The role of actin polymerization in the development of the fetal vertebrate heart during S-looping

By

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ABSTRACT

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Research on fetal cardiac looping is an important because cardiac looping is the first organ to undergo asymmetrical organogenesis. As well, birth defects occur due to errors in the development process, of which congenital heart defects are very common. Most heart defects begin in the looping process that preludes the shape of a mature heart. Therefore, research into heart looping can give light to how these defects develop. The looping process, which occurs between 30 and 56 hours post incubation (stage 10-16 in development), is divided into two processes. The first process, C-looping, is the process where the heart tube, which at first lies along the sagittal at the embryos midline, undergoes dextral rotation and ventral bending to produce a distinct C-shape. Much research has been done on C-looping, as it has been shown C-looping is regulated by factors intrinsic to the heart tube. In S-looping, the atrium moves anterior while the ventricle moves posterior and undergoes dextral rotation, producing the S-shape characteristic of S-looping. Unlike C-looping, little is known about the dynamics behind S-looping. In this thesis, embryonic hearts isolated at stage 12, the stage at the end of C-looping and start of S-looping, were treated with cytochalasin D(CD) and then grown *in vitro*. The results show that most of the embryos exposed to CD did not develop past stage 12. Using fluorescence microscopy, trademark filamentous actin networks were not present as they are in wild type embryos. Therefore, it is speculated that actin polymerization plays a role in bending of S-looping. Although it has been shown that C-looping relies on factors intrinsic to the heart tube, external factors, such as the effect of the splanchnopleurale membrane, should be research as

well since S-looping is a much more complicated process than C-looping. Despite the encouraging results, more data still needs to be obtained to conclude that actin polymerization is essential to S-looping, since low data sets may expose the data to random variation.

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INTRODUCTION

The study of embryogenesis allows scientists to understand the various stages in the anatomical development of an organism from conception until birth. By studying each stage closely, it is possible to link how the preceding stage develops into its succeeding stage[1]. Often, aberrations in the development of each stage can cause a range of minor to major abnormalities, some of which may pose a potential threat to the organism's life. There is both an evolutionary and medical application for the study of fetal anatomical development, because it provides us with insight into the evolutionary history of our past, the frequency of genetic defects since1 in every 33 babies in the United State is born with a defect[1] and a basic understand of heart development is essential.

The development of the fetal vertebrate heart provides a characteristic example of asymmetrical organ development. Since it is the first organ to undergo asymmetrical development, it is the organ most commonly researched when studying the dynamics behind asymmetrical development. Furthermore, congenital heart defects are among the most common birth defects and often occur throughout the looping process. Therefore, researching the dynamics behind fetal heart development can give light to how fetal heart defects form. Much is already known about the first stage in asymmetrical heart development, known as C-looping, which begins at stage HH 10 in development[2]. However, little is known regarding the mechanism of S-looping, which occurs between stage HH 12 and 16 [2]. Therefore, the goal of this experiment is to examine the role of actin polymerization during S-looping of fetal heart development, using the chick as the model organism.

1.1 The Chick as a Model for Fetal Vertebrate Heart Development[1]

The use of humans as a model for early embryogenesis crosses major ethical boundaries, since the isolation required to study the embryo would end human life, raising questions as to what point does human life begin. As a result, a much simpler model organism must be used. In this study, fertilized White-Leghorn chicks are the chosen model organism.

Chicks possess characteristics common among model organisms. Chicks undergo rapid embryonic development, since S-looping begins after roughly 48 hours, whereas S-looping in humans begins much later. As a result, research on chick embryogenesis is more time-effective than if a mammalian model with a longer gestation period was used. Second, the embryogenesis of the chick provides a link between the developments of less biologically complex organisms with the more highly ordered organisms such as mammals. Human embryology is often taught with reference to research done on early embryogenesis in chicks since the early development of many biological systems in chicks, including that of circulatory system, are almost identical to those that occur in mammals. The chick embryo is much more easily isolated, since it must only be removed from the yolk of an egg, while mammalian embryogenesis occurs in the placenta, which would be much more difficult and troublesome to isolate. Finally, chick embryogenesis is transparent and easily manipulated, facilitating observations with regards to embryonic anatomical development and enabling scientists to manipulate genetic and cellular factors believed to be important to, with respect to this experiment, fetal heart development.

As a result, due to its rapid life cycle and reproduction, shared early embryonic anatomical development mechanisms, easy embryonic isolation and transparent embryogenesis, the White-Leghorn chicken is a quintessential model organism for the study of fetal vertebrate heart development.

1.2 Fetal Heart Development Until C-looping (Stage 10 – 33 hours)

Gastrulation is the period during embryonic development following the rapid mitotic cleavage of the oocyte. In gastrulation, the embryonic cells migrate to their appropriate positions within the embryo, known then as the gastrula, to undergo differentiation. The gastrula is composed of three layers: the endoderm, the mesoderm and the ectoderm. As embryonic development progresses, the primitive streak begins to form after 6-7 hours post incubation in chicks[3]. The primitive streak provides the embryo with bilateral symmetry since it forms along the anterior-posterior axis. As the formation of the primitive streak progresses, an organizer region, known in chicks as Hensen's node, forms at stage HH 4 of embryonic development (18-19 hours)[2]. The pre-cardiac region lies within the splanchnic mesoderm, which, during stage HH 5 and 6 of development (19-25 hours), lies symmetrically on both sides of the head process, which has formed anterior to Hensen's node[2]. The splanchnic mesoderm is in direct contact with the endoderm, forming an interface rich in fibronectin[3]. The pre-cardiac cells occupy select patches in the splanchnic mesoderm on both sides of primitive streak[3]. Paracrine signalling then directs these pre-cardiac cells towards the mid-line during stages HH 8 and 9 (26-33 hours). As the pre-cardiac cells approach the midline, they form bi-lateral tubes, which then fuse together into a single chamber tube at the midline along the anterior-posterior axis[3]. Once this tube has formed, the fetal vertebrate heart then begins its asymmetrical organogenesis, known as C-looping[2].

1.3 C-looping (Stages 10-12 / 33 to 45 hours)

C-looping initiates the differentiation of the single-cardiac vessel into the various chambers that encompass the mature vertebrate heart through looping of the symmetrical single-chamber heart tube. At the beginning of stage HH 10, blood enters and is pumped out by the same chamber in the heart tube, since the atrium and ventricles have not yet been separated. During C-looping, the ventral wall of the tube twists to the right and arches outwards (convex), while the dorsal side twists in the same direction and arches inwards (concave)[4]. The arching, known as ventral bending, is influenced by molecular interactions while the rightward torsion is a result of mechanical pressures exerted by the splanchnopleure membrane[4]. Since the focus of this research is to understand the role of molecular and not mechanical processes regarding looping, only ventral bending will be discussed. The region of the tube that arches outwards will become the left and right ventricle, while the posterior end of the tube, which remains along the anterior-posterior axis, will develop into the atrium.

Ventral bending is influenced by asymmetrical gene expression between the left and right sides of the anterior/posterior axis of the tube. This gene expression preludes asymmetrical organogenesis. The cardiac cells on the left side of the axis begin to upregulate NODAL, a protein transiently involved in all asymmetrical organogenesis[3]. NODAL induces the expression of Pitx2c, which is transiently expressed throughout asymmetrical organogenesis whereas the upstream transcription factors are no longer expressed[3]. It is believed Pitx2c plays a crucial role in asymmetrical development since Pitx2c knock-out models fail to undergo ubiquitous L-R asymmetrical organogenesis[3]. However, the focus of this paper is not to explore the function of transcription factors that influence L-R asymmetrical organogenesis, but to explore the role of

actin throughout S-looping. Before researching how actin might control the bending in S-looping, one must first understand how actin controls bending in C-looping, since it is likely that the dynamics of these two processes are similar.

1.4 Role of Actin Polymerization during C-looping

As one of three types of protein filaments that constitute the cytoskeleton, actin is found in almost all cells[5]. From the formation of contractile rings during mitosis to the formation of pseudopodia in phagocytosis, actin plays an essential role in many of the cells' regular functions[5]. Although found almost ubiquitously throughout the cell, actin is most densely localized under the cellular membrane since it plays a major role in cellular structure and formation[5]. Actin filaments actively grow by the addition or removal of actin monomers at either end of the filament[5]. Furthermore, actin has a high affinity for and can interact with a broad range of actin-binding proteins, which explain the wide variety of roles actin plays in the cells. Therefore, actin can be expressed in a variety of both intracellular and intercellular structures and networks.

Since one must study the dynamics of the preceding stage to understand the dynamics of its succeeding stage, it is essential to understand the dynamics of actin's role in C-looping in order to understand the potential dynamics of actin's role in S-looping. It has been shown that actin plays an active role in C-looping, since disrupting the intercellular actin network in the cardiac membrane disrupts looping[6]. However, since actin is expressed in a variety of cellular functions, there are several possibilities explaining how actin controls C-looping. One theory explored actin's association with myosin. Because actin associates with myosin to form contractile structures, it was theorized that these structures formed the necessary contractions

required for ventral bending[7]. However, studies have demonstrated that the actin-myosin complex plays no role in C-looping[7]. Nevertheless, the actin-myosin complex remains a plausible theory and must be taken into consideration when studying the latter stages of looping.

Current studies on actin's role in C-looping suggest that the mechanical forces directed by actin polymerization drive ventral bending. This means that it is not the actin-myosin complex, but rather the highly organized intercellular actin networks that push and pull the fetal vertebrate heart throughout C-looping. Previous studies demonstrated that isolated hearts underwent C-looping in culture[6]. Therefore, the molecular mechanisms that drive C-looping must be intrinsic to the heart tube. Moreover, when the embryo or isolated heart were treated with effective concentrations of actin polymerization inhibitors, Cytochalasin D (CD) and Latrunculin A (LA), heart development was inhibited at the stage CD and LA were added. The effective concentrations of CD and LA were only potent enough to inhibit polymerization, rather than inhibit the cytoskeletal structure of the plasma membrane, strengthening the theory that actin polymerization drives ventral bending. As a result, the current hypothesis explains that ventral bending is controlled by pressures exerted by the growing actin networks which form circumferential contractile rings and are found in a high concentration along the bending perimeter[7]. However, since this experiment was done *in vitro*, there are still limitations to this theory as other *in vivo* forces may also contribute to C-looping. Nevertheless, this theory provides a plausible explanation for the forces that drive C-looping.

1.5 Dynamics of S-Looping[3]

While there has been extensive research behind the forces that drive C-looping, very little is known about S-looping. Early S-looping begins at stage 12 and continues until stage 16, at

which point septation begins to differentiate the atrium and ventricle into left and right. Early S-looping is characterized by a two chamber heart, where the blood enters the atrium through the sinus venosus and is pumped out by the ventricles through the ventral aorta. As S-looping progresses, there is both bending and rotation of the heart. Both the bending and the rotation of the fetal heart move the atrium, which at stage 12 was posterior to the ventricle, anterior to the ventricle, as seen in mature mammalian hearts. The ventricles, found anterior to the atrium at stage 12, twist ventrally and posteriorly. The bend that separates the atrium and the ventricle after C-looping becomes sharper while another bend forms posterior to the atrium. The S-shape that gave rise to the name "S-looping" is created by the change in anterior/posterior position of the atrium and ventricle, since the atrium is still connected to venules, which remain posterior to the atrium, and since the ventricles are still connected to the aortic arch, which remains anterior to both the ventricles and atrium (Figure 1 show a steady progression of S-looping from stage 12 to stage 16).

While ventral bending in C-looping is driven primarily by forces exerted through actin polymerization, it is naïve to assume these forces continue to be the only acting force driving bending throughout S-looping. Many more factors may come into play as the chick matures and the heart's shape becomes more complex since the finally positioning in S-looping is a precursor to the mature heart. In fact, current unpublished research proposes the splanchnopleure membrane may also control bending during S-looping, since removal of the SP-membrane produced a compacted C-shaped heart. Therefore, any research done on C-looping dynamics, such as myosin-inhibition, should be repeated since S-looping is proving to be an independent process from C-looping. Nevertheless, it should be expected that inhibition of actin polymerization either alters or inhibits proper S-looping.

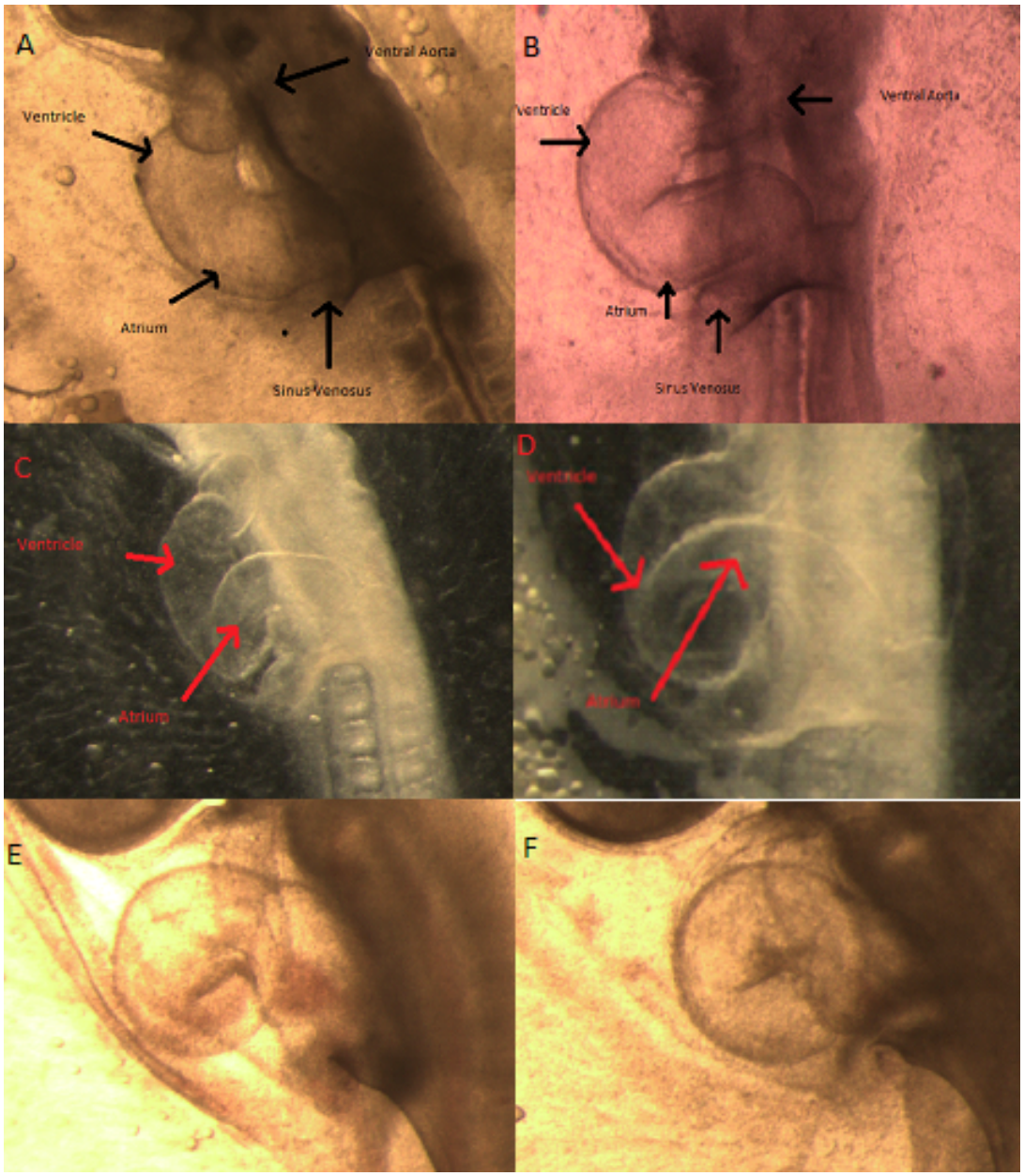

Figure 1: Progression of S-Looping from chicks isolated at at stages 12-16: (A) Fetal heart at beginning of C-looping. Heart demonstrates distinct C shape with ventricle positioned anterior to atrium. (B) The ventral bend from C-looping becomes more pronounced. The atrium begins to migrate anteriorly. (C) A new distinct bend appears posterior to the atrium as the atrium continues to migrate anterior to the ventricle. The ventricle rotates 90 degrees dorsally and migrates posterior. (D) Atrium now positioned anterior to ventricle, which now occupies the transverse plane, creating distinct S-loop shape. (E) The heart tube beings to coil into its final structure. (F)Heart tube has looped into its final compact shape before septum formation and valve formation begin.

1.6 Phalloidin as a Molecular Marker for Actin

In order to qualitatively observe actin's role in S-looping, one must use an actin marker to observe the highly-organized actin networks under microscope. Otherwise, the actin filaments would appear invisible. Common molecular markers used in molecular biology consist of fluorophores attached to proteins with a high-affinity for the target structure researchers wish to observe. Phalloidin, a toxin isolated from the death cap, binds with a high-affinity to F-actin and as such is often the chosen molecular marker when studying actin. Alexa fluoro 488 is the fluorophore attached to phalloidin. The number '488' refers to the wavelength of light emitted by the Alexa fluorophore when it fluoresces. As a result, the actin filaments will appear green under fluorescence microscopy.

EXPERIMENTAL

2.1 Wildtype Embryo Phalloidin Staining

Fertilized White-Leghorn Chicks were incubated at 33°C for the allotted time to reach the target stage, as noted by Bellairs and Osmand. Using a ring of filter paper, the embryo was removed from the top of the egg[8]. The isolated embryos were positioned ventral-side up, and another ringed filter paper was placed on top to sandwich the embryo and a metal ring was placed on top. The embryos were fixed overnight with 4 % paraformaldehyde. Photos of the embryo's body and the embryo's heart were then taken. After fixation, the embryos' were dissected out of the SP membrane with forceps and placed individually in a 24-well plate. They were washed three times on a rocker with 1X PBS. The first two washes were done for five minutes and the third wash for thirty minutes. The embryos were then washed in a solution of BSA and SDS Triton X for thirty minutes. They were then stained in BSA and SDS Triton X

with phalloidin overnight at 4°C. After phalloidin staining, the embryos were washed three times in 1X PBS on a rocker at 4°C. The first two washes were done for five minutes and the third wash was done for an hour. Each embryo was mounted on a slide. The slides were then exposed to confocal fluorescence microscopy at magnifications of 5X and 20X.

2.2 Cytochalasin D treatment and phalloidin staining

Since C-looping in chicks ends at stage 12, the fertilized chick eggs were incubated at 37°C for 48 hours. Using a ring of filter paper, the embryo was removed from the top of the egg and placed in 2 mL medium of chick media + streptomycin/penicillin. Half of the isolated chicks were treated with 2μL of CD, only enough to inhibit actin polymerization but not to affect cytoskeleton contractions. The other half of the embryos were left untreated. Both treated and untreated chick embryos were then placed back in the incubator for another 12 hours in bags with an air composition of 95% O_2 and 5% CO_2. Although 12 hours in vivo develops well past S-looping, *in vitro* conditions are far less optimal and so the embryos develop slower. After 12 hours, the chick embryos were taken out and the chick media was removed. The embryos were then fixed overnight. Then, the embryos were dissected out of the ring of filter paper using forceps and placed in individual wells in a well plate. They were washed three times on a rocker with 1X PBS. The first two washes were done for five minutes and the third wash for thirty minutes. The embryos were then washed in a solution of BSA and SDS Triton X for thirty minutes. They were then stained in BSA and SDS Triton X with phalloidin overnight at 4°C. After phalloidin staining, the embryos were washed three times in 1X PBS on a rocker at 4°C. The first two washes were done for five minutes and the third wash was done for an hour. Each

embryo was mounted on a slide. The slides were then exposed to confocal fluorescence microscopy at magnifications of 5X and 20X.

RESULTS

Chicks isolated *in vivo* demonstrate expected development of the heart tube throughout S-looping (figure 1). Similarly, actin staining with phalloidin shows an elaborate intercellular actin network throughout the heart tissue. At stage HH 13, under X the inner curvature of the heart begins to bend, initiating the anterior migration of the atrium and the posterior migration of the heart (Figure 2). However, the C-loop is still visible. Actin networking is filamentous with higher concentrations of actin networks forming in areas where the heart tube is changing bending (Figure 2). At stage HH 14, under 5X, the ventricle continues posterior migration as it undergoes a 90 degree dextral rotation until it occupies the transverse plane (Figure 3). A 20X, trademark circumferential actin contractile rings appear on the outer curvature of the looping heart tissue (Figure 3). At stage HH 15, under 5X it is clear that the atrium has finally migrated anterior to the ventricle (Figure 4). Similarly, the dextral rotation and posterior migration of the ventricle gives rise to the characteristic S-shape of S-looping (Figure 4). At 20 X, although the actin contractile rings are not as evident, there is still elaborate actin networking throughout the heart tissue (Figure 4).

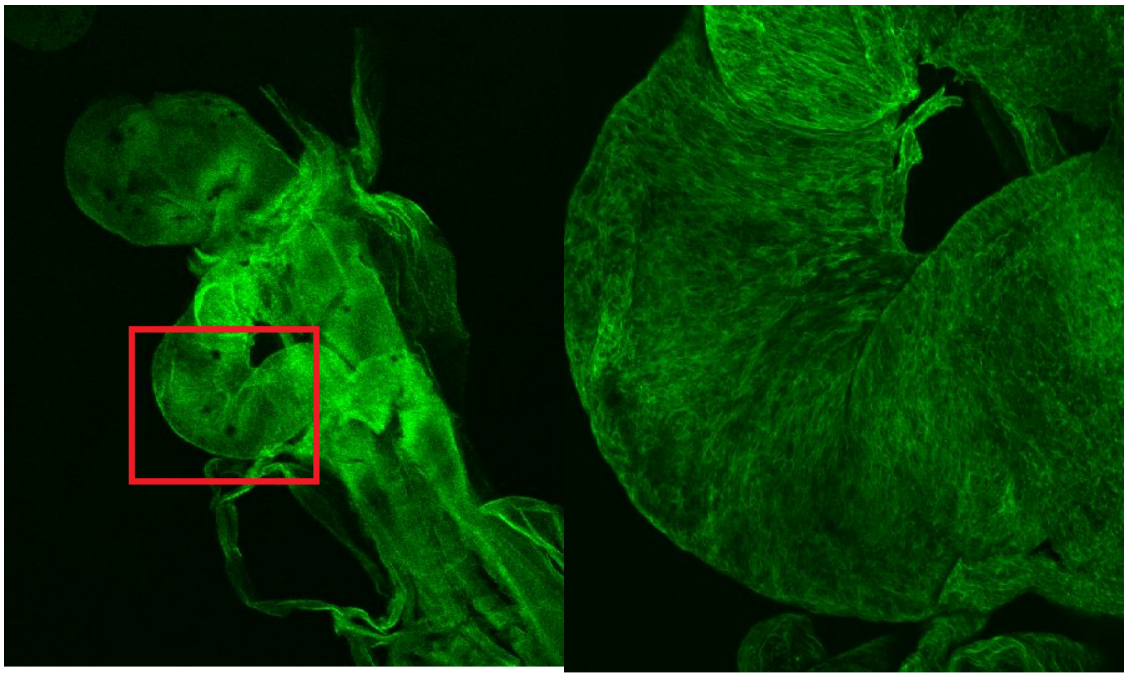

Figure 2: Embryo (5X) and embryonic heart (20X) at stage HH 13: At 5X, the inner curvature of the heart begins to bend as it initiates S-looping. At 20X, actin filaments in the heart tissue form concise intercellular networks throughout.

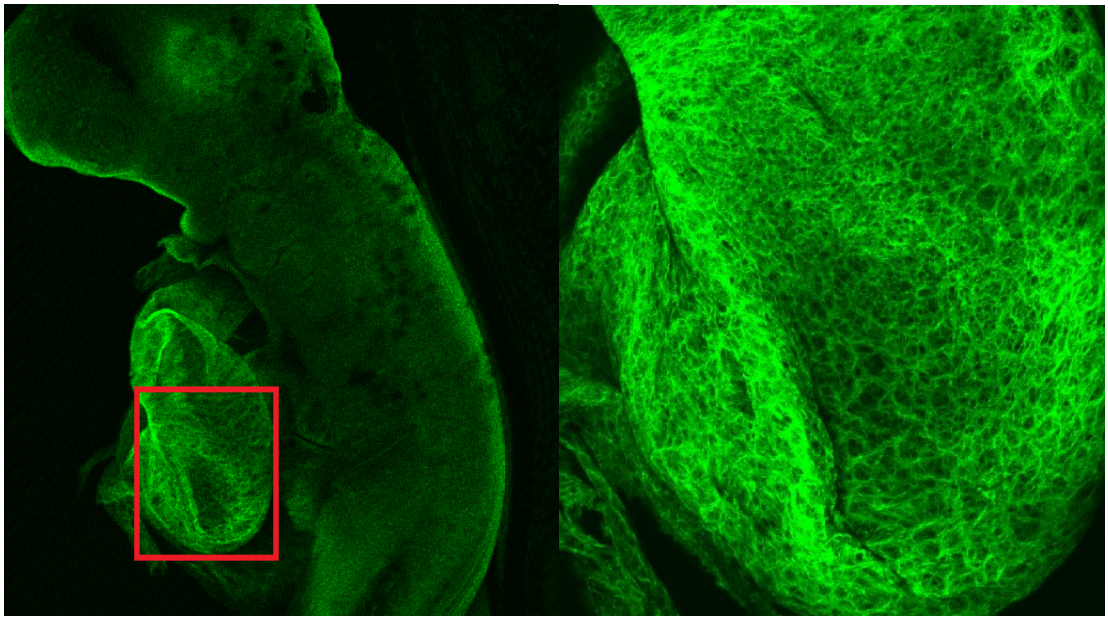

Figure 3: Embryo(5X) and embryonic heart(20X) at stage HH 14: At 5X, the embryonic heart demonstrates trademark posterior migration and 90 degree dextral rotation of the ventricle. Atrium continues to migrate anteriorly. Also both the ventricle and the atrium appear to be level along transverse plane. At 20X, contractile ring formation believed to play a role in bending are found all over the heart tissue.

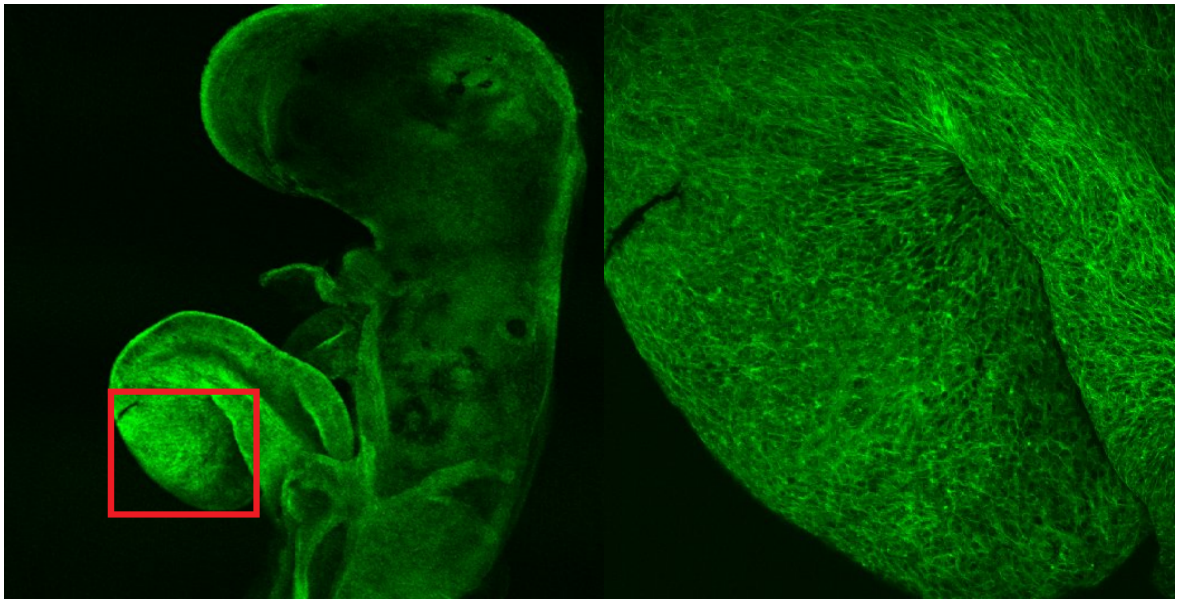

Figure 4: Embryo(5X) and embryonic heart(20X) at stage HH 15. A 5X, The atrium has completely migrated anterior to ventricle, as the posterior migration and dextral rotation of the ventricle gives rise to the characteristic S-shape of S-looping. At 20X, trademark actin networks are found ubiquitously in the heart tissue.

When 1μM of CD was added to the embryo and then allowed to incubate at $37^{\circ C}$ for 12 hours, 11 out of 15 chicks that were stained with CD failed to develop past stage HH 12. In contrast, 12 out of 15 chick embryos that were not exposed to the CD did develop past that given stage in development. For example, two chicks isolated at the same stage in development, which is roughly stage HH 12 at 48 hours, where one is exposed to actin and the other is left untreated, will result in the untreated embryo to reach stage HH 15 in development after 12 hours where the other one will stop at stage HH 12 (**Figure 5**). The embryonic hearts at 20X, where one was treated and untreated, show a sharp contrast in actin network integrity. The untreated chick's heart, which is at stage HH 14, demonstrates a developed a ubiquitous filamentous actin network in the heart tissue (**Figure 6**). Similarly, the untreated chick's heart shows characteristic actin

contractile rings throughout the heart tissue (**Figure 6**). Unlike the untreated chick, the treated chick's heart failed to develop past stage HH 12, when it was exposed to CD, and still demonstrates the characteristic C-loop shape of stage HH 12 (**Figure 6**). Furthermore, the heart does not show a filamentous structured actin network as shown in untreated hearts (**Figure 6**). The actin that is present appears as circular blobs deposited ubiquitously at higher concentrations around the perimeter of the fetal heart (**Figure 6**).

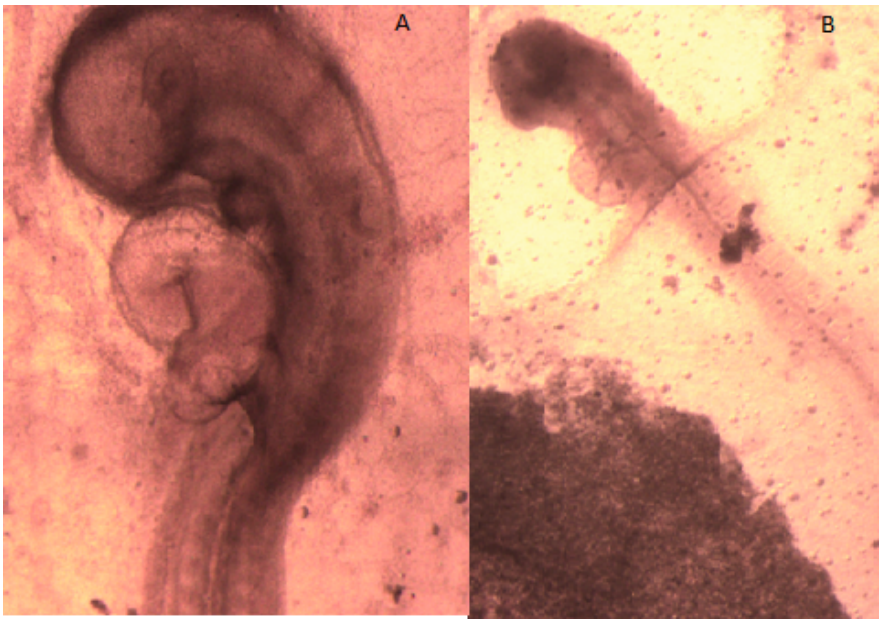

Figure 5: Treated and untreated chick embryonic development comparison: (A) The untreated chick isolated at stage HH 12 and then grown for 12 hours in chick media demonstrates continous development, although slightly delayed in vitro. (B) The treated chick isolated at stage HH 12 and then grown for 12 hours in chick media+CD does not develop past its stage of isolation.

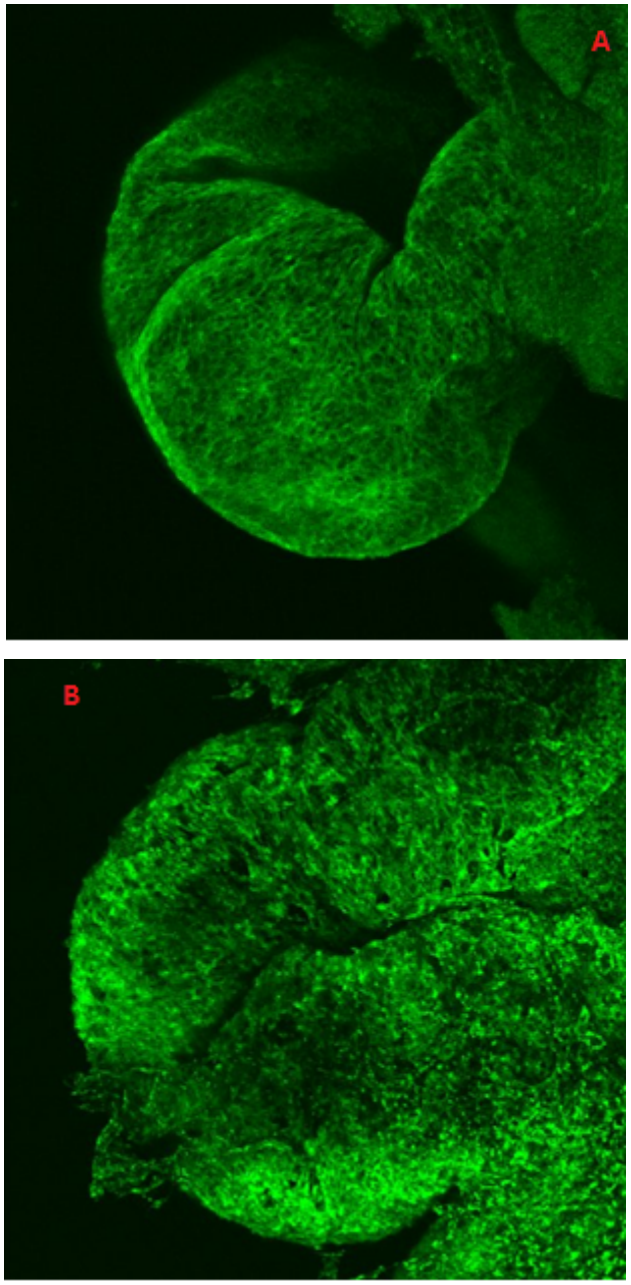

Figure 6: Phalloidin staining in a treated and an untreated fetal heart: (A) The untreated heart demonstrates a nicely structured filamentous actin network that stretches ubiquitously throughout the heart tissue. (B) The embryonic heart treated with CD demonstrates a lack of progression in development post-staining (at stage 12), as it shows the C-loop shape characteristic of stage 12. There is no structured filamentous actin network. However, the actin still appears as circular blobs surrounding the perimeter.

DISCUSSION

Because treatment with the actin polymerization inhibitor, CD, inhibited development past the stage of treatment and that highly disrupted actin networks were observed, the data suggests that actin polymerization plays a vital role in S-looping.

4.1 Role of actin polymerization in S-looping

Actin works through the polymerization and depolymerisation of its monomeric subunits, which stack together rings of actin monomers (globular actin) to create F-actin (polymer)[5]. This explains the intercellular network of actin since the polymeric F-actin would naturally appear as a filamentous actin network. As a result, addition of CD inhibits the formation of these actin networks. In C-looping, it was the inhibition of these networks that inhibited bending that produces the distinct C-shape in C-looping. However, because CD does not inhibit monomeric actin from forming small polymeric rings, we observe the small circular blobs of actin in the treated fetal hearts. Furthermore, in the CD treatments, actin appears most densely around the perimeter of the heart. Since higher densities of actin are found at the perimeter, where bending occurs, this strengthens the argument that actin polymerization is a driving force in S-looping. Conclusively, with the obtained data, it is plausible to suggest that inhibiting actin polymerization inhibits S-looping altogether.

4.2 Other potential factors driving S-looping

Another factor that may affect S-looping is gene expression. Because actin has so many roles in the cell, it can interact with a vast array of proteins and is therefore involved in a vast amount of signalling pathways. As shown in C-looping, the NODAL pathway finishes with the

expression of Pit2x, which when knocked-out, is shown to inhibit C-looping[3]. It is certain that either this pathway or other pathways are being expressed produce the genetic force behind C-looping. In many congenital heart defects, errors in the signalling pathway result in major defects. For example, in Hypoplastic Heart syndrome, where one of the ventricles is severely developed, it is believed that an error in the Gap junction protein Alpha 1 signalling pathway causes underdevelopment[3]. Therefore, understanding the genetic pathways that are associated with actin polymerization should be a key focus for future research in S-looping.

Current studies at Union College suggest that the SP membrane plays a role in S-looping, both through bending and rotation of the heart tube. Unlike C-looping, where removal of the SP membrane only delays development[9], removal of the SP membrane causes the heart to adopt a collapsed structure with no distinct shape. This example hints that factors not found to play a role in C-looping may play a role in S-looping since the heart develops a more complex shape. As such, future research should be done both on the effect of the actin-myosin contractions and the effect of heart beat, since a beating heart provides fresh oxygen to the actin which can then be used to produce ATP. The development of final S-looped shape is essential to the proper development of the fetal heart since the structure of the heart at the end of S-looping is the structure through which valve formation and septum formation occur. Errors in proper looping can impact septum formation, which in severe cases can cause heart murmurs. As a result, further study into other factors behind S-looping must be done to understand and potentially fix all congenital heart defects.

4.3 Limitations

The greatest limitation in this experiment was recreating the conditions in vivo conditions in vitro, since external factors may also influence S-looping. For example, liquid surface tension has enough force to alter both rotation and bending, and if the chick is exposed to the surface, the force from this tension can alter heart formation. Because this was not accounted for, it could explain why three of the untreated chicks did not develop past stage 12. The inability to recreating exact in vivo conditions explains why despite the embryos being incubated in total 60 hours, they only progressed to stage 14 or 15, which usually requires only 56 hours of incubation for development. One final limitation is the lack of sufficient data to certify that inhibition of actin polymerization inhibits develop past the stage of treatment. Having a low data collection exposes the results to random variation. Therefore, while 30 embryos are enough to speculate actin polymerization's effect, more samples will need to be obtained to strengthen this conclusion.

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