial resolution (∼ 10 µm) and relatively good penetration depth (up to ∼ 2 mm) (Mesud Yelbuz et al., 2002). Therefore, OCT is ideal for imaging semitransparent chick embryos at early stages (Mesud Yelbuz et al., 2002). Stacks of cross-sectional images were obtained with a commercial OCT system (Thorlabs, Newton, NJ), and 3D tissue
geometries were reconstructed using the image analysis software Volocity (PerkinElmer, Waltham, MA) and ImageJ (NIH).

### 2.3.3 Stiffness measurements

To measure tissue stiffness, embryos were transferred individually to a large bath of PBS at room temperature and tested using a custom-built microindentation device (Zamir et al., 2003). Tissue stiffness was measured at five locations around the AIP — medial, left/right lateral, and left/right mediolateral sites (see Fig. 2.4A). Briefly, a microindenter was attached to a calibrated cantilever beam and treated with bovine serum albumin to prevent adhesion to the indented tissue. Using customized Matlab (Mathworks, Natick, MA) codes, motion of the indenter was driven by a piezoelectric actuator, and tissue deformation was recorded and analyzed afterward to calculate local force-deflection curves for the tissue (Zamir et al., 2003). The tangential slope of the force-deflection curve at a representative deflection of 10 µm was used as a local measure of tissue stiffness. For each location, three consecutive indentations were made to ensure a repeatable response.

To investigate how contraction inhibition would affect tissue stiffness, ideally, microindentation tests should be carried out in the same embryo before and after exposure to Bleb. Such an approach, however, sometimes was impractical, for repeated indentations often caused damage to the tissue. In that case, stiffness in either control or Bleb-treated embryos was measured instead. Our results show that the variability introduced by these single measurements is not significant.

### 2.3.4 Visualizing microindentation with OCT

To visualize and qualitatively analyze the tissue deformation near the indenter, we performed microindentation tests on HH8 embryos \( (n = 3) \) using our OCT system (see Fig. 2.8A and Appendix Fig. A.1). Due to limited space, the entire microindentation setup could not be transferred to the OCT platform. Thus, using pulled glass micropipettes, we fashioned microindenters of similar (cylindrical) geometry and attached them to a hand-driven micromanipulator (Sutter Instrument, Novato, CA). Real-time OCT images of the deforming tissue was recorded while the indenter was moving along its axis aligned with
the imaging plane. (Stiffness was not measured in these experiments.) Image stacks were processed later using image analysis software ImageJ. Leading edge (deforming tissue entering fluid space) and trailing edge (tissue space occupied by fluid) of the deformation were identified via image processing algorithm (Appendix A.1.1 and Fig. A.1; see also Fig. 2.8A).

2.3.5 Strain measurements

To quantify tissue deformation around the AIP, we tracked the movements of fluorescent tissue labels during culture. Prior to culture, DiI (D282, Life Technologies, Carlsbad, CA) labels were injected into the tissue via pulled glass micropipettes using a pneumatic pump (World Precision Instruments, Sarasota, FL) and a micromanipulator (Voronov et al., 2004). To measure tissue movements along the tangential direction, five labels were injected in the endoderm evenly around the AIP (see Fig. 2.6A).

Bright field and fluorescence images were acquired at the beginning of culture and approximately every 2 hr thereafter using a fluorescence microscope (Leica Camera Inc., Allendale, NJ) and a high magnification camera (Canon USA Inc., Melville, NY). Label tracking was performed later using image analysis software ImageJ. The distance between adjacent endodermal labels were measured over time, and the tangential stretch ratio (λ) was computed as the current length divided by the reference length. Due to photobleaching and tissue movements, some labels became either invisible or indistinguishable from others after approximately 6 hr. Thus, we chose to inject labels at various stages and quantify endodermal contraction during the following 6 hr. Since computing the cumulative stretch ratio by multiplying incremental stretch ratios (Ramasubramanian et al., 2006) could accumulate measurement errors, this method is not suitable for our analysis. Instead, we calculated the shortening rate (\( \dot{\lambda} = -\dot{\lambda} \)) and assumed (to a first approximation)

\[
\dot{\lambda} \approx -\frac{\Delta \lambda}{\Delta t} \approx \frac{1}{\Delta t} \left(1 - \frac{l}{L}\right),
\]

(2.1)

where \( L \) and \( l \) are the distances between adjacent labels at time \( t \) and \( t + \Delta t \), respectively.

During culture, some lateral and mediolateral labels split into two with one remaining in the endoderm and the other moving with the adjacent mesoderm. Motions of the split label
pair were tracked, and speed of the mesodermal migration relative to the endoderm was computed as the label distance divided by the duration after the splitting occurred.

### 2.3.6 Stress measurements

To estimate tissue tension along the tangential direction, we introduced microsurgical incisions (150–200 µm long) around the AIP using a Gastromaster microdissection device (Xenotek Engineering, Belleville, IL; see Fig. 2.7A,B) (Voronov et al., 2004; Varner and Taber, 2012). The sandwich structure of embryo and filter paper rings was submerged under PBS at room temperature and properly positioned so that the cutting tip was normal to the AIP rim. Immediately after cutting, bright field images of the wound were taken using a high magnification camera attached to the microscope. The opening angle of the cut, which is the angle between two wound edges, was measured using image analysis software ImageJ and used to characterize the tissue tension normal to the cut direction (Varner and Taber, 2012). To minimize the variability among different embryos, we made multiple cuts either at the medial and lateral AIP or at the mediolateral locations in the same embryo (see Fig. 2.7A,B). Cuts at different locations were made in random order and far from each other. Our results show that the change in opening angle caused by introducing a second cut was less than 5 deg.

### 2.3.7 Statistical analysis

Statistical analysis was performed using SigmaPlot software (Systat Software Inc., San Jose, CA). The Holm-Sidak method (one-way, two-way, and three-way ANOVA) was used to compare experimental data among different groups (culture conditions, locations, stages). All experimental measurements are presented as mean ± SD, with statistical significance assumed for \( p < 0.05 \).
2.4 Computational methods

2.4.1 Theory for modeling morphogenesis

The finite-element method has been used to simulate a variety of morphogenetic processes. In the present study, we developed two types of finite-element models to explore how tissue geometry, material properties, and active contraction contribute to the observed gradients in tissue stiffness and tension around the AIP (see Figs. 2.4 and 2.7). The framework for modeling morphogenesis is based on the growth theory of Rodriguez et al. (1994). Here, we briefly summarize the basic idea.

Consider a pseudoelastic body, which transforms from the reference configuration at $t_0$ to the current configuration at $t$ ($t > t_0$). The total deformation is described by the deformation gradient tensor $\mathbf{F}$, which can be decomposed as

$$\mathbf{F} = \mathbf{F}^* \cdot \mathbf{M},$$

where $\mathbf{M}$ is the morphogenesis tensor and $\mathbf{F}^*$ is the elastic deformation gradient tensor. Growth and active contraction are simulated through $\mathbf{M}$, which defines the stress-free configuration for each material element after the simulated morphogenetic processes. Mechanical stress is generated through $\mathbf{F}^*$, which includes the elastic response to external loads as well as the enforcement of geometric compatibility between material elements after growth and contraction. The Cauchy stress tensor $\sigma$, which is assumed to depend only on $\mathbf{F}^*$, can be computed as

$$\sigma = \frac{1}{J^*} \mathbf{F}^* \cdot \frac{\partial W}{\partial \mathbf{E}^*} \cdot \mathbf{F}^{*\text{T}},$$

where $W(\mathbf{E}^*)$ is the strain-energy density function, $J^* = \det \mathbf{F}^*$ is the elastic volume ratio, and $\mathbf{E}^* = (\mathbf{F}^{*\text{T}} \cdot \mathbf{F}^* - \mathbf{I})/2$ is the Lagrangian elastic strain tensor with $\mathbf{I}$ being the identity tensor and T denoting the transpose.

2.4.2 Models for microindentation

As shown below, we observed a gradient in AIP stiffness, which decreased bilaterally with distance from the embryonic midline (see Fig. 2.4B). Stiffness depends on both material
properties and geometry. To investigate the relative contributions of tissue geometry and material properties to the stiffness gradient, we developed 3D finite-element models to simulate microindentation around the AIP using COMSOL Multiphysics (v3.5, COMSOL Inc., Burlington, MA).

**Model Geometry.** The model geometry was constructed from OCT cross sections of the AIP taken from an HH8+ embryo (Fig. 2.2). At each stage, the most significant difference in tissue stiffness was observed between the medial and lateral regions of the AIP (see Fig. 2.4B,C). Thus, we manually segmented the AIP from OCT cross sections at these two locations using ImageJ and imported each into COMSOL (Fig. 2.2B,C). By sweeping the 2D cross sections through 3D space, separate models with pseudo-embryonic geometries were created for microindentation at these two locations (Fig. 2.2′B,C′).

**Boundary and Contact Conditions.** In each model, plane symmetry is assumed about the embryonic midline, and a cylindrical indenter is placed at the indentation site normal to the tissue surface (Fig. 2.2′B,C′). For simplicity, we assume that the indenter tip is adherent to the tissue and prescribe a linear function for the axial displacement history at its far end. In addition, a small portion of neural tube is included with its dorsal midline on cranial-caudal oriented rollers to simulate the support by the relatively stiff vitelline membrane. All other boundaries in the model are taken as traction free.

**Material Properties.** As a first approximation, we neglect any material differences between the endoderm and mesoderm and model the entire tissue as a uniform, hyperelastic, nearly incompressible material. Considering that material nonlinearity is relatively mild at early stages (Zamir et al., 2003; Rémond, 2006; Varner and Taber, 2012), we take the strain-energy density function in the neo-Hookean form

\[
W = \frac{\mu}{2}(\tilde{I}_1 - 3) + U(J^*),
\]  

(2.4)

where \(\mu\) is the small-strain shear modulus, \(\tilde{I}_1 = J^* - 2/3 \text{tr}(\mathbf{F}^*T \cdot \mathbf{F}^*)\) is a modified strain invariant, and \(U(J^*)\) is a penalty function for nearly incompressible material. Here, we choose \(U(J^*) = p(1 - J^*) - p^2/(2\kappa)\), where \(p\) is a penalty variable, and \(\kappa \gg \mu\) is the bulk modulus.
Figure 2.2: **Finite-element models for microindentation around AIP.** (A) Bright field image of HH8+ embryo with two indentation sites around the AIP: medial and lateral. (B, C) Medial (B) and lateral (C) OCT cross sections of the AIP with segmented geometries shown in red and blue, respectively. Arrows indicate indentation locations. (B’, C’) Segmented sections ((B) and (C)) were imported into COMSOL and swept through 3D space to create pseudo-embryonic geometries. These models were used to simulate microindentation at the medial (B’) and lateral (C’) AIP. Due to the left-right symmetry about the embryonic midline (dash-dot line in (A)) at this stage, only half of each geometry is shown. The dorsal side of the neural tube (NT) is placed on rollers to simulate the presence of the relatively stiff vitelline membrane. Scale bar: 200 µm.

The indenter is modeled as a Hookean material with the material constants chosen typical for Silica glass ($E = 73.1$ GPa, $\nu = 0.17$) from the COMSOL Material Library.

For each model, the unknown shear modulus $\mu$ was determined by solving an inverse problem. We iteratively adjusted the value for $\mu$ so that the numerical force-deflection curve given by the model matches our experimental data. During indentation, the indenter force is calculated by integrating the normal axial stress over the cross-sectional area of the indenter. Because the indenter itself deforms only negligibly, the prescribed axial displacement is considered as the tissue deflection.
2.4.3 OV model with endodermal contraction

To investigate how endodermal contraction and material properties affect tissue strain and stress around the AIP, we created a model for the OVs using the commercial finite-element code ABAQUS (v6.10, SIMULIA, Providence, RI). Model geometry was constructed in ABAQUS/CAE, and material properties and cytoskeletal contraction were defined through the ABAQUS user subroutine UMAT (Young et al., 2010).

Model Geometry and Boundary Conditions. For simplicity, since the cross sections of the OVs after HH10 are relatively circular (see Fig. 2.3A’ and also Fig. 2.5B’), the OVs are approximated by a torus (Fig. 2.3). Geometric dimensions of the model (Fig. 2.3B,B’) are taken from images of a representative HH10 embryo (Fig. 2.3A,A’). At this stage, the endoderm contacts approximately a quarter of the mesoderm, and each tissue layer takes about half of the wall thickness in the region of contact (Fig. 2.3A’,B’). Plane symmetry is enforced at the embryonic midline, and only half of the model is shown. Considering the fact that relatively little tension is present at the lateral regions of the AIP (see Fig. 2.7A–D), we assume that the lateral end of the model is free. In addition, a toroidal coordinate system \( \{R, \Theta, \Phi\} \) and the corresponding unit base vectors \( \{e_R, e_\Theta, e_\Phi\} \) are defined following the undeformed model geometry (Fig. 2.3B,B’), with \( \Theta \) increasing from the medial (\( \Theta = 0 \)) to the lateral locations (\( \Theta = \pi/2 \)).

Material Properties. For both tissue layers, we take the strain-energy density function \( W \) (Eq. 2.4) from our microindentation model but replace the penalty function with \( U(J^*) = \kappa \left((J^* - 1)/2 - \ln J^* \right)/2 \). The material properties for each tissue layer have not been reported. Thus, we consider two cases, in which the shear modulus \( \mu \) of the endoderm is either uniform or linearly varying around the AIP and take

\[
\mu = \mu_0 + (\mu_1 - \mu_0) \cdot \bar{\Theta},
\]

where \( \mu_0 \) and \( \mu_1 \) are endodermal shear moduli at the medial (\( \bar{\Theta} = 0 \)) and lateral (\( \bar{\Theta} = 1 \)) AIP, respectively, with \( \bar{\Theta} = \Theta/\left(\pi/2\right) \) being the normalized tangential arc length. As shown later, results of our microindentation tests suggest that \( \mu \) decreases with distance from the medial AIP and stays relatively unchanged during the studied stages (see Figs. 2.8C,F
Figure 2.3: **Torus model for omphalomesenteric veins (OVs) with endodermal contraction.** (A) HH10 embryo with a relatively straight heart tube (HT) connected to bilateral OVs. Due to the left-right symmetry about the embryonic midline (dash-dot line), only half of the AIP is considered. (A’) As shown in a mediolateral OCT cross section of the AIP (yellow line in (A)), the endoderm (ENDO, red) contacts approximately a quarter of the mesoderm (MESO, blue). (B, B’) Curved tube model for the OV with cross-sectional dimensions (B’) taken from (A’). Endodermal contraction (approaching arrows in (B)) is specified in the undeformed toroidal coordinate system \( \{R, \Theta, \Phi\} \). Symmetry is enforced at the medial AIP, and the lateral end is free. Scale bars: 200 \( \mu \)m.
and 2.4C). Thus, we choose $\mu_1/\mu_0 = 1$ for the uniform case and 0.5 for the gradient case (see Fig. 2.9B). As the mesoderm is taken passive (see below), we assume that the mesodermal modulus $\mu_m$ is uniform and take $\mu_m = \mu_0$ as a first approximation. Results of the sensitivity analysis for this model suggest that varying mesodermal and endodermal moduli over relatively large ranges changes the strain and stress distributions quantitatively but not qualitatively (see Appendix A.1.2 and Fig. A.2A–D).

**Contraction Simulation.** Previous results indicate that the endoderm (not the mesoderm) is the primary contractile tissue layer and that the contraction is relatively isotropic within the tissue plane (Voronov et al., 2004; Varner and Taber, 2012). Thus, we assume that contraction occurs only in the endoderm with the corresponding morphogenesis tensor $M$ defined as

$$M = G^{-2}e_\theta e_\theta + G(e_\phi e_\phi + e_\phi e_\phi) \quad (0 < G \leq 1)$$

(2.6)

where $G$ is the contraction parameter, and the constant volume constraint $\det M = 1$ is satisfied. Similar to the shear modulus $\mu$, we consider two cases and assume that $G$ takes the form

$$G = G_0 + (G_1 - G_0) \cdot \theta,$$

(2.7)

where $G_0$ and $G_1$ are contraction constants at the medial ($\theta = 0$) and lateral ($\theta = 1$) AIP, respectively. Here, we choose $G_0 = G_1 = 0.7$ for the uniform contraction case and $G_0 = 0.7$, $G_1 = 1$ for the gradient contraction case (see Fig. 2.9C). (A reversed gradient in endodermal contraction ($G_1 < G_0 \leq 1$) causes tissue tension to peak at the mediolateral AIP, contradicting our experimental findings (see Fig. 2.7).) The amount of contraction ($G = 0.7$) was estimated based on our measurement of the endodermal shortening rate from HH10 to HH11+ ($\dot{\Lambda} \approx 0.04\text{hr}^{-1}$, 6–7 hr, see Fig. 2.6C). Effects of the contraction gradient were examined in the sensitivity analysis for this model (see Appendix A.1.2 and Fig. A.2E,F).

### 2.5 Experimental results

Our recent studies suggest that prior to HH10, endodermal contraction is responsible for bringing the originally separate heart fields toward the embryonic midline, where they fuse
to form the HT (Varner and Taber, 2012; Rémond et al., 2006). At HH8, we also found that this contraction is accompanied by gradients in tissue stiffness and tension that decrease from the medial to lateral locations around the AIP, which may play a mechanical role in patterning heart fields. After looping begins at HH10, however, contraction is no longer necessary, as looping and OV fusion appear to progress relatively normally when contraction is inhibited (Rémond et al., 2006; Rémond, 2006). To better understand the implications of these findings, we measured tissue stiffness, strain, and stress around the AIP during stages HH8+ to HH11, which include the period from just before the HT is created to just after looping begins.

2.5.1 Stiffness around AIP

Using microindentation tests, we measured tissue stiffness at the medial, mediolateral, and lateral locations around the AIP between HH8+ and HH11 (Fig. 2.4A; n ≥ 6 for each stage). Since the nonlinearity of tissue force-deflection curves is relatively mild at these early stages (Rémond, 2006; Ramasubramanian et al., 2008; Varner and Taber, 2012) (see also Fig. 2.8C,F), all the stiffness data presented here are for a representative indentation depth of 10 µm. For each stage, tissue stiffness decreased from the medial AIP toward both lateral locations and was relatively symmetric about the embryonic midline, except for HH10+/11-, where the mediolateral location was stiffer on the right side (Fig. 2.4B; p < 0.001, two-way ANOVA). Over time, the lateral stiffness remained relatively constant, while the medial stiffness peaked at HH9, decreased afterward until HH10, and stayed relatively unchanged thereafter (p < 0.001, two-way ANOVA).

To investigate how cytoskeletal contractility contributes to AIP stiffness, we repeated the measurements in embryos exposed to 50 µM Bleb for 1 hr (n ≥ 5 for each stage). Between HH8+ and HH10, the AIP stiffness for each location decreased significantly compared to the corresponding control, and the amount of reduction was approximately 40–60% for the medial and mediolateral locations, although the stiffness gradient remained (Fig. 2.4C; p < 0.001, three-way ANOVA). As shown by OCT images of the AIP cross sections, tissue geometry did not change significantly after exposure to Bleb (Fig. 2.5). This result suggests that the reduction in AIP stiffness after Bleb exposure can be attributed mainly to tissue relaxation caused by contraction inhibition. Because the AIP stiffness in Bleb-treated embryos was relatively symmetric before HH10, we combined the data for the
Figure 2.4: Stiffness measurements around AIP. (A) Using microindentation, tissue stiffness was measured at five locations (arrows) around the AIP of chick embryos: L = lateral, M = medial, ML = mediolateral. (B) Tissue stiffness around the AIP at a representative indentation depth of 10 µm from HH8+ to HH11. At each stage, AIP stiffness decreased from the medial site toward the lateral sites and was relatively symmetric about the embryonic midline, except for HH10+/11-, where the right ML was stiffer than the left ML. The gradient in AIP stiffness peaked at HH9 and gradually decreased afterward (p < 0.001, two-way ANOVA). (C) After embryos exposed to 50 µM (−)-blebbistatin (Bleb) for 1 hr, the AIP stiffness between HH8+ and HH10 decreased significantly compared to the corresponding control (B). However, the gradient in stiffness remained (p < 0.001, three-way ANOVA). Considering the left-right symmetry of AIP stiffness before HH10, we combined the data for the two ML and the two L locations. (D) For comparison, the stiffness gradient is defined as the difference in AIP stiffness between the M and L locations. The passive stiffness gradient (Bleb) remained relatively constant over time, while the active stiffness gradient (difference between control and Bleb) peaked at HH9 and decreased afterward, following the trend of the total stiffness gradient (control; p < 0.001, one-way ANOVA). (* p < 0.001, ∧ p < 0.005, ♯ p < 0.01; ** denotes p < 0.001 for the annotated data compared with any other data of the same kind at a different stage.) Scale bar: 200 µm.
Figure 2.5: Cross sections around AIP obtained via optical coherence tomography (OCT). (A, B) Bright field images of HH9 (A) and HH10 (B) chick embryos. Using microindentation, tissue stiffness was measured at five locations (arrows) around the AIP: L = lateral, M = medial, ML = mediolateral. At HH10, the heart tube (HT) is caudally connected to the omphalomesenteric veins (OVs). (A’, B’) OCT cross sections of the AIP at the indentation sites before (–) and after (+) embryos exposed to 50 µM (–)-blebbistatin (Bleb) for 1 hr. Treatment of Bleb relaxed the tissue somewhat, but did not significantly change geometries of the AIP (NT = neural tube). Note that the curvatures at the M and ML AIP decreased from HH9 to HH10. Scale bar: 200 µm.
two mediolateral and the two lateral locations. Overall, AIP stiffness stayed relatively unchanged for each location over time, except that the stiffness for the mediolateral location increased slightly from HH9+/10- to HH10 ($p < 0.001$, three-way ANOVA).

For comparison, the stiffness gradient is defined as the difference in AIP stiffness between the medial and lateral locations. To separate contraction from other contributing factors (e.g., tissue geometry and passive material properties), we define the active gradient as the difference between the total (control) and passive (Bleb-treated) gradients (Fig. 2.4D). The passive stiffness gradient remained relatively constant over time, while the active stiffness gradient peaked at HH9 and decreased afterward, following the trend of the total stiffness gradient ($p < 0.001$, one-way ANOVA).

Taken together, these results indicate that the active stiffness gradient along the AIP increases to a peak at HH9 and then decreases. The passive stiffness gradient remains relatively constant during these stages. These results also suggest that the changing active stiffness gradient is caused primarily by changes in myosin-II-based cytoskeletal contraction.

### 2.5.2 Tissue movements and deformation around AIP

Previous studies have shown that before HH9, mesoderm along the AIP migrates over the contracting endoderm, gaining speed as it approaches the embryonic midline (Abu-Issa and Kirby, 2008; Varner and Taber, 2012). Here, we investigate whether this changes after the onset of looping at HH10.

To quantify tissue movements and deformation, fluorescent labels were injected around the AIP of embryos harvested between HH8+ and HH10, and label motions were tracked during 6 hr of culture (Fig. 2.6A; $n \geq 5$ for each stage). In all embryos, regardless of their initial stages, lateral and mediolateral labels moved toward the medial label, which stayed along the embryonic midline. Meanwhile, some labels split into two, with the mesodermal (more anterior) label moving relatively faster than the endodermal label (Fig. 2.6A). These observations are qualitatively consistent with our previous study at earlier stages prior to HH9 (Varner and Taber, 2012), suggesting that endodermal contraction and mesodermal migration continue to occur after this time.
Figure 2.6: **Tissue movement and deformation around AIP.** (A) HH9 (upper panels) and HH10 (lower panels) chick embryos were cultured for 6 hr with fluorescent labels (green dots) injected around the AIP to track tissue movements (L = lateral, M = medial, ML = mediolateral). During culture, L and ML endodermal labels (arrowheads) moved toward the medial AIP, and some split into two, suggesting the mesoderm (arrows) migrates over the underlying contracting endoderm. (B) Mesodermal migration speed relative to the endoderm, as measured by tracking the distance between split labels, decreased significantly after HH10 (*p < 0.001, one-way ANOVA). (C) Endodermal shortening rate (decreasing rate of endodermal stretch ratio) decreased significantly from HH8+ to HH10 and stayed relatively constant afterward (*p < 0.001, one-way ANOVA). However, no statistically significant difference was found between different locations within each stage. (D) Plot of endodermal shortening rate vs. AIP stiffness gradient. Note the change in behavior after HH9. Arrows indicate the time course. Scale bar: 200 µm.
On the other hand, these tissue motions slowed down after HH9 (Fig. 2.6B,C; see also arrowheads at 6 hr in Fig. 2.6A). For comparison, we computed the mesodermal migration speed and endodermal shortening rate $\dot{\Lambda}$ (i.e., decreasing rate of endodermal stretch ratio, see Eq. 2.1). Since no statistically significant difference was found between various locations within each stage ($p > 0.05$, two-way ANOVA), we combined the data for all the locations. The results show that the average endodermal shortening rate decreased significantly from HH8+/9- ($\dot{\Lambda} = 0.116 \pm 0.035 \text{ hr}^{-1}$) to HH10 ($\dot{\Lambda} = 0.038 \pm 0.035 \text{ hr}^{-1}$) and stayed relatively constant afterward ($p < 0.001$, one-way ANOVA). Similarly, mesodermal migration speed relative to the endoderm also decreased significantly from 13.4±5.0 μm/hr before HH10 to 5.0 ± 2.1 μm/hr after HH10 (* $p < 0.001$, one-way ANOVA).

To examine whether there is a correlation between tissue movement and stiffness, we plotted endodermal shortening rate vs. AIP stiffness gradient for controls (see above; Fig. 2.6D). Our data show that the behavior of endodermal shortening rate after HH9 was generally consistent with the AIP stiffness gradient, i.e., they both decreased from HH9 to HH10 and stayed relatively unchanged afterward. From HH8+ to HH9, however, the AIP stiffness gradient increased a little, while $\dot{\Lambda}$ decreased slightly, although the differences between these two stages were not statistically significant.

Taken together, these results show that the endoderm shortens at a rate that decreases before HH10 and remains relatively constant afterward. Notably, the endodermal shortening rate decreases by almost three-fold from the time the cardiogenic mesoderm begins to fuse at HH9 until looping begins at HH10.

### 2.5.3 Tissue stress around AIP

As discussed above, endodermal contraction affects the mesodermal movements and tissue stiffness around the AIP, which both depend on the mechanical stress within the tissue. To better understand the mechanical changes that occur around the AIP, we examined tissue stress using microsurgical cuts.

In general, the opening angle in control embryos decreased from the medial AIP to the lateral locations for each stage (Fig. 2.7A,C; $n \geq 5$ for each cut at each stage; $p < 0.001$, two-way ANOVA). The spatial distribution of the opening angle was relatively symmetric.
Figure 2.7: **Tissue stress around AIP.** (A,B) To examine tissue stress, microsurgical cuts were introduced around the AIP of chick embryos (L = lateral, M = medial, ML = mediolateral). (A,C) In control embryos, the M cuts opened significantly more than the L cuts; no significant difference were found in opening angle between the left and right ML cuts at HH9, while the right ML cuts opened significantly more than the left ML cuts at HH10 and HH11, suggesting that left-right asymmetry develops in AIP tension once looping begins. (B,D) Compared to controls, embryos treated with 50 µM (−)-blebbistatin (Bleb) for 1 hr exhibit less tension around the AIP, as shown by the decrease in opening angle. The spatial distribution of opening angle in Bleb-treated embryos was relatively symmetric from HH9 to HH11 (p < 0.001, three-way ANOVA). (E) For comparison, tension gradient is defined as the difference in opening angle between M and L cuts. With HH8+/9- data included from Varner and Taber (2012), both the total (control) and active (difference between control and Bleb) tension gradients around the AIP peaked at HH9 and decreased significantly afterward, while the passive (Bleb) gradient remained relatively unchanged from HH9 to HH11 (p < 0.001, one-way ANOVA). (F) Plot of AIP tension gradient vs. stiffness gradient. Note the change in behavior after HH9. Arrows indicate the time course. (* p < 0.001, # p < 0.01; ** and ^&^ denote p < 0.001 and p < 0.005, respectively, for the annotated data compared with any other data of the same kind at a different stage.) Scale bars: 200 µm.
about the medial AIP at HH9, while the right mediolateral cuts opened significantly more than the left mediolateral cuts afterward. This result suggests that left-right asymmetry in tissue tension develops around the AIP once looping begins. Compared to HH9, the opening angle stayed relatively unchanged for the lateral cuts, increased somewhat for the right mediolateral cuts, but decreased for the medial and left mediolateral cuts at later stages. Overall, the spatial differences in the opening angle between the medial and mediolateral cuts, as well as between the medial and lateral cuts decreased significantly after HH9.

To examine how contraction affects tissue stress, the opening angle was also measured in embryos treated with 50 µM Bleb (n ≥ 5 for each cut at each stage). After 1 hr of exposure, less tension was present around the AIP, as shown by the decrease in opening angle compared to controls (Fig. 2.7B,D; p < 0.001, three-way ANOVA). In Bleb-treated embryos, the opening angle decreased symmetrically about the embryonic midline and stayed relatively unchanged from HH9 to HH11.

For comparison, we define the AIP tension gradient as the difference in opening angle between the medial and lateral cuts and also include the data for HH8+/9- from Varner and Taber (2012). Our results show that both the total (control) and active (difference between control and Bleb) tension gradients peaked at HH9, decreased significantly from HH9 to HH10, and stayed relatively unchanged after HH10 (Fig. 2.7E; p < 0.001, one-way ANOVA). The passive (Bleb) gradient, however, increased a little before HH9 and stayed relatively constant afterward. Our data also show that the AIP tension gradient correlates with the stiffness gradient, as they changed synchronously during these stages (Fig. 2.7F).

Taken together, these results show that the active AIP tension gradient increases to a peak at HH9 and decreases afterward, consistent with the trend in the AIP stiffness gradient. These data suggest that changes in tangential tension around the AIP are regulated by myosin-II-based cytoskeletal contraction and likely play an important role in altering the tissue stiffness.
2.6 Computational results

Complementary to laboratory experiments, computational models are efficient and useful for elucidating underlying mechanisms during morphogenesis, as intuition can be misleading when trying to interpret the experimental results of complicated problems. To investigate how tissue stiffness, strain, and stress around the AIP are affected by material properties, active contraction, and geometry, we developed finite-element models.

2.6.1 Models for microindentation around AIP

Somewhat surprisingly, a stiffness gradient was still present around the AIP even after treatment with Bleb (see Fig. 2.4C). This residual gradient could be attributed to differences in either tissue geometry or mechanical properties or both. To characterize tissue geometry in living embryos, we captured OCT sections at locations corresponding to each indentation site around the AIP (see Fig. 2.2B,C and also Fig. 2.5A’,B’). These images show marked differences in both the curvature and thickness of the tissue under the indenter.

To test whether geometric differences alone could account for the observed gradient, we constructed 3D finite-element models to simulate the microindentation tests around the AIP. Since the largest difference in passive stiffness was observed between the medial and lateral regions of the AIP (see Fig. 2.4C), we created separate models for these two cases. In both models, we used the segmented OCT image corresponding to the indentation site of interest to create the swept model geometry (see Fig. 2.2B’,C’).

As a test of our modeling results, we also performed microindentation on the OCT platform and characterized the tissue deformation under the indenter using an image processing algorithm (see Appendix Fig. A.1). As shown in OCT cross sections of the deforming tissue (Fig. 2.8A,D), the leading edge (red pixels) of the deformation represents regions where the deforming tissue had entered into space previously occupied by only fluid, whereas the trailing edge (blue pixels) represents regions where tissue had been replaced by fluid. Our models for microindentation at the medial and lateral AIP produced tissue deformation patterns that are qualitatively consistent with those observed using OCT (Fig. 2.8B,E). This was not the case for preliminary 2D models, nor simple extruded 3D models, which did not include a crescentic AIP (data not shown).
Figure 2.8: **Results from finite-element models for microindentation around AIP. (A,D)** OCT cross sections through the indentation planes at the medial (A) and lateral (D) AIP, as the indenter (green lines) first comes into contact with the tissue (undeformed) and after indentation (deformed). Red and blue pixels indicate the leading edge and trailing edge of the deformation, respectively (see text and Appendix Fig. A.1 for details; NT = neural tube). (B,E) Undeformed and deformed cross-sectional shapes obtained from models at the medial (B) and lateral (E) AIP. The algorithm used to analyze the OCT images was used to characterize the leading and trailing edges of the model deformation. Both models qualitatively match the tissue deformations observed experimentally. (C,F) The simulated force-deformation curve at the medial (C) and lateral (F) AIP. $\mu = 65$ Pa yields force-deformation curve that approximately matches experimental data after blebbistatin treatment (black lines) for microindentation at the medial AIP (red line in (C)), but not for that at the lateral site (solid blue line in (F)). When the material modulus is reduced to $\mu = 15$ Pa, however, the simulated force-deformation curve at the lateral AIP (dashed blue line in (F)) matches the experimental data. These results suggest a gradient in passive shear modulus toward the medial AIP. Scale bars: 200 $\mu$m.
At the medial AIP, our simulated force-deflection curve roughly matches our experimental data if the shear modulus $\mu$ is taken as 65 Pa (Fig. 2.8C). Here, we use Bleb-treated experimental data for comparison, since we are interested in the residual stiffness gradient. When $\mu = 65$ Pa is included in the model for the lateral AIP, however, the simulated force-deflection curve is much stiffer than that observed experimentally (Fig. 2.8F). But if $\mu$ is reduced to 15 Pa, the simulation result more closely matches the experimental force-deflection curves.

These results suggest that differences in mechanical properties, not geometry, are responsible for the residual stiffness gradient around the AIP after contraction is inhibited by Bleb. We estimate an approximately four-fold decrease in passive tissue modulus between the medial and lateral regions of the AIP at HH8+.

2.6.2 OV model with endodermal contraction

Previously, we used a simplified 2D model to determine that endoderm is the primary contracting tissue layer during HT formation (Varner and Taber, 2012). To more thoroughly investigate how material properties and endodermal contraction contribute to the stage-dependent strain and stress distributions around the AIP, we use a 3D toroidal model for the OVs. The geometry is based on an OCT cross section of the mediolateral AIP at HH10 (see Fig. 2.3).

As suggested by our stiffness measurements, the shear modulus ($\mu$) and contraction ($G$) of the endoderm are taken either uniform or decreasing from the medial AIP to the lateral regions (Fig. 2.9A–C). In general, contraction causes the endoderm to shorten in the tissue plane while increasing its thickness, generating tensions ($\sigma_{\theta\theta}, \sigma_{\phi\phi} > 0$) and negative strains ($E_{\theta\theta}, E_{\phi\phi} < 0$) along both longitudinal and circumferential directions (Fig. 2.9D,E). Meanwhile, the adjacent passive mesoderm is compressed longitudinally ($E_{\theta\theta}, \sigma_{\theta\theta} < 0$) and stretched circumferentially ($E_{\phi\phi}, \sigma_{\phi\phi} < 0$) by the contracting endoderm.

For comparison, the circumferential components of Lagrangian strain and Cauchy stress (i.e., $E_{\phi\phi}$ and $\sigma_{\phi\phi}$) in the endoderm are plotted along the AIP (see curved arrow in Fig. 2.9A). For uniform modulus and contraction, both $E_{\theta\theta}$ and $\sigma_{\theta\theta}$ decrease from the medial AIP.
Figure 2.9: Results from finite-element model for omphalomesenteric vein (OV) with endodermal contraction. (A) Endoderm (ENDO) and mesoderm (MESO) in undeformed OV model. (B,C) Elastic modulus ($\mu$, (B)) and contraction stretch ratio ($G$, (C)) of the endoderm are taken either uniform or linearly varying with $\bar{\Theta}$. (D,E) Lagrangian strains (D) and Cauchy stresses (E) are plotted on the surface of the deformed model for uniform material modulus and uniform contraction of the endoderm. (F,G) Distributions of longitudinal strain ($E_{\Theta \Theta}$, (F)) and stress ($\sigma_{\Theta \Theta}$, (G)) along the center line of the endoderm (curved arrow in (A)). The numerical results given by the model for uniform contraction are qualitatively consistent with our experimental data. Increasing contraction from $G = 0.7$ (solid black line) to 0.5 (dashed black line) matches the strain measurements (from HH10 to HH11+, circles in (F)) reasonably well, suggesting that endodermal contraction is relatively uniform around the AIP.
(\bar{\theta} = 0) to the lateral locations (\bar{\theta} = 1) (Fig. 2.9F,G). Increasing the amount of contraction increases both the strain magnitude and stress gradient. More importantly, the strain values given by the model (G = 0.5, dashed black line in Fig. 2.9F,G) are in relatively good agreement with those measured from HH10 to HH11+ (see circles in Fig. 2.9F), and the stress distribution is consistent with the AIP tension gradient revealed by microsurgical cutting (see Fig. 2.7A,C). For a gradient in contraction with uniform \( \mu \), \( E_{\theta\theta} \) increases toward the lateral sites and the tension gradient decreases, whereas a gradient in modulus with uniform \( G \) changes the strain and stress distributions relatively little (Fig. 2.9F,G; see also Appendix Fig. A.2C–F). These results suggest that contraction of the endoderm is relatively uniform, although the passive material properties may vary around the AIP, consistent with our measurements (see Figs. 2.8C,F and 2.4C). In addition, results of a sensitivity analysis show that varying the geometric parameters of this model changes the strain and stress distributions quantitatively but not qualitatively (Appendix Fig. A.3).

Taken together, these modeling results suggest that the experimental gradients along the AIP are produced by uniform contraction of the endoderm in conjunction with a gradient in passive modulus, which decreases with distance from the embryonic midline.

### 2.7 Discussion

At a fundamental level, morphogenesis is accomplished through the action of mechanical forces, as the simple geometry of the early embryo deforms to give rise to the intricate 3D structures of the adult organism (Blanchard and Adams, 2011). As one of the most fundamental force-generation mechanisms, cytoskeletal contractility provides major intrinsic driving forces for epithelial morphogenesis (Wozniak and Chen, 2009; Martin, 2010). Within animal cells, meshworks of actin microfilaments are cross-linked with myosin motor proteins. These motors then work to pull antiparallel actin fibers toward one another and generate contractile force (Martin, 2010). In a host of model organisms, contractility has been shown to drive both cell shape changes (Martin et al., 2009; Solon et al., 2009; Blanchard et al., 2010) and cell intercalation (Skoglund et al., 2008; Fernandez-Gonzalez and Zallen, 2009; Rauzi et al., 2010; Fernandez-Gonzalez and Zallen, 2011), as well as higher order structures like multicellular rosettes (Blankenship et al., 2006) and actomyosin purse strings observed during Drosophila dorsal closure (Kiehart, 2000; Hutson et al., 2003).
Cytoskeletal contraction not only exerts forces, but also modifies the mechanical properties of the tissue, as actively contracting cells and tissues stiffen (Wakatsuki et al., 2000, 2001; Rémon et al., 2006; Zhou et al., 2009). This phenomenon has led other researchers to speculate about a possible role for mechanical feedback in regulating cytoskeletal contraction (Taber, 2009; Davidson, 2011). Moreover, recent experimental work in *Drosophila* has indicated a possible role for mechanical tension in regulating myosin II dynamics during early embryonic development (Fernandez-Gonzalez and Zallen, 2009; Pouille et al., 2009). These observations muddle our understanding of the role that contractility plays during development.

Previous work in our lab has suggested that cytoskeletal contraction of the endoderm along the AIP is responsible for pulling the bilaterally situated cardiogenic mesoderm toward the embryonic midline before HH9 (Fig. 2.1A,A′) (Varner and Taber, 2012). This contraction is required for cardiogenic mesodermal fusion and HT formation (Rémon, 2006; Varner and Taber, 2012). After the HT is created at HH10, however, contraction is not necessary for either the progression of fusion or the subsequent cardiac c-looping (Fig. 2.1B,B′) (Rémon et al., 2006; Rémon, 2006). The obvious question to ask here is why endodermal contraction is required before but not after HH10. To explore this question, we studied in some detail the mechanical behavior of the tissue around the AIP from HH8+ to HH11.

### 2.7.1 Tissue mechanics around AIP

At each studied stage, our experimental data indicate that tissue stiffness and tangential tension decrease with distance from the embryonic midline along both arms of the AIP (Figs. 2.4B, 2.7A,C). The tangential strain rate and, therefore, strain relative to HH8+ show that the endoderm shortens during these stages with relatively uniform strain distributions (Fig. 2.6A,C). Our computational model shows that these stress and strain patterns can be caused by a uniform active contraction in the endoderm combined with resistance provided by a passive mesoderm (Figs. 2.8 and 2.9). The tension gradient then would cause the stiffness gradient, since in-plane tension in a membrane would increase its transverse stiffness (Fig. 2.7F) (Zamir and Taber, 2004b). In addition, tracking the motions of injected labels show that the mesoderm migrates over the endoderm toward the embryonic midline during these stages (Fig. 2.6A,B).
To investigate the contribution of cytoskeletal contraction to the observed gradients, AIP stiffness and tangential tension were also measured in embryos with cytoskeletal contraction inhibited. Bleb exposure drops the AIP stiffness and tension considerably, but the gradients remain (Figs. 2.4C and 2.7B,D). The results of our computer models for microindentation suggest that this residual stiffness gradient is likely produced by a spatial variation in the passive tissue modulus (Fig. 2.8C,F). What causes this variation in passive properties is unknown. We speculate that differential amounts of cross-linking within the extra-cellular matrix, which have been observed between the mesoderm and endoderm (Linask and Lash, 1986; Drake et al., 1990; Wiens, 1996; Stainier, 2004), may be responsible for generating this residual gradient.

Our results are consistent with those of previous studies. The endodermal shortening rate we measured between HH10 and HH11+ (Fig. 2.6C) agrees relatively well with the strain data of Ramasubramanian et al. (2006). Our microindentation tests revealed a 2–3 fold decrease in tissue stiffness after exposure to Bleb (Fig. 2.4B,C), which is consistent with those previously reported in the heart and brain of chick embryos at later stages (Rémont, 2006; Ramasubramanian et al., 2008; Filas et al., 2011). We estimated that the passive shear modulus of the AIP varies from 65 Pa at the middle of the AIP to 15 Pa at lateral locations (Fig. 2.8C,F); these values are on the same order as those measured in dorsal isolates of early *Xenopus laevis* embryos (Zhou et al., 2009) and HH12 chick hearts (Zamir and Taber, 2004a).

More importantly, the measured stiffnesses and stresses changed with time in a relatively consistent manner during the studied stages. The maximum active stiffness and stress (opening angle), which occurred at the midline, increased from HH8+ to HH9, decreased immediately after HH9, and then remained relatively constant after HH10, just as looping began (Figs. 2.4B,D and 2.7C,E). Moreover, after HH10, the distributions of these quantities began to exhibit left-right asymmetry, with both becoming greater near the center of the right OV than the left OV (Figs. 2.4B and 2.7A,C). These trends are consistent with the idea that increased tension is the cause of the increased stiffness (Fig. 2.7F) (Zamir and Taber, 2004b).

In contrast to these trends, prior to HH9, the average strain rate decreased until HH10, after which it remained relatively constant like the stress and stiffness (Fig. 2.6C,D). The
reason for this behavior is unknown, but it could be caused by increased resistance to OV deformation exerted by connections to tissue at the ends of the OVs.

2.7.2 Morphogenetic implications of results

Taken together, the results of our study suggest that the endoderm around the AIP undergoes a relatively uniform cytoskeletal contraction that increases in magnitude until HH9 and then decreases afterwards. From this finding and our previous results (Varner and Taber, 2012), we speculate that this contraction affects the motion of cardiogenic mesoderm in two main ways.

First, before the onset of looping at HH10, endodermal contraction pulls (conveys) the primary heart fields toward the embryonic midline (Varner and Taber, 2012), where they fuse to create the HT (Fig. 2.10A,D,E). Second, this contraction establishes a tension gradient, which produces a stiffness gradient in the endoderm, with the greatest tension and stiffness occurring at the midline (Fig. 2.10B,C). We speculate that mesodermal cells crawl up this stiffness gradient (durotaxis) to facilitate their motion toward the midline (Fig. 2.10C,D) (Lo et al., 2000; Discher et al., 2005). The tension gradient also may induce an adhesion gradient with similar effects (Ridley et al., 2003; Parsons et al., 2010).

As mentioned earlier, previous studies have shown that contraction is necessary for normal heart development before but not during c-looping (Rémond et al., 2006; Varner and Taber, 2012). Before looping, we speculate that contraction is needed to bring the heart fields together, so they can fuse to create and lengthen the HT (Fig. 2.10A–E) (Varner and Taber, 2012). If contraction is blocked during this time, looping is disrupted (Varner and Taber, 2012). After this time, however, the fusion process is far enough along to allow mesodermal cells to fuse without contraction through filopodia-mediated “zippering” (Fig. 2.10D–F) (Millard and Martin, 2008). Since contraction is no longer required, we reason that it is down regulated to save energy after HH9. In addition, the decrease in OV strain rate during fusion may indicate increased resistance to OV deformation by surrounding tissues, making convection less effective (Fig. 2.6C).

Finally, our results suggest that differential contraction around the AIP may be one of several mechanisms that help ensure the normal rightward rotation of the looping heart.
Figure 2.10: Mechanical regulation around AIP. (Modified from Abu-Issa and Kirby (2008).) (A–D) Before HH9, (A) contraction (white arrows) of endoderm (blue arch) around the AIP brings the originally separate cardiac fields toward the embryonic midline (dashed line), causing the lateral-to-medial gradients in (B) tissue tension and (C) active stiffness. (D) Meanwhile, endodermal contraction and AIP stiffness gradient drive mesoderm to move toward the medial AIP (black arrows) via cell convection and migration, respectively. On the other hand, mesodermal movements alter the AIP geometry and passive stiffness gradient. (E) Once the bilateral cardiac fields meet at the embryonic midline, they begin to fuse (green arrows) to create the primitive heart tube (HT) at HH10-. (F) At HH10 when formation of the HT is completed and looping begins, mesodermal fusion can occur independently without endodermal contraction. (OV = omphalomesenteric vein)
(Voronov et al., 2004; Taber et al., 2010). The increased stress and stiffness in the right OV after HH10 may indicate that contraction becomes stronger in the right OV than in the left OV during looping (Figs. 2.4B and 2.7A,C). This unbalanced force may pull the heart toward the right side of the embryo.

In conclusion, the present study provides a possible answer to the question posed at the outset: Why is cytoskeletal contraction required for normal cardiac morphogenesis before but not after looping begins? Our results indicate that endodermal contraction is needed at the earliest stages of heart development to draw the bilateral fields of cardiogenic mesoderm toward the midline, where they fuse to create the initially straight HT. After looping begins, however, fusion can continue by filopodial “zippering” without the need for tissue contraction. This work bridges the gap between the mechanics of HT formation and cardiac looping, adding new insight into these two important morphogenetic events.
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Chapter 3

Bending of the Looping Heart: Differential Growth Revisited

3.1 Summary

In the early embryo, the primitive heart tube (HT) undergoes the morphogenetic process of c-looping as it bends and twists into a c-shaped tube. Despite intensive study for nearly a century, the physical forces that drive looping remain poorly understood. This is especially true for the bending component, which is the focus of this paper. For decades, experimental measurements of mitotic rates had seemingly eliminated differential growth as the cause of HT bending, as it has commonly been thought that the heart grows almost exclusively via hyperplasia before birth and hypertrophy after birth. Recently published data, however, suggests that hypertrophic growth may play a role in looping. To test this idea, we developed finite-element models that include regionally measured changes in myocardial volume over the HT. First, models based on idealized cylindrical geometry were used to simulate the bending process in isolated hearts, which bend without the complicating effects of external loads. With the number of free parameters in the model reduced to the extent possible, stress and strain distributions were compared to those measured in embryonic chick hearts that were isolated and cultured for 24 hr. The results show that differential growth alone yields results that agree reasonably well with the trends in our data, but adding active changes in myocardial cell shape provides closer quantitative agreement with stress.

The geometry of the 3D model described in Section 3.5.5 was created by Jiang Yao, Jonathan Young, and Renato Perucchio, based on OCT data provided by Benjamen Filas. Gang Xu conducted preliminary experiments of hyaluronidase culture.
measurements. Next, the estimated parameters were extrapolated to a model based on realistic 3D geometry reconstructed from images of an actual chick heart. This model yields similar results and captures quite well the basic morphology of the looped heart. Overall, our study suggests that differential hypertrophic growth in the myocardium is the primary cause of the bending component of c-looping, with other mechanisms possibly playing lesser roles.

### 3.2 Introduction

For more than a century, researchers have studied the physical mechanisms of cardiac looping (Patten, 1922; Stalsberg, 1970; Männer, 2000; Taber, 2006). During looping, the initially straight heart tube (HT) transforms into a curved tube, laying out the basic pattern of the future four-chambered pump. This paper focuses on the first phase of this process, called c-looping, as the HT bends and twists into a c-shaped tube (Patten, 1922; Männer, 2000). Experiments have suggested that the bending and torsional components of c-looping are driven primarily by forces intrinsic and extrinsic to the HT, respectively (Butler, 1952; Manning and McLachlan, 1990; Voronov et al., 2004; Latacha et al., 2005; Taber, 2006). Although the external forces that drive cardiac torsion are now becoming clear, the internal forces that cause the HT to bend remain poorly understood (Männer, 2000; Voronov et al., 2004; Latacha et al., 2005; Rémond et al., 2006; Taber, 2006).

Some of the early theories for bending of the HT focused on growth. Patten (1922) proposed that the HT is forced to bend simply because it outgrows the distance between its constrained ends. This idea was contradicted three decades later, however, when Butler (1952) found that the isolated HT bends in culture (see also Manning and McLachlan (1990)). Others speculated that differential growth causes bending (Davis, 1927), but Sissman (1966) and Stalsberg (1969) measured mitotic rates in the myocardium (MY) and found no clear spatial patterns. Differential growth was thus ruled out as a causal mechanism because it was generally thought that the embryonic heart grows primarily by hyperplasia (more cells), rather than by hypertrophy (bigger cells) as it does after birth (Grossman, 1980; Ieda et al., 2009). Finally, Manasek et al. (1984) postulated that growth (or swelling) of the
acellular cardiac jelly (CJ), combined with the constraint of a relatively stiff dorsal mesocardium (DM), drives bending of the HT, but later experiments showed that looping occurs even when CJ is chemically removed (Baldwin and Solursh, 1989; Linask et al., 2003).

These results led most researchers, including us, to conclude that growth does not play a major role in looping. Other potential bending mechanisms include differential contraction (Itasaki et al., 1991), differential adhesion (García-Castro et al., 2000; Linask et al., 2003), and active changes in myocardial cell shape (Manasek et al., 1972; Latacha et al., 2005). Considering all of the various possibilities in light of both old and new data, we recently postulated that HT bending is caused by active cell-shape changes driven by the forces of actin polymerization (Latacha et al., 2005). This hypothesis, however, has not yet undergone definitive testing.

A recent study by Soufan et al. (2006) has caused us to reconsider growth as a potential driver for HT bending. These investigators constructed detailed maps of cell proliferation and cell size in the embryonic chick heart (Soufan et al., 2006). They found that relatively little cell division occurs in the HT during c-looping (stages 10 to 12- of Hamburger and Hamilton (1951)), although relatively high mitotic rates were found in the DM and parts of the omphalomesenteric veins (primitive atria). These results support the generally accepted view that cellular hyperplasia does not bend the HT. In contrast, they found clear patterns of cellular hypertrophy, with cells at the outer curvature (OC) of the looped tube becoming significantly larger than those at the inner curvature (IC). This pattern is consistent with differential growth being a possible mechanism for bending.

Here, we use experiments with isolated embryonic chick hearts, along with computational models, to explore differential hypertrophic growth as a mechanism for the bending component of c-looping. Isolated hearts bend in culture without the complicating effects of external loads, which alter the shape of the HT near its ends (Butler, 1952; Manning and McLachlan, 1990). Our results indicate that patterns of growth measured by Soufan et al. (2006) are sufficient to cause the observed deformation, as quantified by measured morphogenetic strains. However, experimental wall stress distributions are not completely consistent with those given by the model, suggesting that other mechanisms also may be involved. Further study indicated that HT bending is driven primarily by differential growth, with CJ swelling, DM tension, and active changes in myocardial cell shape playing secondary roles.
We suggest that these results add new understanding to this important problem in cardiac development.

3.3 Background

3.3.1 Structure of the early heart tube

At Hamburger-Hamilton (HH) stage 10 (33–38 hr of a 21-day incubation period) (Hamburger and Hamilton, 1951), the chick heart is a relatively straight tube consisting of three layers (Fig. 3.1A,A’)—an outer two-cell-thick layer of MY, a one-cell-thick inner layer of endocardium, and a relatively thick middle layer of CJ (extracellular matrix). The HT is connected cranially to the outflow tract (conotruncus), and its posterior end gradually lengthens during c-looping by fusion of the two lateral omphalomesenteric veins. The MY layer is initially anchored to the foregut of the embryo by the DM, which ruptures as the HT loops. An endodermal membrane called the splanchnopleure presses against the ventral surface of the HT.

During approximately the next 12 hr (to HH12), the HT gradually bends ventrally and twists rightward, transforming into the shape of the letter ‘c’ as the original ventral and dorsal sides become the OC and IC, respectively (Fig. 3.1C,C’). Although bending and twisting are coupled to some extent, studies have shown that the torsional component of c-looping is caused by external forces exerted by the splanchnopleure and the omphalomesenteric veins, while bending is driven by forces generated within the HT (Voronov et al., 2004; Butler, 1952; Manning and McLachlan, 1990).

3.3.2 Possible bending mechanisms during c-looping

Over the years, several hypotheses have been proposed for the mechanism of heart bending (Taber, 2006). Some of the most prominent hypotheses are summarized below.
Figure 3.1: **Cardiac c-looping in chick embryo.** (A–C) SEM images of embryonic chick hearts during c-looping (ventral view). The originally straight heart tube (HT) at HH10 in (A) bends ventrally and rotates rightward, transforming into a c-shaped tube at HH12 in (C). Note that artificial labels (red dots) along the ventral midline of the HT at HH10 move to the outer curvature of the HH12 heart. (A′–C′) Rotation of the HT is shown by the orientation of the elliptical lumen (red arrowheads) in OCT cross sections taken midway along the length of the HT (yellow dashed lines in (A)–(C)). AIP = anterior intestinal portal, AT = atrium, CJ = cardiac jelly, CT = conotruncus, DM = dorsal mesocardium, EN = endocardium, IC = inner curvature, LU = lumen, MY = myocardium, SPL = splanchnopleure, OC = outer curvature, OV = omphalomesenteric vein, VE = ventricle. Scale bars: 200 µm. (See Appendix A.2.1 for the embryo preparation protocol for SEM.)
**CJ Swelling with DM Constraint.** Cardiac jelly is secreted by the MY and can change its volume in response to alterations in ambient osmolarity (Nakamura and Manasek, 1978). Manasek et al. (1984) speculated that CJ swelling pressure inflates the HT with a relatively stiff DM locally restricting longitudinal extension along the dorsal side, causing the HT to bend ventrally. This idea was later supported by two pieces of evidence: (1) the MY is in a state of tension, likely due to CJ pressure (Voronov et al., 2004; Zamir and Taber, 2004); and (2) the DM at the IC is 2–3 times stiffer than the rest of the HT (Zamir et al., 2003). However, other studies seemingly contradicted this hypothesis by showing that dissolving the CJ with hyaluronidase does not prevent looping (Baldwin and Solursh, 1989; Linask et al., 2003). On the other hand, these studies were done on whole embryos in which the results are complicated by the effects of torsion, and the lack of CJ made the hearts appear flaccid and abnormally shaped. Here, to help clarify this issue, we repeated these experiments using isolated hearts and obtained similar results.

**DM Tension.** Experiments have shown that the MY and DM are under tension in the HT during c-looping (Voronov et al., 2004; Zamir and Taber, 2004) (see also Fig. 3.8C). Taber et al. (1995) speculated that pre-existing tension in the DM causes the dorsal side of the HT to shorten as the DM ruptures, resulting in ventral bending of the HT. The authors used a mathematical model to demonstrate the plausibility of this mechanism, but they also suggested that other mechanisms probably are involved.

**Cytoskeletal Contraction.** Myosin-based contraction serves as a fundamental force-generating process during morphogenesis (Martin, 2010; Wozniak and Chen, 2009; Varner and Taber, 2012). Recent studies have shown, however, that c-looping in both whole chick embryos and isolated hearts does not require either sarcomeric contraction, which causes the heartbeat, or non-sarcomeric contraction, which drives morphogenesis (Rémont et al., 2006). Nevertheless, contraction likely plays a role in the formation of the HT before looping begins (Rémont et al., 2006; Varner and Taber, 2012), and Nerurkar et al. (2006) showed that contraction can play a backup role in cardiac torsion when normal loads supplied by the splanchnopleure are removed. Hence, it remains possible that cytoskeletal contraction also has some effect on bending, and we explore this prospect here.
**Active Cell-Shape Change.** It has been observed that myocardial cells undergo changes in shape during c-looping (Manasek et al., 1972), but it is not clear whether these shape changes are actively generated and cause bending or whether they are a passive response to bending driven by some other mechanism. Manasek et al. (1972) speculated that the former is true. Using scanning electron microscopy (SEM), they found relatively small cells randomly orientated in the prelooped HT; in the looped heart, myocardial cells at the OC were larger in surface area with arbitrary orientations, whereas those near the IC were elongated in the circumferential direction. We used computer modeling to illustrate how these changes in cell shape can cause the HT to bend (Taber et al., 1995; Latacha et al., 2005) and suggested that these shape changes may be driven by actin polymerization, because drugs that inhibit polymerization prevent looping (Latacha et al., 2005). However, since blocking actin polymerization can affect a variety of cellular activities (Rosenblatt et al., 2004; Spector et al., 1989; Ornelles et al., 1986; Ingber et al., 1995), these results are not conclusive.

**Differential Growth.** As mentioned in the Introduction, researchers in general have ruled out differential growth as a possible cause of looping after failing to find significant differences in cell proliferation and death over the HT (Sissman, 1966; Stalsberg, 1969, 1970). Recently, however, Soufan et al. (2006) found that, whereas cells proliferate at a relatively slow and uniform rate in the HT, cells increase in volume significantly during c-looping in spatial patterns that are consistent with a causal role for ventral bending. (The significant cell proliferation found in the DM and omphalomesenteric veins is more likely to affect torsion than bending (Voronov et al., 2004; Nerurkar et al., 2006; Ramasubramanian et al., 2008).) Their results show that from HH10 to HH12-, when most of the bending occurs, myocardial cell volume increases 2–3 times more on the ventral side than on the dorsal side of the HT. A main goal of this paper is to reevaluate hypotheses for bending in light of these new findings.

In all, we have given a brief review of five proposed mechanisms for the bending component of c-looping. It is important to emphasize that evolution has led to redundancy in morphogenesis, and normal looping may require multiple mechanisms (Stalsberg, 1970; Taber, 2006). In the current study, we consider these mechanisms individually as well as collectively.
3.4 Experimental methods

3.4.1 Preparation and culture of the embryonic heart

Fertile white Leghorn chicken eggs (Sunrise Farms, Catskill, NY) were incubated in a humidified atmosphere at 38 °C for 36–48 hr to yield embryos at HH stages 10 to 12. Embryos were extracted from the eggs using filter paper rings (Waterman, No.2) (Voronov and Taber, 2002) and rinsed in PBS. The sandwich structure of embryo and filter paper rings was covered with liquid culture media to a depth of approximately 5 mm to eliminate surface tension artifacts (Voronov and Taber, 2002), and a stainless steel ring was placed on top of the paper rings to hold them in place.

To eliminate the complicating effects of external loads, as well as to allow better access for manipulation, we studied bending in isolated hearts. First, the splanchnopleure was removed using a fine glass needle. Then, the cranial, caudal and dorsal connections of the HT to surrounding tissues were severed with microscissors (Fine Science Tools, Foster City, CA), and the isolated heart was transferred by pipette to a sterile culture dish (Fisher Scientific, 35 mm) containing media. To prevent cell adhesion and spreading over the stiff plastic substrate, the culture dish was covered with a relatively soft agarose gel (0.3% agar, Sigma, Sigma-Aldrich, St. Louis, MO) (Latacha et al., 2005). Culture dishes were then sealed in plastic bags filled with a humidified mixture of 95% O₂ and 5% CO₂, and put into an incubator for continued 24-hr culture (Voronov and Taber, 2002).

For controls, we used media consisting of 89% Dulbecco’s modified Eagle’s medium (DMEM, Sigma), 10% chick serum (Sigma), and 1% antibiotics (Voronov and Taber, 2002). For chemical perturbation, one of the following drugs was added to the culture media: (–)-blebbistatin (Bleb, 30 µM, Sigma), a myosin-II inhibitor, to inhibit cell contractility (Rémond et al., 2006); cytochalasin D (CytoD, 100 nM, Sigma) to block actin polymerization (Latacha et al., 2005); or ovine hyaluronidase (Hyal, 20 UTR/mL, Sigma) to dissolve the CJ (Baldwin and Solursh, 1989; Linask et al., 2003). To prevent Bleb from being photoinactivated, tin foil was used to cover the media and dishes during culture and manipulation. In this study, we used 176 isolated chick hearts: 81, 52, 35, and 8 for the control group and hearts treated with Bleb, CytoD, or Hyal, respectively. In some samples,
to examine the short-term effects of drug perturbations, culture was stopped briefly at 1 hr and resumed immediately after images were taken.

### 3.4.2 Optical coherence tomography

Optical coherence tomography (OCT) is a noninvasive imaging technique that can provide sub-surface structural information of living tissues with high spatial resolution (∼ 10 µm) and relatively good penetration depth (up to ∼ 2 mm) (Mesud Yelbuz et al., 2002). Therefore, OCT is ideal for imaging the early development of chick embryos (Mesud Yelbuz et al., 2002). Images were obtained with a commercial OCT system (Thorlabs, Newton, NJ), and 3D tissue geometries were reconstructed using image analysis software (Volocity, PerkinElmer, Waltham, MA).

### 3.4.3 Strain measurements

Morphogenetic strains in the MY were measured by tracking the movements of fluorescent tissue labels. Prior to heart isolation, DiI (D282, Life Technologies, Carlsbad, CA) labels were injected into the MY via pulled glass micropipettes using a pneumatic pump (PicoPump PV830, World Precision Instruments, Sarasota, FL) and a micromanipulator (Sutter Instrument, Novato, CA) (Voronov et al., 2004). To quantify myocardial deformation along the longitudinal direction, three labels were evenly placed along either the ventral or dorsal side of the HH10 HT (see Fig. 3.6A). To do this, we first removed the splanchnopleure over the heart in the intact embryo and injected ventral labels along the midline of the HT. Then, we dissected the DM on one side to expose the dorsal side to the injection apparatus and injected labels near the remnant of the DM. Lastly, the DM on the other side was severed, and the conotruncus and omphalomesentric veins were cut to remove the HT from the embryo.

Images were acquired immediately after isolation and every few hours thereafter during culture using a fluorescence microscope (Leica Camera Inc., Allendale, NJ) and a high magnification camera (Canon USA Inc., Melville, NY). Label tracking was performed later using image analysis software (ImageJ, NIH). Arc lengths between adjacent labels were
measured over time, and longitudinal stretch ratios were computed as the current length divided by the reference length immediately after isolation.

Radial and circumferential strains in the MY were estimated from OCT time-lapse images. Since the myocardial wall thickness was relatively uniform around the HT (see Fig. 3.4B), cross sections midway along the length of the HT were selected as representative sections for analysis. Through image cropping, reslicing and thresholding (ImageJ), the myocardial wall was traced and used to calculate its average circumference and thickness. Average radial and circumferential stretch ratios in the MY were computed by dividing the average myocardial thickness and circumference during culture by their values immediately after isolation. In hearts treated with Hyal for 24 hr, however, this analysis was not feasible because the cross section collapsed, making the inner boundary of the MY ambiguous (see Fig. 3.4B).

3.4.4 Stress measurements

Myocardial stresses were estimated by introducing microsurgical incisions in the tissue (Zamir and Taber, 2004). The isolated heart was submerged under PBS and held by a glass micropipette with a small suction (see Fig. 3.8A). Small linear cuts were made in the MY using a Gastromaster microdissection device (Xenotek Engineering, Belleville, IL). To avoid the complication of stress alteration introduced by a second cut, only one cut was made in each heart. Immediately after cutting, OCT was used to image the wounds, which were relatively elliptical in the myocardial plane (see Fig. 3.8C). The geometry of the cut opening was obtained using image analysis software (Volocity and ImageJ), and the aspect ratio of the cut (opening width divided by cut length) was used to characterize the tension in the tissue normal to the cut direction (Zamir and Taber, 2004). Longitudinal and circumferential stresses correspond to circumferential and longitudinal cuts, respectively (see Fig. 3.8C).

3.4.5 Statistical analysis

Statistical analysis was performed using SigmaPlot software (Systat Software Inc., San Jose, CA). The Holm-Sidak method (one-way or two-way ANOVA) was used to compare strain
and stress data among different groups. All experimental measurements are presented as mean ± SD, with statistical significance assumed for $p < 0.05$.

### 3.5 Computational methods

#### 3.5.1 Modeling of morphogenesis

For more than 30 years, computational models have been used to simulate various morphogenetic processes (Wyczalkowski et al., 2012), including some used to test other hypotheses for c-looping (Taber et al., 1995; Nerurkar et al., 2006; Ramasubramanian et al., 2006, 2008). Several of these models, as well as the present model, are based on the theory for finite volumetric growth of Rodriguez et al. (1994). The detailed theoretical framework has been described previously (Rodriguez et al., 1994), and only a short summary is given here.

Consider an elastic body that is initially in a stress-free reference configuration $\beta$ (to a first approximation) when it is created in the embryo (Fig. 3.2). We imagine that the body is first cut into infinitesimal pieces, which undergo morphogenesis (growth, cell-shape change,
etc.) defined by the morphogenesis tensor \( \mathbf{M} \) (analogous to the growth tensor \( \mathbf{G} \) in Rodriguez et al. (1994)). This yields an intermediate stress-free configuration \( \mathcal{B} \). Finally, re-assembly and loading of the body through the elastic deformation gradient tensor \( \mathbf{F}^{*} \) gives the current configuration \( \mathcal{b} \). If the distribution of \( \mathbf{M} \) is geometrically incompatible, then the unloaded body \( \mathcal{B}_R \) will contain residual stress. The total deformation gradient tensor is given by

\[
\mathbf{F} = \mathbf{F}^{*} \cdot \mathbf{M}.
\]  

(3.1)

In this theory, the Cauchy stress tensor \( \mathbf{\sigma} \) is a function of \( \mathbf{F}^{*} \). The constitutive relation for a compressible material can be expressed as

\[
\mathbf{\sigma} = \frac{1}{J^*} \mathbf{F}^{*} \cdot \frac{\partial \mathbf{W}}{\partial \mathbf{E}^{*}} \cdot \mathbf{F}^{*T},
\]

(3.2)

where \( \mathbf{W} \) is the strain-energy density function, \( J^* = \det \mathbf{F}^{*} \) is the volume ratio, and \( \mathbf{E}^{*} = (\mathbf{F}^{*T} \cdot \mathbf{F}^{*} - \mathbf{I})/2 \) is the Lagrangian strain tensor, with \( \mathbf{I} \) being the identity tensor and \( T \) denoting the transpose. Computational models were developed using the commercial finite-element code ABAQUS (v. 6.9, SIMULIA, Providence, RI). With \( \mathbf{F} \) being a solution variable and \( \mathbf{M} \) specified, Eqs. (3.1) and (3.2) were implemented using a user subroutine UMAT (Dassault Systems Simulia Corporation, 2009), as described by Young et al. (2010).

### 3.5.2 Cylindrical model for heart bending

To simulate bending of the isolated chick heart, two types of models are considered. First, to explore basic behavior, we used a cylindrical tube model for the HT. Then, we constructed a model based on realistic heart geometry. This subsection discusses the tubular model; the other model is considered later.

The idealized model for the HT at HH10 is a straight cylinder consisting of a relatively thin outer layer of MY filled with a core of CJ (Fig. 3.3A–C). The lumen is omitted, as it collapses when blood pressure is lost upon isolation. The cross-sectional dimensions are scaled according to representative OCT images. Here, it is important to note that the outflow tract and a small portion of the omphalomesenteric veins are incorporated as part of the HT, since they are part of the isolated heart in the experiments. The myocardial layer is further divided into three regions of interest — ventral myocardium (VMY), dorsal
Figure 3.3: Finite-element models for bending of isolated heart. (A–C) Idealized cylinder model. Due to symmetry, only the top half of the model is shown, i.e., the heart is actually about 1 mm long. (A′–C′) Model based on realistic geometry. The undeformed geometries and meshes are shown in lateral (A,A′), ventral (B,B′), and cross-sectional views (C,C′). In both models, the heart tube consists of ventral myocardium (VMY), dorsal myocardium (DMY), dorsal mesocardium (DM), and cardiac jelly (CJ). The outflow tract and the remnant omphalomesenteric veins are considered part of the heart tube in the cylinder model, but they are included as passive structures in the realistic geometry model. The lumen is included in the realistic model but not in the cylinder model. Shown separately in (B) and (B′), a global cylindrical coordinate system \( \{R, \Theta, Z\} \) and principal directions \( \{e_R, e_\Theta, e_Z\} \) are defined in the undeformed configurations. Symmetry planes of the cylinder model are shown as dash-dot lines in (B) and (C).
myocardium (DMY), and DM. Note that the ventral and dorsal sides become the OC and IC, respectively, of the bent HT.

Because of symmetry, only a quarter of the HT is modeled, with symmetry conditions enforced and no external loads. In addition, a cylindrical coordinate system \( \{ R, \Theta, Z \} \) is defined in the undeformed tube with \( \Theta \) being the circumferential angle relative to the DM (Fig. 3.3C). The mesh consists of quadratic brick elements with reduced integration (C3D20R). We used a moderately fine mesh (21,662 nodes, 4,785 elements) that roughly follows the undeformed cylindrical geometry.

### 3.5.3 Material properties

Mechanical properties for MY and CJ were previously determined for the HH12 heart, and an exponential strain-energy density function was proposed (Zamir and Taber, 2004). At stage 12, material nonlinearity is relatively mild, and studies suggest that it increases during development (Zamir et al., 2003; Zamir and Taber, 2004; Rémond et al., 2006). Since the present study focuses on earlier stages (HH10 to 12), we assume that the properties for both MY and CJ are nearly linear and take the strain-energy density function in the neo-Hookean form

\[
W = C \left( \bar{I}_1 - 3 \right) + \frac{1}{D} \left[ \frac{1}{2} (J^* - 2) - \ln J^* \right],
\]

(3.3)

where \( C \) and \( D \) are material constants, and \( \bar{I}_1 = J^* - 2/3 \) tr\( C^* \) is a modified invariant of the elastic right Cauchy-Green deformation tensor \( C^* = F^* T \cdot F^* \). For relatively small values of \( D \), this form of \( W \) includes slight material compressibility as fluid flows in and out of soft tissues when they deform.

From the results of Zamir and Taber (2004), we take \( C_{MY} = 4C_{CJ} = 13 \) Pa \( \equiv C_0 \) and \( D_{MY} = D_{CJ} = 0.1 \). Unless stated otherwise, the material properties in the DM are taken the same as those in the MY. In this paper, all stresses are reported as dimensionless values normalized by \( C_0 \). As discussed later, choosing an exponential form for the strain-energy density function does not significantly change the results.
3.5.4 Looping simulation

With the morphogenesis tensor $\mathbf{M}$ specified as a function of position and time, the simulation for bending of the HT consists of a series of five sequential steps: CJ swelling, DM dissection, MY contraction, MY growth, and active MY cell-shape change. In each step, we assume that morphogenetic processes occur primarily along principal directions relative to the tube geometry and take

$$\mathbf{M} = M_R e_Re_R + M_\theta e_\theta e_\theta + M_Z e_Z e_Z.$$  \hspace{1cm} (3.4)

where $M_I$ and $e_I$ are the morphogenetic stretch ratio and the unit vector along direction $I$ of the undeformed cylinder ($I = R, \Theta, Z$). Below, we discuss the rationale and specialize $\mathbf{M}$ for each simulation step. The following subsection then details how experimental data were used to determine the values for the $M_I$ in each step.

**CJ Growth.** During looping, CJ continues to be created by myocardial cells, and it may also swell due to changes in osmolarity (Nakamura and Manasek, 1978). Together, these processes are simulated by isotropic growth, i.e.,

$$M_j = M_j (e_Re_R + e_\theta e_\theta + e_Z e_Z) \quad (M_j > 1), \hspace{1cm} (3.5)$$

where $M_j$ is the CJ growth parameter, with $\det M_j = M_j^3$ being the relative volumetric increase of CJ. Growth of CJ inflates and stretches the MY both circumferentially and longitudinally.

The value taken for $M_j$ is based on results from Zamir and Taber (2004), who used cutting experiments and modeling to estimate the residual strain (relative to the zero-stress state) in the MY of the HH12 chick heart. They found that the residual stretch ratio is approximately isotropic in the myocardial plane with a value of about 1.3. In our experiments, the circumference of the HT remained relatively constant during looping, suggesting that CJ undergoes little additional growth as the isolated heart bends (see Fig. 3.4B). We therefore set $M_j = 1.3$ (Table 3.1) at $t = 0$ and hold it constant thereafter (unless noted otherwise).
Table 3.1: Morphogenetic parameters of finite-element models

<table>
<thead>
<tr>
<th>Model</th>
<th>$M_j$</th>
<th>$M_t$</th>
<th>$M_c$</th>
<th>$M_g$</th>
<th>$M_s$</th>
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<tr>
<td>Cardiac Jelly Growth Model</td>
<td>1.3→2.0</td>
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<td></td>
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</tr>
<tr>
<td>Dorsal Mesocardial Tension Model</td>
<td>1.3</td>
<td>0.3</td>
<td>1.0</td>
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<td></td>
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<tr>
<td>Myocardial Cell-Shape Change Model</td>
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<td>1.0</td>
<td></td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Myocardial Differential Growth Model</td>
<td>1.3</td>
<td>1.0, 0.9</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline Model</td>
<td>1.3</td>
<td>0.8</td>
<td>1.0, 0.9</td>
<td>1.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

† Initial cardiac jelly growth $M_j = 1.3$ used in all models as the initial condition. Cardiac jelly growth model includes additional growth of 1.5 to generate bending during looping, and the total growth is $1.3 \times 1.5 \approx 2.0$.
‡ $M_c = 1.0$ for blebbistatin-treated heart and 0.9 for control.

DM Dissection. Until about HH11, the HT is attached to the foregut of the embryo through the DM (Taber, 2006; Männer, 2000), which is in a state of tension (see Fig. 3.8C). When the heart is removed from the embryo, the DM is severed, and the remnants of the DM shorten, similar to a stretched rubber band when its ends are released. This effect is simulated by shortening the zero-stress length of the DM as given by

$$M_t = e_{eR} + e_{e\theta} + M_t e_{eZ} \quad (M_t < 1),$$

where $M_t$ is the DM tension parameter defined as the stress-free DM length divided by its length before dissection.

Unfortunately, the value of $M_t$ is neither available in the literature nor trivial to measure experimentally. This is because the DM is not accessible for label injection in the HH10 embryo, unless one side of the DM is dissected to allow the HT to rotate lateral side up. This dissection, however, disturbs the stress in the DM. Hence, the value of $M_t$ was estimated by matching the longitudinal stress and the deformed HT shape between our model and experiments. This gave $M_t = 0.8$ for control hearts (see Table 3.1 and Appendix Fig. A.6A–D).

MY Contraction. In previous work, we have found that the MY undergoes a general cytoskeletal contraction in response to the removal of compressive loads normally exerted by
the splanchnopleure (Nerurkar et al., 2006; Filas et al., 2011). In contrast to the sarcomeric contraction that generates the heartbeat, cytoskeletal contraction is likely produced by actomyosin fibers localized at or near cell borders (Martin, 2010; Wozniak and Chen, 2009). Our experimental observations of short-term Bleb-exposure suggest that contraction decreases the length and diameter of the HT while increasing the thickness of the myocardial wall (see Fig. 3.5). Consequently, we assume that this contractile response is isotropic at the tissue level within the plane of the MY as observed, for example, during dorsal closure in the Drosophila embryo (Martin, 2010). Simulating contraction as negative growth, we take

\[ \mathbf{M}_c = M_c^{-2} \mathbf{e}_R \mathbf{e}_R + M_c \left( \mathbf{e}_\theta \mathbf{e}_\theta + \mathbf{e}_Z \mathbf{e}_Z \right) \quad (M_c \leq 1), \]  

which satisfies the cellular incompressibility condition \( \det \mathbf{M}_c = 1 \).

Since Bleb inhibits actomyosin contraction, we set \( M_c = 1 \) (passive tissue) to simulate the Bleb-treated heart with the other parameters remaining unchanged (Table 3.1). The value of \( M_c \) for the control case was chosen from our experimental estimates of radial strain caused by contraction. As discussed later, compared to the heart treated with Bleb, the MY of control hearts had approximately 30% more radial thickening (see Fig. 3.6C). We assume this difference can be attributed to cytoskeletal contraction of the MY and choose \( M_c = 0.9 \) \((M_c^{-2} \approx 1.3)\) for the control heart (see Table 3.1).

**MY Growth.** Experimental evidence indicates that significant cell proliferation occurs during c-looping in the foregut near the DM and in parts of the omphalomesenteric veins, which eventually are incorporated into the HT (Abu-Issa and Kirby, 2008; Linask et al., 2003; Soufan et al., 2006). However, relatively little proliferation occurs within the MY of the HT from HH10 to HH12-, when most of the bending occurs (Sissman, 1966; Stalsberg, 1969; Soufan et al., 2006). The mitotic rate begins to increase significantly in the HT only as c-looping nears completion at HH12 (Soufan et al., 2006). Hence, we neglect the effects of cell division on cardiac bending and assume, to a first approximation, that all growth in the MY can be attributed to cellular hypertrophy.

The specific form of the morphogenesis (growth) tensor \( \mathbf{M}_g \) is chosen based on existing data and our own experimental observations. Soufan et al. (2006) found that between HH10 and HH12-, myocardial cells at the IC grow relatively little, while those at the OC become 2–3 times larger than those at the IC. Accordingly, we assume that a dorsal-to-ventral gradient
exists in the spatial pattern of myocardial growth and take a linear distribution around the circumference, i.e.,

\[ G(\Theta) = \det \mathbf{M}_g = G_{IC} + (G^{OC} - G_{IC}) (\Theta / \pi), \]  

(3.8)

where \( G(\Theta) \) is the volumetric-growth function, with \( G_{IC} = 1 \) and \( G^{OC} = 3 \) at the IC \( (\Theta = 0) \) and OC \( (\Theta = \pi) \), respectively (see Fig. 3.3C).

However, since Soufan et al. (2006) measured only changes in volume without considering the possibility of anisotropic growth, the spatial distribution of each component of \( \mathbf{M}_g (G_R, G_\Theta, G_Z) \) remains unknown. Thus, we must make additional assumptions.

First of all, as discussed later, our measurements show that the myocardial wall thickness increases almost uniformly during bending of the isolated heart while its circumference remains essentially constant (see Figs. 3.4B and 3.6C). These observations suggest that growth in the radial and circumferential directions is relatively uniform, and it follows that the differences in cell size measured by Soufan et al. (2006) reflect dimensional changes occurring primarily in the longitudinal direction. These considerations lead us to propose a morphogenesis tensor for MY growth of the form

\[ \mathbf{M}_g = G_R \mathbf{e}_R \mathbf{e}_R + G_\Theta \mathbf{e}_\Theta \mathbf{e}_\Theta + \frac{G(\Theta)}{G_R G_\Theta} \mathbf{e}_Z \mathbf{e}_Z, \]  

(3.9)

where \( G_R \) and \( G_\Theta \) are uniform growth parameters, \( G/(G_R G_\Theta) = G_Z \), and \( \det \mathbf{M}_g = G(\Theta) \) is the volumetric growth given by Eq. (3.8).

Various possibilities exist for the values of \( G_R \) and \( G_\Theta \). At first thought, it may seem that the measured change in myocardial thickness dictates the value of \( G_R \). However, this matter is complicated by the fact that both circumferential and longitudinal growth can cause myocardial thickening, because the CJ constrains myocardial expansion, causing the MY to become compressed with an accompanying thickness increase. Moreover, since the inner myocardial circumference changes little during culture, radial growth alone would increase circumferential tension near the outer myocardial surface as it grows outward. Our results indicate that the amount of radial growth needed to match our data would increase myocardial circumferential stress to an extent that cannot be overcome by other factors, contrary to our observations that show a decrease in myocardial tension during bending (see
Fig. 3.8 and Appendix Fig. A.5). These considerations lead to the alternative assumption \( G_R = G_\Theta \equiv M_g \), leaving \( M_g \) as the only free growth parameter (with \( G(\Theta) \) known).

We do not know how to justify this assumption based on microstructure. However, we note that, like the mature heart, the embryonic heart adapts to changes in loading conditions by altering its radius and wall thickness (Clark et al., 1989). Hence, the increased CJ volume may trigger a response similar to that of elevated end-diastolic volume, i.e., the unloaded circumference grows. In addition, the increased wall tension caused by CJ pressure induces an increase in myocardial thickness that tends to return wall stress toward normal levels. Taking \( G_R = G_\Theta \) then represents the simplest assumption.

A parameter study was used to determine \( M_g \). As shown below, the best match to the bending strains in control hearts is given by taking \( M_g = 1.3 \) (see Table 3.1 and Appendix Fig. A.6E,F).

Active MY Cell-Shape Change. According to Manasek et al. (1972), myocardial cells at the OC increase in apical surface area but remain randomly oriented during c-looping, while cells near the IC become elongated and aligned in the circumferential direction. The changes in cell surface area measured by these investigators are generally consistent with the volumetric data of Soufan et al. (2006). Taken together, these results suggest that cells near the IC become shorter longitudinally than those near the OC. Manasek et al. (1972) speculated that these cell-shape changes drive rather than being a consequence of looping, and results obtained by Latacha et al. (2005) suggest that polymerizing actin filaments cause the observed changes in cell morphology.

These effects are included in the model through the shape-change tensor

\[
M_s = e_R e_R + S e_\Theta e_\Theta + S^{-1} e_Z e_Z,
\]

which satisfies the constraint \( \det M_s = 1 \), i.e., changes in cell shape occur isovolumetrically. The shape-change function \( S \) is assumed to depend on \( \Theta \). The SEM images of Manasek et al. (1972) suggest that the gradient in cell morphology increases toward the IC, and thus we take

\[
S(\Theta) = (M_s - 1) \cdot [(\Theta/\pi) - 1]^2 + 1 \quad (M_s > 1),
\]
where \( M_s \) is the shape-change parameter. From Eq. (3.10), we can consider \( S^2(\Theta) \) as the change in the aspect ratio of circumferential to longitudinal length for a cell, which monotonically decreases from \( M_s^2 \) at the IC (\( \Theta = 0 \)) to unity at the OC (\( \Theta = \pi \)).

To estimate \( M_s \), we quantified cell shapes from the SEM images of Manasek et al. (1972) and chose \( M_s = 1.3 \) for the baseline model for control hearts (see Table 3.1, Appendix A.2.2, and Fig. A.4).

**Timing of Events.** The steps outlined above are assumed to occur sequentially, with CJ swelling occurring before dissection of the DM. This sets the reference state (\( t = 0 \) hr) for experimental correlations from the beginning of the culture period. The MY then contracts after the heart is isolated. Finally, MY growth and cell-shape changes occur over the next 24 hr of culture. The total morphogenesis tensor is given by

\[
M = M_s \cdot M_g \cdot M_c \cdot M_t \cdot M_j.
\] (3.12)

**Evaluation of Strain and Stress.** For comparison with acquired data, we quantify the myocardial strains and stresses in the same way as in our experiments (see Experimental Methods). Briefly, longitudinal stretch ratios were calculated as the changes in myocardial length (on the outer surface) at the OC (\( \Theta = \pi \)) and near the IC (\( \Theta = \pi/6 \)) of the HT, respectively, where our labels were placed in the experiments. Radial and circumferential stretch ratios were computed from the average myocardial thickness and circumference, respectively, at the cross-sectional plane of symmetry (see Fig. 3.3C). To evaluate myocardial stresses, we introduced virtual cuts in the cylinder model by freeing nodal connections on the plane of the cut. All cuts were made approximately 150 \( \mu m \) long and 75 \( \mu m \) deep with their centers located on the cross-sectional plane of symmetry. The OC and IC cuts were located at the center of the VMY (\( \Theta = \pi \)) and DMY (\( \Theta = \pi/3 \)), respectively, where we made the cuts in the experiments. The tension in the MY perpendicular to the cut was quantified by the aspect ratio of the cut opening. It is important to note that the stretch ratios and decreases in cut aspect ratios are defined relative to the configuration at the beginning of culture, i.e., after CJ growth and DM dissection.
3.5.5 Model based on realistic heart geometry

After using the idealized cylinder model to determine the morphogenesis tensor for each simulation step, we extrapolated these results to a heart model based on realistic 3D geometry.

We reconstructed the geometry for an isolated HH10 heart from a stack of OCT cross sections (Fig. 3.3A′–C′). Anatomic structures, such as the MY, CJ, and lumen, were segmented out from the images. Then, voxel surfaces at the interface of two entities were extracted and smoothed to remove any sharp changes in curvature at the voxel edges and vertices, which could cause stress concentrations and convergence difficulties. After adjusting the position of each vertex by its weighted average over neighboring vertices, the triangular mesh on the congruent surface was further smoothed and coarsened using the PATRAN Mesh-on-Mesh routine (PATRAN, MSC Software, Santa Ana, CA). Finally, the closed triangular surface mesh was converted to a solid tetrahedral mesh using ABAQUS/CAE. This model consists of 11,914 nodes and 51,405 elements (C3D4), and the MY contains at least two layers of elements in the thickness direction.

For convenience, the model was partitioned into the following segments: CJ, DM, DMY, VMY, outflow tract, and remnants of omphalomesentric veins (Fig. 3.3A′,C′). Unlike the cylinder model, the lumen space was included, and frictionless contact conditions were defined to model the collapse of the lumen due to the loss of blood pressure and myocardial contraction. The parameters from the cylinder model were used to compute average input parameters, which were taken as uniform in each region. Such an approximation has its drawbacks, as stress concentrations arise due to the mismatch between adjacent regions. Hence, to achieve a finer spatial variation and to reduce stress concentrations, the MY was further partitioned into 24 subregions.

Principal directions (\(e_R, e_\theta, e_Z\)) were defined for each element following anatomic features of the undeformed HT. For boundary conditions, the cranial end of the outflow tract was fixed while all other boundaries were free. Material properties for both MY and CJ were adopted from the cylinder model (see Eq. (3.3)). As an approximation, the outflow tract and the remnant omphalomesenteric veins were taken as passive, while the rest of the MY layer was assumed to undergo the same morphogenetic processes (contraction, growth,
cell-shape change, etc.) as defined by the parameter values extrapolated in each region from those given by the cylinder model.

3.6 Experimental results

To test the plausibility of the differential growth hypothesis for cardiac bending, we conducted experiments on isolated chick hearts. Measurements were made from images acquired at various times during a 24-hr culture period from about HH10. First, we explored the fundamental idea that myocardial growth can drive HT bending. Then, we collected strain and stress data to be used in testing our computational models.

3.6.1 Perturbations of HT growth and bending

To determine whether growth of the MY is required for HT bending, we compared the amount of growth in hearts that bend normally to growth in hearts in which bending is perturbed. For this purpose, we note that looping is inhibited by exposure to relatively low doses of CytoD or latrunculin A, which block actin polymerization (Latacha et al., 2005). In contrast, looping in both whole embryos and isolated hearts continues unimpeded when actomyosin contraction is blocked by BDM, Y-27632, ML-7, or Bleb after looping begins (Rémond et al., 2006). Hence, if differential growth provides the main driving force for bending, then it follows that actin inhibitors should have a greater effect on HT growth than myosin inhibitors.

Published studies suggest that this is generally the case for various cell types. Since cell division requires the formation and contraction of an actomyosin ring, both actin and myosin inhibitors reduce hyperplasia (Rosenblatt et al., 2004; Spector et al., 1989). But whereas blocking actin polymerization decreases hypertrophy (Ornelles et al., 1986; Ingber et al., 1995), inhibiting contraction via Bleb, for example, has relatively little effect on increases in cell size (Földes et al., 2011). In fact, rather than dividing into two cells, stem-cell-derived cardiomyocytes exposed to Bleb often end up with two nuclei following mitosis (Li et al., 1997; Földes et al., 2011). Taken together, these results are consistent with the
Figure 3.4: **Bending of isolated hearts in different culture conditions.** (A) Ventral view of a representative HH10 chick embryo before heart dissection. (B) Lateral views and OCT cross sections of hearts isolated at HH10 (0 hr) and cultured for 24 hr in various conditions: control, 30 µM (-)-blebbistatin (Bleb), 20 UTR/mL hyaluronidase (Hyal), and 100 nM cytochalasin D (CytoD). Bending occurred in all cases except the CytoD-treated hearts. Significant thickening of the myocardial wall was observed in the cross sections of bent hearts (solid arrowheads), while the myocardium thickened less in the CytoD-treated heart (hollow arrowheads). DM = remnant dorsal mesocardium, IC = inner curvature, OC = outer curvature. Scale bar: 200 µm in (A) (same for (B)).

Figure 3.5: **Short-term exposure to blebbistatin reveals effects of myocardial contraction in isolated hearts.** Bright-field images (lateral view) and OCT cross sections are shown for control and blebbistatin-treated hearts (Bleb, 30 µM). After 1 hr, the circumference and length of control hearts decreased, while the myocardial wall thickness increased (yellow arrowheads). However, these geometric quantities remained relatively unchanged (red arrowheads) in Bleb-treated hearts, where cytoskeletal contraction was blocked. These results suggest that myocardial contraction contributes to the observed increase in wall thickness in control hearts. Scale bar: 200 µm.
view that inhibiting actin polymerization hinders both tissue growth and looping more than inhibiting contraction. However, we still need to verify this for embryonic hearts.

To check for a correlation between myocardial growth and heart bending, we cultured isolated HH10 hearts in media containing 100 nM CytoD or 30 μM Bleb. After 24 hr of culture, CytoD prevented significant bending in 89% of the hearts (n = 35), while all of the Bleb-treated hearts (n = 52) bent approximately the same amount as controls (n = 81; Fig. 3.4). (Effectiveness of drug treatments was verified by diminished but not abolished heartbeat and reduced tension in the MY.) OCT cross sections near the center of the HT showed that the thickness of the myocardial wall increased by a factor of about 2.9 in control hearts, but only 2.2 and 1.5 in Bleb-treated and CytoD-treated hearts, respectively (Figs. 3.4B and \( \lambda_r \) in 3.6C). Changes in cross-sectional area of the MY showed similar trends (Fig. 3.7). As discussed below, the difference in myocardial thickness and cross-sectional area between control and Bleb-treated hearts is likely caused by contractile effects rather than growth (see Figs. 3.5, 3.6B,C, and 3.7), and since both drugs inhibit contraction, decreased growth likely accounts for the further reduction in thickness in CytoD-treated hearts. These results suggest a link between cell growth and looping but do not rule out other possible contributing factors.

As a side issue, since CJ expansion can affect growth in the MY though mechanical feedback (Taber and Perucchio, 2000), we examined the effects of removing the CJ in isolated hearts. Previous studies suggest that looping occurs in intact embryos devoid of CJ, although the heart becomes flaccid (Baldwin and Solursh, 1989; Linask et al., 2003). We exposed isolated hearts to 20 UTR/mL ovine Hyal. After 24 hr of culture, all hearts (n = 8) were bent significantly (Fig. 3.4B). Despite the noticeable overall shrinkage of the HT, the cross-sectional area of the myocardial wall increased similarly to control and Bleb-treated hearts (Figs. 3.4B and 3.7), consistent with results for whole embryos (Baldwin and Solursh, 1989). We reason that shrinkage of the HT is caused by the loss of CJ pressure, but the myocardial growth that drives bending is unaffected by CJ removal.

Taken together, the results of these drug perturbation experiments support the hypothesis that differential growth plays a role in heart bending through myocardial hypertrophy. However, these experiments do not address the issue of how growth causes the HT to bend. That is the objective of the following experiments and modeling.
Figure 3.6: Morphogenetic strains measured during bending of isolated heart. (A) Fluorescent labels were injected on the ventral (v) and dorsal (d) sides of the HT to measure longitudinal strains. (B) Longitudinal stretch ratios ($\lambda_{zv} > 1$, $\lambda_{zd} < 1$) indicate ventral elongation and dorsal shortening in both control hearts and hearts treated with 30 µM (−)-blebbistatin (Bleb). Linear regressions (solid lines) suggest longitudinal stretch ratios change linearly with time (for control and Bleb-treated hearts, $R^2 = 0.9839, 0.9157$ for $\lambda_{zv}$ and $R^2 = 0.7831, 0.8409$ for $\lambda_{zd}$, respectively). (C) Summary of all myocardial stretch ratios after 24-hr culture: radial $\lambda_r$, circumferential $\lambda_\theta$, longitudinal on the ventral side $\lambda_{zv}$, and longitudinal on the dorsal side $\lambda_{zd}$. The myocardial wall thickened less in Bleb-treated hearts than that in control, and even less in hearts treated with 100 nM cytochalasin D (CytoD; * $p < 0.001$, one-way ANOVA). Note that longitudinal stretch ratios were not measured in CytoD-treated hearts, where bending was inhibited. Scale bar: 200 µm.
3.6.2 Morphogenetic strains

Average longitudinal stretch ratios relative to HH10 were computed along the ventral and dorsal sides of the HT by tracking the motions of three tissue labels on each side during culture (Fig. 3.6A). Consistent with the results of Butler (1952), the ventral side of the heart elongated, while the dorsal side shortened as the heart bent. In control hearts \((n = 13)\), the stretch ratio on the ventral side \((\lambda_z^v)\) increased from unity to \(1.45 \pm 0.18\), while the dorsal value \((\lambda_z^d)\) decreased to \(0.73 \pm 0.12\) after 24 hr (Fig. 3.6C). These values are relatively consistent with those reported by Butler (1952), which were based on length measurements of a single heart. The deformation rates during culture remained relatively constant (Fig. 3.6B). In Bleb-treated hearts \((n = 8)\), \(\lambda_z^v\) increased more \((1.61 \pm 0.23)\), but the dorsal side shortened approximately the same amount as controls \((\lambda_z^d = 0.73 \pm 0.19)\). Since bending was inhibited by CytoD (see Fig. 3.4B), longitudinal stretch ratios were not measured in CytoD-treated hearts.

We computed average radial \((\lambda_r)\) and circumferential \((\lambda_\theta)\) stretch ratios in the MY from measured myocardial thickness and circumference, respectively. After 24 hr, the change
in circumference was relatively small and nearly the same for all three groups ($\lambda_\theta \approx 1.1$; Figs. 3.6C and 3.7), suggesting that the CJ volume increased relatively little during culture. In contrast, compared to controls ($\lambda_r = 2.88 \pm 0.36$), the wall thickened significantly less in Bleb-treated ($\lambda_r = 2.19 \pm 0.21$) and even less in CytoD-treated hearts ($\lambda_r = 1.52 \pm 0.13$) (Fig. 3.6C; $n \geq 6$ for each group; $p < 0.001$, one-way ANOVA). Since Bleb specifically inhibits actomyosin contractility, we attributed the differences in $\lambda_r$ between control and Bleb groups to myocardial contraction, i.e., as the lumen closes in isolated hearts (see Fig. 3.4B), the circumference of the MY decreases while its thickness increases due to near incompressibility (Fig. 3.5). Because some boundaries of the myocardial wall became indistinguishable without CJ (see Fig. 3.4B), stretch ratios were not computed for Hyal-treated hearts. However, the measured areas of the myocardial cross sections suggest that losing CJ pressure does not significantly affect growth of the MY (Fig. 3.7).

### 3.6.3 Myocardial stresses

Circumferential and longitudinal microsurgical cuts near the OC or the IC were used to probe myocardial stress in the HT (Fig. 3.8C). We used the aspect ratios $\alpha_{z}^{OC}$, $\alpha_{\theta}^{OC}$, $\alpha_{z}^{IC}$ and $\alpha_{\theta}^{IC}$ of the cut openings to characterize the stresses $\sigma_{zz}^{OC}$, $\sigma_{\theta\theta}^{OC}$, $\sigma_{zz}^{IC}$ and $\sigma_{\theta\theta}^{IC}$, respectively.

After isolation at HH10 ($t = 0$ hr), all cuts opened immediately after being made (Fig. 3.8C). The aspect ratios indicate that initial longitudinal stress near the IC ($\alpha_{z}^{IC} = 0.46 \pm 0.03$) was considerably smaller than the other three measured stresses ($\alpha_{z}^{OC} = 0.65 \pm 0.08$, $\alpha_{\theta}^{OC} = 0.65 \pm 0.13$, $\alpha_{z}^{IC} = 0.73 \pm 0.14$) (Fig. 3.8B; $n = 5$ for each type of cut). The difference between $\alpha_{z}^{IC}$ and $\alpha_{z}^{OC}$ was statistically significant ($p = 0.011$, two-way ANOVA), suggesting that $\sigma_{zz}^{IC} < \sigma_{zz}^{OC}$ at $t = 0$ hr. Interestingly, additional data show that $\alpha_{z}^{OC}$ (and $\sigma_{zz}^{OC}$) was significantly larger in the isolated heart than in the intact heart at HH10 (Fig. 3.9; $n = 6$), consistent with an increase in OC tension that would be expected if the DM shortens and bends the heart somewhat after dissection. Here, it is important to note that the cuts in the isolated HH10 hearts were made just after removal from the embryo, before the active contractile response became significant.

At the end of the culture period ($t = 24$ hr; Fig. 3.8B,C), the stresses at the OC of control hearts dropped by nearly 50% ($\alpha_{z}^{OC} = 0.36 \pm 0.08$, $\alpha_{\theta}^{OC} = 0.35 \pm 0.09$; $n = 7$ for each type of cut) relative to those in hearts at HH10 ($p < 0.001$, two-way ANOVA). Cuts at the
Figure 3.8: Residual stresses in the myocardium as revealed by microsurgical cuts. (A) Linear cut in heart tube cultured for 24 hr. Opening of the circumferential cut indicates tensile longitudinal stress. Cut aspect ratio ($\alpha$), defined as opening width ($w$) divided by cut length ($l$) in the local myocardial plane (see panel C), characterizes tension in the myocardium. (B) Aspect ratios for cuts made near the outer curvature (OC) or inner curvature (IC) of control hearts before (0 hr) or after (24 hr) culture. Subscripts $z$ and $\theta$ denote longitudinal and circumferential tensions, respectively (* $p < 0.001$, ** $p = 0.011$). (C) Opening (red dashed ellipses) and closure (blue dashed lines) of representative cuts show residual stress states in control hearts before (0 hr) and after (24 hr) culture. Outward and inward arrows indicate tension and compression, respectively. Since cuts made near the IC of bent hearts usually do not open, aspect ratios were reported as 0 in B. Scale bars: 200 $\mu$m.
Figure 3.9: **Immediate effects of heart isolation on myocardial stress.** In HH10 hearts of whole embryos, circumferential and longitudinal cuts were made on the ventral side, which becomes the outer curvature (OC) of the looped heart. Cut aspect ratios indicate that, immediately after isolation, longitudinal stress (shown by $\alpha^\text{OC}_z$) in the myocardium increased significantly (*$p = 0.011$, two-way ANOVA), while circumferential stress (shown by $\alpha^\text{OC}_\theta$) changed little. These results are consistent with the idea that tension in the dorsal mesocardium bends the heart after dissection.

IC hardly opened at all (Fig. 3.8C; $n \geq 5$ for each type of cut). These results indicate that both longitudinal and circumferential myocardial tensions decrease throughout the MY as the HT bends.

In some hearts, circumferential cuts were made in remnants of the DM (Fig. 3.8C). We did not measure the aspect ratio for these cuts, because access to the DM was sometimes difficult as it became packed tightly inside the bent HT. But generally speaking, strong longitudinal tension seemed to be present along the DM before and after culture ($n = 4$ for each), supporting one of the assumptions in our model.

It is also worth noting that exposure to Bleb or CytoD further diminished myocardial stresses during culture (data not shown). Therefore, we often used cutting to test the efficacy of these drug treatments.

Taken together, these results indicate the following: (1) tension was present everywhere in the MY of the straight HH10 heart before culture; (2) after culture for 24 hr, the tension decreased significantly or even disappeared in control hearts; and (3) treatment with Bleb or CytoD relaxed almost all the initial tension.
3.7 Computational results

As described above, two types of finite-element models were developed (Fig. 3.3). Our strategy is (1) to investigate possible bending mechanisms individually using a cylinder model; (2) to obtain a baseline cylinder model that can best recapitulate our experimental data; and (3) to extend our analysis to a model based on realistic heart geometry. As in the experiments, morphogenetic stretch ratios in all models were calculated relative to the configuration at the onset of culture, i.e., following CJ growth and dissection of the DM.

3.7.1 Single-mechanism models

To better understand fundamental behavior and limitations, we first created a separate model for each bending mechanism. Each model is based on idealized cylindrical geometry and contains one free morphogenetic parameter (see Eqs. (3.5)–(3.11)) that changes linearly in time as suggested by the time-history plot of longitudinal stretch ratios (see Fig. 3.6B). The input value of each model-specific parameter was adjusted to match our strain data as much as possible (Table 3.1 and Fig. 3.10B). To avoid the complication of myocardial contraction, only Bleb-treated hearts ($M_c = 1$) are considered in this subsection. Please note that our comparisons focus on stresses near the middle of the HT, where our measurements were made.

CJ Growth with DM Constraint. In both whole embryos and isolated culture, the HT apparently always bends with the DM located along the IC, consistent with the CJ swelling hypothesis of Manasek et al. (1984). This mechanism requires the DM (i.e., IC of the HT) to be stiffer than the MY, as verified by the microindentation measurements of Zamir et al. (2003). Here the DM is represented by a relatively narrow region of the outer cell layer along the dorsal side of the HT (Fig. 3.3A,C). Guided by published stiffness data for HH12 chick hearts (Zamir et al., 2003), we set the DM modulus to five times of that of the MY. In this single-mechanism model, we took $M_j = 1.5$ relative to $t = 0$ hr (the total growth is $M_j = 1.3 \times 1.5 \approx 2.0$) to match the longitudinal strain on the ventral side of the HT (Table 3.1 and $\lambda_{zv}$ in Fig. 3.10B).
Figure 3.10: **Single-mechanism cylinder models for bending of blebbistatin-treated heart.** (A) Deformed shapes and stress distributions shown in lateral and cross-sectional views. Because of symmetry, only the top half of the model is shown. The initial configuration for each model includes cardiac jelly (CJ) growth that occurs prior to HH10. Simulations then include one of the following mechanisms: additional CJ growth with dorsal mesocardium (DM) constraint; DM tension; active cell-shape changes in the myocardium (MY); or MY differential growth. Only the differential growth model produces deformation and stresses comparable to experimental results (DMY = dorsal myocardium, VMY = ventral myocardium, IC = inner curvature, OC = outer curvature). (B) Experimental and numerical stretch ratios in the MY: radial \( \lambda_r \), circumferential \( \lambda_\theta \), longitudinal on ventral side \( \lambda_{zv} \), and longitudinal on dorsal side \( \lambda_{zd} \). The differential growth model yields the closest agreement with experiment.

Similar to a previous model based on this idea (Taber and Perucchio, 2000), the HT bends as expected (Fig. 3.10A). However, although the longitudinal stretch at the OC matches our data, the IC also elongates, contrary to our measured IC shortening (\( \lambda_{zd} \) in Fig. 3.10B). This behavior leads to relatively modest bending (Fig. 3.10A).

The stress patterns predicted by this model also are not consistent with our experimental data. Without the DM, CJ growth would generate myocardial tension in both the circumferential and longitudinal directions. With the DM, superimposed bending increases this tension at the OC and decreases it at the IC, whereas our experiments indicate that stresses decrease throughout the MY as the HT bends (see Fig. 3.8B,C). Hence, along with the present and other published studies of Hyal-treated hearts (Baldwin and Solursh, 1989; Linask et al., 2003) (see Fig. 3.4B), these results suggest that CJ swelling can contribute to the bending, but other factors likely play a more prominent role.
**DM Tension.** Until about HH11, the DM connects the dorsal side of the HT to the foregut of the embryo (Taber, 2006; Männer, 2000). Since our experiments indicate that the DM is initially under tension (see Fig. 3.8C), this structure would shorten when it ruptures during normal looping or is severed for isolated heart experiments. This shortening, modeled as negative longitudinal growth of the DM, causes the HT to bend ventrally with the DM located along the IC. In agreement with trends in our measurements, the computed longitudinal strain decreases at the IC and increases a little at the OC (Fig. 3.10B). However, whereas the longitudinal myocardial stress near the IC (not in the DM) decreases as in cultured hearts, the OC stress increases (Fig. 3.10A), contrary to our experimental data (see Fig. 3.8B,C). In addition, even very strong DM shortening ($M_t = 0.3$) does not generate enough bending, as reflected in $\lambda_{zv}$ (Fig. 3.10A,B).

**Active Cell-Shape Change.** As discussed below Eq. (3.11), we stipulate active changes in cell shape to simulate the observed circumferential elongation and longitudinal shortening of myocardial cells near the IC. These shape changes cause the HT to bend ventrally (Fig. 3.10A). Here, we choose $M_s = 1.7$, which corresponds to a maximum increase in cell aspect ratio of approximately 2.9 (much larger than our estimate of $M_s = 1.3$ from the cell-shape data of Manasek et al. (1972)). With this value, the model matches the longitudinal stretch ratio on the dorsal side ($\lambda_{zd}$) but not on the ventral side ($\lambda_{zv}$) of the HT (Fig. 3.10B). In addition, the CJ restricts the increase in myocardial circumference, causing circumferential tension to decrease, in agreement with our data, but the longitudinal tension in the MY increases in regions near the IC and OC (Fig. 3.10A), contrary to our data. Taken together, these results suggest that a bending mechanism based on active changes in myocardial cell shape alone is not consistent with all of our newly acquired data.

**Differential Growth.** Unlike the other mechanisms, our model shows that differential growth based on the measurements of Soufan et al. (2006) is capable of not only generating sufficient bending, but also relaxing the initial tensions in the MY (Fig. 3.10A,B). For Bleb-treated hearts, the differential growth model yields results that agree reasonably well with all of our experimental strain and stress data (Fig. 3.10).

Next, we explored whether differential growth alone can also reproduce our experimental results for control hearts, which undergo cytoskeletal contraction after isolation (Fig. 3.11).
Figure 3.11: **Differential growth model for bending of control hearts.** (A) Deformed shapes and myocardial stresses. Cardiac jelly growth (initial state) is followed by myocardial (MY) contraction and growth. (DM = dorsal mesocardium, IC = inner curvature, OC = outer curvature) (B) Experimental and numerical stretch ratios (Radial $\lambda_r$, circumferential $\lambda_\theta$, ventral longitudinal $\lambda_zv$, and dorsal longitudinal $\lambda_zd$). Stretches given by differential growth model are similar to those given by baseline model, all of which agree reasonably well with experimental data. (C) Experimental and numerical changes in cut aspect ratios ($\Delta \alpha_z$ and $\Delta \alpha_\theta$ correspond to decreases in longitudinal and circumferential stress, respectively). Differential growth model captures experimental decrease in OC longitudinal stress but not magnitude of change in other stresses. The baseline model, which includes dorsal mesocardium dissection and active MY cell-shape changes, gives better agreement.
For the same amount of growth, myocardial contraction was added \( M_c = 0.9 \), see Eq. (3.7)). Although the nearly incompressible CJ keeps the myocardial circumference relatively unchanged, contraction causes the MY to shorten longitudinally and to thicken radially (Fig. 3.11A). All strains agree reasonably well with our data (Fig. 3.11B), but contraction elevates myocardial tensions \( \sigma_{zz} \) and \( \sigma_{\theta\theta} \) so that they remain unrealistically high near the IC after bending is complete (Fig. 3.11A,C). Taken together, these results suggest that differential growth is the primary driver of bending in isolated hearts, with other mechanisms possibly playing a lesser role.

In summary, all of the studied mechanisms for the bending component of c-looping cause the HT to bend with the DM located along the IC, as observed both in intact embryos and in isolated heart culture. However, of the various proposed mechanisms, our models indicate that only differential growth is capable of capturing the regional changes in stress and strain in Bleb-treated hearts. As a step toward understanding the situation in ovo, we next combine mechanisms to develop a baseline model for isolated hearts cultured under control conditions.

### 3.7.2 Baseline model

Our simulation for bending of the isolated heart under control conditions consists of five steps: (1) CJ growth, which also occurs in ovo; (2) DM dissection (to isolate the HT from the embryo); (3) MY contraction, which is a response to the removal of normal external loads; (4) MY differential growth; and (5) active changes in MY cell shape. As described above, the parameter values in the model were chosen using a combination of existing data and manual iteration to obtain agreement between model predictions and our experimental results (Table 3.1). It is important to note that the primary driving mechanism in our model, differential growth, is based on the measurements of Soufan et al. (2006).

Results are shown for each step of the model in Fig. 3.12A,C,D. As expected, the initial tensions generated by CJ growth are almost uniform in the MY, except near the ends of the HT. Shortening of the DM upon dissection then causes the HT to bend a little toward the ventral side, increasing \( \sigma^\text{OC}_{zz} \) and decreasing \( \sigma^\text{IC}_{zz} \) (defined in the DMY region near the IC, not at the DM where tension is large, see Fig. 3.12C). Next, MY contraction shrinks the outer layer slightly and uniformly elevates tension in both directions by approximately
Figure 3.12: **Baseline cylinder model for bending of isolated heart.** (A) This model includes five steps: cardiac jelly (CJ) growth, dorsal mesocardium (DM) dissection, myocardial (MY) contraction, MY differential growth, and active MY cell-shape change. Deformed shape and longitudinal stress distribution after each step in simulation are shown (lateral view; DMY = dorsal myocardium, VMY = ventral myocardium, IC = inner curvature, OC = outer curvature). (B) MY contraction is turned off to simulate bending of hearts treated with blebbistatin (Bleb). (The scale and legend are the same as in A.) (C,D) Stress distributions after each step along the MY circumference at center of the heart tube. (E) Experimental and numerical stretch ratios in the MY for control and Bleb-treated hearts: radial $\lambda_r$, circumferential $\lambda_\theta$, longitudinal on ventral side $\lambda_{zv}$, and longitudinal on dorsal side $\lambda_{zd}$. Model results are shown after the differential growth and cell-shape change steps. (F) Decrease in MY tension after 24-hr culture as characterized by decrease in aspect ratios of cut ($\Delta \alpha = \alpha(0\text{ hr}) - \alpha(24\text{ hr})$). Although most bending is produced by differential growth, cell-shape change significantly lowers the circumferential stress ($\sigma_{\theta\theta}$), especially near the IC (panels (D) and (F)).
30%–50%. Differential growth then produces considerable bending while lowering tension levels globally. To this point, the strains and stresses given by the model show the correct trends (see control, differential growth in Fig. 3.12E,F) with one notable exception: the myocardial stresses near the IC (\(\sigma_{zz}^{IC}\) and \(\sigma_{\theta\theta}^{IC}\)) decrease much less during the simulation than our cutting data suggest (see \(\Delta\sigma_z^{IC}\) and \(\Delta\sigma_{\theta}^{IC}\) in Fig. 3.12F).

Introducing MY cell-shape changes further reduces myocardial stresses to levels consistent with our data while increasing the amount of bending somewhat (Fig. 3.12A,C–F; see control, cell-shape change in E,F). Taken together, these results suggest that most of the bending is caused by differential growth, but cell-shape changes may provide a supplementary mechanism. This model represents our baseline cylindrical model for bending of isolated hearts under control conditions.

To test our model, we use it to simulate bending of Bleb-treated hearts (Fig. 3.12B). The only change we make here is to remove the contraction step by setting \(M_c = 1\). Notably, all of the predicted stretch ratios match the data quite well, with \(\lambda_r\) showing better agreement than in the control case (Fig. 3.12E). The decrease in stress also agrees reasonably well, though the drop in longitudinal stress is under predicted (Fig. 3.12F).

The baseline model includes five primary morphogenetic parameters: \(M_j\) (CJ growth), \(M_t\) (DM tension), \(M_c\) (MY contraction), \(M_g\) (MY growth), and \(M_s\) (MY cell-shape change). As discussed in the Computational Methods Section, the values for all of these parameters except for \(M_t\) were deduced rather directly from published data or our newly acquired data and are considered “known”. The value of \(M_t\) was chosen to make the change in longitudinal stress match approximately the experimental trends and is considered “free”. In addition, the components of \(M_g\), i.e., \(G_R\), \(G_\theta\), and \(G_Z\), are based on the assumption \(G_R = G_\theta\) with \(G = G_RG_\thetaG_Z\). Hence, we explored the sensitivity of the computational results to the values of \(M_t\), \(G_R\), and \(G_\theta\).

In general, the results of this sensitivity analysis show that the solution is relatively robust (Appendix A.2.3). The magnitudes of the stresses and strains change, but the qualitative trends remain the same. Hence, we conclude that the behavior of the model is relatively insensitive to the precise values of the free parameters.

In this study, we used the neo-Hookean form of strain-energy density function (Eq. (3.3)), which is relatively linear compared to the exponential form used in Zamir and Taber (2004).
Figure 3.13: **Model for bending of control heart based on realistic geometry.** Lagrangian strains \( (E_{zz}, E_{\theta\theta}) \) and myocardial stresses \( (\sigma_{zz}, \sigma_{\theta\theta}) \) are shown in the initial (0 hr) and final (24 hr) shapes of the heart (lateral view). To help visualize the bending deformation, the passive outflow tract and remnant omphalomesenteric veins are separated from the rest of the heart tube by dashed lines. When growth of cardiac jelly (CJ) occurs, the lumen decreases significantly, as shown in cross-sectional view. (DM = dorsal mesocardium, MY = myocardium, DMY = dorsal myocardium, VMY = ventral myocardium)

To evaluate how material nonlinearity affects the final results, we reran the simulation with the exponential strain-energy density function. The difference between the results given by these two models is very little (Appendix Fig. A.7).

### 3.7.3 Model based on realistic heart geometry

Models based on simple geometries are useful for studying general behavior of biomechanical systems. They often provide qualitatively correct results and can be surprisingly accurate quantitatively. Nevertheless it is important to examine the effects of realistic geometry when feasible. Hence, we developed a model for a representative isolated HH10 chick heart based on 3D geometry reconstructed from a stack of OCT images (Fig. 3.3A′–C′). The simulation includes the same morphogenetic steps and model parameters as the cylinder model with one important difference: the realistic-geometry model includes the
outflow tract and remnants of the omphalomesenteric veins explicitly as passive segments, whereas these structures are incorporated into the cylindrical model as part of the active HT. Hence, in comparing results between the two models and with experiments, the reader should focus on the HT located between the dashed lines in Fig. 3.13.

In general, the myocardial stresses and strains produced by this model are qualitatively consistent with those given by the baseline cylinder model (Fig. 3.13). Notably the initial tensions in the MY decrease significantly during bending, with only a small amount left at the OC (except near the boundaries between adjacent subregions, where stress concentrations occur). We found similar agreement for single-mechanism simulations (results not shown). Because the end segments in this model are taken as passive, the final deformed shape of the model appears to be bent less than control hearts after 24-hr culture (compare with Fig. 3.6A). Note, however, that the model strongly resembles hearts cultured for a shorter period of time (see 8 hr heart in Fig. 3.6A).

We also note that CJ growth reduces the lumen space included in this model (see myocardial cross sections in Fig. 3.13). In fact, contraction closes the lumen almost completely (see Fig. 3.4B). This supports our simplification of neglecting the lumen in the cylinder model.

3.8 Discussion

The heart is the first functioning organ to develop in the vertebrate embryo. Immediately after the HT forms, cardiac looping begins with the heart undergoing dramatic changes in morphology while simultaneously beginning to pump blood to the developing embryo (Taber, 2006; Männer et al., 2008). Looping represents the first large-scale marker of left-right asymmetry in the embryo, and abnormalities during this process often lead to congenital heart defects (Ramsdell, 2005; Männer, 2009). For these reasons, cardiac looping has long intrigued developmental biologists.

Despite decades of study, however, looping has remained a poorly understood biophysical process (Patten, 1922; Stalsberg, 1970; Männer, 2000; Taber, 2006). For c-looping in particular, misperceptions have contributed to this state of affairs. For example, it has been known for more than 60 years that the directionality of the c-looped HT is caused by torsion rather than rightward bending (Butler, 1952). Nevertheless, many researchers apparently
did not appreciate the significance of this fact (Taber, 2006), leading Männer (2000) to emphasize that looping comprises both bending and torsional components. One consequence is that, while torsion may depend on differences between the anatomic left and right sides of the heart (Voronov et al., 2004; Taber, 2006; Ramasubramanian et al., 2008), dorsal-ventral differences should be more important for bending (Butler, 1952; Manasek et al., 1972; Latacha et al., 2005). All of the bending mechanisms considered here are based on dorsal-ventral variations within the HT.

Another possible misconception led indirectly to the present study. It has been known that the heart grows primarily by cardiomyocyte hyperplasia before birth and by hypertrophy after birth (Grossman, 1980), and a recent study suggests that the “on-off switch” for cell division is controlled by myocardial fibroblasts (Ieda et al., 2009). Accordingly, when early studies found no significant spatial patterns of cellular proliferation in the looping HT, researchers essentially eliminated differential growth from their list of potential looping mechanisms (Sissman, 1966; Stalsberg, 1969). Recent studies have challenged this view, however, by showing that some cellular hypertrophy occurs before birth (Soufan et al., 2006) and some hyperplasia occurs after birth (Mollova et al., 2013).

As mentioned in the Introduction, Soufan et al. (2006) carefully measured cell size and proliferation rates in the embryonic chick heart and reconstructed 3D maps of myocardial growth throughout the looping stages. Their measurements indicate that hypertrophic growth dominates hyperplastic growth in the MY during the time when most of the bending of c-looping occurs. Significantly, they found that cells located at the OC of the HT become 2–3 times larger than those near the IC. This finding suggests that differential growth may be a mechanism for bending after all.

Examining this idea from a mechanics perspective, we have found that differential myocardial growth likely is the primary mechanism that drives bending of the HT during c-looping. Evidence supporting this conclusion is discussed below.

### 3.8.1 Hypertrophic myocardial growth correlates with bending.

Prior experiments have shown that blocking actin polymerization inhibits c-looping in both intact embryos and isolated heart culture (Latacha et al., 2005), while inhibiting actomyosin
contraction has little or no effect on looping once it begins at HH10 (Rémond et al., 2006). It is well documented that an intact actin network is required for both cellular hypertrophy and hyperplasia. Exposure to actin polymerization inhibitors, such as cytochalasins and latrunculins, can prevent protein synthesis and cytokinesis, which are important for increasing cell size and cell division, respectively (Ornelles et al., 1986; Ingber et al., 1995; Rosenblatt et al., 2004; Spector et al., 1989). On the other hand, actomyosin contraction is only required by the latter, as treatment with the myosin inhibitor Bleb often results in binucleated cells without affecting cell size (Li et al., 1997; Földes et al., 2011). Hence, we reasoned that if hypertrophic differential growth drives looping, then inhibiting actin polymerization but not contraction should decrease myocardial growth rates as well as looping progression.

Consistent with this idea, our measurements of myocardial thickness, circumference, and cross-sectional area show that myocardial growth in control, Bleb-treated, and Hyal-treated hearts, all of which bent, was significantly greater than in hearts exposed to CytoD, which did not bend (Figs. 3.6 and 3.7). These results suggest a correlation between myocardial growth and bending. Although we did not measure mitotic rates or changes in cell size, we postulate that both Bleb and CytoD decrease myocardial hyperplasia but only the latter also decreases myocardial hypertrophy, in agreement with previous studies (Ornelles et al., 1986; Ingber et al., 1995; Rosenblatt et al., 2004; Spector et al., 1989; Li et al., 1997; Földes et al., 2011). Further work is needed, however, to test this hypothesis at the cell level.

### 3.8.2 Differential myocardial growth is the primary bending mechanism.

It is not surprising that the dorsal-ventral gradient in myocardial hypertrophy measured by Soufan et al. (2006) can cause the HT to bend with the DM located along the IC, as observed experimentally (Fig. 3.4B). However, as shown by our single-mechanism models, several other processes can produce similar shapes (Fig. 3.10A). Hence, we used stress and strain measurements to help determine the most plausible mechanism(s).

Of the various individual looping mechanisms, our models show that differential growth comes the closest to matching all of our data (Figs. 3.10 and 3.11). In particular, a model based on growth alone captures the increase in myocardial wall thickness, the respective
increase and decrease in longitudinal strain at the OC and IC, and the overall reduction in myocardial tension given by the experiments. Quantitative comparison of results reveals, however, that this model underestimates the temporal decay in myocardial tension near the IC of control hearts. Matching this result required adding active changes in cell shape (see below), establishing our baseline model (Fig. 3.12).

In summary, our baseline model predicts the correct trends in stress and strain distributions, as well as curvature of the deformed HT (Fig. 3.12). This is not a trivial result, as the model contains relatively few free parameters. Initial geometry and material properties were taken from representative data (Zamir and Taber, 2004); CJ growth \( M_j \) was determined from myocardial tensions estimated by local tissue dissection (Zamir and Taber, 2004); MY contraction \( M_c \) was determined from myocardial thickening of control relative to Bleb-treated hearts; volumetric MY growth \( G = \det M_g \) was taken from the measurements of Soufan et al. (2006); and active cell-shape changes \( M_s \) were estimated from data in Manasek et al. (1972). Only the morphogenetic parameters defining DM tension \( M_t \) and MY growth anisotropy \( G_R \) and \( G_\Theta \) or the assumed \( M_g \equiv G_R = G_\Theta \) were determined by fitting model results to experimental data. Moreover, sensitivity analysis shows that the values of these free parameters affect the final results quantitatively but not qualitatively (Appendix A.2.3).

To test our baseline model further, we used data from Bleb-treated hearts. For the same parameter values, our model with contraction turned off predicts reasonably well the results from Bleb experiments (Fig. 3.12B,E,F). Finally, when the same morphogenetic parameters are input into a model based on realistic heart geometry, the same trends occur, illustrating the robustness of our model (Fig. 3.13).

3.8.3 Other mechanisms contribute to the bending process.

Since looping plays a central role in cardiac development, multiple backup mechanisms probably have evolved over time (Stalsberg, 1970; Taber, 2006). In previous work, we have found that myocardial contraction may provide one such mechanism for the torsional component of c-looping (Voronov et al., 2004; Nerurkar et al., 2006; Ramasubramanian et al., 2008). For bending, active contraction in the DM when it ruptures may increase DM tension in both whole embryos and isolated hearts (Taber et al., 1995). Although this
contraction may contribute to the bending. Bleb exposure has shown that contractility is not necessary for normal looping (Rémond et al., 2006).

Cardiac jelly pressure may provide another backup mechanism. During the 1980s, Manasek and colleagues postulated that growth of CJ drives both bending and torsion during c-looping (Nakamura and Manasek, 1978; Manasek et al., 1984). In their view, CJ inflates the HT with the DM locally constraining longitudinal expansion, causing the HT to bend. They also speculated that spiraling fibers in the MY cause the HT to twist as it inflates (Manasek et al., 1984). Since that time, several new pieces of evidence have added credence to their hypothesis, including experiments showing that CJ can swell substantially via osmotic pressure (Manasek et al., 1984) and that the IC (where the DM is located) is stiffer than the rest of the MY (Zamir et al., 2003). In addition, physical and computer models have shown that their proposed mechanism is plausible (Manasek et al., 1984; Taber and Perucchio, 2000). However, other experiments have indicated that the heart loops in chick embryos when CJ is digested by Hyal (Baldwin and Solursh, 1989; Linask et al., 2003). Here, we further confirmed this result for bending of isolated chick hearts (Fig. 3.4B). While these results show that CJ is not necessary for HT bending, our model suggests that constrained CJ growth still contributes to the bending process and also increases myocardial tension (Fig. 3.10A), which helps to stabilize the HT as a structure, especially in whole embryos where cytoskeletal contraction does not normally occur (Nerurkar et al., 2006; Fillastre et al., 2011). Without this added tension, the MY may buckle during looping (Appendix Fig. A.5).

Another possible backup mechanism is active changes in cell shape. This mechanism was originally proposed by Manasek et al. (1972), who found that myocardial cells elongate in the circumferential direction near the IC and spread near the OC of the looping HT. Computer models, including the single-mechanism model presented here, show that such shape changes are consistent with the observed bending pattern (Taber et al., 1995; Latacha et al., 2005) (see also Fig. 3.10). Considering this and other available studies (prior to the time that Soufan et al. (2006) was published), we speculated that polymerizing actin filaments generate myocardial cell-shape changes that cause the HT to bend (Latacha et al., 2005). However, when we include shape changes based on measured changes in cell aspect ratio from Manasek et al. (1972), our single-mechanism model produces considerably less bending than observed in isolated hearts (Fig. 3.10). The model also shows, however, that
myocardial cell-shape changes can significantly lower circumferential stress, making this mechanism an essential component of our baseline model (Figs. 3.10A and 3.12).

Taken together, our results suggest that locally constrained CJ growth, DM tension, and active changes in myocardial cell shape all contribute to the bending process, but they likely play a more minor role than differential growth. If myocardial growth is perturbed, however, these backup mechanisms may upregulate to restore normal looping.

3.8.4 Limitations

Our quantitative analyses of myocardial growth and cell-shape change are based on experimental studies of hearts from whole embryos (Soufan et al., 2006; Manasek et al., 1972). Although the tissue growth estimated from isolated hearts appears to be consistent with these data, our numbers warrant further study using detailed measurements at the cell level. Such measurements, which are beyond the scope of the present work, are needed for isolated hearts under both control and perturbed conditions. It also would be extremely useful to acquire time-lapse data from living samples during looping.

Studying bending in isolated hearts has several advantages over conducting experiments with whole embryos. Devoid of the complicating effects of torsion, isolated hearts are not affected by mechanical loads external to the HT and can be manipulated relatively easily for strain and stress measurements. However, it is important to note that the shape of the heart in ovo is affected by boundary conditions at the ends of the HT. On the other hand, as shown by Flynn et al. (1991), freeing one end of the looped heart in the embryo allows it to assume a bent configuration similar to that seen in isolated culture. This observation suggests that the intrinsic bending mechanism in ovo is the same as that in isolated hearts cultured in vivo.

Another consequence of isolation is that (non-sarcomeric) cytoskeletal contraction occurs in the MY in response to the removal of compressive loads normally exerted by the splanchnopleure (Nerurkar et al., 2006; Filas et al., 2011). However, our model suggests that, although this contraction increases myocardial tension considerably, it has little effect on bending (Fig. 3.12). Since this contractile response does not occur in ovo, it may be more appropriate to consider Bleb-treated hearts as “controls” and those with this elicited
contraction as “perturbed” hearts. Notably, our baseline model captures the behavior of both reasonably well.

Although the final shape of the heart is relatively consistent between individuals within a given species, there is quite a bit of variability in heart morphology during development. The geometry of our realistic-geometry model is based on a single representative chick heart at HH10. In general, the specific geometry of a given heart would affect the trends in the numerical results quantitatively, but not qualitatively.

Our model provides one possible solution to the bending problem. While this solution fits a relatively wide range of supporting data, other solutions involving the same or other mechanisms may exist. Perhaps cell-cell adhesion, for instance, plays a role (García-Castro et al., 2000; Linask et al., 2003). The built-in redundancy in morphogenesis combined with the relatively high variability in the normal form of the developing embryo (von Dassow and Davidson, 2007), especially at early stages, makes finding a unique solution impractical. The best we can do is to insist that model results are consistent with trends in the experimental data under various conditions.

In conclusion, our study on bending of isolated hearts has provided new evidence supporting differential hypertrophic myocardial growth as the driving mechanism for the bending component of c-looping. Other morphogenetic processes likely play a lesser role. Future work is needed to further test this hypothesis, for example, by quantifying cell-level growth and shape changes in living hearts under various experimental conditions.
References


Chapter 4

Bending and Twisting the Embryonic Heart: A Computational Model for C-Looping Based on Realistic Geometry

4.1 Summary

The morphogenetic process of cardiac looping transforms the straight heart tube into a curved tube that resembles the shape of the future four-chambered heart. Although great progress has been made in identifying the molecular and genetic factors involved in looping, the physical mechanisms that drive this process have remained poorly understood. Recent work, however, has shed new light on this complicated problem. After briefly reviewing the current state of knowledge, we propose a relatively comprehensive hypothesis for the mechanics of the first phase of looping, termed c-looping, as the straight heart tube deforms into a c-shaped tube. According to this hypothesis, differential hypertrophic growth in the myocardium supplies the main forces that cause the heart tube to bend ventrally, while regional growth and cytoskeletal contraction in the omphalomesenteric veins (primitive atria) and compressive loads exerted by the splanchnopleuric membrane drive rightward torsion. A computational model based on realistic embryonic heart geometry is

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4The model geometry was created by Jiang Yao, Jonathan Young, and Renato Perucchio, and the OCT data was provided by Benjamen Filas. The contraction inhibition experiment was conducted by Judy Fee. Section 4.4.3 was drafted by Jiang Yao.
used to test the physical plausibility of this hypothesis. The behavior of the model is in reasonable agreement with available experimental data from control and perturbed embryos, offering support for our hypothesis. The results also suggest, however, that several other mechanisms contribute secondarily to normal looping, and we speculate that these mechanisms play backup roles when looping is perturbed. Finally, some outstanding questions are discussed for future study.

4.2 Introduction

Cardiac looping is a fundamental unsolved problem during early heart development. Looping represents the first major morphological sign of left-right asymmetry in the vertebrate embryo. In addition, looping abnormalities likely underlie some of the cardiac malformations that occur in as many as 1% of liveborn and 10% of stillborn human births (Harvey, 1998), and such defects may result in numerous spontaneous abortions during the first trimester (Srivastava and Olson, 1997; Ramsdell, 2005). During the last 30 years, most research on this problem has focused, with considerable success, on genetics and molecular signaling (Harvey, 2002), while interest in biophysical mechanisms waned. As a result, the physical processes that create the looped heart tube (HT) have remained poorly understood.

During the last decade, we have used a combination of experiments and computational modeling to explore the mechanics of the first phase of looping, called c-looping, as the initially straight HT bends and twists into a c-shaped tube normally directed toward the right side of the embryo (Patten, 1922; Männer, 2000). Employing an engineering approach, we have identified several of the forces that are involved in c-looping and have proposed hypotheses for how these forces are integrated to produce a looped heart (Voronov et al., 2004; Latacha et al., 2005; Taber, 2006; Taber et al., 2010; Shi et al., 2014). Computational models with simplified heart geometry have been instrumental in testing the physical plausibility of our hypotheses for bending or rotation alone, but a realistic model for the entire c-looping process has not yet been published. Models are important complements to laboratory experiments, as intuition can be misleading when trying to interpret the results of highly nonlinear problems such as looping, which involves multiple intrinsic and extrinsic forces as well as dramatically changing 3D geometry.
Here, we present the first relatively comprehensive computational model for the early HT and use it to explore a new hypothesis for the mechanics of c-looping. This model extends and integrates our previous models for looping, which simulated bending and torsion separately (Taber et al., 1995; Taber and Perucchio, 2000; Voronov et al., 2004; Rama-subramanian et al., 2006, 2008; Shi et al., 2014). The model is built on a foundation of experimental data and is based on the fundamental principles of soft tissue mechanics, including large deformation, growth, and active cytoskeletal contraction. Novel features include contact between the HT and splanchnopleuric membrane and realistic 3D geometry reconstructed from images of an embryonic chick heart (acquired via optical coherence tomography, OCT). For the same set of physical parameters, the model captures reasonably well the morphology of the looping heart under both control and mechanically perturbed conditions. This study lays the foundation for future patient-specific models for cardiac morphogenesis.

4.3 Background

The problem of cardiac looping has a long and tortuous history. Since the pioneering study of Patten (1922) nearly a century ago, researchers have proposed numerous hypotheses for the mechanisms of c-looping, but few have survived the test of time. (See Taber (2006) and Shi et al. (2014) for recent reviews.) Here, we briefly summarize our current thinking on this topic.

When it is first created in the chick embryo at stage HH10 of Hamburger and Hamilton (1951), the heart is a relatively straight tube consisting of an outer layer of myocardium, a middle layer of matrix called cardiac jelly (CJ), and an inner layer of endocardium (see Fig. 3.1A,A’). The HT is connected caudally to the omphalomesenteric veins (OVs), cranially to the conotruncus (outflow tract), and dorsally to the dorsal mesocardium (DM), which attaches the entire length of the HT to the foregut. In addition, the splanchnopleuric membrane (SPL) presses against the ventral side of the HT and wraps around the caudal sides of the OVs at the anterior intestinal portal (AIP) (Männer, 2000; Taber, 2006).

During c-looping, the HT undergoes a combination of ventral bending and rightward torsion (rotation) (Männer, 2000; Voronov et al., 2004; Taber, 2006). These deformations
transform the ventral and dorsal surfaces of the initially straight HT into the convex outer curvature (OC) and concave inner curvature (IC), respectively, of the curved tube (Männer, 2000; Voronov et al., 2004) (see Fig. 3.1C,C’). In 3D space, the looped heart acquires a helical shape (Bayraktar and Männer, 2014). During this process, the OVs gradually fuse to lengthen the HT, and the DM ruptures so only the ends of the HT remain connected to the embryonic foregut.

Studies suggest that the bending and torsional components of c-looping are driven by different sets of physical forces. While bending is caused mainly by forces generated within the HT, torsion is driven primarily by external loads (Butler, 1952; Voronov and Taber, 2002; Voronov et al., 2004; Latacha et al., 2005; Ramasubramanian et al., 2008). Proposed bending mechanisms include buckling as the HT outgrows the allotted distance between its ends (Patten, 1922; Bayraktar and Männer, 2014), dorsally constrained longitudinal stretching as CJ swells and inflates the HT (Manasek et al., 1984), differential hyperplastic growth of the myocardium (Stalsberg, 1969), active changes in myocardial cell shape (Manasek et al., 1972; Latacha et al., 2005; Auman et al., 2007), differential cytoskeletal contraction (Itasaki et al., 1991; Taber et al., 1995), and bending forces exerted on the HT by remnants of the DM after it ruptures (Taber et al., 1995).

Recently, we have shown that the bending component of c-looping can be attributed primarily to differential hypertrophic growth, although CJ swelling, active myocardial cell-shape change, and DM tension may play more minor roles. This idea is consistent with recent results of Soufan et al. (2006), who found that myocardial cells near the OC of the HT increase in volume during c-looping considerably more than cells near the IC. Prior to the study of Soufan et al. (2006), it was generally thought that the heart grows primarily by cellular hyperplasia before birth and hypertrophy after birth (Grossman, 1980), leading most researchers to rule out differential growth as a mechanism for looping when no clear mitotic patterns were found in the HT (Sissman, 1966; Stalsberg, 1969). In fact, this is one reason why we had previously supported a bending mechanism based on active changes in cell shape (Taber et al., 1995; Latacha et al., 2005; Ramasubramanian et al., 2006).

Interestingly, one of the forces involved in torsion of the HT was suggested more than 60 years ago by Butler (1952), who speculated that the left OV exerts a torque on the heart as it grows larger than the right OV. Recent studies support this hypothesis, as reducing the size of the left OV by dissection or inducing the right OV to grow larger than the left
OV produces abnormal leftward looping (Voronov et al., 2004; Kidokoro et al., 2008). Other data suggest that proliferating cells on the left side of the DM normally provide a rightward push on the HT that determines looping direction (Linask et al., 2005), and cytoskeletal contraction in the OVs near the AIP also may be involved (Voronov et al., 2004). It is likely that multiple redundant mechanisms contribute to torsion of the heart, thus reducing the incidence of leftward looping, which is a major source of congenital heart defects (Ramsdell, 2005).

We have found, however, that the OVs normally cause only a relatively small amount of rightward torsion. Once looping direction is defined by asymmetric growth in the OVs or proliferative cellular forces in the DM, the SPL supplies a surface pressure that pushes the HT dorsally into its fully twisted position (Voronov and Taber, 2002; Voronov et al., 2004; Linask et al., 2005; Kidokoro et al., 2008).

Based on these prior studies, the main forces that drive normal c-looping in our proposed model are differential growth in the HT and OVs, and pressure exerted by the SPL. Differential growth causes the HT to bend (Fig. 4.1A) and the OVs to push against the HT, initiating a slight rightward twist (Fig. 4.1B). Then, SPL pressure enhances the torsion as the HT continues to bend (Fig. 4.1C). The model also includes growth of CJ, tension in the
DM, active changes in myocardial cell shape, cytoskeletal contraction around the AIP, and elongation of the HT caused by OV fusion.\(^5\)

### 4.4 Material and methods

#### 4.4.1 Preparation and culture of the embryonic heart

The methodology for preparation and culture of the chick embryo is adopted from Voronov and Taber (2002). Briefly, fertile white Leghorn chicken eggs (Sunrise Farms, Catskill, NY) were incubated in a humidified atmosphere at 38°C for 33–48 hr to yield embryos at HH stages 10 to 12 (Hamburger and Hamilton, 1951). Embryos were extracted from the eggs using filter paper rings and rinsed in PBS. To eliminate surface tension artifacts, the sandwich structure of embryo and filter paper rings was covered with liquid culture media consisting of 89% Dulbecco’s modified Eagle’s medium (DMEM, Sigma), 10% chick serum (Sigma), and 1% antibiotics to a depth of approximately 5 mm. A stainless steel ring was placed on top of the paper rings to hold them in place. Culture dishes were then sealed in plastic bags filled with a humidified mixture of 95% O\(_2\) and 5% CO\(_2\), and put into an incubator for continued culture.

#### 4.4.2 Measurement of HT rotation

Most experimental data used to inform and test our model come from previous studies. To this we add measurements of HT rotation as a function of time. Cardiac rotation was defined by the rotation of the lumen near the center of the HT. Images of the heart were acquired hourly from HH10- via OCT (Thorlabs, Newton, NJ) (Mesud Yelbuz et al., 2002), and 3D tissue geometry was reconstructed using image analysis software (Volocity, PerkinElmer, Waltham, MA). Through image cropping, reslicing, and thresholding (ImageJ, NIH), the lumen in the chosen cross section was traced and fit to an ellipse. The rotation angle (\(\alpha\)) is defined as the angle between the long axis of the fitted ellipse and

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\(^5\)In this chapter, the term contraction refers to cytoskeletal contraction based on nonmuscle myosin II, not the sarcomeric contraction that causes the heartbeat.
the embryonic dorsal-ventral axis (see Fig. 4.4A). All experimental measurements are presented as mean ± SD, and statistical analysis was performed using SigmaPlot software (Systat Software Inc., San Jose, CA).

4.4.3 Computational model geometry

A finite-element model was developed to simulate c-looping of the HT. The geometry was reconstructed from OCT images using Matlab (Mathworks, Natick, MA), PATRAN (MSC Software, Santa Ann, CA), and ABAQUS (SIMULIA, Providence, RI). Constitutive relations (material properties) and morphogenetic processes were defined via the ABAQUS user subroutine VUMAT (see below) with local material orientations determined using customized Matlab code. Simulations were carried out using the commercial finite-element code ABAQUS/Explicit, and some post-processing tasks were automated by execution of customized Python codes.

The initial geometry of our model was reconstructed from a stack of OCT images of an HH10- heart (Fig. 4.2). Briefly, the SPL, foregut wall, myocardium, CJ, and lumen were segmented out from the images. (The endocardium was omitted in the current model.) Then, we extracted voxel surfaces at the interface of two entities and removed any voxel edges and vertices with sharp changes in local curvature, which could cause stress concentrations and convergence issues. The voxel surfaces were smoothed using Matlab to adjust the position of each vertex by its weighted average over the neighboring vertices. Then, the congruent and manifold triangular surface mesh was further smoothed and coarsened using the PATRAN Mesh-on-Mesh routine. Finally, the closed triangular surface mesh for the heart was converted to a solid tetrahedral mesh using ABAQUS/CAE.

Considering the geometric differences between the heart and surrounding membranes, our model includes two types of elements. The myocardium and CJ consist of 68,504 tetrahedral (C3D4) elements, and the SPL and foregut membranes consist of 10,231 triangular (M3D3) elements. The entire model contains 21,710 nodes. Testing with denser meshes showed that the chosen mesh is accurate enough for the present purposes.
Figure 4.2: **Finite-element model for embryonic heart.** Model geometry in undeformed configuration was reconstructed from OCT images of a representative HH10 heart. (A) The complete model (ventral view) consists of the heart (white dashed line; see also (B)), which is sandwiched between the splanchnopleure (SPL) and the ventral wall of the foregut (FG). The caudal end of the SPL is attached to the caudal side of the omphalomesenteric veins (OVs) around the anterior intestinal portal (AIP), and the other membrane boundaries are fixed (black solid lines). (B) The heart model (ventral view) includes the heart tube (HT), conotruncus (CT), and the left and right OVs (LOV and ROV). The ends of the CT and OVs are constrained to move along the normal directions on their boundary surfaces (circular discs with arrows). (C) The dorsal mesocardium (DM), a narrow region along the dorsal side of the HT (side view), is free except near its cranial and caudal ends (braces), where it is anchored to the FG. The cranial end is on cranial-caudal oriented rollers (double-headed arrow), and the caudal end slides freely in the cranial-lateral plane (crossed double-headed arrows). (A′) Transverse cross section of the model (black dashed line in (A)). Frictionless contact is enforced between the heart and the two external membranes (SPL and FG). In the HT, the myocardium (MY) wraps around the cardiac jelly (CJ), which encloses the lumen. Note that the long axis of the lumen lies approximately along the dorsal-ventral direction at HH10+. (B′) Frontal section of heart in (B). The MY and CJ are divided into regions for prescribing morphogenetic processes (colored stars, circles, square, diamond, and triangle; see text for details) in local radial, circumferential, and longitudinal directions ($e_R, e_\theta, e_Z$).
4.4.4 Theory for modeling morphogenesis

The analysis of the model is based on a finite-element implementation of a biomechanical theory for large deformation and growth of soft tissue (Rodriguez et al., 1994). We have used this theory in Chapter 3 to simulate various morphogenetic processes. Here, we briefly discuss the basic idea; further details can be found in Section 3.5.1.

Briefly, the total deformation of a pseudoeelastic body is described by the deformation gradient tensor $F$, which maps material points from the reference configuration at HH10- to the deformed configuration at a later time. Growth and active contraction are simulated through the morphogenesis tensor $M = F^* - 1 \cdot F$, which defines the local zero-stress configuration after the simulated processes. The elastic deformation gradient tensor $F^*$ generates mechanical stress by enforcing geometric compatibility between material elements and accounting for the elastic response to applied loads. The Cauchy stress tensor $\sigma$ is assumed to depend only on $F^*$ through the constitutive relation

$$\sigma = \frac{1}{J^*} F^* \cdot \frac{\partial W}{\partial E^*} \cdot F^*^T,$$

where $W(E^*)$ is the strain-energy density function, $J^* = \det F^*$ is the elastic volume ratio, and $E^* = (F^*^T \cdot F^* - I)/2$ is the Lagrangian elastic strain tensor with $I$ being the identity tensor and $T$ denoting the transpose.

4.4.5 Material properties

Mechanical properties are based on the microindentation measurements of Zamir and Taber (2004). These authors characterized the myocardium and CJ of HH12 chick hearts as homogeneous isotropic materials using a strain-energy density function in the form

$$W = \frac{A}{B} (e^{B(\tilde{I}_1 - 3)} - 1) + \frac{1}{D} \left( \frac{J^*^2 - 1}{2} - \ln J^* \right),$$

where $A$ and $B$ are material constants, and $\tilde{I}_1 = J^*^{-2/3} \text{tr}(F^*^T \cdot F^*)$ is a modified strain invariant. Since another study found no significant difference in end-diastolic stiffness between HH10 and HH12 hearts (Rémond, 2006), we assume that the material properties
remain relatively unchanged throughout c-looping and take the above strain-energy density function for all tissues in our model.

For myocardium and CJ, we used the mean values of the mechanical parameters reported by Zamir and Taber (2004), i.e., $A_{MY} = 13.0 \text{ Pa}$, $B_{MY} = 0.57$, $A_{CJ} = 3.2 \text{ Pa}$, $B_{CJ} = 0.39$, and $D_{MY} = D_{CJ} = 0.01$. Although material properties for the SPL have not been measured, it is known that the SPL contracts and is under significant tension during looping (Voronov and Taber, 2002). Since contraction generally causes an increase in tissue stiffness, we chose $A_{SPL} = 2A_{MY} = 26.0 \text{ Pa}$, $B_{SPL} = B_{MY} = 0.57$, $D_{SPL} = D_{MY} = 0.01$ for the SPL as well as the foregut. A sensitivity analysis shows that varying the value of $A_{SPL}$ over a relatively large range has little effect on the overall deformation (e.g., rotation of the HT) or qualitative trends in strain and stress distributions (see Appendix Fig. A.9).

### 4.4.6 Boundary and contact conditions

The membrane representing the SPL wraps around the caudal side of the OVs and adheres to the OVs in the region of contact (Fig. 4.2A,B). Otherwise, the SPL and foregut are taken as fixed along their margins (Fig. 4.2A). The HT is sandwiched between these membranes, with frictionless contact enforced between them (Fig. 4.2A’).

For the heart, the cranial end of the conotruncus and the lateral ends of the OVs are allowed to move only along the normal directions of their boundary surfaces (Fig. 4.2B). Along the dorsal side of the HT, a narrow region is defined as the DM, which is free except at its cranial and caudal ends, where it is anchored to the foregut (Fig. 4.2C). The cranial end of the DM is supported by cranial-caudal oriented rollers, while its caudal end is on 2D rollers in the cranial-lateral plane to allow lateral rotations. These boundary conditions simulate growth and remodeling of surrounding tissues to accommodate growth of the heart. Finally, frictionless contact is imposed on the interior surface of the CJ to allow the lumen to collapse as CJ grows and swells (Fig. 4.2A’,B’).
4.4.7 Local material orientations

To implement anisotropic morphogenetic processes for each anatomic region, the model geometry was used to define local radial ($e_R$), circumferential ($e_\Theta$), and longitudinal ($e_Z$) directions for each element (Fig. 4.2B).

For the SPL and foregut, the radial direction ($e_R$) is along the normal to the surface. Since the morphogenesis tensor is taken as isotropic in the local tangential plane of these membranes (see below), $e_\Theta$ and $e_Z$ were arbitrarily chosen as any two orthogonal unit vectors within the element plane.

To define the normal direction in the heart, $e_R$ was calculated as the weighted average of the exterior and interior surface normals by distances of the element centroid to these two surfaces. The longitudinal direction ($e_Z$) is taken along the projection of 3D curve connecting the transverse cross-sectional centroids of the HT or OVs onto the local myocardial plane. The circumference direction ($e_\Theta$) is then the cross product of $e_Z$ and $e_R$.

4.4.8 Looping simulation

The baseline model for the looping HT under control conditions includes the essential morphogenetic processes and parameters contained in our recent model for bending of isolated hearts (Shi et al., 2014) (see Section 3.5.4 for details). Here, that model is extended to include the effects of the OVs and SPL, which drive torsion. This subsection provides details of the simulation procedure for the baseline model; model perturbations are described later.

In general, the morphogenesis tensor is taken in the form

$$M = M_R e_R e_R + M_\Theta e_\Theta e_\Theta + M_Z e_Z e_Z,$$

(4.3)

with the components $M_I$ ($I = R, \Theta, Z$) being specified for each particular mechanism as functions of space and time relative to the reference configuration at HH10-.

Morphogenesis in the HT. Our recent study of cultured HTs in isolation suggests that differential growth in the myocardium is the primary mechanism that drives the bending component of c-looping with CJ growth, DM tension, and active changes in myocardial
Table 4.1: Morphogenetic processes in baseline model

<table>
<thead>
<tr>
<th>Morphogenetic Process</th>
<th>Morphogenesis Tensor</th>
<th>Morphogenetic Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endodermal contraction</td>
<td>( M_c = M_c^2 e_R e_R + M_c (e_\theta e_\theta + e_Z e_Z) )</td>
<td>( M_c = 0.9 \to 0.9 \times 0.7^* ) (SPL, Foregut, and AIP)</td>
</tr>
<tr>
<td>CJ growth</td>
<td>( M_j = M_j (e_R e_R + e_\theta e_\theta + e_Z e_Z) )</td>
<td>( M_j = 1.1 \to 1.1 \times 1.3 ) (HT)</td>
</tr>
<tr>
<td>DM tension</td>
<td>( M_t = e_R e_R + e_\theta e_\theta + M_t e_Z e_Z )</td>
<td>( M_t = 1.0 \to 0.8 ) (DM)</td>
</tr>
<tr>
<td>Myocardial differential growth</td>
<td>( M_g = M_g (e_R e_R + e_\theta e_\theta) + G/M_g^2 e_Z e_Z )</td>
<td>( M_g = 1.0 \to 1.3 ) (HT)</td>
</tr>
<tr>
<td>Myocardial cell-shape change</td>
<td>( M_s = e_R e_R + S e_\theta e_\theta + S^{-1} e_Z e_Z )</td>
<td>( M_s = 1.0 \to 1.3 ) (HT)</td>
</tr>
<tr>
<td>OV fusion†</td>
<td>( M_f = e_R e_R + e_\theta e_\theta + M_f e_Z e_Z )</td>
<td>( M_f = 1.0 \to 1.5 ) (HT)</td>
</tr>
<tr>
<td>Growth in the OVs and CT‡</td>
<td>( M_i = M_i (e_R e_R + e_\theta e_\theta + e_Z e_Z) )</td>
<td>( M_i = 1.0 \to 1.3 ) (CT), 1.6 (LOV), 1.2 (ROV)</td>
</tr>
</tbody>
</table>

* Initial contraction \( M_c = 0.9 \) used as the initial condition at HH10-; additional contraction of 0.7 occurs during looping, and the total contraction is \( 0.9 \times 0.7 = 0.63 \). Same notation also applies to other parameter values.

† \( \theta \in [0, \pi] \) is the circumferential angle relative to the DM.

‡ Longitudinal growth in the HT for modeling OV fusion does not contribute to reported strains.

‡ CT = conotruncus, LOV/ROV = cranial side of left/right OV.

cell shape contributing to a lesser degree (Shi et al., 2014). All of these mechanisms are included in the present model. Here, we briefly state the rationale for the chosen form of \( \mathbf{M} \) for each mechanism as listed in Table 4.1.

The differential growth tensor \( \mathbf{M}_g \) provides a spatial gradient in myocardial hypertrophic growth, which increases from the IC to the OC, consistent with the measurements of Soufan et al. (2006). The myocardial cell-shape tensor \( \mathbf{M}_s \) was determined from SEM images of Manasek et al. (1972), who found that cells at the OC spread out while those near the IC elongate circumferentially during c-looping. Isotropic growth of CJ is given by \( \mathbf{M}_j \), which was deduced from an estimate of myocardial stress in the looped heart (Zamir and Taber, 2004). Finally, the DM tension tensor \( \mathbf{M}_t \) shortens the zero-stress length of the DM by an amount that yields myocardial stress and strain distributions in reasonable agreement with experimental data (Shi et al., 2014).
The spatial distributions of morphogenetic variables and parameters were extrapolated from our study of the isolated heart (Shi et al., 2014). However, differences in looping morphology between isolated and intact hearts required some minor adjustments. The most significant change is that in the present model, we do not include myocardial contraction, which is an adaptive response triggered by the removal of normal compressive loads exerted by the SPL (Nerurkar et al., 2006; Filas et al., 2011). In addition, the CJ growth parameter $M_j$ increases from an initial value of 1.1 at HH10- to 1.43 at HH12 (see Table 4.1), consistent with the 1.3-fold increase suggested by the myocardial residual strain determined by Zamir and Taber (2004). Finally, the initial value $M_j = 1.1$ was chosen to yield the modest myocardial tension present at that stage (Shi et al., 2014).

To implement the morphogenetic functions in a piecewise continuous manner, the HT was further divided into 24 subregions, and the average values of the parameters were uniformly assigned for each subregion. This procedure reduced stress concentrations near boundaries between adjacent subregions.

**Endodermal contraction.** Previous experiments have shown that the endoderm comprising the SPL and the caudal regions of the OVs at the AIP is in a state of relatively isotropic contraction during c-looping (Voronov and Taber, 2002; Voronov et al., 2004; Varner and Taber, 2012). We assume that endodermal contraction occurs isovolumetrically and take the contraction tensor in the form $M_c = M_c^2 e_R e_R + M_c (e_\theta e_\theta + e_Z e_Z)$ with the constant volume constraint $\det M_c = 1$ satisfied. The contraction parameter $M_c$ in these regions was chosen to decrease (i.e., contractile strength increases) from 0.9 at HH10- to 0.63 at HH12 (see Table 4.1). These values were estimated from experimental data provided by Voronov et al. (2004) and Ramasubramanian et al. (2006).

**Growth in the OVs and Conotruncus.** Recent results suggest that asymmetric growth in the OVs plays an important role in determining looping directionality (Voronov et al., 2004; Ramasubramanian et al., 2006, 2008; Kidokoro et al., 2008). Here, we used the limited data that are available to estimate growth in the OVs as well as in the conotruncus. We assume that growth along the cranial sides of OVs (both myocardium and CJ) is isotropic and take $M_i = M_i (e_R e_R + e_\theta e_\theta + e_Z e_Z)$, where $M_i$ is the growth parameter (see Table 4.1). Since the left OV grows noticeably larger than the right OV during c-looping (Stalsberg and DeHaan,
1969; Voronov et al., 2004; Kidokoro et al., 2008), we chose $M_i = 1.6$ for the left OV and $1.2$ for the right OV based on the morphogenetic strains between HH10- and HH11 measured by Ramasubramanian et al. (2006). Notably, the value of $M_i$ has a relatively small effect on HT torsion (see Appendix Fig. A.10). For the conotruncus, which bulges near the end of c-looping (Männer, 2000) (see also Fig. 3.1C), we estimated $M_i = 1.3$ (i.e., $\det M_i = M_i^3 \approx 2$). This value is based on measurements of cardiomyocyte growth and proliferation provided in Soufan et al. (2006), who found little change in cell number but approximately a two-fold increase in cell size in the conotruncus from HH10- to HH12.

**Fusion of the OVs.** During c-looping, the HT lengths as new segments are added to its caudal end by fusion of the bilateral OVs (Voronov et al., 2004; Taber, 2006; Kidokoro et al., 2008; Abu-Issa and Kirby, 2008; Varner and Taber, 2012). The details of this fusion process are not included in the present model. For a first approximation, however, we specify global longitudinal growth of the HT through a morphogenesis (OV fusion) tensor in the form $M_f = e_R e_R + e_{\theta} e_{\theta} + M_f e_Z e_Z$, where $M_f$ is the OV fusion parameter (see Table 4.1). This growth is taken as uniform throughout the myocardium and CJ in the HT. The value $M_f = 1.5$ was estimated from the observed change in length of the HT during c-looping. It is important to note that, since OV fusion adds material only at the caudal end of the HT, this process does not contribute to morphogenetic strains measured by tracking tissue labels. For comparison with experimental data, therefore, we exclude this uniform growth component from our strain calculations given by the model.

**Total Morphogenesis Tensor.** The total morphogenesis tensor for each region is the product of all the individual morphogenesis tensors (Shi et al., 2014). It is important to point out that while some of these tensors share similar mathematical expressions (e.g., $M_j$ and $M_i$, as well as $M_l$ and $M_f$), they represent fundamentally different mechanisms and function in different regions (see Fig. 4.2). In our previous model for isolated hearts, morphogenetic processes were assumed to occur in sequential steps to demonstrate how each individual process affects the results (Shi et al., 2014). Here, we include only two steps — an initial step to reach the reference stage of HH10- and a looping step spanning the entire c-looping process from HH10- to HH12. The first step includes only the initial CJ growth and endodermal contraction; the second step includes everything else (see Table 4.1). All
the morphogenetic parameters are assumed to change linearly over time, as suggested by time plots of measured myocardial strains in isolated hearts (Shi et al., 2014).

**Perturbations.** To test the model, we used it to simulate looping under a variety of experimental perturbations (Voronov et al., 2004; Rémond et al., 2006; Ramasubramanian et al., 2008; Kidokoro et al., 2008). In each case, the parameter values are the same as those in the baseline model, unless stated otherwise. The perturbations include the following:

- To simulate the effects of contraction inhibitors (e.g., blebbistatin), contraction is turned off by setting $M_c = 1$ everywhere.

- To simulate inhibition of OV fusion, the uniform component of longitudinal growth is turned off in the HT by setting $M_f = 1$. Since it is unclear whether the DM ruptures in hearts with OV fusion blocked, we set the DM either free or on cranial-caudal oriented rollers along its length.

- To simulate mechanical perturbations involving dissection, the appropriate parts were removed from the model (e.g., SPL, conotruncus, OVs, or HT), and some boundary conditions were adjusted accordingly to eliminate rigid-body motions. Since contraction is not necessary for normal c-looping, we neglect the possible effects of contraction triggered by dissection (Rémond et al., 2006; Nerurkar et al., 2006; Filas et al., 2011).

### 4.4.9 Comparing numerical and experimental results

To compare the results yielded by our computational model with experimental data, we focus mainly on morphology, which is the most important criterion with the most abundant data. However, since multiple mechanisms can produce similar changes in shape (Stalsberg, 1970; Taber, 2006; Shi et al., 2014), it is essential also to consider other mechanical quantities such as stress and strain. To help visualize the deformation in the model (especially HT rotation), artificial labels are placed along the ventral midline of the HT or the OV junction (e.g., see Fig. 4.9). Morphogenetic Lagrangian strains and HT rotation were
Figure 4.3: **Stress distributions in baseline model for c-looping.** (A) Longitudinal stress ($\sigma_{zz}$) and (B) circumferential stress ($\sigma_{\theta\theta}$) are shown at three looping stages in ventral and transverse cross-sectional views (black dashed lines in (A)), respectively. To help visualize the deformation, white dashed lines divide the heart into regions, and artificial markers along the ventral midline of the undeformed heart tube (HT) indicate rotation. For comparison, ‘⊕’ (red) and ‘⊖’ (blue) signs denote regional myocardial tension and compression for the corresponding stress component, as previously revealed by microsurgery experiments (Voronov et al., 2004; Zamir and Taber, 2004; Shi et al., 2014). Note the rightward rotation of the HT as shown by the motion of the markers in (A) and the lumen orientation in (B). Scale and legend are the same in (A) and (B).

quantified following the same procedures used in experiments. Since tissue stresses are difficult to measure accurately in intact embryos, we focus on the qualitative trends in stress, i.e., whether the tissue is under local tension or compression, rather than numerical values.

### 4.5 Results

In this section, results are first presented for the baseline model. Then, the ability of the model to predict the outcomes of various experimental perturbations is examined.

#### 4.5.1 Baseline model for normal c-looping

In general, the evolving morphology of the HT during c-looping given by the baseline model (Fig. 4.3) agrees with experimental observations relatively well (compare with Fig.
Figure 4.4: Rotation of heart tube during c-looping. (A) Rotation angle $\alpha$ is defined as the angle between the long axis of the elliptical lumen and the embryonic dorsal-ventral axis in a cross section located at the middle of the heart tube (CJ = cardiac jelly, DM = dorsal mesocardium, MY = myocardium). (B) Rotation angle of HT given by the baseline model (dashed black line) agrees relatively well with experimental measurements (solid black line). Torsion of the HT is not affected significantly by inhibition of cytoskeletal contraction (red line), whereas it is hindered when the SPL is removed from the model (blue line).

3.1). At the reference stage of HH10-, the initial CJ growth causes the HT to elongate slightly and the lumen to close slightly. At HH11, the HT is bent significantly with the DM located at the IC. Simultaneously, the HT twists (rotates) toward the right as it is pushed rightward by the rapidly growing left OV and compressed ventrally by the contracting SPL (see circular labels in Fig. 4.3A). In addition, the length and circumference of the HT increase considerably. By the completion of c-looping at HH12, the OC and IC (original ventral and dorsal sides) of the HT have undergone considerable elongation and shortening, respectively, and the HT has fully rotated rightward with the lumen now oriented roughly along the embryonic lateral direction. Relative to HH11, the HT and left OV have grown significantly, and the conotruncus is bent rightward. All of these changes are consistent with normal c-looping in ovo (Männer, 2000; Taber, 2006) (see Fig. 3.1).

To quantify torsion, we measured the rotation angle $\alpha$ of the HT in embryos cultured for 12 hr from HH10- (Fig. 4.4). The rotation angle increased as looping progressed, with most rotation occurring between HH10 ($\alpha = 18.0 \pm 6.5$ deg) and HH11 ($\alpha = 62.5 \pm 10.8$ deg; $n = 5$) (Fig. 4.4B). The temporal plot of rotation angle given by our baseline model agrees with the experimental trend reasonably well (Fig. 4.4B).
Figure 4.5: **Time history of regional longitudinal strains in baseline model.** Longitudinal strains ($E_{ZZ}$) relative to HH10– in seven regions are compared with experimental data from Ramasubramanian et al. (2006). LHT/RHT/VHT = left/right/ventral sides of heart tube, LOV/ROV = cranial half of left/right omphalomesenteric veins, LAIP/RAIP = left/right sides of anterior intestinal portal.

To test the model further, myocardial strains relative to HH10– given by the model are compared with the experimental measurements of Ramasubramanian et al. (2006). Longitudinal strains ($E_{ZZ}$) and circumferential strains ($E_{\theta\theta}$) were averaged over seven regions and three regions, respectively, and plotted as functions of stage (Figs. 4.5 and 4.6). Overall, the general trends given by the model agree reasonably well with the data, although the strain magnitudes are somewhat smaller in the model. The discrepancy is most apparent for $E_{ZZ}$ in the caudal parts of the OVs, where endodermal contraction causes longitudinal shortening, but the counterbalancing effects of CJ growth may have been overestimated.

In the HT, the longitudinal strain, which is closely associated with bending, increases on the original ventral and right sides while it changes relatively little on the left side (mainly due to compression by the SPL). Growth of the myocardium and CJ causes the average circumferential strain to increase in the HT and both OVs. On the cranial side of left OV, the longitudinal strain also increases considerably during looping, but the cranial side
Figure 4.6: **Time history of regional circumferential strains in baseline model.** Average circumferential strains ($E_{\theta\theta}$) relative to HH10- in three regions are compared with experimental data from Ramasubramanian et al. (2006). HT = heart tube, OV = omphalomesenteric vein.

of right OV is initially compressed somewhat in the longitudinal direction by the faster growing left OV.

The trends in stress distributions given by the model also generally agree with those estimated experimentally (Voronov and Taber, 2002; Voronov et al., 2004; Zamir and Taber, 2004; Shi et al., 2014) (Fig. 4.3; $\sigma_{zz}$ and $\sigma_{\theta\theta}$ represent longitudinal and circumferential stresses, respectively). Due to the initial CJ growth, relatively uniform myocardial tension ($\sigma_{zz}, \sigma_{\theta\theta} > 0$) is present everywhere in the HT at HH10-. Endodermal contraction around the AIP generates longitudinal tension ($\sigma_{zz} > 0$) and compression ($\sigma_{zz} < 0$) on the caudal and cranial sides of OVs, respectively. These effects increase in the OVs as looping progresses. In the HT, however, the original myocardial tensions gradually decrease and even disappear in some regions. As discussed in Shi et al. (2014), much of this reduction in tension is caused by the myocardial growth that drives bending. Compression of the HT by the SPL is another contributing factor.

Taken together, our baseline model produces looping morphology, as well as strain and stress distributions, that are qualitatively consistent with available experimental data.
Figure 4.7: **Effects of inhibiting contraction or vein fusion.** (A) Straight heart tube (HT) at HH10-. To help visualize the deformation, artificial markers are placed along the ventral midline. **(B) Baseline (control) model loops to the right at HH12. (C) Baseline model with cytoskeletal contraction turned off in the splanchnopleure, foregut, and the caudal sides of the omphalomesenteric veins (OVs) around the anterior intestinal portal. Consistent with experiments (Rémond et al., 2006), the heart loops relatively normally without contraction. (D,E) Baseline model with simulated OV fusion (uniform longitudinal growth in HT) turned off. Along the dorsal side of the HT, the dorsal mesocardium (DM) is either (D) ruptured (free) or (E) attached to the foregut (roller). Without OV fusion, the heart undergoes less bending and rotation than control (D). With the DM further constrained (E), relatively little rightward rotation occurs, and compression causes the cranial side of the left OV to buckle (arrowheads). (F) Buckled left OV in HH9 embryo exposed to 30 µM (−)-blebbistatin cultured for 20 hr (see Appendix Fig. A.8). Scale bar: 200 µm.

### 4.5.2 Effects of inhibiting contraction and vein fusion

Studies have shown that c-looping is relatively normal when non-muscle myosin II-based contraction is inhibited after looping begins at HH10 (Rémond et al., 2006; Rémond, 2006). Before HH10, however, blocking contraction prevents fusion of the OVs, and the heart does not loop. Rather, the OVs appear to be buckled (Appendix A.3.1 and Fig. A.8), and cardia bifida can occur (Rémond et al., 2006; Rémond, 2006; Varner and Taber, 2012). These effects also occur when OV fusion is blocked by dissection of endoderm at the center of the AIP (DeHaan, 1959; Nadal-Ginard and García, 1972; Kidokoro et al., 2008).

When contraction is turned off in our model ($M_c = 1$), looping morphology is relatively normal with rotation angle history similar to control, although the diameter of the left OV
Figure 4.8: **Effects of removing external constraints on heart tube (HT).** (A–D) Brightfield images of experimental perturbations reprinted from Voronov et al. (2004). (A′–D′) Corresponding finite-element simulations. (A,A′) Straight HT at HH10-. To help visualize rotation, fluorescent labels were injected along the lateral sides of the HT in the experiment, and artificial labels are placed at similar locations in the model. (B,B′) Same heart at HH12 after 12 hr of culture. As the heart rotates rightward, labels on the right (black numbers) and left (white numbers) sides of the HT move toward the dorsal and ventral sides, respectively. The model captures this phenomenon, as the labels originally on the right side now become invisible (dotted circles). (C,C′) Same heart at HH12 after removal of the splanchnopleure (SPL). In both the experiment and model, most rotation disappears as heart untwists, but heart remains bent slightly toward the right. (D,D′) Heart in (C) after transverse dissection of conotruncus (CT, black arrow) and longitudinal dissection of DM (brace in (C)). In both experiment and model, the HT tilts toward the right. In addition, the interventricular groove (white arrowhead) smooths out as the heart unbends. Scale bar: 200 µm.

is smaller than normal (Fig. 4.7A–C; see also Fig. 4.4B). This decrease in vein diameter is caused by the drop in contraction-induced tension around the AIP that normally produces longitudinal compression and a corresponding increase in diameter on the cranial side of the vein.

When OV fusion is turned off ($M_f = 1$), the heart still loops but it bends and rotates less (Fig. 4.7D). This behavior contradicts the experimental findings described above, and we reasoned that this inconsistency can be attributed to the DM not rupturing as it normally does when the HT bends. To simulate an intact DM, we added cranial-caudal oriented rollers along the full length of the DM. This added constraint eliminates almost all HT rotation (Fig. 4.7E), and the left OV buckles on its cranial side (see arrowheads in Fig. 4.7E).
These results agree relatively well with experimental observations (Fig. 4.7F; see also Appendix Fig. A.8).

4.5.3 Effects of mechanical perturbations

Researchers have used various types of mechanical perturbations to explore the roles of external loads in the looping process (Nadal-Ginard and García, 1972; Voronov and Taber, 2002; Voronov et al., 2004; Taber, 2006; Kidokoro et al., 2008; Bayraktar and Männer, 2014). Most of these studies use dissection to disrupt the transmission of stress or to remove neighboring tissue. Similar to prior work with models of simplified geometry (Voronov et al., 2004; Ramasubramanian et al., 2008; Taber et al., 2010), we used results from these experimental studies to test our present model. Each experiment was simulated while keeping all model parameters unchanged.

Voronov et al. (2004) have shown that when the SPL is removed from an HH12 heart, the HT loses most of its rotation (Fig. 4.8A–C; see also Fig. 4.4B). Then, after the conotruncus and DM are severed, the HT tilts to the right (Fig. 4.8D). Our simulations for these dissections produced similar results (Fig. 4.8A′–D′).

In other experiments, Ramasubramanian et al. (2008) removed the SPL and either one or both OVs. After 12 hr of culture, the heart looped leftward when the left OV was removed and rightward when either the right OV or both OVs were removed (Fig. 4.9A–F). These results suggest that looping direction can be determined by unbalanced lateral forces exerted by the OVs, and, without the counterbalancing effects of the dissected vein, the remaining vein pushes the HT to the opposite side. Our model reproduces all of these results reasonably well, including the left looping case, although there are some discrepancies in the morphology of the remaining vein (Fig. 4.9A′–F′).

Ramasubramanian et al. (2008) attributed the observed HT rotation in the absence of the SPL and both OVs to a contractile response that occurs on the right side of the HT when the SPL is removed (Nerurkar et al., 2006; Ramasubramanian et al., 2008). The present model, which does not include this additional contraction, shows that initial geometric left-right asymmetry in conjunction with constraints imposed on the HT also can contribute to this torsion. At HH10-, the right side of the HT is slightly longer than the left side (Kidokoro
et al., 2008) (see also Fig. 4.2B,B'), causing the HT to bend slightly toward the right as it elongates while constrained by the DM at each end. This causes the caudal end of the HT to shift slightly leftward, which is consistent with the physical model results of Bayraktar and Manner (2014), and the continued caudal DM attachment to the foregut converts the bending into a rightward twist.

Finally, Kidokoro et al. (2008) removed the HT and found that the OVs continued to fuse and regrow a portion of the HT, which tilted rightward (Fig. 4.9G,H). Although our model with the HT removed does not include vein fusion, it captures the morphology of the OVs relatively well (Fig. 4.9G',H').

Taken together, these comparisons show that our model is able to predict the outcomes of various mechanical perturbations remarkably well without altering model parameters. Hence, these results support our integrated hypothesis for the physical mechanisms of c-looping.

4.6 Discussion

Cardiac looping has been one of the most studied and perplexing problems in morphogenesis. Although the genetic and molecular aspects of looping are now becoming clear, the mechanistic side of the story has remained poorly understood. Clearly, bending and twisting the HT requires mechanical forces, and researchers have proposed and tested a number of hypotheses for the biomechanics of looping. However, none of these hypotheses are consistent with all available data.

In the past decade, we have examined some of these ideas in considerable detail from a biomechanical engineering perspective. Using a combination of computational modeling and experiments, we have proposed a new hypothesis for the physical mechanisms that cause the HT to bend and twist during c-looping. According to our hypothesis, bending is driven primarily by forces generated within the HT, while twisting is caused mainly by external loads. Specifically, we have found that ventral bending is likely caused by differential hypertrophic growth in the myocardium (Shi et al., 2014), whereas torsion is driven by a combination of unbalanced forces exerted by the OVs on the HT that determine looping directionality and a compressive load applied by the SPL that enhances torsion.
Figure 4.9: Effects of removing omphalomesenteric veins (OVs) or heart tube (HT). (A–H) Bright-field images of experimental perturbations reprinted from Ramasubramanian et al. (2008) (A–F) and Kidokoro et al. (2008) (G,H). (A,C,E,G) HH10- hearts with (A) the left OV, (C) the right OV, (E) both OVs, or (G) the HT removed. For access to the heart, the splanchnopleure was removed first. Black lines denote the cuts. To help visualize rotation, fluorescent labels were injected along the ventral midline of the heart. (B,D,F,H) The same hearts after 12 hr of culture. White dotted lines in (B) and (F) outline the inner curvature of the HT. (A′–H′) Corresponding finite-element simulations. The model predicts all of the final shapes reasonably well, including the leftward looping (B,B′). Note that a portion of the HT (black dotted line in (H)) regrew above the interventricular grooves (arrowheads in (G)) in the experiment through OV fusion, which is not included in the model (panel (H′)). Scale bar: 200 μm.
Separate models for the bending and torsional components of c-looping have demonstrated the feasibility of these hypotheses (Taber et al., 1995; Voronov et al., 2004; Latacha et al., 2005; Nerurkar et al., 2006; Ramasubramanian et al., 2006, 2008; Shi et al., 2014). However, those previous models are based on simplified geometries and do not include coupling between bending and torsion. Hence, one purpose of the present study is to examine these hypotheses using a more comprehensive model that includes both bending and torsion as well as realistic geometry.

Our general strategy was to first develop a baseline model for normal c-looping. Model parameters for the HT are based on previous estimates (Shi et al., 2014), although some were modified slightly to account for differences in morphology between isolated and intact hearts. Forces generated within the HT include CJ swelling, tension in the DM, differential myocardial growth, and active changes in myocardial cell shape (Fig. 4.2B,C,B’). External forces include those exerted on the HT by the SPL and OVs. Vein forces are generated by growth and cytoskeletal contraction on their cranial and caudal sides, respectively (Fig. 4.2B,B’). Our model yields relatively good agreement with normal heart morphology, as well as the qualitative trends in stress, strain, and HT rotation (Figs. 4.3–4.6). Because of significant variability in the normal shape of the heart during looping (von Dassow and Davidson, 2007), we reason that matching spatiotemporal trends is a more realistic approach than insisting on precise quantitative agreement between numerical and experimental results.

Perhaps more importantly, for the same parameter values, the model predicts reasonably well the effects of various mechanical perturbations of the looping process (Figs. 4.7–4.9). According to our hypothesis, looping direction is determined by a left-right difference in lateral forces exerted by the OVs, which push against the HT. Normally, the left OV is larger and exerts more pushing force than the right OV, causing the HT to twist slightly rightward (Voronov et al., 2004; Taber, 2006; Kidokoro et al., 2008; Ramasubramanian et al., 2008; Taber et al., 2010). However, if the left OV is removed or if growth of the right OV is enhanced, the right vein pushes the heart leftward, resulting in left looping (Ramasubramanian et al., 2008; Kidokoro et al., 2008). Experiments also have shown that the SPL causes most of the remaining torsion, since little torsion occurs when the SPL is
removed before HH12 (Voronov et al., 2004; Nerurkar et al., 2006). Our model captures the results from these perturbation experiments quite well (Figs. 4.8, 4.9 and Appendix Fig. A.10E). The behavior of the model also is consistent with the finding that cytoskeletal contraction is not necessary for looping (Rémond et al., 2006; Rémond, 2006) (Figs. 4.7C and 4.4B).

Recently, Bayraktar and Männer (2014) used a physical model to show that a growing tube constrained within the pericardial cavity (formed by the SPL and foregut) buckles into a helical shape consistent with the shape of the c-looped HT. This mechanism for bending extends the original idea of Patten (1922) that the HT buckles as it grows longer within a confined space, but the authors acknowledge that this idea seems inconsistent with bending of isolated hearts (Bayraktar and Männer, 2014). These authors also show that looping direction can be determined by a small initial offset in the lateral position of the caudal end of the HT. The present model does not rule out these mechanisms as possible contributing factors in c-looping, and, in fact, torsion of the HT in both models is caused primarily by constraints imposed by the SPL.

We suggest that recent experimental data support the hypothesis that differential hypertrophic growth drives the bending component of c-looping. For example, our model predicts a decrease in longitudinal stress near the OC as looping progresses (Fig. 4.3A), in agreement with experimental results (Shi et al., 2014) (see also Fig. 3.8). In contrast, this stress would be expected to increase as the tube bends in the physical model of Bayraktar and Männ er (2014).

Bayraktar and Männ er (2014) also speculate that in embryos with cardia bifida, oppositely directed offsets between the left and right hemi-hearts may explain why the convex OCs of these hemi-hearts face the embryonic midline (Nadal-Ginard and García, 1972). Our model suggests an alternative explanation, i.e., that the left OV pushes the left hemi-heart rightward and the right OV pushes the right hemi-heart leftward (Fig. 4.9A′–D′).

Taken together, the present results support the physical plausibility of our integrated hypothesis for c-looping. However, we must note that our model does not include all possible mechanisms that may be involved in this process. For example, it does not explicitly include OV fusion, which is simulated by global longitudinal growth of the HT (see Figs. 4.2 and 4.7D,E). The forces involved in the fusion process may affect looping (DeHaan, 1967; Nadal-Ginard and García, 1972; Rémond et al., 2006; Kidokoro et al., 2008; Varner and
Taber, 2012). Our model also does not include left-right differences in growth of the DM as found by Linask et al. (2005). These authors speculated that a higher proliferation rate on the left side of the DM pushes the HT rightward and vice versa. This could be one of the redundant mechanisms that help make looping a relatively robust developmental process.

Finally, our model does not include mechanical feedback, which is a subject of increasing interest in developmental biology (Belousov, 1998; Nerurkar et al., 2006; Belousov, 2008; Ramasubramanian and Taber, 2008; Taber, 2008, 2009; Pouille et al., 2009; Belousov, 2012; Bayly et al., 2013). For example, we have found that when the SPL is removed at HH10, little torsion occurs prior to HH11. Upon further culture, however, the data suggest that an abnormal contraction on the right side of the HT restores normal torsion by HH12 (Nerurkar et al., 2006; Ramasubramanian et al., 2008). This contraction appears to be a reaction to changes in the normal loads exerted on the heart (Nerurkar et al., 2006; Filas et al., 2011).

In the future, similar models could be used to determine the biophysical links between various genetic mutations and observed looping defects. This would entail conducting parameter studies by changing one model parameter at a time and comparing predicted and observed morphologies. However, it is important to keep in mind that multiple mechanisms can produce similar morphologies (Stalsberg, 1970; Taber, 2006; Shi et al., 2014). To hone in on precise mechanisms, it may be necessary to further develop techniques for measuring stress and strain fields noninvasively (Velduis and Brodland, 1999; Blanchard et al., 2009; Grashoff et al., 2010; Campàs et al., 2014).

In conclusion, our model is consistent with a variety of available data and supports the hypothesis that the bending component of c-looping is driven primarily by differential hypertrophic growth, while torsion is caused by forces exerted by the OVs and SPL. Looping directionality is dictated by unbalanced forces exerted by the OVs, with normal rightward looping caused by the left vein pushing with more force than the right vein. While these may be the primary mechanical forces involved in c-looping, it is likely that other forces also are involved, thus minimizing abnormalities in this crucial morphogenetic process.
References


Chapter 5

Conclusions

In this dissertation, we used a combination of laboratory experiments and computational modeling to investigate the mechanics of the early development of the embryonic heart. Here, we summarize our findings and discuss future directions.

5.1 Summary of results and discussion

Key findings from this work include:

(1) Investigating the role of endodermal contraction in driving the mesodermal movements (and fusion) along the anterior intestinal portal (AIP) helps explain the seemingly perplexing problem why cytoskeletal contraction is required before but not after cardiac looping begins. Our experimental results indicate that the AIP stiffness and tangential tension both decrease bilaterally with distance from the midline and these gradients are mainly caused by myosin-II-based cytoskeletal contraction of the endoderm. Moreover, these gradients peak at HH9 and decrease immediately afterward, consistent with reductions in endodermal shortening and mesodermal migration. Results from our computational models show that the observed gradients in AIP stiffness and tangential tension can be caused by a relatively uniform endodermal contraction in conjunction with a passive modulus gradient and constraints imposed by the mesoderm. These results suggest that contraction of the endoderm along the AIP contributes to the mesodermal movements in two main ways. First, endodermal contraction convects the originally separate cardiogenic fields toward the embryonic midline. Second, contraction generates the observed lateral-to-medial stiffness gradient (by changing tangential tension), which may facilitate directed mesodermal
migration (durotaxis). Both movements, which enable mesodermal fusion to occur at the midline to create the heart tube (HT), depend on endodermal contraction before the onset of looping. After looping begins at HH10, however, the fusion process is far enough along so that it can continue to occur through filopodia-mediated “zippering” without the need for contraction.

(2) Although differential hyperplasic growth has long been ruled out as a possible mechanism for c-looping, recent studies suggest that regional differences in myocardial cell hypertrophy may be responsible for causing bending of the HT. Using computational modeling, as well as experiments with HTs cultured in isolation, we found that our measurements of myocardial strains and stresses are generally consistent with bending caused by previously measured spatial distributions of changes in myocardial cell volume. Quantitative matching the experimental stress, however, requires the addition of other mechanisms, such as active changes in myocardial cell shape. These results suggest that differential hypertrophic growth in the myocardium is the primary driver for the bending component of c-looping, with other mechanisms contributing secondarily.

(3) Using a 3D finite-element model with geometry reconstructed from OCT images of an actual embryonic chick heart, we simulated the entire process of c-looping including both bending and torsional components. The behavior of our model agrees reasonably well with a variety of experimental data obtained from control and mechanically perturbed embryos. The results support our newly proposed looping hypothesis, i.e., that the bending component of c-looping is driven primarily by differential myocardial hypertrophic growth, whereas torsion is mainly caused by external loads exerted on the HT by the splanchnopleure (SPL) and omphalomesenteric veins (OVs). First, asymmetric growth on the cranial sides of the OVs generates unbalanced lateral forces on the caudal end of the HT, which determine looping direction. Then, the SPL provides compressive loads on the ventral side of the HT, which enhance torsion. Nevertheless, we do not rule out other possible mechanisms, which may help ensure normal rightward looping under perturbed conditions.

The results of this dissertation have advanced our acknowledge on the biophysical mechanisms of early cardiac development. Our approach of integrating laboratory experiments and computational modeling can be applied to a wide range of biomechanical problems during morphogenesis. However, our work certainly has many limitations, which warrant future research.
5.2 Future directions

**Strain characterization.** In this dissertation, strains were characterized mainly by tracking fluorescent labels manually injected into the tissue. This technique has many drawbacks. It is somewhat labor-intensive and inefficient, and worst of all, it can damage the tissue, which may perturb development somewhat. In addition, the outcome sometimes is not satisfactory due to photobleaching and tissue movements. Therefore, it may not be ideal for measuring tissue deformation in some problems.

We had also attempted to measure surface strains by attaching polystyrene microbeads coated with adhesive molecules onto the HT (Filas et al., 2007). Unfortunately, we had very limited success in enhancing adhesion (especially on isolated hearts), as beads often detached from the curved tissue surface (e.g., the outer curvature of the HT). (This technique actually works better inside the early brain tube.)

Advances in microscopy and fluorescent tagging proteins have made it possible to overcome these challenges. One way is to insert gene sequences that express fluorescent proteins into the genome of target cells. This method, however, has been primarily limited to model orgasms like zebrafish and *Drosophila* (Hutson et al., 2003; Martin et al., 2009; Solon et al., 2009; Blanchard et al., 2010; Gorfrinkel and Blanchard, 2011). Relatively recently, Sato et al. (2010) created a transgenic quail line. Their work has shed light on the possibility of applying this technique to avian embryos. Another way is to incorporate fluorescent probes, such as Di-8-ANEPPS, into the cell membrane or other subcellular organelles. This membrane dye allows quantifying tissue deformation and cardiac function simultaneously (DiFranco et al., 2005; Pucihar et al., 2009).

**Stress characterization.** In this dissertation, local tissue stress was probed by introducing microsurgical cuts and examining the wound opening/closure. Such an invasive approach often triggers an active contraction around the wound edge, which may alter the local stress state quite significantly (Varner et al., 2010; Rémond, 2006; Wyczalkowski et al., 2013). In addition, using geometric measures of the opening of the cut (e.g., opening angles and aspect ratios) to quantify the tissue tension level sometimes requires the assistance of computer models, as the effects of tissue curvature on wound geometry can be
counterintuitive. Therefore, it would be desirable to develop some noninvasive techniques for stress measurement.

Very recently, using fluorescence microscopy, Campàs et al. (2014) measured intercellular forces in a tissue construct by analyzing the deformation of a fluorescent cell-sized oil microdroplet introduced between cells. In another study, Grashoff et al. (2010) developed a calibrated vinculin-based biosensor to measure focal adhesions at a piconewton (pN) level using single molecule fluorescence force spectroscopy. These ingenious designs will open up possibilities for the next-generation of quantitative experimental biology.

**Integrating multiscale mechanisms.** Another trend in studies on the mechanics of morphogenesis is the emergence of a multiscale approach. Take cell migration as an example. Scientists are no longer satisfied by the classic model of a single cell crawling on a 2D substrate, as the morphology and behavior of a cell migrating in a 3D matrix are drastically different from that on a 2D surface (Friedl et al., 1998; Friedl and Bröcker, 2000; Legant et al., 2010, 2013). They also want to understand how collective cell migration takes place in 3D environment, as well as how cells interact with the matrices and communicate with each other (Friedl and Gilmour, 2009; Ilina and Friedl, 2009; Trepat et al., 2009).

In this dissertation, we have embraced the idea of integrating mechanisms at different scales. In Chapter 3, regional changes in myocardial cell size and shape were estimated from experimental data and incorporated into our computational models for bending of the HT. This approach helps propose new hypotheses for tissue morphogenesis as well as understand spatiotemporal changes in the mechanical environment (stress, stiffness, adhesion) for cells.

**Computational modeling.** In Chapter 2, we used two types of finite-element model to investigate separately how strain and stress distributions around the AIP are affected by tissue geometry, passive material properties, and endodermal contraction. For each model, the geometry was created by sweeping a 2D cross section of the AIP at the indentation site through 3D space. Due to technical difficulties at the time of this project, we were not able to create a comprehensive model that includes all these contributing factors, as well as realistic AIP geometry. Recent advances in modeling technique allow us to reconstruct 3D
tissue geometry from OCT datasets, as discussed in Chapter 4. This new approach may be applicable to a more thorough study of tissue mechanics around the AIP in the near future.

In Chapter 4, we created a finite-element model based on realistic heart geometry to simulate the entire process of c-looping. OV fusion was not explicitly included, and instead, it was modeled as uniform longitudinal growth of the HT. It would be interesting to develop a numerical algorithm for simulating the actual fusion process by tying together the neighboring nodes along the fusing path. Such a technique would allow us to extend our research on cardiac development to later stages.

**Incorporating mechanical feedback.** Ever since the pioneering work of Belousov et al. (1975), mechanical feedback has drawn more and more attention from developmental biologists (Belousov, 1998; Farge, 2003; Shraiman, 2005; Nerurkar et al., 2006; Belousov, 2008; Fernandez-Gonzalez and Zallen, 2009; Taber, 2008, 2009; Pouille et al., 2009; Belousov, 2012; Bayly et al., 2013). Relatively recently, our lab has found that mechanical perturbations lead to changes in myosin-II-based cytoskeletal contractility in the embryonic heart and brain (Nerurkar et al., 2006; Filas et al., 2011). Although mechanical regulation is not included in the studies of this dissertation, our computational models can be easily modified to incorporate mechanical feedback laws.
References


Appendix A

Supplementary Information

A.1 Supplementary information for cytoskeletal contraction around AIP during early heart development

A.1.1 Image processing algorithm for analyzing tissue deformation during microindentation

To analyze indenter displacement and tissue deformation during microindentation tests, we processed real-time OCT images using the image analysis software ImageJ (NIH).

Within each image stack, we first chose a reference frame, which is the frame that the indenter first contacts the tissue (Fig. A.1A). This reference frame was subtracted from subsequent images to show the tissue being deformed under the indenter (Fig. A.1B). The resultant image contains pixels with increased intensity as the tissue deformed (Fig. A.1C). These pixels represent the leading edge of the deformation, where the tissue had moved into space previously occupied by fluid.

Similarly, we repeated this subtraction using inverted images (Fig. A.1A’,B’). The resultant image contains pixels that decreased in intensity (Fig. A.1C’). These pixels represent the trailing edge of the deformation, where tissue was replaced by fluid.

Sections A.1.1 and A.3.1 were drafted by Victor Varner and Judy Fee, respectively. They also provided the corresponding data.
Figure A.1: Characterizing tissue deformation during microindentation using optical coherence tomography (OCT). (A) Sagittal (midline) OCT section through HH8 embryo. The cylindrical indenter, which is aligned within the imaging plane, has just come into contact with the tissue. (B) Subsequent OCT image showing the deformed shape of the indented tissue. (C) Image of deformation leading edge created by subtracting (A) from (B). (A’,B’) Inverted images of (A) and (B). (C’) Image of deformation trailing edge created by subtracting (A’) from (B’). (D) Enlarged composite color image generated by overlaying (C) (red, leading edge) and (C’) (blue, trailing edge) on top of (A). Scale bars: 200 μm.

Both subtracted images were then overlaid on top of the originals to generate the composite image, in which the leading and trailing edges of the tissue deformation are indicated in red and blue, respectively (Fig. A.1D).

A.1.2 Sensitivity analysis of OV model

To investigate how the strain and stress distributions around the AIP are affected by material properties, endodermal contraction, and OV geometry, we conducted a sensitivity analysis for our OV model by varying the following parameters one at a time:

- Relative shear modulus of mesoderm $\alpha = \mu_m/\mu_0$, where $\mu_0$ is the endodermal modulus at the medial AIP ($\Theta = 0$).
• Gradient in endodermal modulus $\beta = \mu_1/\mu_0$, where $\mu_1$ is the endodermal modulus at the lateral AIP ($\bar{\Theta} = 1$).

• Gradient in endodermal contraction $\gamma = (1 - G_1)/(1 - G_0)$, where $G_0$ and $G_1$ are the contraction stretch ratios at the medial and lateral AIP, respectively.

• Torus radius $\rho_0$.

• Average cross-sectional radius $R_0$.

• Thickness of each tissue layer $T_0$.

In general, uniform distributions of endodermal contraction ($G$) and shear modulus ($\mu$) generate negative longitudinal strain ($E_{\Theta\Theta}$) and positive longitudinal stress ($\sigma_{\theta\theta}$) in the endoderm, both of which decrease with distance away from the midline (Fig. A.2A,B). Increasing $\alpha$ over a relatively large range ($\alpha \geq 0.5$) decreases the strain magnitude while elevating the tension level, but their spatial distributions do not change qualitatively. The larger mesodermal shear modulus ($\alpha$) is, the deformation is less uniform and more localized toward the lateral end.

Our results show that increasing the gradient in endodermal modulus (i.e., decreasing $\beta$) has relatively little effects on the strain and stress distributions, except that the strain magnitude and stress both decrease slightly at the mediolateral location (Fig. A.2C,D). On the other hand, the strain distribution is significantly affected by the contraction gradient. For example, $E_{\Theta\Theta}$ decreases with $\bar{\Theta}$ when $\gamma = 1$ (i.e., $G_1 = G_0$, uniform contraction), while it increases with $\bar{\Theta}$ when $\gamma = 0$ (i.e., $G_1 = 1$, no contraction at the lateral end) (Fig. A.2E,F). Our experimental data agree relatively well with the numerical result for uniform contraction (see Fig. 2.9F).

Our results also show that varying the geometric parameters of the model ($\rho_0$, $R_0$, and $T_0$) changes the strain and stress distributions quantitatively but not qualitatively (Fig. A.3).

Taken together, these results show that our OV model is relatively robust. The experimental trends in the strain and stress are consistent with the numerical results for uniform contraction.
Figure A.2: Effects of material moduli and endodermal contraction on strain and stress distributions from omphalomesenteric vein (OV) model. Distributions of longitudinal strain ($E_{\theta\theta}$) and stress ($\sigma_{\theta\theta}$) along the longitudinal direction are shown by changing dimensionless parameters: (A,B) mesodermal modulus ($\alpha = \mu_m/\mu_0$), (C,D) gradient in endodermal modulus ($\beta = \mu_1/\mu_0$), and (E,F) gradient in endodermal contraction ($\gamma = (1 - G_1)/(1 - G_0)$).
Figure A.3: Effects of geometric dimensions on strain and stress distributions from omphalomesenteric vein (OV) model. Distributions of (A,C,E) Lagrangian strain ($E_{\Theta\Theta}$) and (B,D,F) Cauchy stress ($\sigma_{\Theta\Theta}$) along the longitudinal direction (curved arrow in (A)). Sensitivity studies of varying (A,B) the toroidal radius $\rho_0$, (C,D) the cross-sectional radius $R_0$, or (E,F) the thickness of endodermal (ENDO) or mesodermal (MESO) layer $T_0$. Variations in these geometric quantities change the results quantitatively, not qualitatively, showing the robustness of this model.
A.2 Supplementary information for bending of looping heart

A.2.1 Embryo preparation for scanning electron microscopy

Scanning electron microscopy (SEM) has been widely used to characterize surface features and structures of embryonic hearts (Manasek et al., 1972; Waterman, 1972; Männer, 2000; Sedmera et al., 2000). For specimen preparation, we followed the standard procedure summarized in Waterman (1972). Briefly, a buffered fixative of 2% formaldehyde (Sigma) and 2% glutaraldehyde (Sigma) was used to preserve and stabilize the tissue structure. After fixation for 24 hr, the samples were rinsed 2–3 times with PBS and postfixed with 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) for 1 hr. Then, the fixed samples were rinsed in a graded ethanol series (30%, 50%, 70%, 90%, 95%, 100%) for a total treatment of 1 hr and dehydrated in a critical point dryer (Autosamdri-815, Tousimis, Rockville, MD) to prevent tissue collapse and shrinkage. Finally, the dried samples were sputter coated with gold on mounting stubs and examined using an imaging system (Nova NanoSEM 230, FEI, Hillsboro, OR).

A.2.2 Estimation of cell-shape change parameter

To estimate the changes in cell shape during looping, we analyzed myocardial cell shapes and orientations in prelooped (HH10-) and looped (HH11) hearts based on the original cell contour drawings of Manasek et al. (1972). The ventral side of the prelooped heart (the original Figure 6) was divided into left and right halves (Fig. A.4A), while the looped heart (the original Figure 7) was divided into IC and OC regions (Fig. A.4B). Within each region, cell contours were traced manually and fitted with ellipses using image analysis software (ImageJ). For each cell, the aspect ratio (γ) was defined as the major axis of the fitted ellipse divided by the minor axis, and the orientation was taken as the angle between its major axis and the horizontal of the image, which is approximately in the circumferential direction of the HT. The average cell aspect ratio and average orientation were computed for each region and used to quantify the changes in myocardial cell shape during looping.

In the prelooped HH10- heart, myocardial cells are aligned primarily circumferentially and have similar aspect ratio values on the left (LHT; $γ = 1.87 \pm 0.65$, $n = 374$) and right
Figure A.4: **Cell shapes and orientations in prelooped and c-looped chick heart.**

(A) Myocardial cells in HH10- heart. The heart tube (HT) is divided into right (RHT, dark green) and left (LHT, light green) sides. (B) At HH11, the myocardium is divided into two regions — outer curvature (OC, purple) and inner curvature (IC, red). (Original drawings of cell contours are from Manasek et al. (1972).) (C) Myocardial cells located at the IC of the looped HT have significantly larger aspect ratios than those in the other three regions (*p < 0.001, one-way ANOVA). (D) Cell orientation angles (in degrees) with respect to the horizontal in the images suggest that myocardial cells are primarily aligned along the circumferential direction. Scale bar: 100 μm (same for A).
(RHT; $\gamma = 1.80 \pm 0.59$, $n = 364$) sides of the HT (Fig. A.4A,C,D). Although only the ventral surface of the heart is shown in the original figure, myocardial cell size and shape are relatively uniform at this stage, consistent with data of Soufan et al. (2006) and our own experimental observations (data not shown). Therefore, we take 1.8 as the average aspect ratio of myocardial cells at HH10-.

From HH10- to HH11, the ventral and dorsal sides of the initially straight HT become the convex (OC) and concave (IC) sides of the looped heart, respectively. Although most cells remain circumferentially aligned, especially near the IC, cell morphologies are quite different between the OC and IC (Fig. A.4B,C,D). Compared to myocardial cells at HH10-, cells at the OC of the HH11 HT have similar aspect ratios ($\gamma = 1.80 \pm 0.59$, $n = 134$), whereas those near the IC are significantly more elongated in the circumferential direction ($\gamma = 2.11 \pm 0.82$, $n = 248$; $p < 0.001$, one-way ANOVA) (Fig. A.4B,C).

The 1.2-fold increase in average cell aspect ratio near the IC (2.1/1.8 $\approx$ 1.2) corresponds to a 10% increase in circumferential cell length (i.e., $\tilde{S} = 1.1$ according to Eq. (3.10)). Since Manasek et al. (1972) provide no data for the HH12 heart, we assume that changes in shape occur linearly over time and reach $\tilde{S} = 1.2$ at HH12. This calculation, which is based on average cell shapes in the concave half of the HT, probably underestimates $M_s$, which is the maximum value of $S(\Theta)$ at the IC ($\Theta = 0$) (see Eq. (3.10)). Therefore, we choose $M_s = 1.3$ for our baseline model.

### A.2.3 Sensitivity analysis of baseline cylinder model

The baseline model has five morphogenetic parameters ($M_j$, $M_t$, $M_c$, $M_g$, $M_s$), most of which are essentially determined directly from experimental data. However, there are two free parameters (DM tension parameter $M_t$ and myocardial growth parameter $M_g$), as well as an untested assumption ($G_R = G_\Theta$). In the following paragraphs, we explore how results from the baseline model depend on these parameters and the assumption.

To investigate how relative radial ($G_R$) and circumferential ($G_\Theta$) growth affects myocardial strain and stress, we vary $G_R$ while keeping the product $G_R G_\Theta = 1.3^2$ constant. To avoid the complication introduced by other morphogenetic steps, we focus here on the differential growth model. Over a fairly large range of values for $G_R$, the stretch ratios change relatively
Figure A.5: Effects of altering myocardial growth parameters in differential growth model for blebbistatin-treated heart. Radial growth ($G_R$) is specified and circumferential growth ($G_\theta$) is adjusted to keep the product $G_R G_\theta = 1.3^2$ fixed. (A) Changing $G_R$ over a relatively wide range has relatively small effect on stretch ratios. Radial $\lambda_r$ (solid lines), circumferential $\lambda_\theta$ (dashed lines), ventral longitudinal $\lambda_zv$ (dash-dot lines), and dorsal longitudinal $\lambda_zd$ (dotted lines). Experimental data are indicated by horizontal red lines, and vertical blue line indicates $G_R = G_\theta = 1.3$, which is used in our baseline model. (B) Increases in $G_R$ elevate myocardial stresses globally. Boxed number is the baseline value. Due to circumferential compression in the myocardium, the myocardium begins to buckle when $G_R \leq 1$, as shown by the buckled shape of the model (CJ = cardiac jelly, DM = dorsal mesocardium, DMY = dorsal myocardium, VMY = ventral myocardium, IC = inner curvature, OC = outer curvature).
Figure A.6: **Parameter study for baseline cylinder model for control heart.** (A–D) Effects of changing dorsal mesocardium (DM) tension parameter $M_t$. Decrease in $M_t$ (i.e., more DM tension) causes increased magnitude of bending, but stretch ratios remain relatively unchanged. Radial $\lambda_r$ (solid lines), circumferential $\lambda_\theta$ (dashed lines), ventral longitudinal $\lambda_{zv}$ (dash-dot lines), and dorsal longitudinal $\lambda_{zd}$ (dotted lines). Decreasing $M_t$ also causes longitudinal stress ($\sigma_{zz}$) to increase at the outer curvature (OC) and decrease near the inner curvature (IC) outside the DM. (E,F) Effects of changing myocardial growth parameter $M_g$ on stretch ratios and longitudinal stress distribution. Stress increases with $M_g$, i.e., more circumferential and radial growth. In panels B and E, experimental data are shown as horizontal red lines. The default input values ($M_t = 0.8$, $M_g = 1.3$) for our baseline model are indicated by vertical blue lines and boxed numbers.
Figure A.7: Exponential form for the strain-energy density function does not significantly change the results of baseline model. (A) Stretch ratios given by exponential form (Exp, hatched bars) are similar to those from neo-Hookean form (NH, solid bars) for both control and blebbistatin-treated (Bleb) hearts. Differences are less than 3%. (B) These two models give similar distributions of longitudinal stress ($\sigma_{zz}$) at the beginning (0 hr) and end (24 hr) of culture, except in the dorsal mesocardium (DM).

little (Fig. A.5A). Myocardial stresses, however, are affected considerably, as increasing $G_R$ elevates them globally (Fig. A.5B). Interestingly, the model begins to buckle when $G_R \leq 1$, due to circumferential compression in the relatively thin MY. The ratio $G_R/G_\theta = 1$ yields results that agree reasonably well with our experimental data.

In general, increasing the value of $M_t$ increases the amount of bending somewhat, but it has little effect on stretch ratios (Fig. A.6A,B). In addition, this parameter affects myocardial stress quantitatively but not qualitatively (Fig. A.6A,C,D).

In our model, we assume $G_R = G_\theta \equiv M_g$. Since the volumetric cell growth ($G = G_RG_\thetaG_Z = M_g^2G_Z$, see Eq. (3.9)) is given by the experimental data of Soufan et al. (2006), increasing $M_g$ corresponds to more radial and circumferential growth but less longitudinal growth. As a result, radial ($\lambda_r$) and circumferential ($\lambda_\theta$) stretch ratios increase with $M_g$, while longitudinal stretch ratios ($\lambda_zv$, $\lambda_zd$) decrease (Fig. A.6E). In addition, myocardial stresses increase with $M_g$ throughout the MY (Fig. A.6F).

Finally, the results from the model are relatively insensitive to the form taken for the strain-energy density function (Fig. A.7). Since the material properties of the HT are not highly nonlinear, a neo-Hookean form is adequate for the present study.
Taken together, the results of the sensitivity analysis show that our baseline model is fairly robust, as changing values of the morphogenetic parameters within reasonable ranges affects the results somewhat quantitatively, but not qualitatively.

A.3 Supplementary information for computational modeling of c-looping

A.3.1 Contraction inhibition prior to stage 10-

To examine the effects of inhibiting contraction on vein fusion and looping prior to HH10-, embryos ($n > 5$) were harvested at HH9+ (approximately 30 hr of incubation) and cultured in medium containing 30 $\mu$M (-)blebbistatin (Sigma-Aldrich, St. Louis, MO). A stock solution of 30 mM (-)blebbistatin in dimethylsulfoxide was diluted 1:1000 in the culture medium to give the final concentration of 30 $\mu$M. For controls, the same amount of the solvent dimethylsulfoxide was added. To prevent photoinactivation of blebbistatin during culture and manipulation, exposure to light was limited and aluminum foil was used to cover the dishes whenever possible. The medium was replaced with fresh medium and blebbistatin after exposure to light during microscopy. Effectiveness of drug treatments was verified by diminished but not abolished heartbeat and reduced tissue tension.

If myosin II-based contraction is inhibited before HH9, the OVs fail to fuse to create the HT (Rémond et al., 2006; Varner and Taber, 2012). Once looping starts at HH10, however, OV fusion and looping appear normal when contraction is blocked (Rémond et al., 2006; Rémond, 2006). To test our model with OV fusion turned off, we acquired new experimental results for contraction inhibition prior to HH10-.

In control embryos, the OVs fused normally as indicated by the descending AIP and increasing length of the HT (Fig. A.8A–C). After 20 hr of culture, embryos reached HH13+ with c-looping completed (Fig. A.8C). In blebbistatin-treated embryos, the OVs stopped fusing and the AIP did not descend (Fig. A.8D–F). The HT looped slightly, but its length remained relatively constant. Moreover, both OVs enlarged significantly, and the cranial side of left OV appeared to buckle (see arrowheads in Fig. A.8F).
Figure A.8: **Effects of inhibiting contraction before looping begins.** (A–C) HH9+ embryo cultured in control conditions for (A) 0 hr, (B) 7 hr, and (C) 20 hr. Note descending anterior intestinal portal (AIP; asterisk denotes the first pair of somites) as omphalomesenteric veins (OVs) fuse to form the heart tube (HT). (D–F) HH9+ embryo cultured in 30 µM (-)blebbistatin for (D) 0 hr, (E) 7 hr, and (F) 20 hr. AIP did not descend as OVs did not fuse. Instead, the OVs enlarged abnormally, and buckles (arrowheads) appeared along the cranial side of left OV. Scale bar: 200 µm.

Figure A.9: Effects of elastic modulus of splanchnopleure (SPL) on torsion of heart tube (HT). Increasing the dimensionless modulus of the SPL relative to the myocardium ($A'$) has little effect on torsion of the HT.
**A.3.2 Sensitivity analysis of baseline realistic-geometry model**

To investigate further how torsion of the HT is affected by SPL and OVs forces, we varied the parameter values for the material modulus of the SPL ($A_{SPL}$) and growth of the OVs ($M_i$).

When the dimensionless SPL modulus $A' = A_{SPL}/A_{MY}$ increases from 0.5 to 4 ($A' = 2$ for baseline model), the time history plot of rotation angle changes little (Fig. A.9B).

When symmetric growth ($M_i = 1, 1.2,$ and $1.4$) is specified for both OVs, the HT always twists rightward with similar rotation angles (Fig. A.10A–D). This is because in the undeformed model, the left OV is somewhat larger than the right OV, introducing an initial left-right asymmetry. Thus, growing at the same rate, the left OV remains larger than the right OV.

To simulate looping caused by chemically induced overgrowth in the right OV (Kidokoro et al., 2008), we specify $M_i = 1$ for the left OV and $1.8$ for the right OV. The results show that at HH12, the initial geometric bias is overcome by the faster growing right OV, and the HT twists leftward (Fig. A.10E).

Taken together, these results are generally consistent with previous experimental data.
References


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