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Zebrafish Cardiac Development: The Effects of Retinoic Acid on Heart Tube Morphology and Function

by

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This paper was prepared

under the direction of

Professor Rachel Fink

For eight credits

I would like to dedicate this thesis to my parents
who have always encouraged and inspired my curiosity.

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ABSTRACT

The formation of a heart occurs through a series of dynamic developmental events where individual cells differentiate, migrate, fuse, and loop to form a muscular tube which then has the ability to pump blood throughout the body. The zebrafish has been used as a model organism for vertebrate cardiac development due to the transparency of its embryonic tissues. The zebrafish heart is comprised of two chambers, an atrium and a ventricle. During heart cell determination, it is believed that retinoic acid acts as a chemical signal which helps to restrict the fate of pre-ventricle and pre-atrium cells (Hochgreb et al., 2003; reviewed in Simoes-Costa et al., 2005). Research has found that both excess and inhibition of retinoic acid signaling during development causes abnormal heart formation.

This thesis investigates the effects of excess retinoic acid on heart morphology and function by recording real-time videos and time-lapse images of the heart tube. Embryos were treated with varying concentrations of retinoic acid. The percentage of embryos which developed abnormal hearts increased with an increase in retinoic acid concentration. In addition, the stage and length of exposure had a significant effect on the percentage of abnormal hearts which formed.

Three general heart morphologies were observed as a result of excess retinoic acid exposure: Wide Atrium, Linear Heart, and Small Heart. These abnormal heart tube morphologies exhibited variation in normal heart looping. Treated embryos exhibited decreased circulation, retrograde blood flow, and/or no circulation. This thesis proposes a model to explain how excess retinoic acid signaling affects the determination of pre-cardiac cells which then leads to the formation of abnormal heart tube morphologies. It is proposed that insufficient mechanical stimuli due to poor circulation induces abnormal heart looping (Miyasaka et al., 2011).

INTRODUCTION

Definition of the Heart

Due to the vast diversity of heart morphology in the animal kingdom, both evolutionary and developmental biologists have developed a cohesive definition for the heart by evaluating anatomical features in the circulatory system. A heart is defined as a segmented tube with defined chambers comprised of cardiac myocytes (Gilbert, 2010). The myocytes form the myocardium, which is the contracting musculature of the heart. Also, the heart has an inner layer of tissue called the endocardium. The myocytes are specialized cells that are responsible for generating a contractile force which creates a pressure difference in a closed circulatory system. This pressure pumps blood through the heart and into the connecting arteries. The different segments of a heart can be divided into two categories: the inflow tract which includes the sinus venosus and atrium and the outflow tract which is comprised of the ventricle and conus arteriosus (**Figure 1**). The function of the heart is to pump blood throughout the blood vessels allowing for gas exchange to take place in the tissues. This is an adaptation which allows for a larger and more complicated anatomy where simple diffusion of gas is not sufficient (Hoar, 1966).

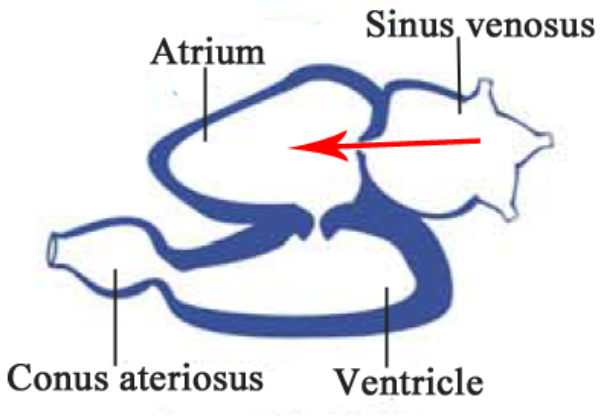


Figure 1: Two-chambered heart. The red arrow indicates the flow of blood into the inflow tract, the sinus venosus and atrium. The blood then flows through the outflow tract, the ventricle and the conus ateriosus. Image modified from Simoes-Costa et al., 2005.

Different organisms have hearts with a varied number of chambers, but the hemodynamic functions of the inflow and outflow segments can still be distinguished (reviewed in Simoes-Costa et al., 2005). The cells in the inflow and outflow tract are both cardiomyocytes which are morphologically and genetically distinct from each other (Keegan et al., 2004). Atrium cells form a thin-walled chamber which is flexible, and the ventricle cells form a thick-walled chamber which is less flexible (Hoar, 1966).

The Mechanics of Heart Muscle Contraction

When the heart muscles contract, they generate a force which creates pressure differences that then drive the flow of the blood through the blood vessels (Vogel, 1992). Cardiac muscle cells are able to generate contractile forces

due to the highly ordered arrangement of thin and thick protein filaments inside the cells (**Figure 2**) (Sehnert et al., 2002).

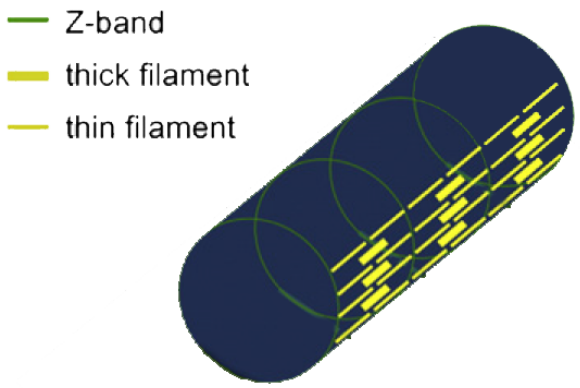


Figure 2: The orientation of the thick and thin filaments in a myofibril. The thick and thin filaments are arranged so that they can overlap and interact with each other. The Z bands (green) indicate the functional unit of the muscle, the sarcomere. Many sarcomeres comprised a myofibril. Image modified from Lin et al., 2012.

Thin filaments are made of actin and thick filaments are comprised of myosin (Lin et al., 2012). The Z bands are comprised of the thick and thin filaments and indicate the functional unit of the muscle called the sarcomere. Many sarcomeres comprise a long rod like structure in muscle cells called a myofibril. These filaments are able to interact with each other by sliding back and forth to generate a contractile force (Lin et al., 2012). The contraction of the heart tube is controlled by changes in ion concentrations across the plasma membrane of the muscle cells (Sehnert et al., 2002). Initially there is an influx of calcium ions into the cardiac cells which then triggers the contraction of the thick and thin filaments which then leads to the contraction of the heart tube in a wave-like motion starting

with the inflow tract. There are populations of cells responsible for controlling the pace at which the heart contracts, and these are located both at the floor of the atrium and at the atrio-ventricular boundary, called the AV canal (Hoar, 1966). This area is also referred to as the pacemaker of the heart.

Variation in Heart Structure

To understand how segmentation and cell differentiation function in hearts of higher vertebrates, biologists have investigated the primitive hearts of the class Gastropoda, the slugs and snails, where the heart is a linear tube segmented into two chambers (**Figure 3A**) (reviewed in Simoes-Costa et al, 2005). In this example, the inflow and outflow tracts are clearly defined as two distinct chambers. In comparison, higher vertebrates have an increased number of segments and heart looping leads to a more morphologically complex heart. For example, zebrafish (*Danio rerio)* have developed an S-shaped heart, where the inflow and outflow tracts are parallel to each other (**Figure 3B**).

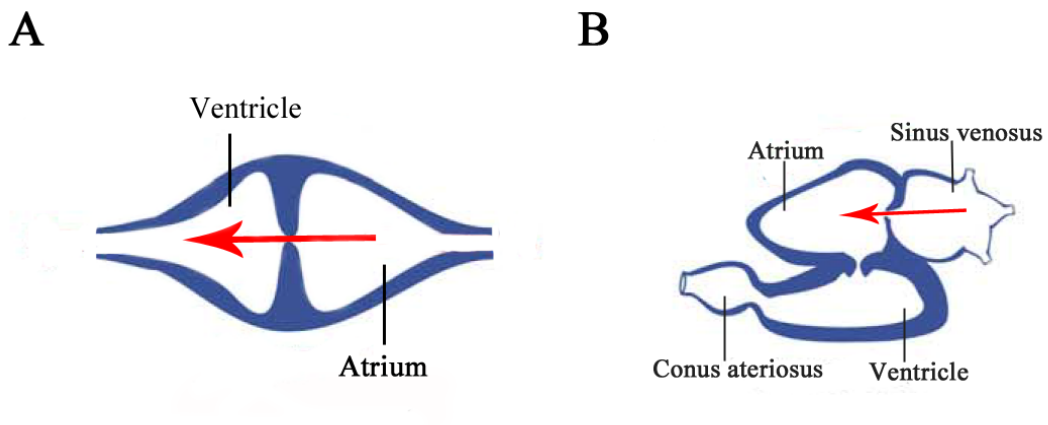


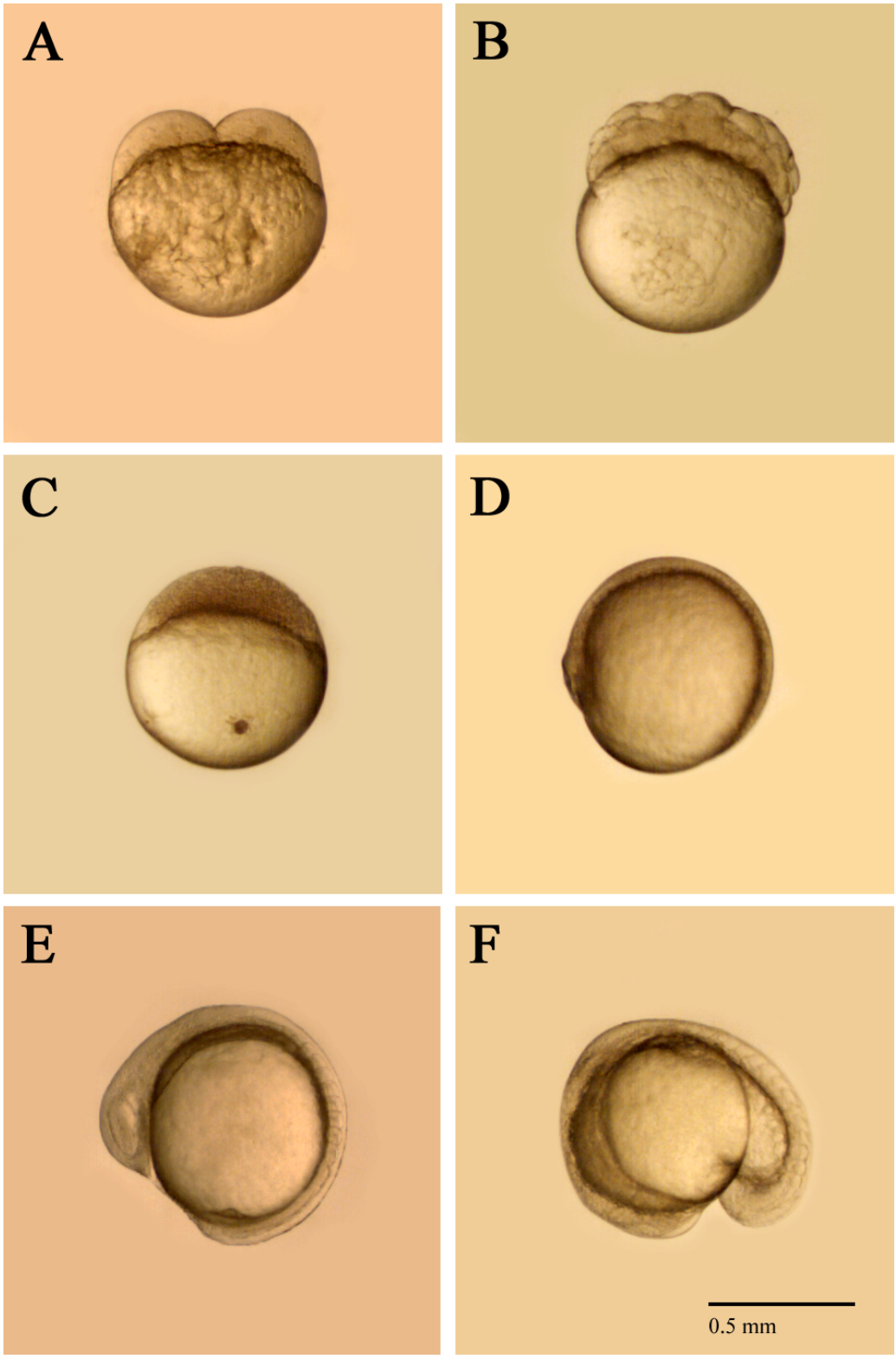
Figure 3: Variation in heart morphology. (A) A linear heart which is present in the class Gastropoda. (B) S-shaped heart in the zebrafish where the atrium and ventricle become parallel. Red arrows indicate the flow of blood through the heart. Image modified from Simoes-Costa et al, 2005.

To understand how the heart has evolved into a segmented structure requires an understanding of the molecular patterning in developing cardiac cells.

Zebrafish as a Model Organism for Cardiac Development

The zebrafish has been used as a model organism for segmented cardiac development due to the transparency of its embryonic tissues which allows for easy imaging of the heart as development proceeds. In addition, zebrafish are hardy organisms, easy to care for and maintain. The embryonic development occurs rapidly and by three days post-fertilization, the heart becomes fully functional. In normal development, first cleavage occurs approximately ¾ hours post-fertilization (hpf) at the animal pole with an incomplete meroblastic division of the two daughter cells (**Figure 4A**) (Gilbert, 2010). Cells continue to divide rapidly (**Figure 4B**), and by approximately 4 hpf, the dome stage contains one

thousand cells positioned on top of the yolk sac (Kimmel et al., 1995) (**Figure 4C**). Also, cardiac cells start to differentiate at this stage. The cells then start to spread via epiboly down from the animal pole towards the vegetal pole while one side of the embryo starts to thicken (**Figure 4D**). This region is called the embryonic shield, and it defines the dorsal side of the embryo at 6 hpf. The segmentation period begins approximately 12 hpf with the formation of somites (**Figure 4E**). At 18 hpf, the embryo is wrapped around the yolk sac and trunk somites have become well developed (**Figure 4F**). By 24 hpf, the yolk sac is reduced in size while the tail has elongated and straightened (**Figure 4G**). Also, the early heart tube is first visible at this stage due to the fact that it has started contracting and circulation has commenced. At 36 hpf, the yolk sac has significantly decreased in size while the embryo has completely straightened (**Figure 4H**). At 55 hpf, the pigmented cells are developing throughout the body and the embryo has developed pectoral fins (**Figure 4I**). At 3 days post-fertilization, the embryo becomes motile and hatches from the chorion, a protective membrane that surrounds the developing embryo (Kimmel et al., 1995) (**Figure 4J**).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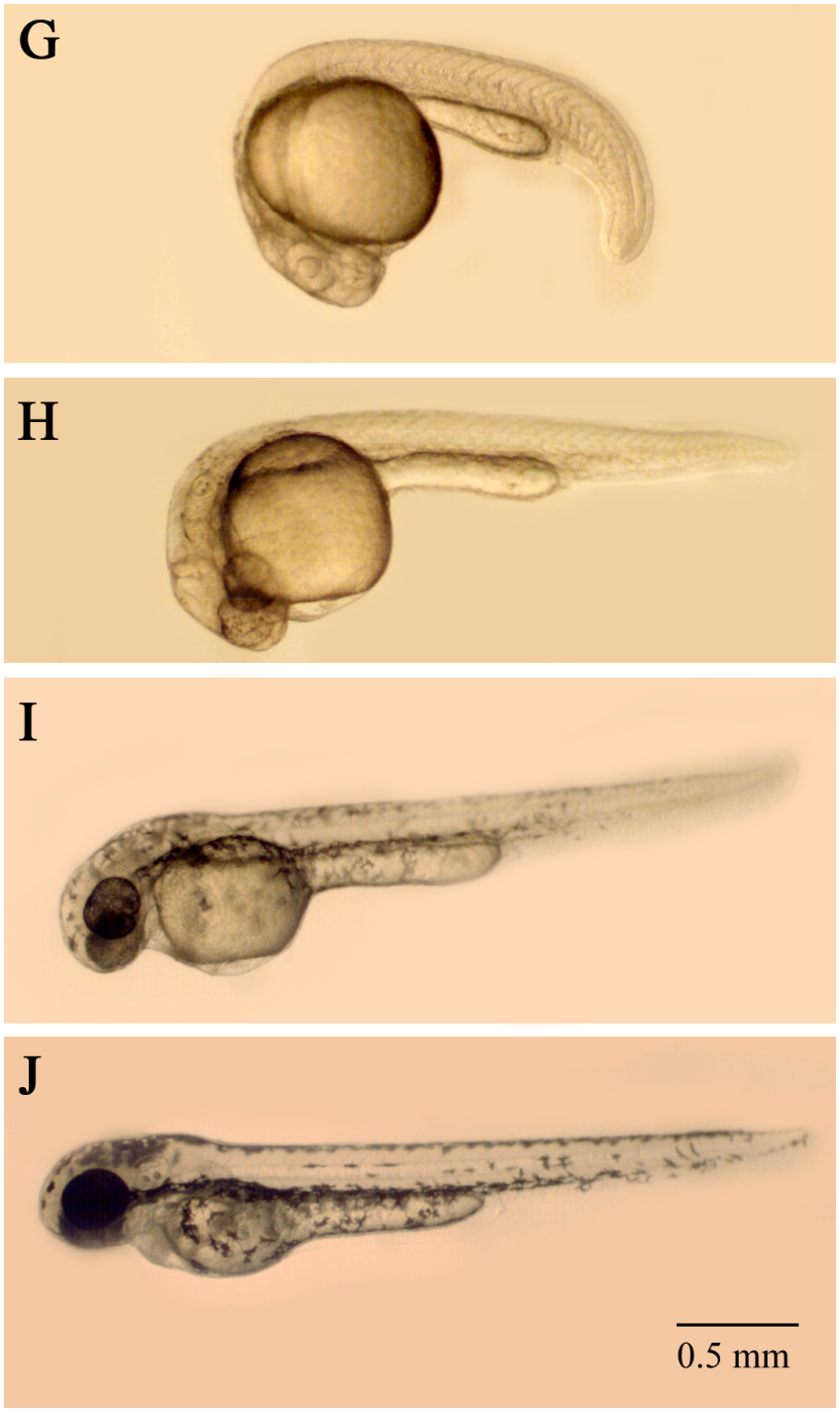

Figure 4: Embryonic development in zebrafish. (A) Two-cell stage, 0.75 hpf. (B) Thirty-two cell stage. (C) Dome stage, 4.3 hpf. (D) 75 % epiboly stage, 8 hpf. (E) 12 hpf. (F) 18 hpf. (G) 24 hpf. (H) 36 hpf. (I) 55 hpf. (J) 3 days post-fertilization.

Early Differentiation of Cardiac Progenitor Cells

At the onset of gastrulation, mesodermal pre-cardiac cells migrate bilaterally towards the animal pole and arrive on either side of the midline (**Figure 5**) (Warga and Kimmel, 1990).

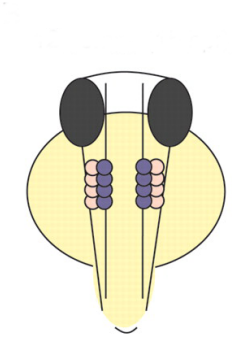

Figure 5: Location of the cardiac precursor cells in the lateral mesoderm. This shows the dorsal view of an embryo where the cardiac precursor cells are positioned in the lateral mesoderm around 16 hpf. Purple marks pre-ventricle cells and pink denotes pre-atrial cells. Image from Bakker, 2011.

The differentiation of these cells into cardiac precursor cells is thought to occur approximately 5 hpf in the bilateral regions of the anterior lateral plate mesoderm (Chen and Fishman, 1996). These bilateral populations of pre-cardiac cells are referred to as the heart fields. Nkx2.5 is one of the first detectible marker proteins present in cardiac precursor cells and works in combination with multiple other cardiac-specific genes to define the heart fields (Chen and Fishman, 1996). It has been experimentally determined that the location of the pre-cardiac cells is under regulation, because when these cells are ablated with a laser, new populations of precursors form from the surrounding mesoderm (Serbedzija et al., 1998). Serbedzija et al. have investigated the reformation of the pre-cardiac cells in

zebrafish after ablation by looking at how the regulation of the heart field allows for only specific cells to reform the progenitor cells because of their position in the lateral mesoderm (1998). When *Nkx2.5* is first expressed in the mesodermal cells, it is not restricted to pre-cardiac cells, but is expressed in a much broader region of mesoderm. The gene *GATA4* also plays an important role in pre-cardiac cell differentiation. There is a specific region of cells in the lateral mesoderm which has both the expression of *Nkx2.5* and *GATA4* which defines the cardiac progenitor cells (**Figure 6**) (1998).

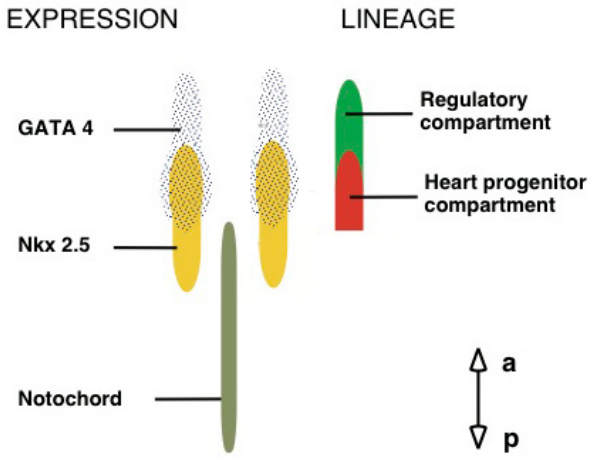

Figure 6: Restricted gene expression in the heart field. This diagram shows the region of cells in the heart field which expresses both *GATA 4* and *Nkx2.5* which then becomes the actual heart progenitor cells (region marked by red). It also shows that it is the cells just anterior to the region of gene expression overlap (region marked in green) which have the ability to replace the pre-cardiac cells when they are ablated. This diagram also shows the position of the notochord in reference to the heart fields. Image modified from Serbedzija et al., 1998.

As development proceeds, Serbedzija et al. found that the expression of *Nkx2.5* stops in the most posterior cells which do not become cardiac cells. They hypothesized that the notochord, which is near to the posterior heart field cells,

may have a regulatory effect on the expression of *Nkx2.5.* This observation led to the conclusion that it is critical that cells have the ability to express both *Nkx2.5* and *GATA4* in order to become a pre-heart cell. Therefore, Serbedzija et al. determined that it is the anterior cells which replace the ablated cells because they have the ability to express both genes. They also speculated that the prechordal plate which is spatially near the anterior cells may help to support the expression of *Nkx2.5*.

The Effects of Retinoic Acid on the Number of Cardiomyocytes

Retinoic acid, a metabolite of vitamin A, is an important signaling molecule known to play a role in early heart development in vertebrates (reviewed in Simoes-Costa et al, 2005). Experiments have shown that retinoic acid is critical to help define the number of cells which will differentiate into cardiomyocytes in the lateral mesoderm (Keegan et. al., 2005; Waxman et al., 2008; Johnson Sorrell and Waxman, 2011; Huang et al., 2011). Retinoic acid accomplishes this by restricting the number of cells which express the gene *Fgf8a*, in the cardiac field (Johnson Sorrel and Waxman, 2011). It has been observed that when the retinoic acid signal is blocked, the number of cells expressing *Fgf8a* in the posterior region of the heart field increases. This, in turn, results in an increased number of cardiac cells. Experiments have shown that these results can be reversed when retinoic-acid-restricted embryos are treated with an *Fgf8a* morpholinos which alters gene expression. Also the gene *hoxb5b*, which is expressed in response to

retinoic acid within the forelimb field, is thought to be important in restricting the number of cardiac cells which differentiate into atrial cells (Waxman et al., 2008). Experiments have shown that when retinoic acid signaling is restricted, there is a reduction in the expression of the gene *hoxb5b* and an increase in the number of atrial cells.

The gene RALDH2 is responsible for the synthesis of retinaldehyde dehydrogenase which is the enzyme involved in the rate-limiting step in retinoic acid synthesis (Begemann et al., 2001). When the expression of RALDH2 is blocked by the inhibitor *4-Diethylaminobenzaldehyde* (DEAB) there is an increase in the number of cardiomyocytes (Waxman el al., 2008*;* Huang et al., 2011). The mutant *neckless* also blocks the expression of this enzyme resulting in similar effects on cardiomyocyte differentiation (Begemann et al., 2001). In addition, the treatment of retinoic acid receptor antagonist BMS189453 leads to an increase in cardiomyocytes by restricting retinoic acid expression (Keegan et al., 2005; Waxman el al., 2008; Huang et al., 2011). Fate mapping tests have been performed with BMS189453-treated cells which have shown that the fate of these cells are transformed due to the fact that they are no longer able to respond to the retinoic acid (Keegan et al., 2005). It was observed that there are twice as many cardiomyocytes generated in BMS189453 treated embryos in the lateral plate mesoderm than in the wild type. It has been determined that the actual size of the heart field is not expanded but rather it is the density of the cells within the field which increases. It has also been documented that it is only the cardiomyocytes

which form the outer myocardium that respond to retinoic acid signaling and that there is not an increase in the number of endocardium progenitor cells in BMS189453-treated embryos. Hearts which have an increase in the number of cardiomyocytes both due to DEAB and BMS189453 have enlarged chambers (**Figure 7**) (Waxman et al., 2008).

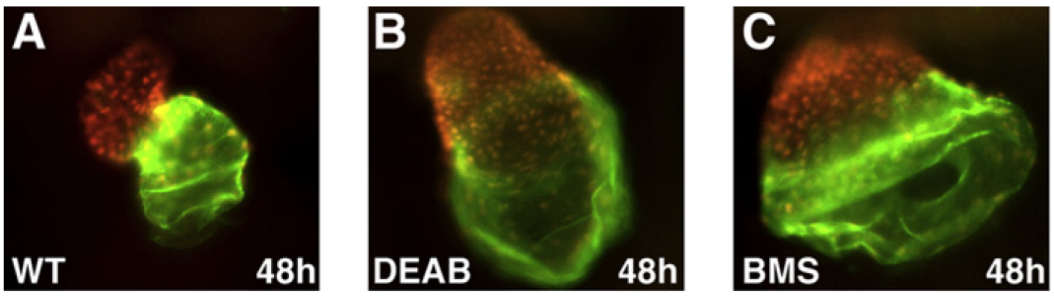

Figure 7: Inhibition of retinoic acid signaling in developing embryos. Frontal view of the heart in embryos 48 hpf. Atrium (green) labeled with the anti-Amhc antibody and ventricle (red) expressing the transgene *Tg(cmlc2:DsRed2-nuc)*. (A) Wild type embryo. (B) Embryo treated with DEAB shows enlarged chambers. (C) Embryo treated with BMS189453 shows enlarged chambers. Image from Waxman et al., 2008.

Retinoic Acid: Molecular Determination of the Chambers

Once the mesodermal cells, which express both *Nkx2.5* and *GATA4* have been determined to form heart cells, a secondary specialization differentiates pre-ventricle from pre-atrium cells. From labeling experiments, it has been determined that pre-cardiac cells in the early blastula do not have a specified fate in the heart and it is not until 40 % epiboly that there are specific molecular ventricle or atrium determinants which restrict cell fate within the heart (Keegan et al., 2004). Experiments in mice and chicks suggest that the restricted synthesis of retinoic acid in the posterior mesoderm is responsible for the differentiation of

pre-ventricle and pre-atrium cells (Hochgreb et al., 2003). Due to the fact that the retinoic acid is restricted to the posterior mesoderm, only the most posterior cells in the cardiac field are exposed to retinoic acid. During early gastrulation, retinaldehyde dehydrogenase marked in orange, is restricted to a region several hundred micrometers away from the cardiac heart field expressing *GATA 4,* in chicks, and *Tbx-5,* in mice, marked in purple (**Figure 8A, 8B, and 8E)**. As development proceeds, the expression of the gene RALDH2 moves anteriorly towards the heart field. Retinoic acid is then present in the most posterior pre-cardiac cells and this contributes to the differentiation of the atrium cells (**Figure 8C, 8D, and 8F**) (Hochgreb et al., 2003). There is evidence to suggest that the use of retinoic acid in heart polarity is conserved within other vertebrates such as amphibians and fish (Drysdale et al., 1997; Stainer and Fishman, 1992). The gene *RALDH2* is also expressed in the lateral mesoderm in the zebrafish, but it is unclear whether or not there is the same anterior wave of *RALDH2* gene expression directly adjacent to the cardiac field which results in the restricted exposure of retinoic acid to only the posterior cells in the cardiac field (reviewed in Simoes-Costa et al, 2005). In experiments looking at heart development in *neckless* mutants, where the expression of *RALDH2* is blocked, there is abnormal heart development. Therefore, this suggests that retinoic acid plays a significant role in heart development in zebrafish (Begemann et al., 2001).

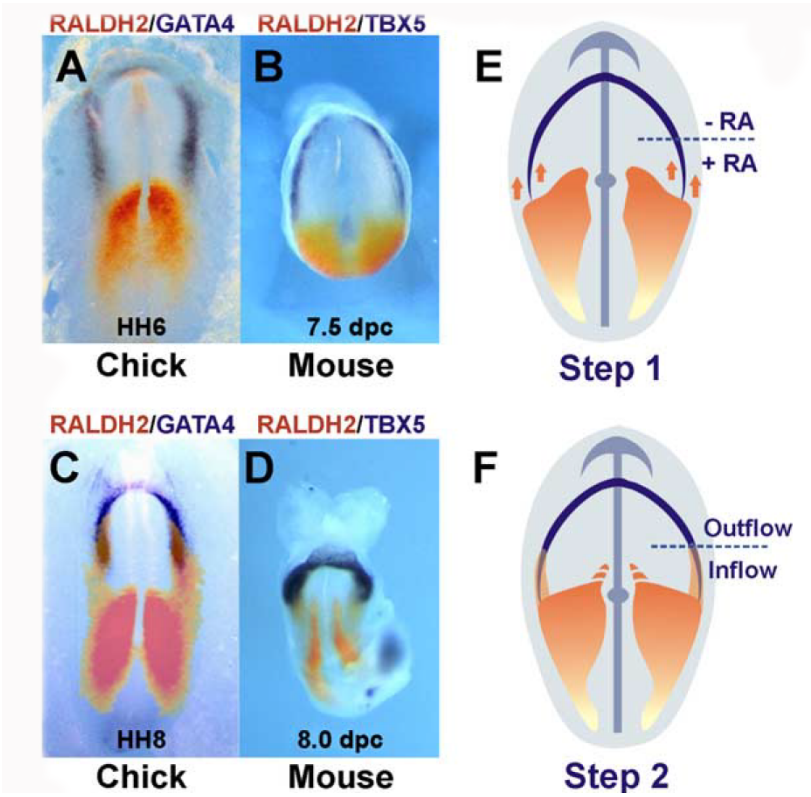

Figure 8: The restriction of retinoic acid to the posterior region of the developing embryo. (A,B.E) show how early in development the enzyme RALDH2 is involved in the rate-limiting step of retinoic acid synthesis (orange) and is expressed posterior to the heart field (purple). In the chick, the heart field is labeled by the expression of the gene *GATA 4* and in the mouse by labeling the gene *TBX5,* which are both heart-field restricted genes. (C,D,F) show the embryos later in development when RALDH2 expression has expanded anteriorly, overlapping with the posterior region of the heart field. Image from Simoes-Costa et al, 2005.

Migration of Cardiac Progenitor Cells

For a single heart to form, the two cardiac fields must migrate and meet at the midline of the embryo. It is thought that endodermal cells provide either a substrate for the pre-cardiac cells to migrate on or a chemical signal which triggers directional migration to the midline (Holtzman et al., 2007). The

migration of these cells has been closely followed using cell tracking technologies and can be broken down into two separate phases (Holtzman et al., 2007). In the first phase, all the cells travel medially, while in a second phase, the most anterior and posterior cells move at an angle to form the circumference of the heart tube. In another cell tracking experiment, it was found that anterior cells and posterior cells do not travel at the same speed (Smith et al., 2008). The anterior cells migrate at a constant rate whereas the posterior cells accelerate and double their speed mid-migration and then return back to their initial speed as they near the midline.

As the cells are migrating, they begin to specialize into epithelial cells by recruiting adherens junction proteins to the apical regions of the cell membrane and cell-cell contacts (Trinh and Stainier, 2004). Also at this time, β- catenin is co-localized near the adherens junction proteins in the basolateral membranes. Both of these molecules are known to play important roles in the maturation of epithelial cells and the formation of tight junctions. In *nat* mutants, there is a lack of organization of adherens junction proteins due to the fact that fibronectin is no longer synthesized and released from the precursor cells and, as a result, the cells are not able to migrate properly (Trinh and Stainier, 2004). In 10% of the mutants, due to the lack of cell migration, two separate hearts form, a condition known as cardia bifida (Chen et al., 1996).

Early Heart Tube Formation

Once the cardiac precursor cells reach the midline, approximately 18 hpf, they come together forming a circular disk (**Figure 9A**), and then form a cone with the pre-ventricle cells concentrated in the center and pre-atrial cells on the periphery (**Figure 9B**) (Smith et al., 2008). The cone then undergoes extension around 20 hpf, with fusion starting at the posterior end of the cone, which then moves anteriorly, as the inflow cells migrate to the left toward the midline. This resulting structure is referred to as cardiac jogging (**Figure 9C**).

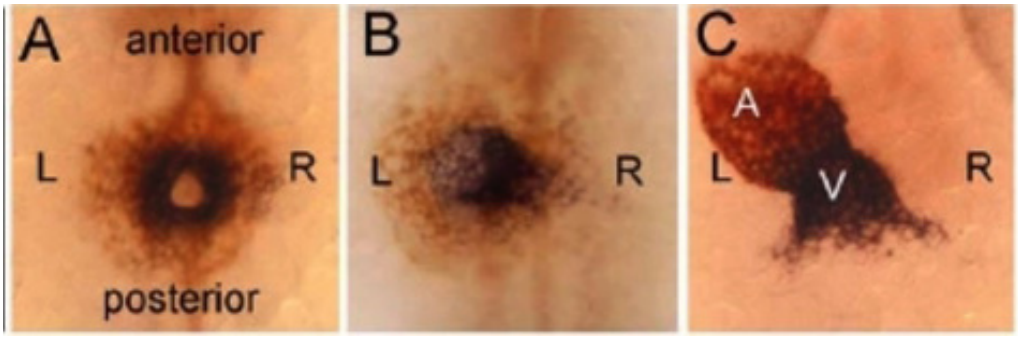

Figure 9: Formation of the heart tube. Double labeling of *ventricular myosin heavy chain* (*vmhc*) in blue and *atrial myosin heavy chain* (*amhc)* in red allows for visualization of the pre-ventricle and pre-atrial cells with probes. (A) At 20 hpf, a disk is formed by the pre-cardiac cells with pre-ventricular cells in the middle and pre-atrium cells on the periphery. (B) As development proceeds, the pre-ventricle cells rearrange to form the apex of a cone. (C) Extension of the cone into a tube occurs simultaneously with cardiac jogging. A= atrium, V = ventricle, L = left, R= right Image from Smith et al., 2008.

The heart tube is fully fused at 26 hpf (Yelon et al., 1999). From 26 hpf through 60 hpf, the heart tube undergoes a significant repositioning in the embryo. Originally the cone and early tube is positioned with the atrium anterior to the ventricle (Stainier and Fishman, 1992). As the head lifts and the yolk sac retreats,

the heart tube is repositioned so that the ventricle is now anterior to the atrium; there is a complete reversal of the orientation of the tube (**Figure 10**).

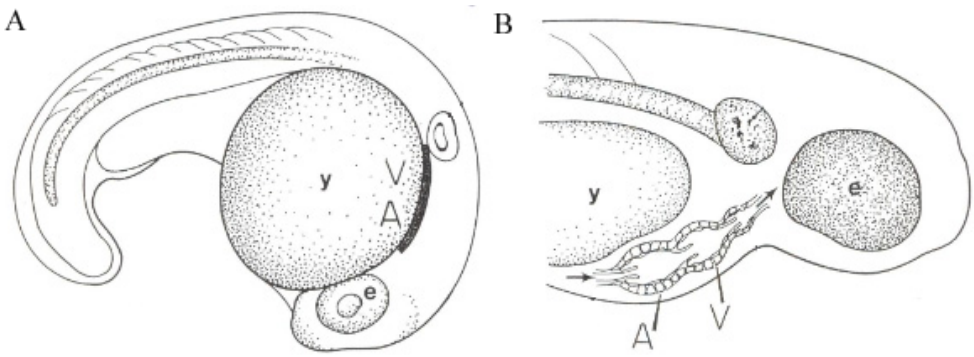

Figure 10: Repositioning of the posterior-anterior axis of the heart tube. (A) Originally the cone and early tube are positioned with the atrium anterior to the ventricle at 19 hpf. (B) As the head lifts and the yolk sac retreats, the heart tube is repositioned so that the ventricle is now anterior to the atrium; there is a complete reversal of orientation of the tube at 60 hpf. A= atrium, V= ventricle, y = yolk sac, e = eye. Images modified from Stainier and Fishman 1992.

Heart Looping

The formation of the S-shaped heart is called heart looping. At 30 hpf, the heart tube is positioned slightly offset from the midline with the venous pole cells at the midline with the atrium cells positioned to the left side of the embryo due to cardiac jogging (**Figure 11A**) (Smith et al., 2008). By 36 hpf, the boundary between the atrium and ventricle called the AV canal starts to bend and constrict. (**Figure 11B**). At 55 hpf, the heart has completely looped and repositioned anteriorly to the ventral side of the embryo (**Figure 11C**) (reviewed in Bakkers, 2011).

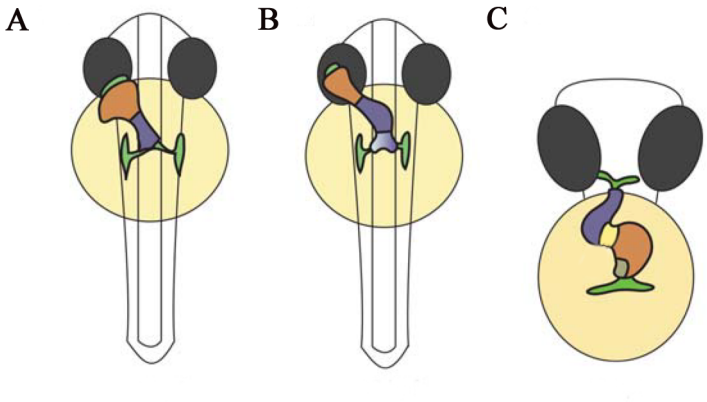

Figure 11: Heart looping forms an S-shaped heart tube. (A) Dorsal view. At 30 hpf the heart tube is linear with the ventricle (purple) positioned at the midline and the atrium (orange) shifted to the left side of the embryo. (B) Dorsal view. At 36 hpf, the tube has begun to bend at the AV canal and constrict. (C) Frontal view. At 55 hpf, the heart tube has completely shifted from the dorsal side of the embryo to the ventral side. The heart has continued to bend so that the ventricle has looped back towards the right side of the embryo. The endocardium is marked in green, the AV canal is marked in yellow, and the pacemaker is marked in brown. Image modified from Bakkers, 2011.

Experiments have found that retinoic acid helps with the determination of the laterality of the heart looping when the ventricle loops to the right (Huang et al., 2011). When embryos which are at the 2-somite stage are exposed to a retinoic acid receptor antagonist, randomized heart looping occurs. Retinoic Acid is an important signal that helps to define the expression of the gene *bmp4*, which is mainly expressed on the left side of the heart field. It has been observed that when *bmp4* is ectopically expressed on the right side of the field, the heart loops to the left instead of the right (Huang et al., 2011). Also, it has been found that mechanical stimuli due to blood flow plays an important role in heart looping in zebrafish and mice (Miyasaka et al., 2011; Nishii et al., 2011). When there is a lack of blood flow, normal heart looping does not occur.

Chamber Ballooning

At the same time that heart looping is occurring, the atrium and ventricle regions become enlarged by ballooning out to form more defined chambers (**Figure 12**) (Auman et al., 2007).

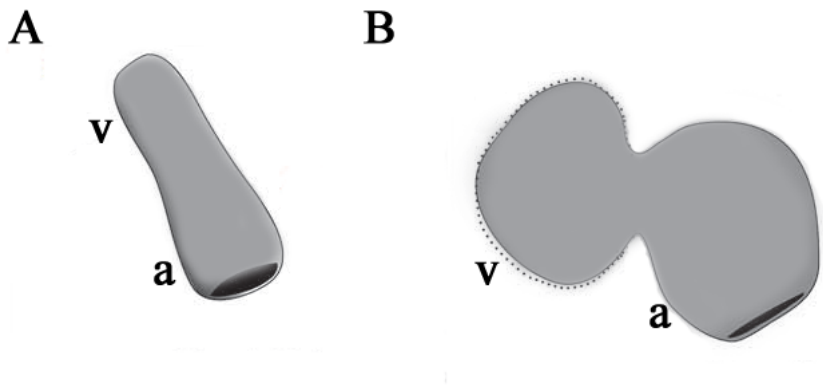

Figure 12: The ballooning out of the chambers. (A) The linear heart tube at 24 hpf. (B) The heart tube which has undergone ballooning out of the chambers at 48 hpf. a = atrium v = ventricle Image modified from Auman et al., 2007.

The process by which this happens is thought to be a combination of restricted cell proliferation and changes in cell shape and structure. Specifically, in the ventricle, experiments have shown that the elongation of the myocytes leads to the formation of the curvature of the enlarged chamber (Auman et al., 2007). Also, in chick embryos, it has been shown that chamber ballooning is a result of both cell enlargement and cell proliferation (Soufan et al., 2006). It is believed that the circulation of blood triggers the elongation of the cells (Auman et al., 2007). Shear forces generated by the endocardium and hemodynamic forces generated by the contraction of the heart, are thought to elicit cell elongation. In

zebrafish the mutant *wea* lacks sarcomeres in the atrial region and as a result, is not able to contract effectively. These mutants have smaller ventricles which leads to poor circulation (Auman et al., 2007). In addition, while cell enlargement is occurring, the maturation of myofibrils takes place and these increase in number and organization (Lin et al., 2012).

Valve Formation

For the heart to function as an effective pump, it must develop a mechanism by which it can prevent retrograde flow where blood flows backward through the circulatory system. The formation of valves helps to solve this problem and facilitate unidirectional blood flow through the vessels. Valves form at three different locations in the heart: the sinus venosus, AV canal, and the conus ateriosus. The formation of the valves at the sinus venosus and the conus ateriosus is not well understood, but the formation of the AV canal valve is well studied in zebrafish. Circulation is initiated at 24 hpf and at this point in development, there are no valves and there is only a single layer of endocardium which lines the inside of the heart tube (Beis et al, 2005). Around 48 hpf, the cells at the AV canal start to change their shape and become distinguishable from the rest of the endocardium. At 90 hpf, these pre-valve cells make a transition from epithelial cells to mesenchyme cells and ingress to form the valves. The structures which form as a result of this transition are called the endocardial cushions. At 5 days post-fertilization, the valves look like two protrusions (Beis et al., 2005).

The Effects of Excess Retinoic Acid on Heart Development

Experiments have shown that excess retinoic acid signaling leads to abnormalities in heart tube morphology. In chick embryos treated with excess retinoic acid, heart tubes had truncated ventricles and enlarged atria (Yutzey et al. 1994). In zebrafish, it was also observed that treatment of excess retinoic acid results in the truncation of the heart tube (Stainier and Fishman, 1992; Waxman and Yelon, 2009). More recently, experiments using molecular markers and cell counting have shown that excess retinoic acid treatment results in a reduction in the number of atrial and ventricular cells. This, in turn, results in a variety of heart tube morphological abnormalities (Waxman and Yelon, 2009). The severity of the tube abnormalities was dependent on the concentration of retinoic acid exposure. Also, it has been proposed that Hox genes play an important role in retinoic acid signaling. Experimentally, when zebrafish embryos overexpressed the gene *Hoxb5b*, the resulting abnormal heart morphologies were similar to hearts treated with excess retinoic acid (Waxman and Yelon, 2009). This observation supports the conclusion that Hox genes help to regulate the concentration of retinoic acid signaling in normal heart development.

Current Investigation

The study of excess retinoic acid signaling and its effects on zebrafish cardiac development presents exciting opportunities to investigate further the role

retinoic acid plays in normal cardiac development. Waxman and Yelon did not consider the effects of excess retinoic acid signaling past 48 hpf; therefore, they were not able to observe the impact retinoic acid had on later developmental processes such as cardiac looping since looping is not complete until 55 hpf (2009). The research presented in this thesis explores the effects of excess retinoic acid on cardiac cell differentiation and how this then affects heart tube function and morphology. Embryos were exposed to retinoic acid at critical stages in heart cell determination and were then examined at 36 hpf, 55 hpf, and 3 days post-fertilization. Real-time and time-lapse recordings were made of the heart tube at these stages, which allowed for the assessment of both heart tube structural abnormalities and function.

MATERIALS AND METHODS

Adult Zebrafish and Embryo Care

Adult fish were maintained in a tank filled with spring water at 27°C with marbles on the bottom. Fish were exposed to 14 hours of light and 10 hours of darkness. They were fed fish flakes and live or frozen brine shrimp daily. Increased embryo production occurred when embryos were fed brine shrimp just before the lights were turned off at night. Embryos were collected daily by siphoning among the marbles at the bottom of the tank. Collected embryos were then washed into spring water and kept in sterile dishes at 27°C in an incubator. Watchmaker forceps were used to surgically remove the chorion which allowed for clearer imaging of embryos.

Immobilization Techniques

Three different methods were tested to try and immobilize the embryos during imaging because by 20 hpf, the embryos developed functional muscles which made imaging difficult.

I. MS-222:

The first method tested was the chemical MS-222 (Sigma) which was used to immobilize the muscles by preventing transmission of nerve impulses (Armstrong et al., 1983). Initially a 0.1mg/ml concentration of the drug was used as suggested by the literature, but the embryos still moved after 4 hours of exposure. The concentration was then increased to 0.2mg/ml and all the

embryos were immobilized within one minute with the heart still beating. Treated embryos were then transferred to a drop of water on a large coverslip surrounded by a circle of Dow Corning high vacuum grease. A small coverslip was placed on top to secure the embryos in place. It was observed that after 4 hours of exposure to the drug, there seemed to be a noticeable decrease in the heart rate in the majority of the embryos. Over 48-72 hours, the heart rate continued to decrease significantly until the heart stopped beating. A more careful investigation of the effects of MS-222 is needed to fully understand the effect it has on the heart rate over time. It became clear that another method of immobilization was needed which did not affect the heart beat in order to observe normal heart development.

II. Agar:

The second method tested was 1.2% agar which was prepared in spring water. The mixture was heated until the agar had fully dissolved. The agar was then maintained in a water bath at 50°C. A small amount of agar was pipetted into a test tube and allowed to cool to 40°C and then dechorionated embryos were added to the test tube. The embryos were then transferred in the agar to the center of a circle of high vacuum grease on a large cover slip. A hair loop was used to position the embryos in the agar before it completely cooled. Once the agar hardened, a drop of water was pipetted on top of the agar and a small coverslip was placed on top to keep the chamber from drying out. This method of immobilization was problematic because the embryos were in a set position

and could not be repositioned as development proceeded. Also, as the embryos developed, they became smashed which did not allow for effective imaging.

III. Methylcellulose:

The third method tested was 3% methylcellulose which was prepared by stirring in spring water overnight. Two different mixtures of methylcellulose were made up with different viscosities: 4000 cps and 1500 cps. There were many bubbles in the methylcellulose; therefore, test tubes of the methylcellulose were frozen and then allowed to thaw at room temperature to remove the bubbles. It was determined that the 4000 cps methylcellulose immobilized the embryos most effectively. To image the embryos, a viewing chamber was prepared with a circle of high vacuum grease applied to a slide with a circle cut out in the middle. Then 4000 cps methylcellulose was pipetted into the circle of grease filling it half way. Dechorionated embryos were then transferred into the methylcellulose. A hair loop was used to position the embryos in the methylcellulose. A drop of water was pipetted on top once the embryos were positioned correctly and a small coverslip was used to keep the chamber from drying out. Between recordings, the viewing chambers were kept in an incubator at 27 °C.

Embryos Treated with Retinoic Acid

A 0.02M stock solution of *trans*-retinoic acid (Sigma) was prepared in DMSO. The stock solution was stored at -20 °C in the dark, and diluted in spring

water to give final concentrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M retinoic acid. Twenty-one experiments were performed where embryos were treated with retinoic acid (**Table 1**).

As a control for the effect of DMSO, embryos were treated with DMSO for two hours. Embryos were exposed to concentrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M retinoic acid at 50% epiboly and dome stage for 1 hour. In another series of experiments, embryos were treated with 10^{-6} M retinoic acid at the dome stage for 2 hours. Because retinoic acid is a polar molecule, it is unclear exactly how much retinoic acid the embryos were exposed to because the dilutions were made in water (Stainier and Fishman, 1992). After exposure time, the embryos were washed back into spring water and were immobilized in methylcellulose. Twenty-one experiments were performed using retinoic acid and there were approximately 15 embryos per experiment. In general, approximately 50% of embryos died within the first 24 hours after collection for both control and retinoic acid treated embryos.

Table 1: Summary of retinoic acid experiments.

Concentration Of Retinoic Acid	Number Of Experiments	Stage of Treatment with Retinoic Acid	Exposure Time
DMSO	1	Dome Stage	1 hr
10^{-8}	1	50% Epiboly	1 hr.
10^{-8}	1	Dome Stage	1 hr.
10^{-7}	3	50% Epiboly	1 hr.
10^{-7}	3	Dome Stage	1 hr.
10^{-6}	8	Dome Stage	1 hr.
10^{-6}	3	Dome Stage	2 hr.
10^{-5}	2	Dome Stage	1 hr.

Embryo Preparation for Fluorescence Microscopy

An effort was made to better visualize the heart tube when it was positioned deep inside the embryo, obscured by the yolk sac. By using two fluorescent markers: Mito Tracker Red which labels mitochondria, and $diOC_6$ which labels mitochondria and endoplasmic reticulum. If heart muscle cells had high numbers of mitochondria, these fluorescent probes might show preferential staining. The embryos were exposed to 20µL of each stain mixed with spring water for 4 minutes. Stained embryos were placed in 0.2mg/ml MS-222. The

embryos were then transferred to a large cover slip surrounded by a circle of vacuum grease. A small coverslip was placed on top to secure the embryos in one place.

Microscopes and Image Formatting

DIC images were taken using a Nikon Diaphot inverted compound microscope, equipped with a Pixelink camera and software. For fluorescence imaging a Nikon Eclipse inverted compound microscope equipped with MetaVue camera and software was used. Images were also taken with an Olympus SZH stereo microscope equipped with a Pixelink camera and software. ImageJ 64 was used to convert real-time recordings into image stacks for processing into movies. ImageJ was also used to horizontally flip images and recordings, crop images and recordings, and take measurements. Photoshop was used to crop images, outline the heart tube in red, clean up the background, add labels, modify contrast, and create montages.

RESULTS

Normal Heart Development

I. Real-Time Recordings

A) Normal Heart Development at 30 hpf

The heart tube was first visible at 30 hpf even though it was positioned deep inside the embryo. This was possible because at this stage in development the heart tube is fully fused and has begun to contract. When embryos were examined at 30 hpf, the early heart tube was positioned posterior and dorsal to the left eye (**Figure 13 and Movie 1**). The heart tube looked like a straight linear tube without defined chambers. There seemed to be only a single contraction in the future atrium region. A few blood cells could be seen moving through the tube with each contraction.

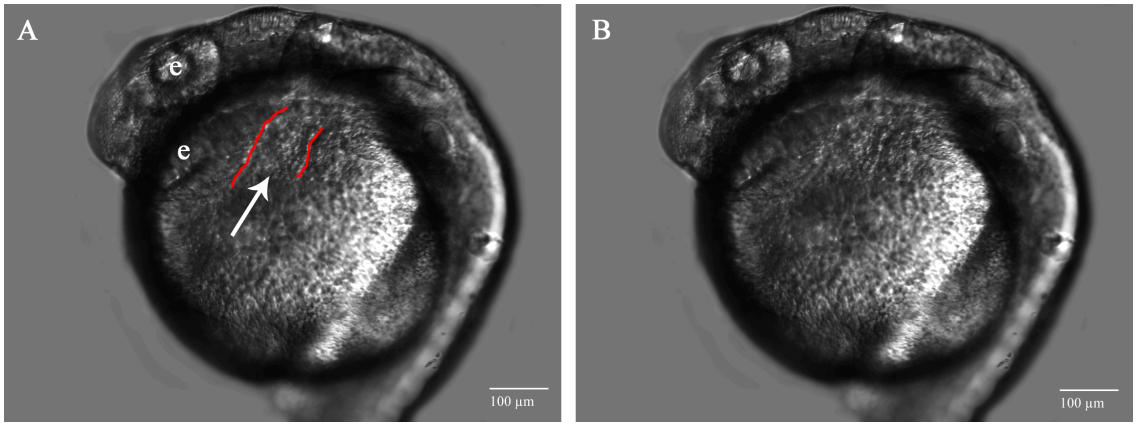

Figure 13: Normal heart development from a left dorsal view at 30 hpf. (A) The heart is a small linear tube at this stage, and is only able to pump a few blood cells per contraction. For each heartbeat there is only a single contraction in the future atrium region. The heart tube is visible at this stage in development dorsal and posterior to the left eye as indicated by the red lines. Due to the fact that the tube is deep inside the embryo, it is hard to see if cardiac jogging has occurred. The arrow indicates the flow of blood through the tube and the two eyes are marked by e. (B) Image of the same embryo without markings.

B) Normal Heart Development at 36 hpf

Examination of embryos at 36 hpf revealed that the heart tube had changed its position in the embryo and had become located more anterior and ventral to the left eye (**Figure 14 and Movie 2**). The tube became more elongated; therefore making it easier to see that the tube contracts in a wave-like motion. An initial large contraction occurred in the future atrium region and was followed by a second and smaller contraction in the ventricle region. The atrium and the ventricle do not become visually distinct until 55 hpf. The rate of contraction and the number of blood cells moving through the heart tube with each contraction had increased from embryos at 30 hpf. Cardiac jogging was observed at this stage, where the ventricle region is shifted to the right side of the embryo creating a bent tube. It was observed that there was a single layer of endocardium which indicates that the valves have not formed yet. Where the future ventricle region bent to the right, the two sides met as the tube contracted to prevent the retrograde blood flow where blood cells flow backward in between contractions. Blood cells were visible at this stage moving across the yolk sac on the ventral side of the embryo and then entered the atrium (**Figure 15**). Blood flowed from both the right and left side of the yolk sac and met before entering the tube.

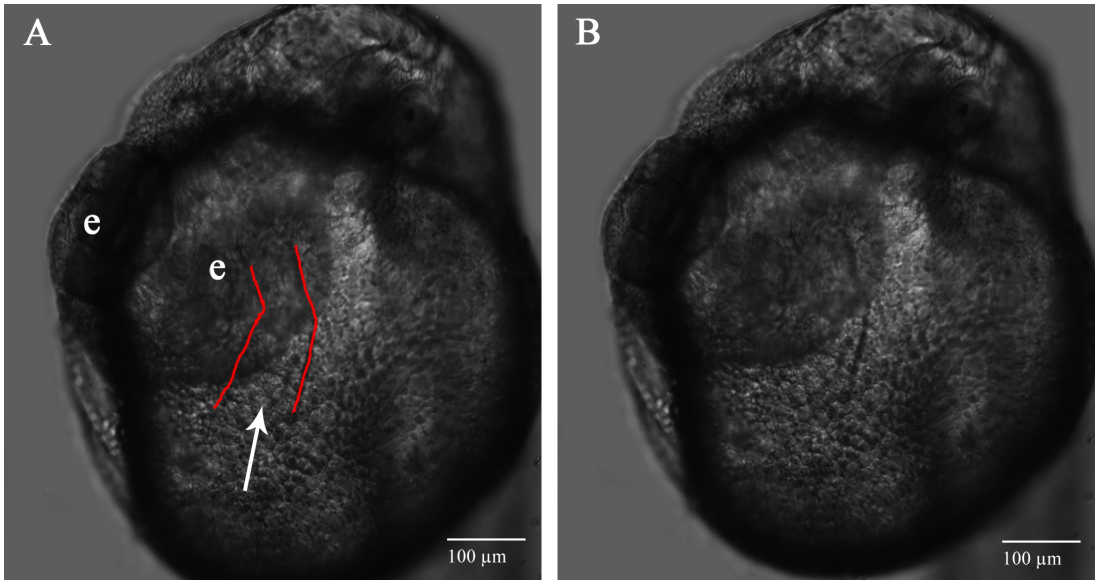

Figure 14: Normal heart development from a left rostral view at 36 hpf. (A) The heart has become elongated and cardiac jogging is visible where the ventricle is bent towards the right side of the embryo. Circulation has increased from 30 hpf and more blood is being pumped through the tube with each contraction. An initial large contraction occurs in the future atrium region and is followed by a second, smaller contraction in the ventricle region. The position of the eyes are marked by e and the arrow indicates the flow of blood through the heart. (B) Image of the same embryo without markings.

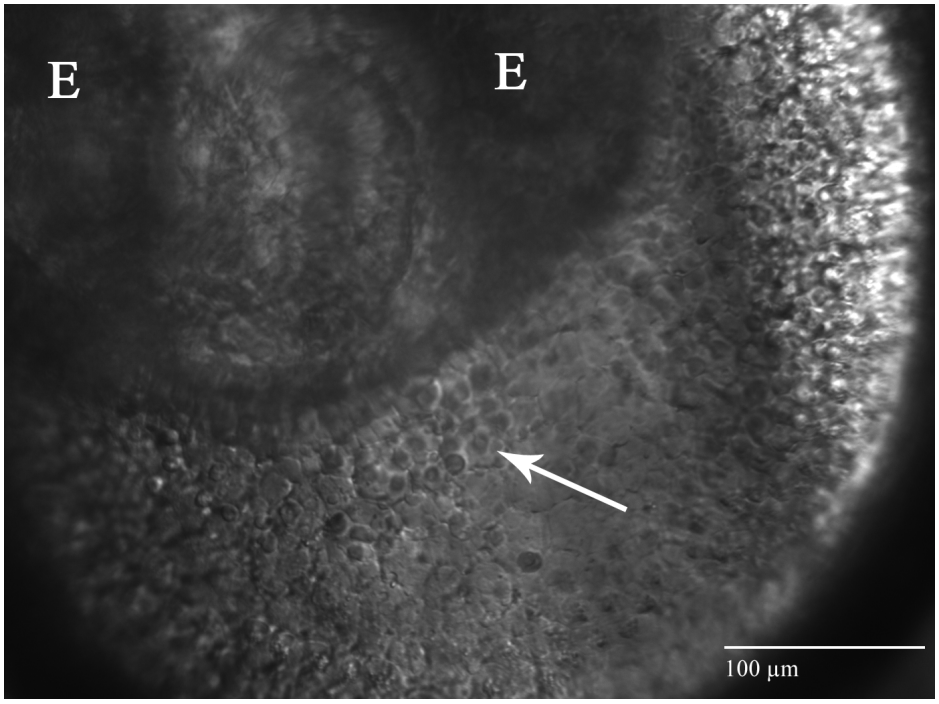

Figure 15: Blood cells moving on the yolk sac at 36 hpf from a frontal view. Blood cells move from both sides of the yolk sac into the inflow tract of the heart tube. The arrow indicates blood cells on the surface of the yolk sac and E marks the position of the eyes.

C) Normal Heart Development at 55 hpf

At 55 hpf, the heart tube had shifted downwards towards the ventral side of the embryo. Also, heart looping had taken place where the ventricle looped back to the left side of the embryo forming an S-shaped tube (**Figure 16 and Movie 3).** The enlargement and ballooning out of the chambers was also observed which contributed to the distinction between the atrium and the ventricle. The heart contracted faster and pumped more blood with each contraction than at 36 hpf. A steady stream of blood cells could be seen moving through the tube with each contraction. Thickening of the endocardium was observed at the AV canal

which indicated the beginning of valve formation. However, because the heart was beating so quickly, it was very difficult to visualize the AV canal. The contraction pattern of the heart tube had also changed, no longer moving with a wave-like motion. For each heartbeat, there was a distinct contraction in the atrium, which was then followed by a separate contraction in the ventricle.

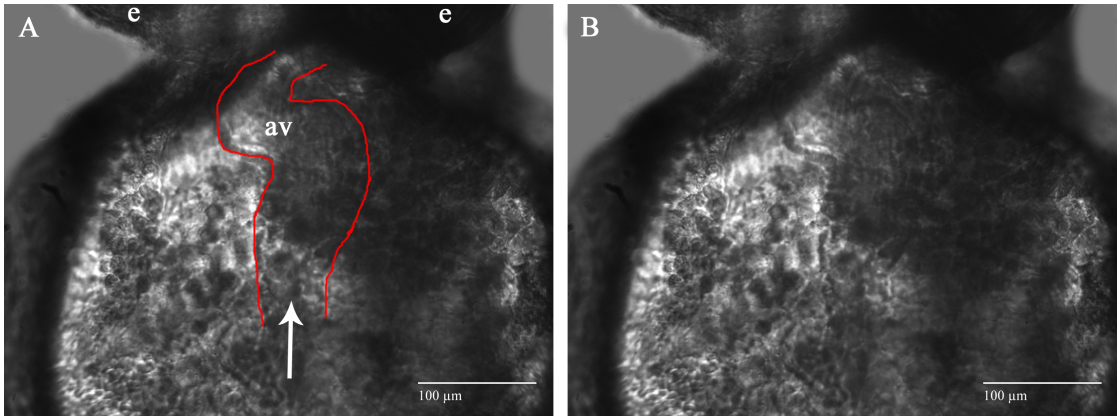

Figure 16: Normal heart development from a frontal view at 55 hpf. (A) The heart tube has undergone heart looping where the ventricle loops back toward the left side of the embryo creating an S-shaped tube. The atrium and the ventricle have become more defined due to the ballooning out of the chambers. The circulation has greatly increased at this stage with a steady stream of blood flowing through the tube with each contraction. Red lines outline the structure of the tube. The AV canal is marked by av and the arrow indicates the flow of blood through the tube. The position of the eyes is marked by e. (B) Image of the same embryo without markings.

D) Normal Heart Development at 3 Days Post-fertilization

Embryos examined at 3 days post-fertilization showed that both the atrium and the ventricle continued to balloon out and become more defined. Also the chambers became more separated at this stage as the AV canal narrowed. The S-shape of the heart tube became more defined as the inflow tract curved toward the right and the outflow tract curved more to the left. The circulation had also

become stronger and the heart tube contracted faster (**Figure 17 and Movie 4**). It was difficult to visualize the heart tube from a direct frontal view due to the formation of pigment on the ventral side of the embryo. At this stage in development, the embryo had hatched out of the chorion and had started to swim around.

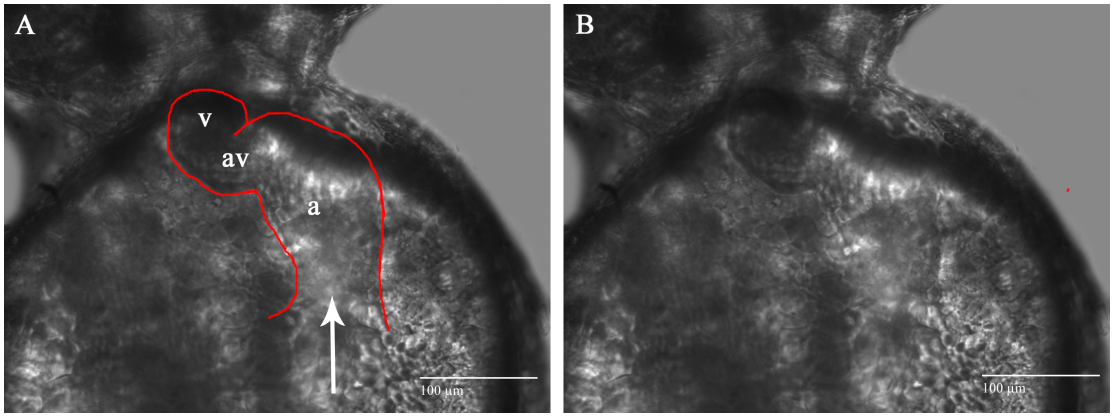

Figure 17: Normal heart development from a frontal view at 3 days post-fertilization. (A) The chambers of the heart tube have continued to become enlarged with ballooning out of the chambers. The AV canal (av) has narrowed, which helps to separate the atrium (a) and the ventricle (v) which have become significantly more defined. Also, the chambers are contracting faster and pumping more blood cells, increasing the amount of circulating blood throughout the embryo. Red lines outline the structure of the tube and the arrow indicates the flow of blood. (B) Image of the same embryo without markings.

II. Time-Lapse Recordings

There is a significant repositioning of the anterior-posterior axis of the heart tube as the embryo develops. Between 30 hpf and 60 hpf, the heart tube moves from the dorsal side of the embryo to the ventral (**Figure 18 and Movie 5**). This is due to the fact that the yolk sac recedes posteriorly as the head lifts.

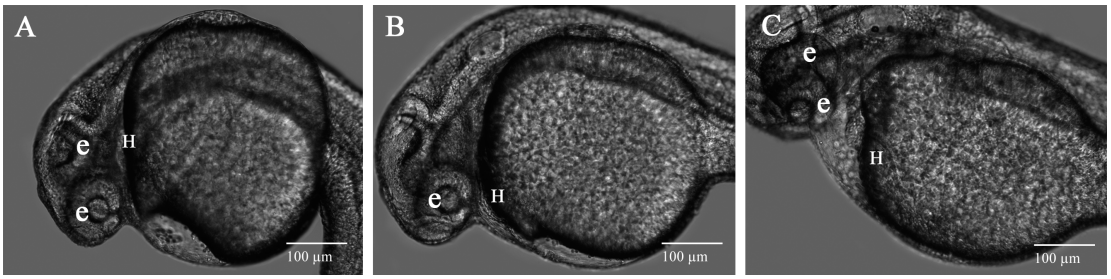

Figure 18: Repositioning of the heart tube from the left side of the embryo. (A) The embryo at approximately 30 hpf, when the heart tube starts to beat and is positioned on the dorsal side of the embryo. (B) At 48 hpf, the heart tube has repositioned directly posterior to the eye. (C) At 3 days post-fertilization, the heart tube has reached the ventral side of the embryo. H indicates the location of the heart tube and e marks the position of the eyes.

Embryos Treated with Retinoic Acid

I. General Trends

Embryos were treated with varying concentrations of retinoic acid in DMSO: 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M. Embryos were exposed for one hour at the dome stage, approximately 4 hpf. Control embryos were treated with DMSO for two hours and 100% of the embryos exhibited normal heart development. It was observed that with an increase in the concentration of retinoic acid, there was an increase in the percentage of embryos which developed abnormal hearts or no detectible heart (**Table 2**). The term abnormal is used to describe several different heart morphologies which varied with the developmental stage of the embryo. As heart development proceeded from the initial fusion of the heart tube to heart looping, there was a large variation in whether or not development was normal. There were heart tubes that exhibited abnormal cardiac jogging, chamber size and differentiation, heart looping, and contraction patterns.

Table 2: The effects of retinoic concentration on the number of abnormal hearts.

Concentration of Retinoic Acid (M)	Stage of Embryo When Retinoic Acid Was Applied	Percent With Abnormal Or No Heart
10^{-8}	Dome Stage	0%
10^{-7}	Dome Stage	26%
10^{-6}	Dome Stage	48%
10^{-5}	Dome Stage	100%

Several different heart abnormalities were observed as a result of retinoic acid exposure. These abnormal hearts fell into four distinct morphological categories: Wide Atrium, Linear Heart, Small Heart, and No Heart. Embryos which were exposed to 10^{-8} M retinoic acid all developed normal hearts. Embryos which were treated with either 10^{-7} M or 10^{-6} M retinoic acid exhibited Wide Atrium, Linear Heart and Small Heart. Embryos exposed to 10^{-5} M demonstrated such a significant truncation of the heart tube that there was no detectible heart (**Table 3**).

Table 3: Heart tube morphologies seen in embryos treated with retinoic acid at the dome stage for 1 hour.

	Number Of Embryos With Heart Tube Morphology At 55 hpf				
Concentration Of Retinoic Acid	**Wide Atrium**	**Small Heart**	**Linear Heart**	**No heart**	**Normal**
10^{-7}	3	1	5	0	25
10^{-6}	15	12	14	0	43
10^{-5}	0	0	0	15	0

Other morphological features were abnormal as a result of excess retinoic acid including reduction of the anterior head, truncation of the tail, both enlargement or reduction of the yolk sac, reduction of the eyes, and hyperpigmentation in the head (**Figure 19**).

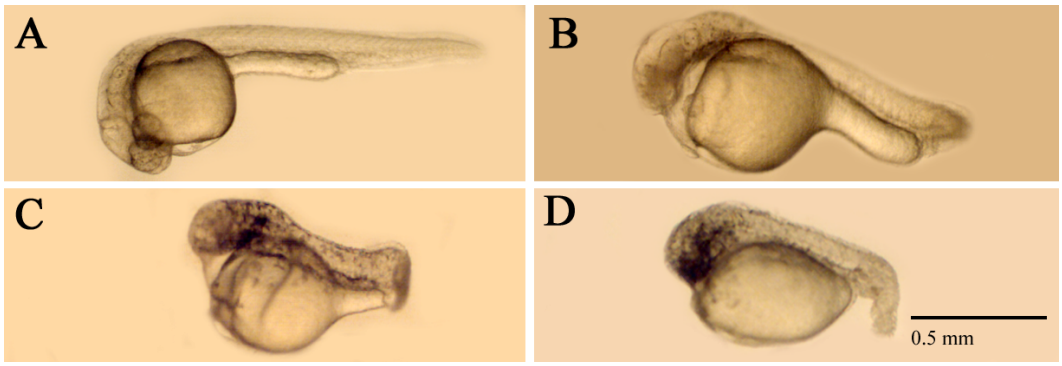

Figure 19: The effects of retinoic acid on morphology. All images are of embryos at 36 hpf. (A) Normal development. (B) Embryo treated with 10^{-6} M retinoic acid for 1 hour shows truncation of the tail and the reduction of anterior head and eyes. (C) Embryo treated with 10^{-6} M retinoic acid for 2 hours shows more severe effects than 1 hour exposure. (D) Embryo treated with 10^{-5} M retinoic acid for 1 hour shows the most severe effects on morphology. There is significant truncation of the tail and head and hyperpigmentation in the head.

It was also observed that when embryos were exposed to the same concentration of retinoic acid at different stages, embryos exposed at an earlier stage had a higher incidence of abnormal heart formation. When embryos where treated with 10^{-7} M retinoic acid at 50% epiboly, only 12% of embryos had abnormal hearts, whereas when embryos were treated at dome stage, 26% of embryos developed abnormal hearts. Also, the length of exposure had a significant effect on the percentage of embryos which developed abnormal hearts. Embryos which were treated with 10^{-6} M retinoic acid for 1 hour at dome stage were compared with embryos treated with the same concentration for 2 hours. It was found that only 54% of embryos had abnormal hearts when exposed for 1 hour, whereas 97% of embryos had abnormal hearts when exposed for two hours. Overall, embryos treated for a longer period of time exhibited more morphological abnormalities (**Figure 19B and 19C**).

II. Embryos treated with 10^{-6} M Retinoic Acid.

When embryos were treated at 10^{-6} M concentration of retinoic acid, 48% of embryos exhibited abnormal heart tubes. Embryos were examined at 36 hpf, 55 hpf, and 3 days post-fertilization.

A) 36 hpf in 10^{-6} M Retinoic Acid

At 36 hpf, it was observed that there was abnormal heart development. In normal development, circulation has started at this stage, but in many of the embryos treated with retinoic acid, there was very poor or no circulation due to

abnormal heart tube morphologies. Examined embryos were found to have two types of heart morphologies at 36 hpf: Wide Atrium, and Small Heart.

Wide Atrium: Embryos showed an increase in the size of the atrium region. There were two variations in the degree of enlargement. First, one group of embryos had a moderate enlarged opening to the inflow tract (**Figure 20 and Movie 6**). A second group of embryos had a more significant enlargement of the atrium region along with an enlarged opening at the inflow tract (**Figure 21 and Movie 7**). There was variation in how effective the abnormal heart tubes were at pumping blood. In some embryos, when the tube contracted, blood cells moved back and forth in response to pressure differences but were not pumped through the tube. In other embryos there was retrograde blood flow where blood cells flowed backward in between contractions. In both groups of embryos, there seemed to be an initial large contraction in the future atrium region followed by a very small contraction in the future ventricle region compared to control embryos. Also, neither type of Large Atrium demonstrated normal cardiac jogging. The enlarged tubes remain linear at this stage unlike normal development where the future ventricle is shifted to the right. In addition, some of the embryos developed abnormally deep channels which the blood flowed through on the yolk sac.

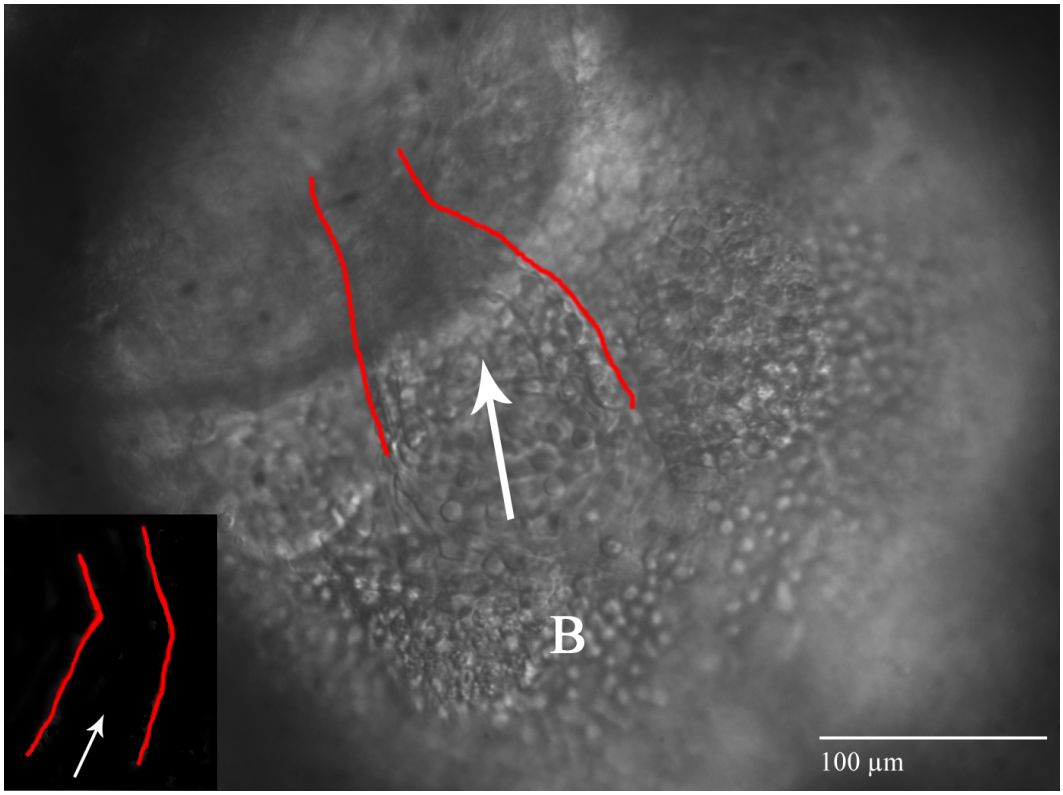

Figure 20: Less severe Wide Atrium at 36 hpf in an embryo treated with 10^{-6} M retinoic acid. This image shows the heart tube from a frontal view. The red lines outline how the heart tube does not demonstrate normal cardiac jogging and remains linear. The white arrow shows normal direction of blood flow through the tube. B shows blood cells which have collected on the yolk sac due to ineffective pumping. Inset shows the normal heart tube for comparison.

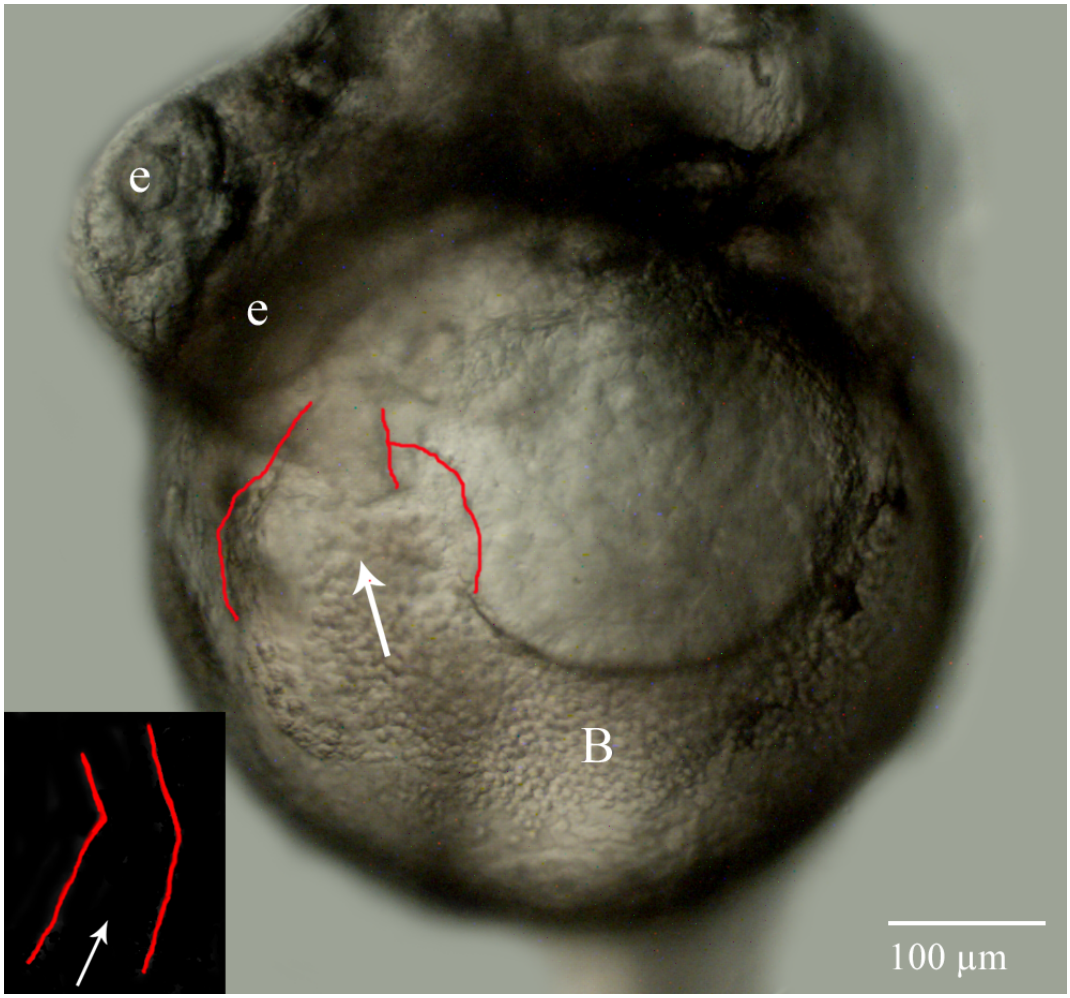

Figure 21: More severe Wide Atrium at 36 hpf in an embryo treated with 10^{-6} M retinoic acid. This image shows the heart tube from the right ventral view. The heart tube has an enlarged opening to the future atrium, indicated by the red lines. Also, the tube does not undergo normal heart jogging. Blood only flows from one side of the yolk sac which is abnormal compared to control embryos where blood flows in from both sides. The embryo has developed an abnormally deep channel which the blood flows through. The white arrow indicates the direction of blood flow through the tube. B marks blood cells moving on the yolk sac and e marks eyes. Inset shows the normal heart tube for comparison.

Small Heart: Another group of embryos showed a significant reduction in the size of both the atrium and the ventricle. These abnormal heart tubes were not able to pump blood, resulting in embryos with no circulation (**Figure 22 and**

Movie 8). In some embryos, the future atriums and ventricles were only very thin rods of cells. It was unclear whether or not these hearts had an internal opening large enough to allow blood to flow through the tube. There seemed to be normal cardiac jogging in the few ventricle cells present. There was essentially only a single contraction in the atrium region and no secondary contraction in the very thin ventricle region. In another group of embryos, the heart tube was a shapeless mass of cells which did not have a tube-like structure. The area surrounding the heart tubes was filled with excess fluid. This may be due to the fact that the heart was not able to pump. In both the Wide Atrium and Small Heart embryos there were large cavities filled with fluid on the yolk sac due to poor circulation (**Figure 23**).

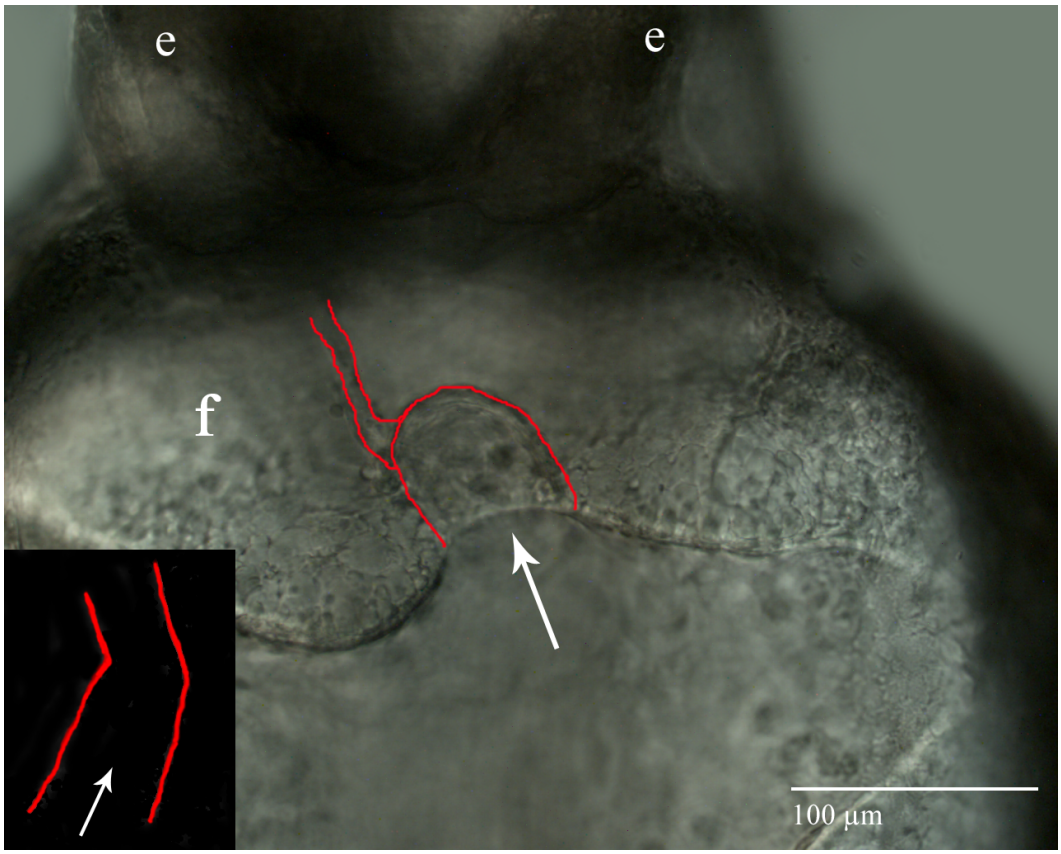

Figure 22: Small Heart at 36 hpf in an embryo treated with 10^{-6} M retinoic acid. This image shows the heart tube from a frontal view. Both the atrium and the ventricle are greatly reduced in size as indicated by the red lines. There is no circulation and a large amount of fluid has collected around the tube shown by f. The arrow shows the normal direction of blood flow through the tube. There is no clear opening to the tube. Inset shows the normal heart tube for comparison.

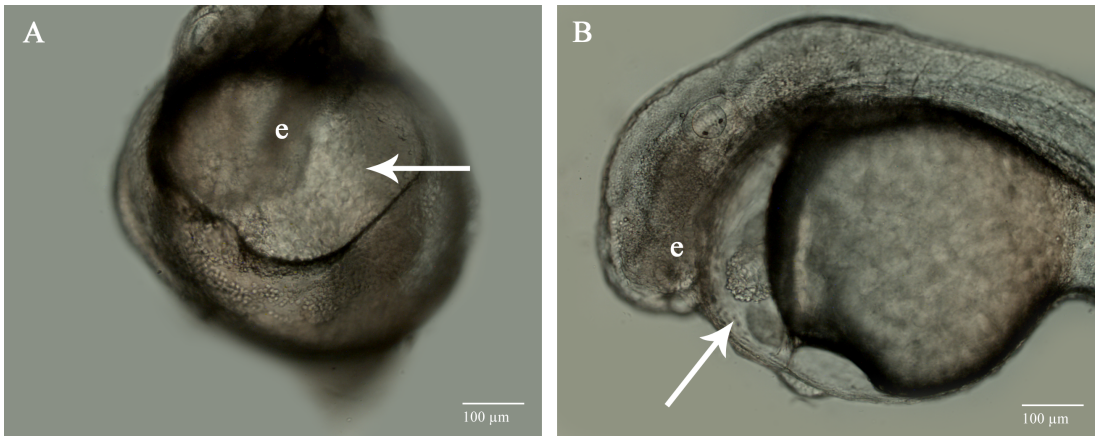

Figure 23: Fluid collection due to poor circulation at 36 hpf in an embryo treated with 10^{-6} M retinoic acid. (A) Frontal view of the embryo and (B) left side of the embryo. The arrows indicate the fluid which has collected around the heart tube and the e marks the normal location of the left eye. Abnormal eye development is noted.

B) 55 hpf in 10^{-6} M Retinoic Acid

Embryos were also examined at 55 hpf and it was observed that there continued to be abnormal heart development. There were three distinct heart morphologies at 55 hpf, Wide Atrium, Linear Heart, and Small Heart. In general, some of the abnormal heart tube morphologies which initially had no circulation started to pump blood cells.

Wide Atrium: Embryos at this stage continued to have enlarged atriums and also developed truncated ventricles where ballooning out was not observed. As seen in 36 hpf embryos, there was a variance in the degree of enlargement of the atrium region. In one group of embryos, there was an abnormal heart tube which only had a wide opening to the inflow tract as seen at the 36 hpf (**Figure 24 and Movie 9**). There was a second abnormal heart tube morphology where the

embryo had an enlarged atrium which abnormally ballooned out and had a wide opening (**Figure 25 and Movie 10**). The ventricle region did not undergo normal heart looping and remained linear. Occasionally, a Wide Atrium embryo exhibited normal heart looping (**Figure 26 and Movie 11**). Both the atrium and the ventricle regions seemed to contract separately as seen in normal development. There was a variation in whether or not these two morphologies developed a heart tube which could pump blood. In many of the embryos, the contraction of the heart tube seemed ineffective and blood cells simply moved back and forth in response to pressure changes, failing to flow through the tube. Also in these embryos, large numbers of blood cells could be seen sitting on the surface of the yolk sac where they seemed to have collected due to the fact that the heart could not pump effectively. Also, many of these embryos exhibited decreased and retrograde blood flow.

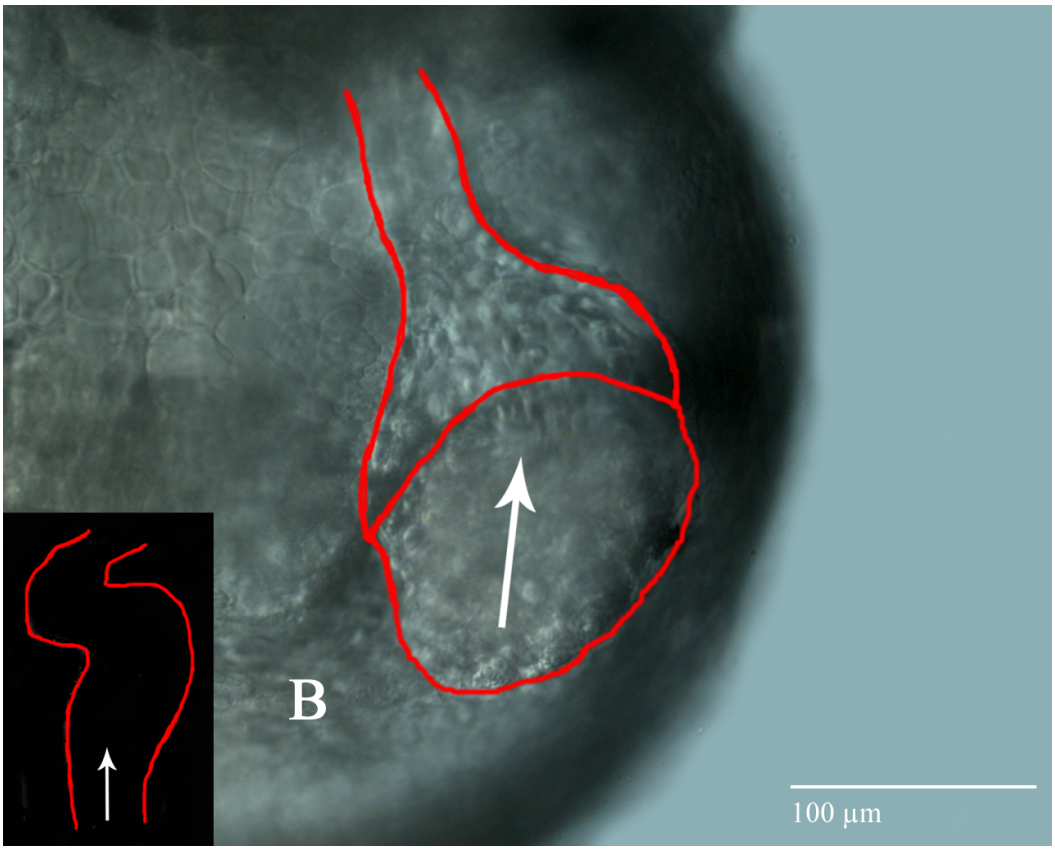

Figure 24: Wide Atrium with enlarged opening at 55 hpf in an embryo treated with 10^{-6} M retinoic acid. The heart tube is shown from a frontal view of the embryo. The atrium has a very wide opening as shown by the red lines. The heart tube does not demonstrate normal heart looping but remains linear. The ventricle also does not undergo normal ballooning out. The white arrow indicates the flow of the blood through the heart tube and B indicates the blood flow from only one direction on the yolk sac which is abnormal. In normal development, blood flows in from both directions. Inset shows the normal heart tube for comparison.

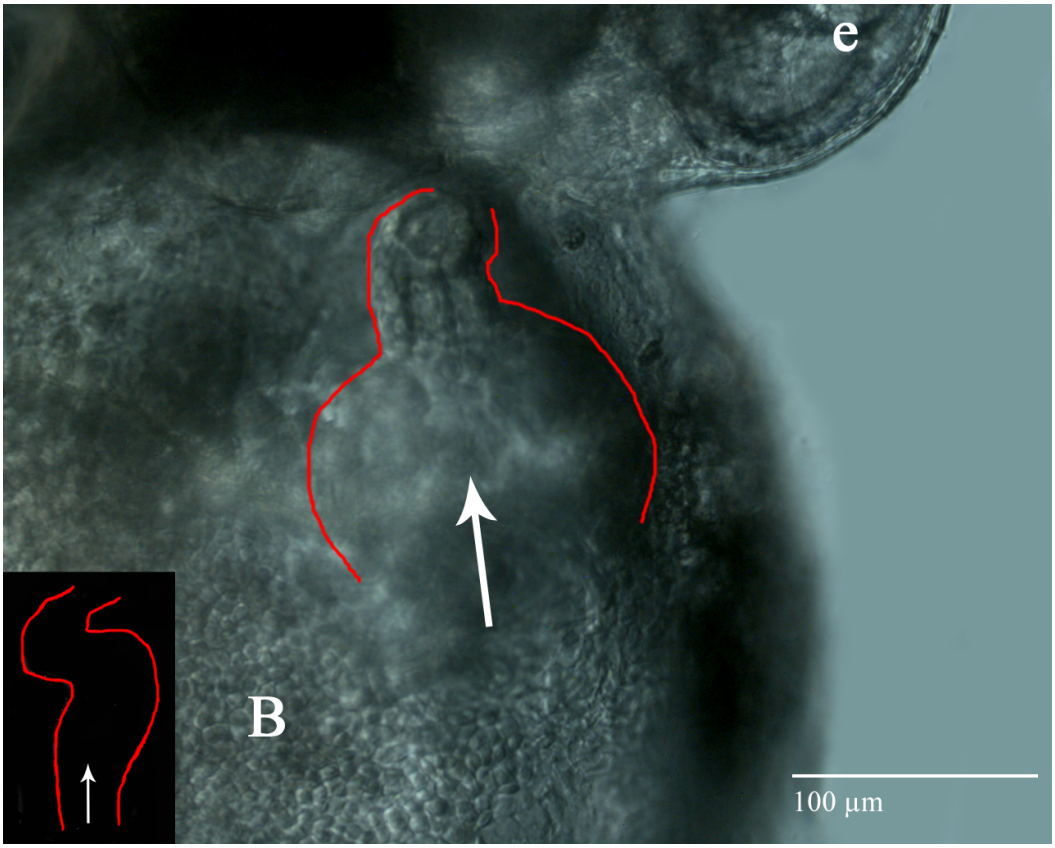

Figure 25: Wide Atrium at 55 hpf in an embryo treated with 10^{-6} M retinoic acid. The heart tube is shown from a frontal view of the embryo. The atrium is enlarged as indicated by the red lines. The ventricle has remained narrow and has not undergone cardiac looping or ballooning out. The arrow indicates the flow of blood, e marks the position of the eye, and B marks the blood cells flowing into the inflow tract. Inset shows the normal heart tube for comparison.

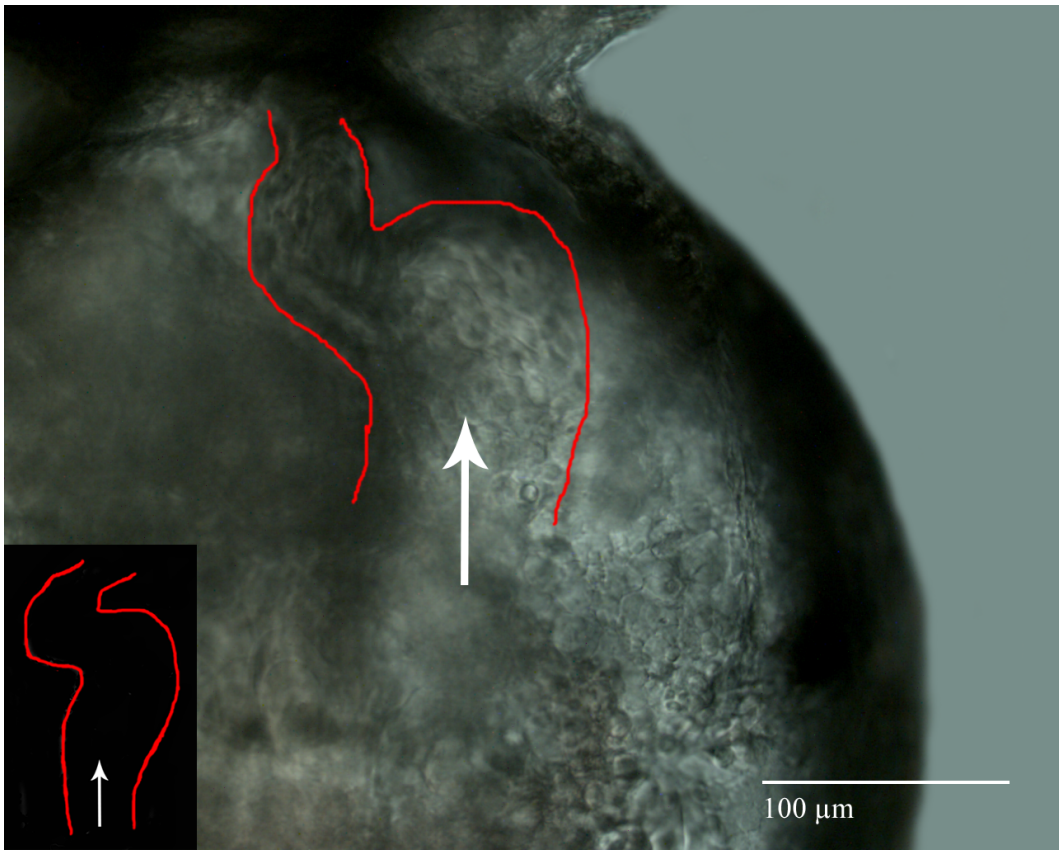

Figure 26: Wide Atrium with heart looping at 55 hpf in an embryo treated with 10^{-6} M retinoic acid. The heart tube is shown from a frontal view of the embryo. The atrium region is enlarged and there is a wide opening to the atrium as indicated by the red lines. The ventricle has remained narrow and undergoes heart looping. The arrow indicates the flow of blood. Inset shows the normal heart tube for comparison.

Linear Heart: Another group of embryos had linear hearts which did not undergo normal heart looping. They remained straight with only the ventricle slightly shifted to the right due to cardiac jogging. Some of the hearts underwent ballooning out of the atrium (**Figure 27 and Movie 12**), and some remained narrow with no ballooning out of the chambers (**Figure 28 and Movie 13**). The circulation in both of these heart morphologies seemed to be impaired. Less blood was pumped through the heart tube with each contraction as compared to normal

development. Also, the heart tube seemed to contract at a slower rate in some of the Linear Heart embryos.

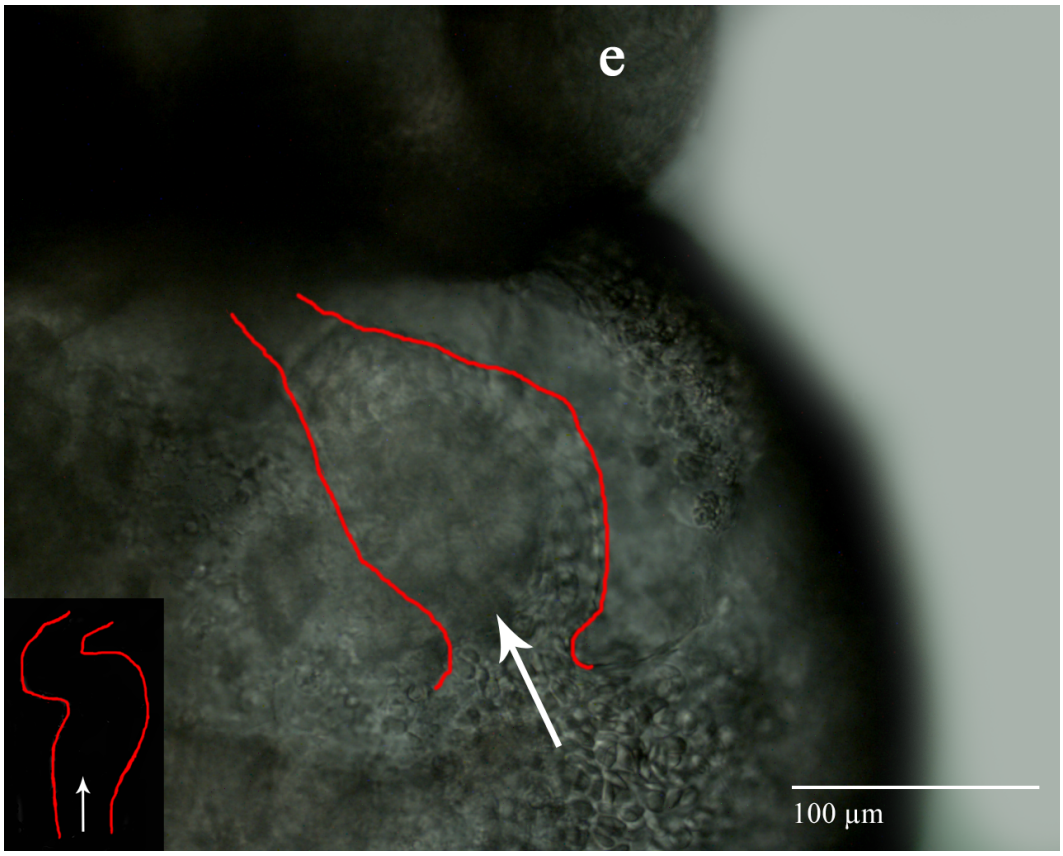

Figure 27: Linear Heart tube at 55 hpf in an embryo treated with 10^{-6} M retinoic acid. The atrium is enlarged and ballooned out as seen in normal development. Normal heart looping has not taken place. There is a decrease in the number of cells being pumped through the heart as compared with normal development. The red lines outline the structure of the heart tube. The arrow indicates the flow of blood through the tube. The e indicates the position of the left eye. There is normal cardiac jogging. Inset shows the normal heart tube for comparison.

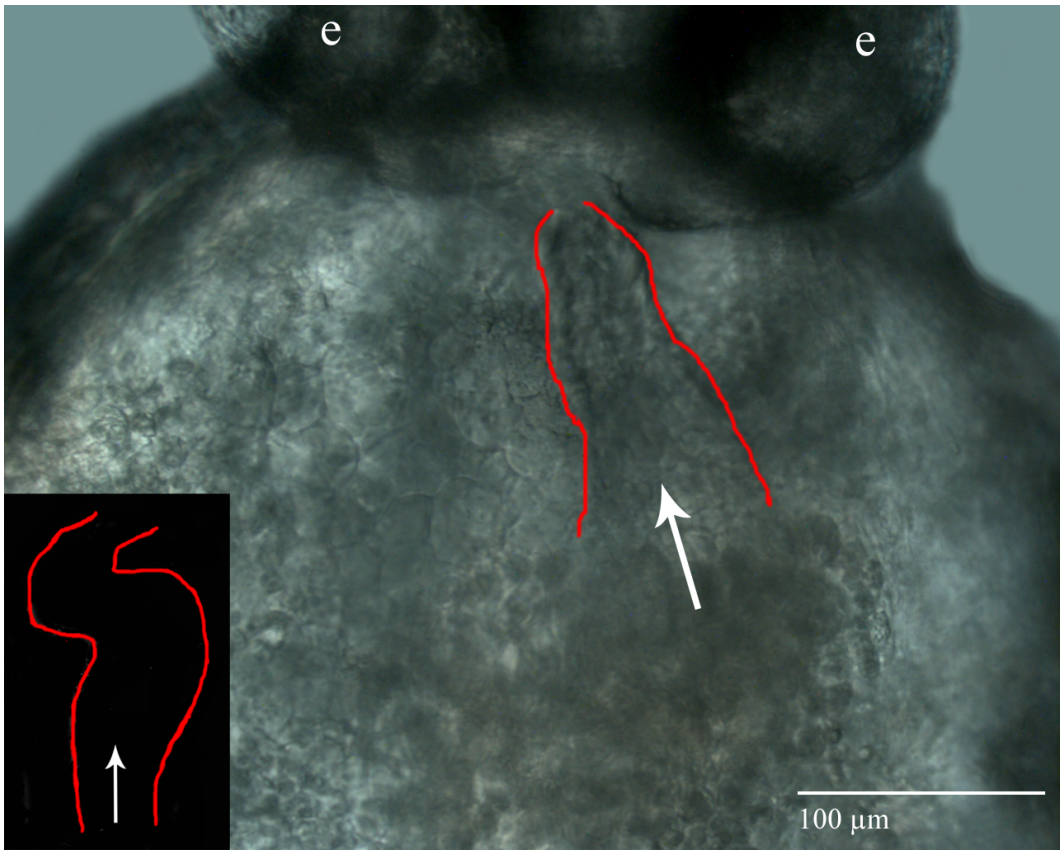

Figure 28: Linear Heart tube at 55 hpf in an embryo treated with 10^{-6} M retinoic acid. The heart tube remains straight and does not undergo heart looping as indicated by the red lines. This heart tube is not able to pump blood as effectively, and less blood flows through the tube with each contraction. The arrow indicates the direction of flow through the tube. E indicates the location of the eyes. Inset shows the normal heart tube for comparison.

Small Heart: Another group of embryos had reduced atriums and ventricles. These embryos did not have circulation and often had irregular contraction patterns that resembled quivering motions instead of regular contractions (**Figure 29 and Movie 14**). In many of the embryos, the atrium region can not be distinguished from the ventricle region. It was not clear whether there was an opening in the heart tube which would allow for the flow of blood.

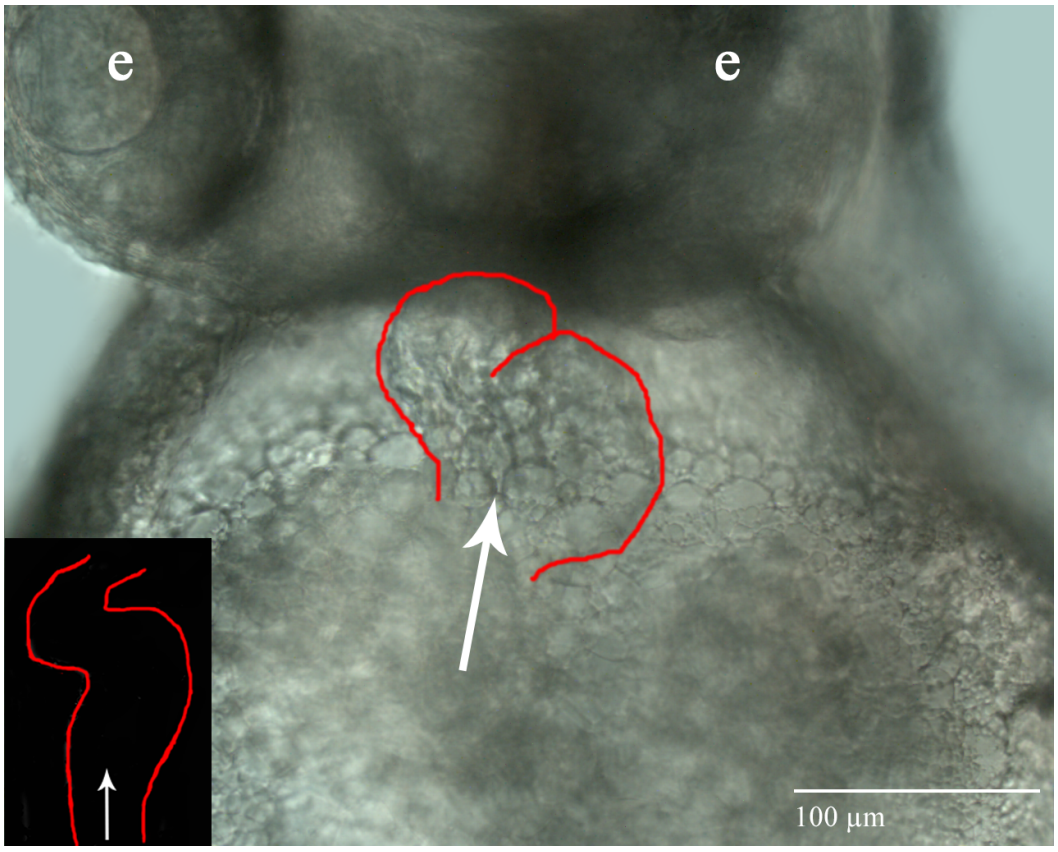

Figure 29: Small Heart with reduced atrium and ventricle at 55 hpf in an embryo treated with 10^{-6} M retinoic acid. Both the atrium and the ventricle are truncated as indicated by the red lines. In many of the embryos it is difficult to distinguish between the atrium and the ventricle cells. This heart tube morphology is not able to pump blood cells. The arrow indicates the normal flow of blood through the tube. The e indicates the location of the eyes. Inset shows the normal heart tube for comparison.

C) 3 days post-fertilization in 10^{-6} M Retinoic Acid

Embryos were also examined at 3 days post-fertilization, and it was observed that there continued to be abnormal heart development. There were three distinct heart morphologies: Wide Atrium, Small Heart, and Linear Heart. In general, some of the abnormal heart tube morphologies which had continued to have no circulation started to pump blood cells.

Wide Atrium: There were two distinct heart morphologies which had a wide atrium. There was one group of embryos which had enlarged atriums and did not undergo normal heart looping (**Figure 30 and Movie 15**). There was another group which had a heart tube morphology where the atrium was enlarged and the heart tube did undergo heart looping (**Figure 31 and Movie 16**). This result suggests that normal heart looping may be delayed in some of the embryos. Both of these heart tube morphologies were able to pump blood effectively by this stage and many blood cells can be seen moving through the heart tube with each contraction.

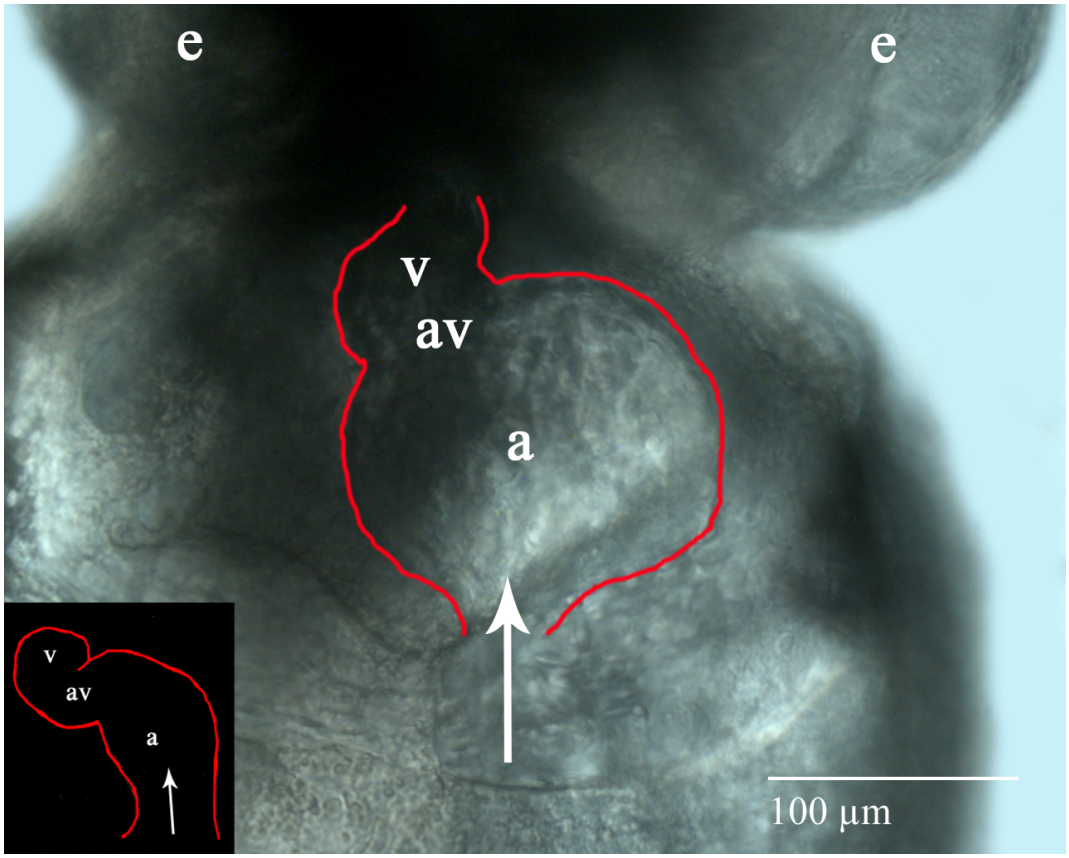

Figure 30: Wide Atrium at 3 days post-fertilization in an embryo treated with 10^{-6} M retinoic acid. The heart tube is shown from a frontal view of the embryo. The tube has not undergone normal heart looping where the ventricle is shifted to the right side of the embryo as indicated by the red lines. The arrow indicates the flow of blood through the tube. The e indicates the location of the eyes, and a indicates the atrium, av the AV canal, and v the ventricle. Inset shows the normal heart tube for comparison.

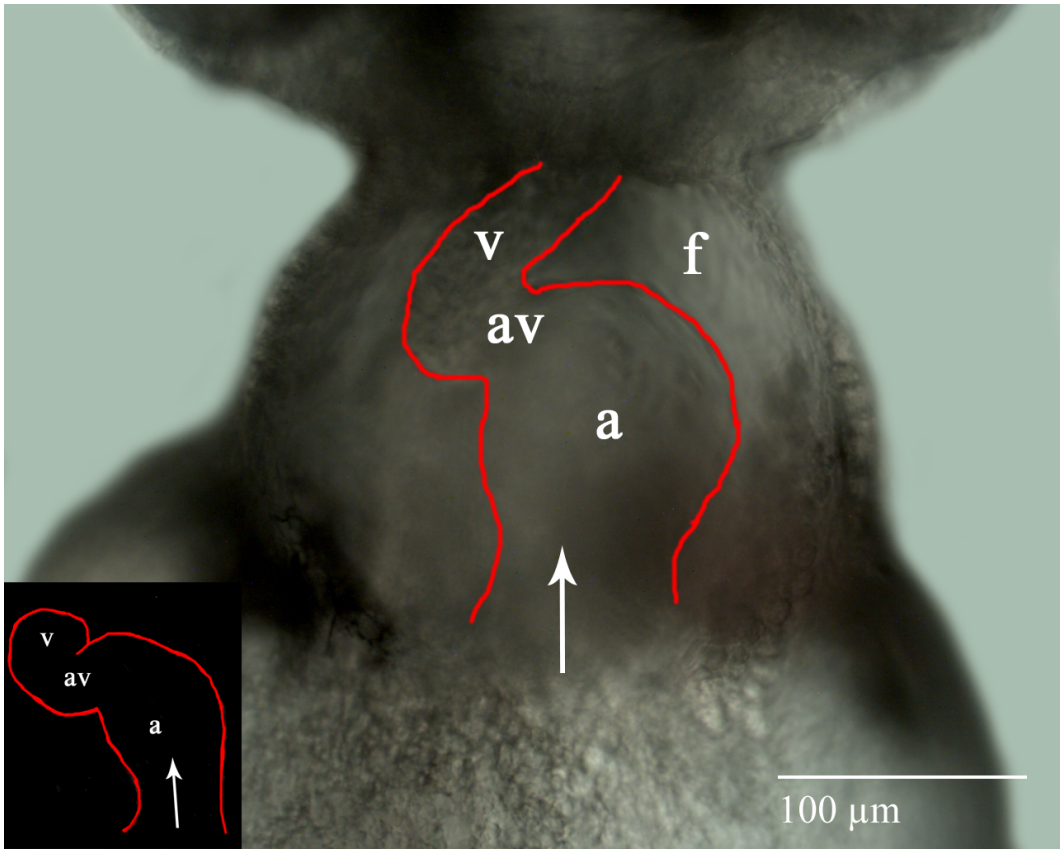

Figure 31: Wide Atrium with heart looping at 3 days post-fertilization in an embryo treated with 10^{-6} M retinoic acid. The heart tube is shown from a frontal view of the embryo. The heart tube has undergone normal heart looping where the ventricle is shifted to the right side of the embryo as indicated by the red lines. The arrow indicates the flow of blood through the tube. The e indicates the location of the eyes, a indicates the atrium, av the AV canal, and v the ventricle. The f shows where fluid has collected around the heart tube. Inset shows the normal heart tube for comparison.

Small Heart: A group of embryos at this stage continued to have both truncated atriums and ventricles (**Figure 32 and Movie 17**). These abnormal heart tubes did not seem to be able to pump blood. The contraction patterns of the atrium and ventricle varied, some had a distinguishable atrium and ventricle

contraction whereas others had more random motion. There was not a clear opening to the heart tube.

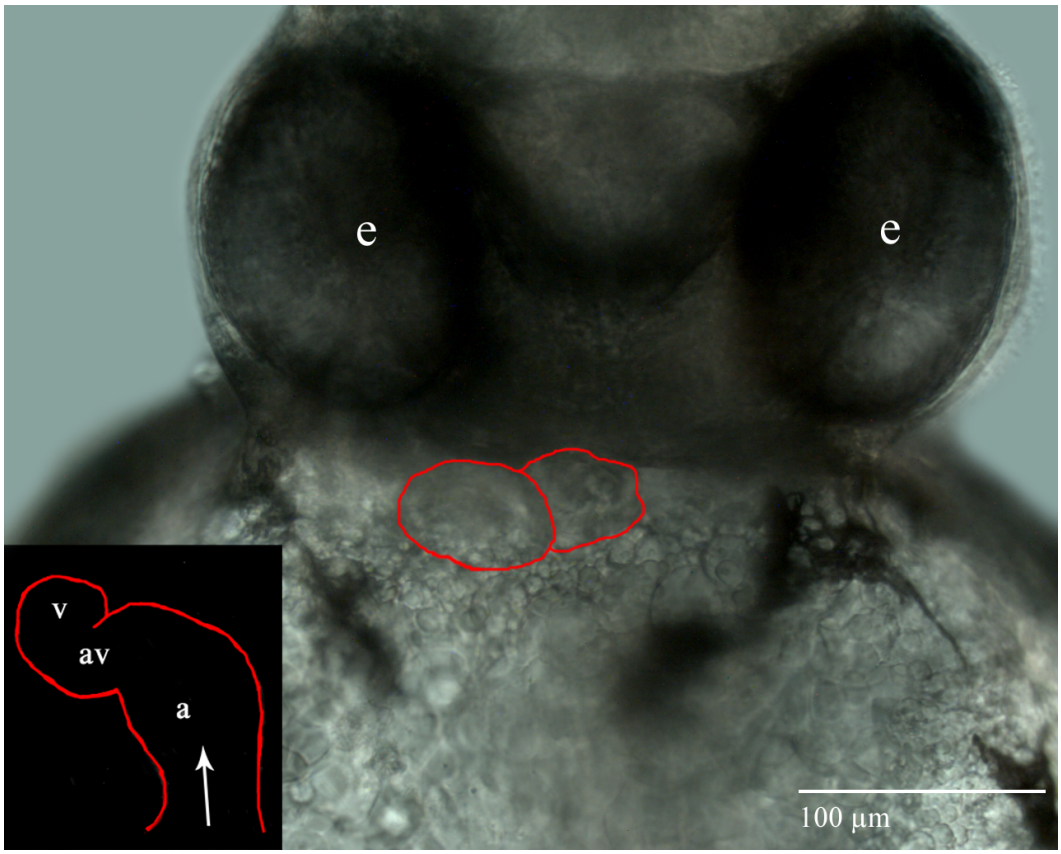

Figure 32: Small Heart at 3 days post-fertilization in an embryo treated with 10^{-6} M retinoic acid. The heart tube is shown from a ventral view of the embryo. Both the atrium and the ventricle are greatly reduced and as a result, the heart tube can not pump blood. The red lines outline the structure of the heart tube. The arrow indicates the flow of blood through the tube and the e indicates the location of the eyes, a indicates the atrium, av the AV canal, and v the ventricle. Inset shows the normal heart tube for comparison.

Linear Heart: Another group of embryos did not undergo heart looping and the heart tube remained straight (**Figure 33 and Movie 18**). The heart tube showed variation in chamber size. Some heart tubes had some ballooning out of the chambers, and some remained relatively small. In general, there seemed to be

two separate contractions in the atrium and the ventricle. These heart tubes tended to have decreased circulation when compared with normal development at this stage.

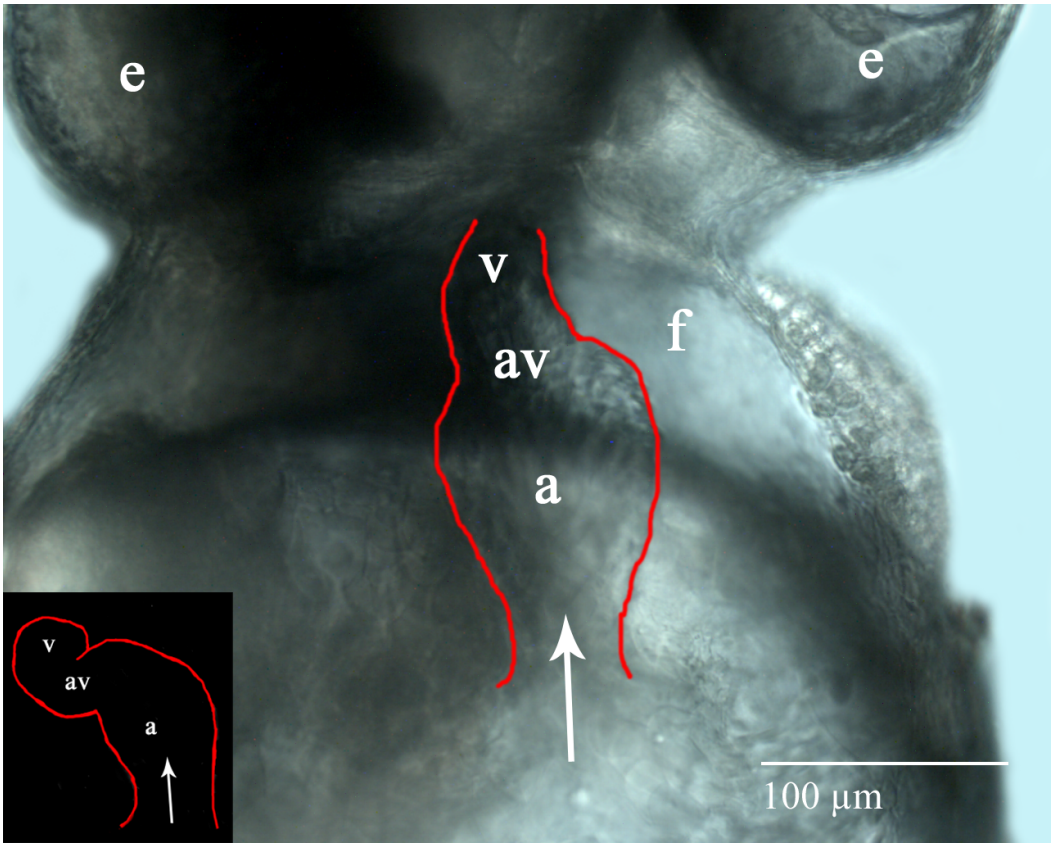

Figure 33: Linear Heart at 3 days post-fertilization in an embryo treated with 10^{-6} M retinoic acid. The heart tube is shown from a ventral view of the embryo. The heart tube does not undergo normal heart looping and there is a variance in whether or not the chambers balloon out. The red lines indicate the structure of the heart tube. The arrow indicates the flow of blood through the tube and the e indicates the location of the eyes, a indicates the atrium, av the AV canal, and v the ventricle. Inset shows the normal heart tube for comparison.

III. Embryos Treated with 10^{-7} M Retinoic Acid

A) Embryos Treated with Retinoic Acid at 50% Epiboly

When embryos were treated with 10^{-7} M retinoic acid at 50% epiboly for 1 hour, 12% of the embryos developed abnormal hearts. Two distinct heart morphologies were observed: Wide Atrium and Linear Heart. **Wide Atrium:** In embryos where the atrium region was greatly expanded there tended to be no circulation at 36 hpf and cells simply moved back and forth in response to pressure changes. Usually by 55 hpf or 3 days post-fertilization circulation did commence. These embryos did not undergo normal heart looping and the tube seemed to shrink inside and become very narrow. **Linear Heart:** In embryos where the heart tube was straight and narrow, the heart tube was less effective at pumping blood. Only a few blood cells moved through the heart tube with each contraction. The Linear Heart heart tube morphology did not undergo normal ballooning out and heart looping.

B) Embryos Treated with Retinoic Acid at Dome Stage

When embryos were treated with 10^{-7} M retinoic acid at dome stage for 1 hour, 26% of embryos exhibited abnormal heart tube morphologies. There were three distinct abnormal heart morphologies: Wide Atrium, Linear Heart, and Small Heart. **Wide Atrium:** The atrium was enlarged with a wide opening. This morphology had no circulation at 36 hpf, but by 3 days post-fertilization, circulation was strong. There was a variation in whether or not embryos underwent normal heart looping. **Linear Heart:** The heart remained straight and

did not undergo normal heart looping by 55 hpf, but still had a strong circulation. There was not normal ballooning out of the chambers. Heart tubes remained linear at 3 days post-fertilization. **Small Heart:** There was a mass of quivering cells and the heart tube was not able to pump blood cells.

IV. Embryos Treated with 10^{-6} M Retinoic Acid for 2 hours

When embryos were treated with 10^{-6} M retinoic acid for 2 hours at dome stage, 97% of embryos exhibited abnormal hearts or no hearts. Four distinct heart tube morphologies were observed as a result of exposure to this concentration of retinoic acid: Wide Atrium, Small Heart, Linear Heart, and No Heart. **Wide Atrium:** The atrium was enlarged and the ventricle was truncated. These heart tubes had essentially no circulation at 36 hpf and no blood cells could be seen moving through the heart tube with each contraction. Also many of these abnormal hearts did not undergo normal heart looping and/or ballooning out of the chambers by 3 days post-fertilization. One group of embryos were remarkable because the heart tubes had enlarged atriums and significantly reduced ventricles simultaneously (**Figure 34 and Movie 19**). This heart tube morphology was only seen in embryos treated for 2 hours. **Small Heart:** Both the atrium and the ventricle were significantly truncated. There was no circulation in these embryos and contraction patterns were irregular. **Linear Heart:** The heart remained straight and did not undergo normal heart looping, but still had a strong circulation. **No Heart:** A significant number of embryos did not have a detectible heart.

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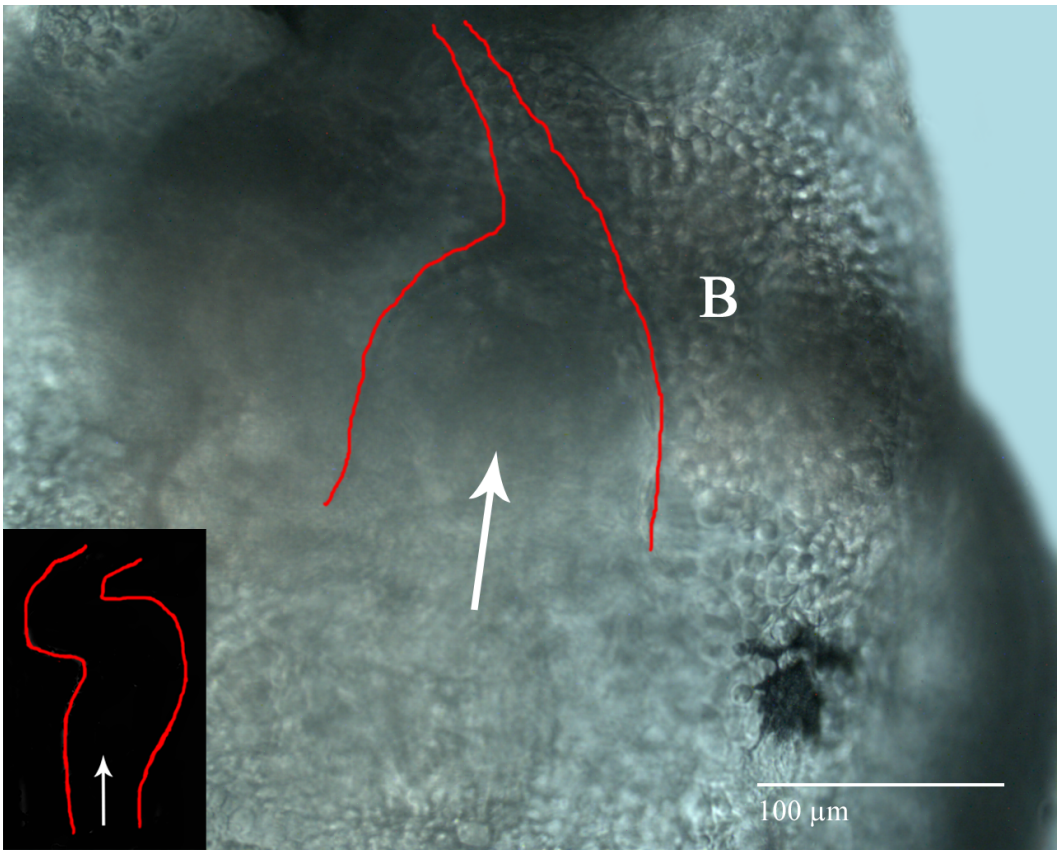

Figure 34: Wide Atrium at 55 hpf treated with 10^{-6} M retinoic acid for 2 hours. The atrium is greatly expanded and the ventricle is truncated as indicated by the red lines. The heart tube is contracting but no blood cells are being pumped through the heart tube. Blood cells surround the heart tube and move back and forth in response to pressure changes. The arrow indicates the flow of blood through the heart tube and B marks the position of the blood cells. Inset shows the normal heart tube for comparison.

V. Embryos Treated with 10^{-5} M Retinoic Acid

When embryos were treated with 10^{-5} M retinoic acid 100% of embryos did not have a detectible heart.

VI. Summary of Retinoic Acid Results

Twenty-one experiments were performed where embryos were treated with excess retinoic acid (**Table 4**).

Table 4: Summary of results from retinoic acid experiments

Concentration Of Retinoic Acid	**# Of Experiments**	**Stage of Treatment with Retinoic Acid**	**Exposure Time**	**# Of Embryos W/ Abnormal Heart**	**# Of Embryos W/ Normal Heart**	**Percentage of Abnormal Hearts**
DMSO	1	Dome Stage	2 hr.	0	20	0%
10^{-8}	1	50% Epiboly	1 hr.	0	0	0%
10^{-8}	1	Dome Stage	1 hr.	0	60	0%
10^{-7}	3	50% Epiboly	1 hr.	2	15	12%
10^{-7}	3	Dome Stage	1 hr.	9	25	26%
10^{-6}	8	Dome Stage	1 hr.	40	43	48%
10^{-6}	3	Dome Stage	2 hr.	68	2	97%
10^{-5}	2	Dome Stage	1 hr.	15	0	100%

Reynolds Number Calculation

The retrograde flow exhibited by many of the abnormal heart tube morphologies indicated that inertial effects were not influencing blood flow through the heart tube. A Reynolds Number calculation was performed for five normal heart tubes at 55 hpf, and it was determined that the Reynolds Number was very low, confirming that inertial effects were negligible (**Table 5**). Because

the structures measured were so small (length of the atrium), the Reynolds Number for the abnormal heart tube morphologies would be essentially the same, therefore they would also not be influenced by inertial effects.

Table 5: Determination of Reynolds Number

Length of Tube (Meters)	Velocity (Meters/Second)	Reynolds Number
4.2×10^{-4}	8.40×10^{-4}	0.396
3.62×10^{-4}	7.24×10^{-4}	0.294
3.82×10^{-4}	7.64×10^{-4}	0.330
5.35×10^{-4}	1.07×10^{-3}	0.643
4.02×10^{-4}	8.04×10^{-4}	0.363

Sample calculation of Reynolds Number:

$Re = \rho v L / \mu$

ρ = density of water (1000 kg/m^3)
v = velocity
L = length of atrium
μ = dynamic viscosity of water (8.9×10^{-4} Pa X s)

$Re = (1000\ kg/m^3)\ (8.40 \times 10^{-4})\ (4.2 \times 10^{-4}) / (8.9 \times 10^{-4}\ Pa \times s) = 0.396$

Fluorescence Markers

In an effort to visualize the tube when it is positioned deep inside the embryo obscured by the yolk sac, two fluorescent stains were used: Mito Tracker Red which labels mitochondria, and $diOC_6$ which labels mitochondria and endoplasmic reticulum. If heart muscle cells had high numbers of mitochondria,

these fluorescent probes might show preferential staining. It was observed that there was staining in many individual cells scattered throughout the entire embryo, but no heart cells were labeled. The yolk sac had the most labeled cells. In general, it was found that $diOC_6$ stained more cells than Mito Tracker Red (**Figure 35A and 35B**). Embryos were then stained with $diOC_6$ for 10 and 20 minutes to see if more cells could be visualized with prolonged exposure to the stains (**Figure 35C and 35D**). It was found that with increased exposure time there was an increase in the number of cells stained in the 10 and 20 minute embryos, but the heart cells were still not labeled with increased exposure.

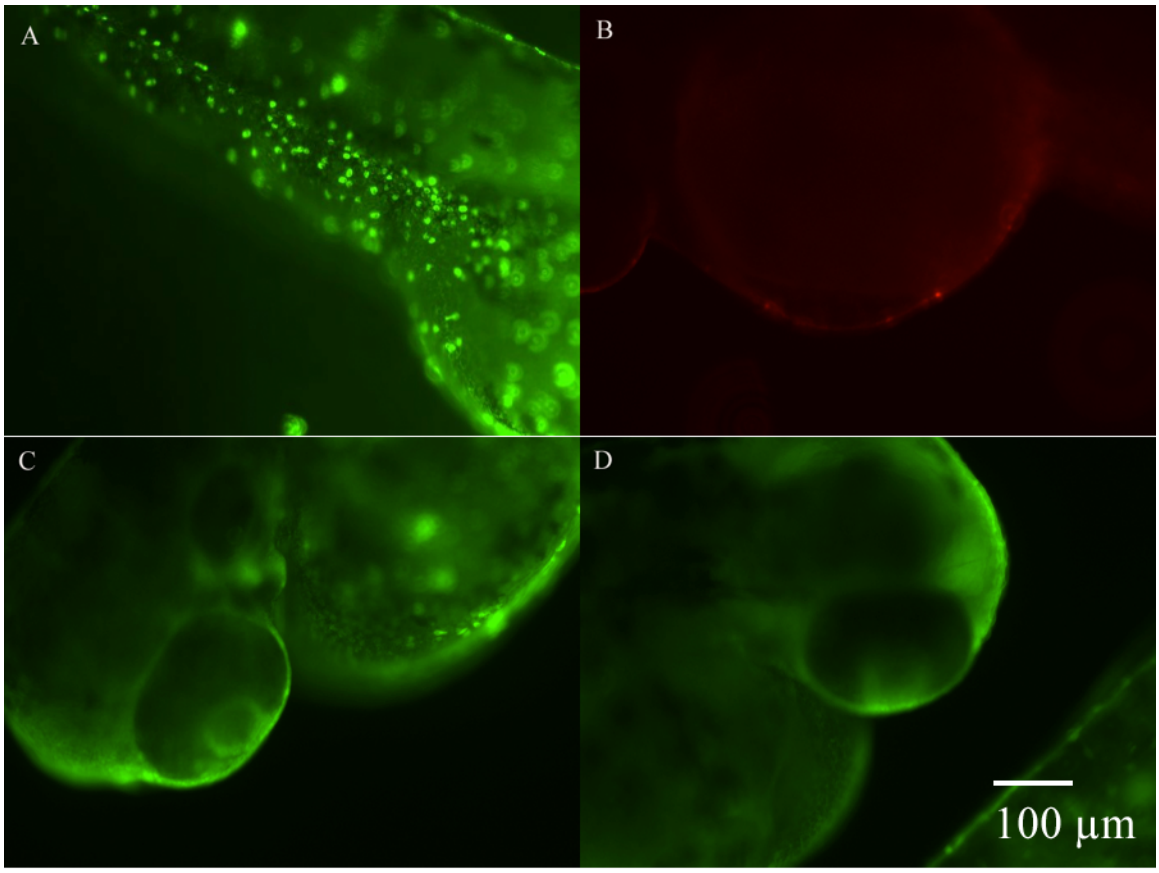

Figure 35: Fluorescence staining with $diOC_6$ and Mito Tracker Red. The embryos (approximately 30 hpf) were treated with 20µL concentration of $diOC_6$ (A) and Mito Tracker Red (B) in spring water for 4 minutes. Stained embryos were then placed in 0.2mg/ml MS-222 and put on a slide. Embryos were stained with $diOC_6$ for 10 minutes (C) and 20 minutes (D) to see if more heart cells were labeled with an increased exposure time.

DISCUSSION

Normal Development and Fluorescence

The early heart tube is positioned deep inside the zebrafish embryo and because of this placement it was difficult to visualize the tube. It was not until 30 hpf that the heart tube could be seen contracting with blood cells moving through it. At 36 hpf, the heart tube had transitioned from the dorsal left side of the embryo and moved towards the frontal side, making it much easier to visualize. By 55 hpf, the heart tube was positioned on the front/ventral side of the embryo and had undergone normal heart looping to form an S-shaped tube with ballooned out chambers. By 3-days post-fertilization, the heart tube was fully functional with defined chambers. These results are consistent with the described developmental stages in the literature (Auman et al., 2007; Smith et al., 2008; Bakkers, 2011). The image quality relied heavily on the proper positioning of the embryos. It was often difficult to focus on the heart tube even though the embryos were transparent. The attempt to visualize the heart cells using fluorescent markers was ineffective heart cells were not selectively stained. In future investigations staining the heart tube cells for atrium and ventricle specific markers as seen in Waxman and Yelon would help to better visualize the formation of the heart tube (2009). It would be particularly helpful at the earlier stages when the heart tube is positioned deep inside the embryo.

The time-lapse imaging of embryos demonstrated the transition of the heart tube from the dorsal side of the embryo to the ventral side. During filming

on the microscope, embryos experienced room temperatures of approximately 23 °C; this was significantly colder than the incubator (27 °C). Because of this temperature variation, there was delayed development compared with previous studies (Kimmel et al., 1995). To achieve more accurate recordings, it would be important to maintain the microscope room at 27 °C. Also, it was difficult to keep the embryos from moving over long observation periods even if they were anesthetized with MS-222. When embryos where immobilized in agar they were crushed as they developed which did not allow for effective imaging and could have impaired development. Methylcellulose was effective at holding the embryos for real-time recordings, however, for time-lapse imaging, the embryos were not immobilized. An investigation into alternative immobilization techniques is necessary to obtain better time-lapse recordings.

Retinoic Acid: General Trends

The results presented here indicate that excess retinoic acid signaling affects normal heart development. Control embryos which were treated with DMSO exhibited 100% normal heart development. More specifically, it was observed that heart development is affected by the concentration of retinoic acid. As the concentration of retinoic acid increased, the percentage of embryos with abnormal heart morphology or no detectible heart increased. Also, the length of exposure time seemed to have a significant effect on the severity and number of abnormal heart tubes that developed. When embryos were treated for 2 hours at

the dome stage with 10^{-6} M retinoic acid, 97% of embryos had abnormal hearts or lack of detectible hearts, whereas when embryos were treated with the same concentration for only 1 hour, only 48% of embryos had abnormal hearts. Also, it was observed that the stage at which retinoic acid treatment is performed was significant because when embryos were treated with the same concentration at both dome stage and 50% epiboly, the earlier-stage embryos had 26% abnormal hearts, whereas in the older stage embryos, only 12% developed abnormal heart tubes.

There were three different levels of severity of abnormal heart morphology. Level 1 was the most severe case where there was a trend of heart tube truncation with increased retinoic acid exposure due to either increased concentration, in the 10^{-6} and 10^{-5} M treatments, or length of exposure, in the 2 hours as compared to the 1 hour treatments. The highest concentrations and the longest exposure time seemed to produce the most heart tube morphologies which were called Small Hearts. The Small Hearts had significantly reduced atriums and ventricles (**Table 6**). If exposure time was increased to 2 hours or concentration was raised to 10^{-5} M retinoic acid, these treatments caused such a significant truncation of the heart tube, that the heart tube could not be detected. Both of these results suggest that the most severe effect of retinoic acid was to decrease the number of cardiomyocytes. This is consistent with the findings of Waxman and Yelon, who found that excess retinoic acid at high concentrations eliminates both atrium and ventricle cells (2009).

Table 6: Embryos Treated with Retinoic Acid at Dome Stage.

		Number Of Embryos With Heart Tube Morphology At 55 hpf				
Embryos Treated for 1 hour	**Concentration Of Retinoic Acid**	**Wide Atrium**	**Small Heart**	**Linear Heart**	**No Heart**	**Normal**
	10^{-7}	3	1	5	0	25
	10^{-6}	15	12	14	0	43
	10^{-5}	0	0	0	15	0
Embryos Treated for 2 hours	10^{-6}	20	14	0	34	2

Level 2 is an intermediate severity which was only seen in the embryos treated for 2 hours with 10^{-6} M retinoic acid (**Table 6**). This heart tube morphology had simultaneous significant reduction of the ventricle and enlargement of the atrium. This heart tube morphology was not able to pump blood and did not undergo normal heart looping. Because the total number of these embryos was small, they were included within the Wide Atrium embryos. To more fully understand this heart tube morphology, a closer investigation of the 2 hour treatments at 10^{-6} M retinoic acid is necessary.

Level 3 gave rise to the Linear Hearts and the Wide Atriums. These two heart morphologies were present when 10^{-7} and 10^{-6} retinoic acid was used, and with 2-hour exposure time, but not at the 10^{-5} M treatment. The 10^{-5} M treatment was the most extreme case where all of the embryos had no detectible heart

(**Table 6**). (2009). The Wide Atrium and the Linear Heart heart tube morphology presented in this investigation, may be similar to the LA and the MA heart tube morphology described by Waxman and Yelon (2009). Because Waxman and Yelon did not examine embryos after 48 hpf, they did not observe that the MA and LA heart tubes did not undergo normal heart looping. It is possible that the heart tubes seen by Waxman and Yelon, if followed for a longer time, would not have undergone normal heart looping. Both the Wide Atrium and the Linear Heart morphologies described in this investigation exhibited variation in normal heart looping thus, suggesting that retinoic acid plays an important role in normal heart looping.

It is important to recognize that the sample size in each of the treatments varied significantly. This was due to the fact that roughly 50 % of the embryos died within the first 24 hours after being collected in both the control and the retinoic acid treated embryos. In future studies, it would be important to try to increase the rate of survival of the embryos by putting the embryos in 10% Hank's Solution which may improve survival as described by Stainier and Fishman, 1992. Small sample sizes make it difficult to determine whether or not the effects seen with retinoic acid are statistically significant. In future experiments if there were enough embryos, a more quantitative description of the effects of retinoic acid could be made.

It is also important to note that some of the structural irregularities in the heart tubes could be the result of the variability in retinoic acid concentration

because the hydrophobic retinoic acid did not fully dissolve in water when the dilutions were made as discussed in the Methods and Materials. In future experiments it would be important to investigate whether there was a more effective way to administer the retinoic acid.

Retinoic Acid Affects Chamber Differentiation

The abnormal heart tube morphologies observed in this investigation indicate that retinoic acid plays an important role in the differentiation of cardiac cells. Waxman and Yelon saw that with excess retinoic acid signaling there was an increase in the number of atrial cells while the ventricle cell number remained the same in LA heart tubes, and that in the MA heart tubes there was a decrease in the number of ventricle cells while there was a normal number of atrium cells (2009). These results suggest that excess retinoic acid does not cause ventricle cells to be converted to atrium cells. However, the results presented in this investigation suggest that the effects of retinoic acid on heart tube morphology are more complicated than what Waxman and Yelon described. As presented previously, when embryos are treated with 10^{-6} M retinoic for 2 hours, both the atrium and ventricle cell populations are affected simultaneously. **Image 36 and Movie 20** clearly show that there can be an enlargement of the atrium and a significant reduction in the size of the ventricle simultaneously. This is not necessarily evidence to support the transformation of ventricle cells to atrium cells, but clearly both cell populations are being affected at the same time.

Perhaps, there is another population of cells, that when under the influence of excess retinoic acid, have the potential to become cardiac cells and can be transformed into atrial cells. This however, does not explain the reduction in the number of ventricle cells. The fate of the ventricle cell is a question that could be answered by future investigations.

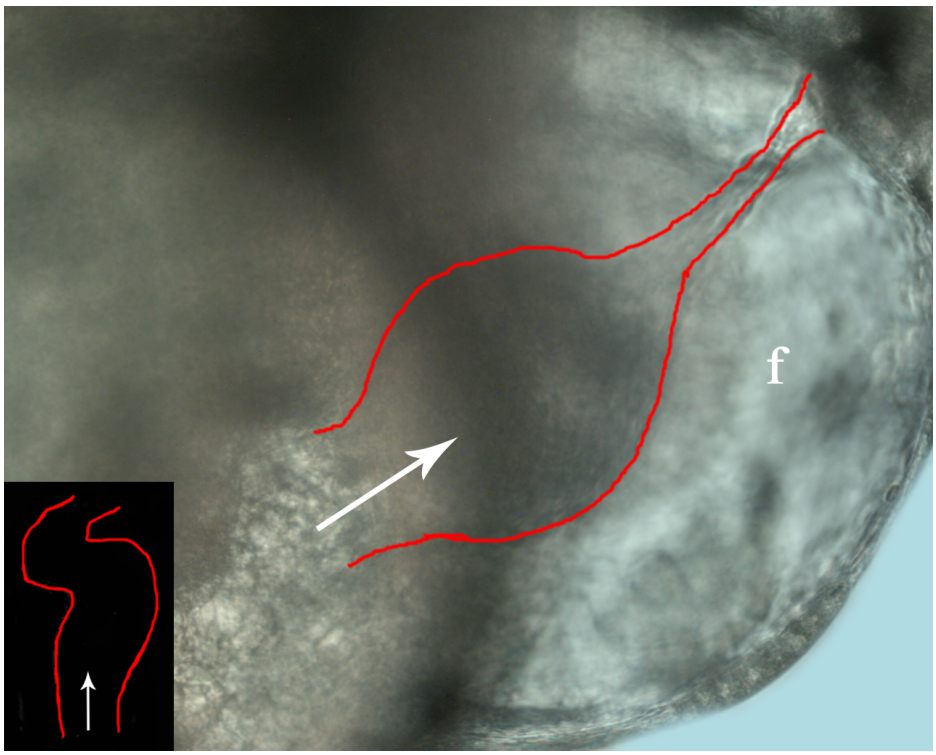

Figure 36: Wide Atrium with a truncated ventricle from the right side at 55 hpf treated with 10^{-6} M retinoic acid for 2 hours. The atrium is greatly expanded and the ventricle is truncated as indicated by the red lines. The arrow indicates the flow of blood through the heart tube and f indicates fluid collecting around the heart tube. Inset shows the normal heart tube for comparison.

To further understand how excess retinoic acid is affecting the ventricle cells, it would be important to have a method for counting the number of atrium and ventricle cells. The term truncated is used in this investigation to describe

heart tubes with ventricles which are smaller in size in comparison with normal development. However, the use of truncated is limited due to the fact that there is no way to determine if the truncated ventricles appear smaller due to the fact that the myocytes have not become elongated during ballooning out, or if there has been an actual reduction in the number of ventricle cells. In **Figure 36** it is clear there is an actual reduction in the number of cells, however, in many of the embryo it is not as obvious.

Chick embryos treated with excess retinoic acid also show an increase in the number of cells expressing atrium-specific markers resulting in a heart tube with an enlarged atrium and truncated ventricle (Yutzey et al., 1994; Hochgreb et al., 2003). In addition, when chick embryos were treated with an antagonist for the retinoic acid receptor, there was an increase in the size of the atrium and truncation of the ventricle (Hochgreb et al., 2003). It is important to note that this result is different from the heart tube morphologies in zebrafish which were treated with a retinoic acid receptor antagonist resulting in the expansion in the number of both the atrium and the ventricle cells (Waxman et al., 2008).

In both chick and mouse embryos the regional restriction of retinoic acid signaling to the posterior heart field is thought to lead to the distinction between pre-atrium and pre-ventricle cells (reviewed in Simoes-Costa et al., 2005). Experiments have found that in mutant *RALDH2* mice, the atrium is significantly truncated suggesting that retinoic acid is a necessary signal for atrium cell differentiation (Niederreither et al., 2001). Further evidence supporting the

relationship between retinoic acid and atrium cell differentiation was observed when the expression of the gene, *Tbx5*, which is an atrium specific marker, is reduced in *RALDH2* mutant mice. This suggests that retinoic acid signaling is critical for the normal formation of the atrium in mice. Similar results were also seen in chicks, where atrium-specific determinants were expressed in cells which also expressed *RALDH2,* indicating that retinoic acid has the same function in chicks and mice (Hochgreb et al., 2003). It has been suggested that ventricle cell formation may be the default because when there is no retinoic acid signaling, ventricle cells fail to differentiate.

Lack of Hemodynamic Stimuli Affects Heart Looping

The Wide Atrium, Linear Heart, and Small Heart heart tube morphologies showed decreased and/or no circulation. The calculation of the Reynolds Number showed that inertial effects were not influencing the flow of blood through the heart tube; therefore, this helps to explain why there was retrograde blood flow in some of the abnormal heart tubes which had decreased circulation. Experiments have shown that normal morphological development of the heart tube is influenced by hemodynamic pressures generated by the flow of blood through the heart tube (Miyasaka et al., 2011). This is called mechanotransduction, where external mechanical forces produced by blood flow act as a signal to regulate gene expression inside the heart tube cells. A molecular investigation of the effects of hemodynamic stimuli in the heart tube have shown

that in zebrafish, there is the expression of the microRNA, miR-143, in the ventricle and the outflow tract of the developing heart tube only when there is blood circulation (Miyasaka et al., 2011). When circulation is stopped, there is no expression of miR-143. When miR-143 expression is decreased by stopping the heart tube from contracting, abnormal heart tube development occurs (Miyasaka et al., 2011). As a result, the heart tubes have dilated atriums, ventricles which do not undergo ballooning out, and abnormal heart looping at 48 hpf. One of the explanations presented for these embryos which do not undergo normal heart looping due to excess retinoic acid signaling is that the ventricle cells become transformed into cells which are more atrium-like (Miyasaka et al., 2011). It is important to note that the excess retinoic acid signaling seen by Miyasaka et al. is distinct from the excess retinoic acid signaling performed in this investigation. Miyasaka et al. stopped the heart tube from beating at 24 hpf, when it is then speculated that excess retinoic acid signaling was induced (2011). This is at a much later stage in development than when the embryos where treated with retinoic acid at dome stage in the current investigation. Retinoic acid signaling is important in heart development at multiple stages during heart development.

Miyasaka et al. present evidence to support a transition where ventricle cells are transformed into atrium cells. This transformation was found when the expression of the microRNA, miR-143 was decreased (2011). This molecule has been found to have a negative regulatory effect on the expression of the gene, *RALDH2*. The inhibition of miR-143 indirectly causes excess retinoic acid

signaling through the lack of negative regulation. Normally, a gradient of retinoic acid signaling is established in the heart tube at 36 hpf, but due to the fact that miR-143 is not being expressed, this gradient is altered. The gene, *atrial myosin heavy-chain* (*amhc*), is an atrium-specific marker, and in the heart tubes where miR-143 expression has been inhibited, cells in the ventricle region also expressed this gene (Miyasaka et al., 2011). This indicates that the ventricle cells may have been transformed to be more atrium like. If this transformation has taken place, this could explain why the heart tube morphologies Wide Atrium and Linear Heart did not undergo normal heart looping in many of the embryos. Due to the lack of mechanical stimuli as a result of poor or no circulation in many of the abnormal heart tubes, the ventricle cells may not have been expressing miR-143, thus altering the retinoic acid gradient in the heart tube. This then led to ventricle cells becoming more atrium like and as a result, heart looping did not occur. The use of atrium and ventricle specific fluorescent markers as described by Waxman and Yelon, could help to confirm this speculation (2009).

Experiments have shown abnormal heart looping in mutant mouse embryos which have defects in the contractile mechanisms of the heart cells (Nishii et al., 2011). These mutants had dilated atria irregularities in the walls of the heart tube. However, in the zebrafish mutant, *silent heart***,** the muscle in the heart tube does not contract due to the fact that there is a lack of thin filaments and the thick filaments are loosely arranged in the sarcomeres (Sehnert et al., 2002). In these embryos, normal heart development was observed even though the

heart tube could not contract. Clearly, there is a controversy as to the effects of hemodynamic stimuli on heart looping. Therefore, further research is needed.

Insufficient hemodynamic stimuli could also explain why there was a variation in whether or not the Wide Atrium heart tube morphology underwent normal heart looping. Both at 55 hpf and 3 days post-fertilization, there was a group of embryos which had enlarged atriums and underwent heart looping. Some of the embryos may have received a strong enough signal from blood flow through the heart tube to trigger normal heart looping. The observed variation in heart looping opens up an opportunity to further investigate the time period during which cardiac cells have the ability to respond to a mechanical signal and then undergo heart looping. It would be interesting to test whether there is a window of time during heart development when the cardiac cells are able to respond to this signal. Also, experiments could be designed to investigate the strength of the mechanical signal to determine how much circulation is needed to produce effective mechanical stimuli. The strength and length of the signal may play an important role in whether or not the heart tube undergoes looping, by affecting the number of ventricle cells present in the heart tube. It was observed that in some of the embryos which did not have circulation at 55 hpf that circulation was initiated by 3 days post-fertilization. However, close attention was not paid to whether or not linear heart tubes at 55 hpf, subsequently underwent heart looping later on in development when circulation was initiated. In future investigations it would be important in investigate whether or not the initiation of circulation would then

induce heart looping after 55 hpf. To better explain the variations in heart looping observed in this study, a closer investigation of blood flow and its effect on heart looping is necessary.

Insufficient Hemodynamic Stimuli Affects Cell Shape

The observed abnormal heart tube morphologies presented in the current investigation suggest that excess retinoic acid has an effect on cell shape. The process of chamber ballooning out and heart looping is mediated by changes in cell shape (Auman et al., 2007). It is thought that the elongation and the enlargement of the ventricle cells contribute to the formation of the ballooned out chambers and when circulation is reduced, cardiomyocytes do not undergo normal elongation and expansion. The surface area of developing ventricular myocytes has been measured at 24, 36, and 48 hpf, and it has been observed that the cell surface area does increase between these stages (Lin et al., 2012). Also, experiments have shown that the remodeling of cell shape in the ventricle is triggered by the actual physical force of blood cells moving against the endocardium which is then transferred to the myocardium, causing the cardiomyocytes to be stretched with changes in pressure (Lin et al., 2012). The cardiac cells are thought to increase in size as a result of this stimulus. There are two populations of cardiomyocytes in the heart tube which are molecularly distinct and undergo elongation and expansion allowing for the formation of the curvature of the chambers (Auman et al., 2007). Also, as the size of the

cardiomyocytes increases during chamber ballooning, it is thought that myofibril maturation occurs (Lin et al., 2012). Between the stages of 24, 36, and 48 hpf, developing embryos showed increased striation in the cardiac muscle. It is thought that the cell shape changes during this time period in development are associated with myofibril maturation. Therefore, it is possible that the Wide Atrium and the Linear Heart heart tube morphologies did not undergo heart looping due to the fact that the cardiomyocytes did not undergo the cell shape changes as a result of poor or no hemodynamic stimuli. In future experiments, it would be important to investigate whether or not the heart cells were changing their shape. The use of fluorescent markers as described by Waxman and Yelon, 2008, might allow for better visualization of the individual cells in the heart tube. In addition, the fixation of embryos could allow for better visualization of individual cells. Also, many of the heart tubes exhibited abnormal contraction patterns which may have been due to the lack of myofibril maturation. In the abnormal heart tubes, the ventricle cells seemed to not contract as strongly. In chick embryos, excess retinoic acid signaling was also found to negatively affect myofibril maturation (Dickman and Smith, 1996).

Mechanical Stimuli in the Endothelium of Blood Vessels

The exact mechanism by which endocardial cells sense the mechanical stimuli in the heart tube is unknown, however, this has been more generally studied in the endothelium of blood vessels. It is thought that mechanical stimuli

might increase the permeability of macromolecules and change lipoprotein accumulation (reviewed in Resnick et al., 2003). Integrins, which are membrane proteins involved in linking the inside of the cell with the extracellular matrix, have been demonstrated to transfer mechanical signaling from outside the cell to the cytoskeleton (reviewed in Ingber, 1997). Local stress applied to integrins, such as deformation of blood vessel membranes due to blood flow, may cause cytoskeletal rearrangements in the cell which are then transmitted to the nucleus affecting gene expression. This disruption then changes the kinetics of the molecules associated with the cytoskeleton (reviewed in Ingber, 1998). This is an example of how a cell can use mechanical stimuli to alter the internal molecular environment of the cell. A similar mechanism could be found in the endocardium, but further research is needed to understand how the flow of blood, generating a shear stress force can then influence gene expression.

Proposed Model for Excess Retinoic Acid Signaling

Based on the results presented in this thesis and in combination with what other researchers have found when studying heart development in zebrafish, a model can be proposed to explain how excess retinoic acid affects heart tube morphology and function. When embryos are exposed to excess retinoic acid at the dome stage, which is a critical time in determination of cardiac cells, this alters the number of cardiac cells which will differentiate into either atrium or ventricle cells (Waxman and Yelon, 2009). As a result of changes in the

differentiation patterns in cardiac cells, the heart tube which forms is abnormally shaped: Wide Atrium, Linear Heart, Small Heart, and No Heart. These abnormal heart tube morphologies vary in their ability to pump blood cells which results in insufficient hemodynamic stimuli by the blood flow through the heart tube. As a result of the lack of mechanical signaling, the cells in the myocardium do not undergo normal enlargement and myofibril maturation which alters the chambers size and contractibility (Auman et al., 2007; Miyasaka et al., 2011; Lin et al., 2012). As a result, normal heart looping does not take place which also contributes to chamber formation. A model is presented here summarizing the effects of excess retinoic acid (**Figure 37**).

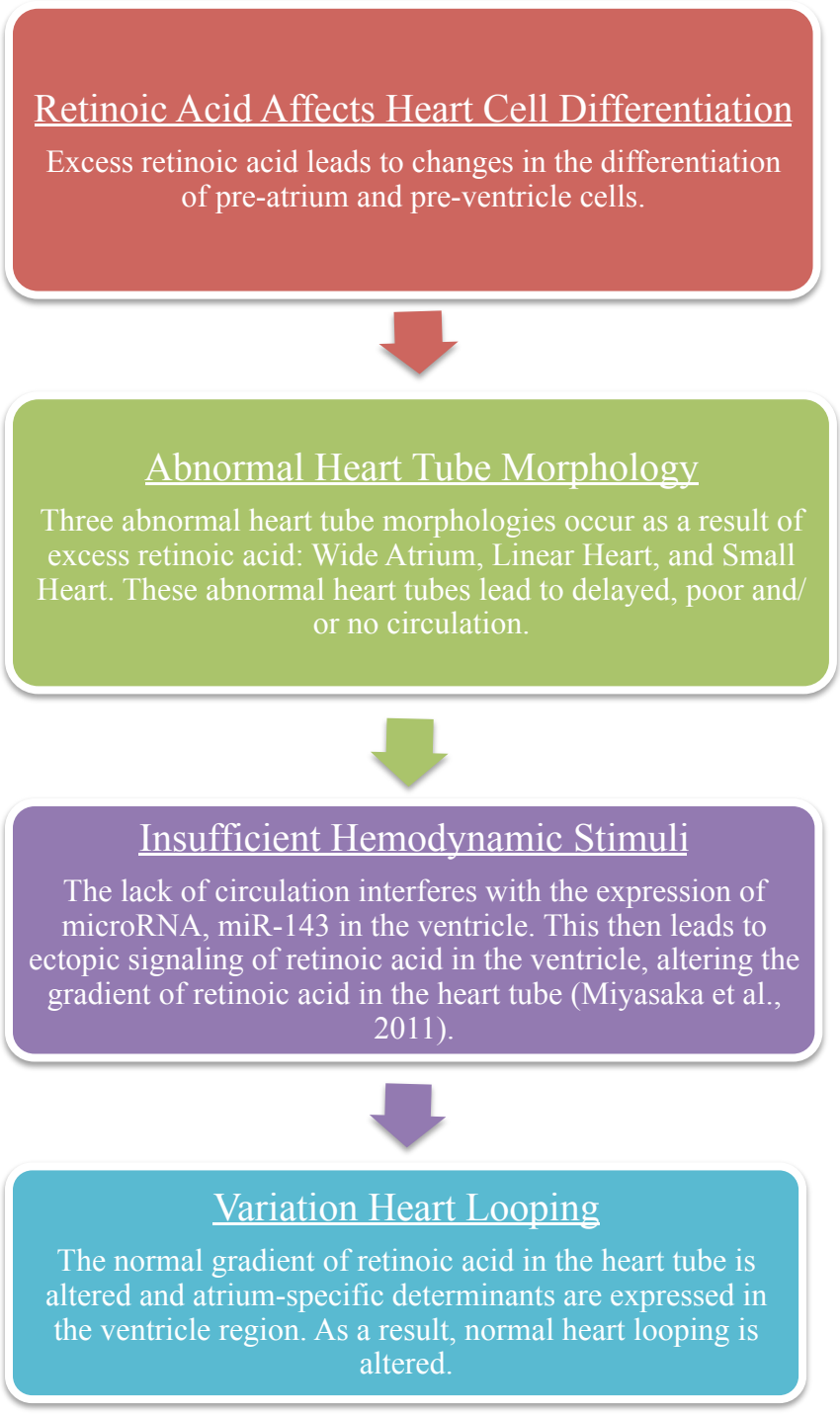

Figure 37: The effects of excess retinoic acid on heart tube morphology and function.

Development of the Inflow Tract

In both the Small Heart and Wide Atrium heart tube morphologies, the inflow tract of the heart tube seemed to be structurally altered. In Small Heart embryos, most of the heart tubes were not able to pump blood cells effectively, which may have been the result of a closed off opening to the inflow tract. In Wide Atrium embryos, the opening of the inflow tract was greatly expanded. In avian embryos, experiments have found that retinoic-acid-deficient embryos develop heart tubes which are closed off and have deletion of the atrial cells (reviewed in Zile, 2010). This heart tube morphology also results in no circulation. Zile described how researchers have found that when retinoic-acid-deficient embryos were exposed to retinoic acid at critical periods during development, normal heart development occurred. Perhaps in zebrafish, excess retinoic acid has a similar effect mediating the connection between veins which connect to the atrium. The Wide Atrium embryo may be a less severe response to retinoic acid exposure resulting in an expanded opening to the inflow tract. The Small Heart embryo could be the more extreme response to retinoic acid where there was a significant reduction in the size of the atrium which also did not appear to have a clear opening into the atrium. This idea is again supported by Waxman and Yelon, when they found that high concentrations of excess retinoic acid caused the deletion of the atrium and the ventricle as a severe response, and that moderate concentrations of excess retinoic acid caused only the enlargement of the atrium as a less severe response (2009).

Many of the embryos also exhibited abnormalities in the veins which connected to the inflow tract of the heart tube. In some of the embryos treated at 10^{-6} M retinoic acid, blood only flowed from one side of the yolk sac, instead of normal blood flow from both sides. In addition, many of the embryos exhibited a large amount of fluid and blood cells on the surface of the yolk sac and around the heart tube. In many of the embryos, there did not seem to be defined vessels leading into the heart tube. This is evidence to support the idea that excess retinoic acid signaling has an effect on the connection between the inflow tract and the veins. In **Movie 19**, blood cells can be seen moving freely around the heart tube as it contracts. There were no clear vessels that contained blood and the blood cells seem to pool around the yolk sac and heart tube. In addition, some of the embryos developed abnormally deep channels which the blood flowed through on the yolk sac. This further suggests that retinoic acid effects the formation of the veins connected to the heart tube. Investigations in mouse embryos have shown that *RALDH2* mutants have decreased endothelial cell maturation and poor blood vessel formation on the yolk sac indicating that retinoic acid is also important in vessel formation (Lai et al., 2003).

The Implications of Retinoic Acid Signaling in Human Cardiac Development

In humans, 10% of early miscarriages are thought to occur due to severe heart abnormalities, which may be related to abnormal retinoic acid signaling (reviewed in Zile, 2010). There has been no direct observation that these heart

abnormalities are similar to the defects presented here. Further, there are many heart diseases in humans which are a result of heart cell enlargement and proliferation (Soufan et al., 2006). This suggests that investigation into the effects of retinoic acid signaling may be important in understanding heart development in humans.

Conclusions

The results presented in this investigation show how the signaling molecule, retinoic acid, plays an important role in heart cell differentiation. Real-time and time-lapse recordings allowed for the assessment of heart tube structure and function as development proceeded. These recordings visually documented a new important connection between how excess retinoic acid signaling leads to insufficient mechanical stimuli in the heart tube resulting in abnormal heart looping. In addition, these results have created many new important opportunities to further investigate how retinoic acid effects heart development. The investigation of the effects of excess retinoic acid signaling on heart development in zebrafish has highlighted how developmental processes rely on a complicated cascade of events in order for normal development to occur.

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