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Interrogating Cardiovascular Morphogenesis in Zebrafish Through Small Molecule Perturbation

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The thesis of Richard Shehane is approved and it is acceptable in quality and form for publication on microfilm and electronically.

Chair

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Dedication

This thesis is dedicated to my loving wife Colleen.

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ABSTRACT OF THE THESIS

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Congenital heart disease can originate with errors in embryonic cardiac morphogenesis. A critical step in heart formation is the migration of bilateral populations of cardiomyocytes to the midline where they fuse to initiate heart tube assembly. Although some genes have been shown to influence cardiac fusion, our understanding of this aspect of cardiac morphogenesis remains incomplete. The identification of small molecules that perturb cardiac fusion could provide further insight into the pathways regulating the early steps of heart formation.

Through a small-scale screen of 276 compounds, we have identified two compounds, ketanserin and scopoletin, that disrupt early cardiac morphogenesis. Treatment of embryos with ketanserin results in an increase in the circumference of the myocardial ring at the completion of cardiac fusion, accompanied by defects in the sheet of endocardial cells that will later line the heart tube. Ketanserin treatment also results in blood regurgitation at the atrioventricular (AV) valve and ectopic expression of atrioventricular canal (AVC) markers in the ventricle. Embryos treated with scopoletin exhibit a dysmorphic myocardial ring and defects in endocardial sheet integrity. In addition, FACS analysis revealed that scopoletin treatment leads to an increased number of erythrocytes in the early embryo.

This work demonstrates the utility of small molecule screening for the identification of novel modulators of cardiac fusion and for the elucidation of potential connections between the regulation of cardiac fusion and other cardiovascular processes. Future studies will illuminate further details about the precise molecular mechanisms by which ketanserin and scopoletin can influence cardiovascular development.

1.

INTRODUCTION

Congenital heart disease is a common type of birth defect that causes thousands of infant deaths each year [1]. However, the origins of these defects are not well understood. Many types of congenital heart disease are thought to result from defects in early cardiac morphogenesis [2]. Therefore, developing a greater understanding of how the heart forms is important for elucidating the causes of cardiac birth defects.

In all vertebrates, early heart formation begins with the specification of bilateral populations of cardiac progenitors in the lateral plate mesoderm [3]. These progenitors will eventually give rise to the myocardium, which forms the muscular component of the atrium and ventricle, and the endocardium, which forms the lining of the cardiac chambers and the cardiac valves. Once specified, the bilateral populations of cardiomyocytes migrate to the midline of the developing embryo and then subsequently undergo fusion and extension, resulting in the formation of the primitive heart tube. The tube then loops to form distinct cardiac chambers separated by an atrioventricular valve [4]. Problems during any one of these stages could lead to cardiac birth defects. In this thesis, we sought to gain a deeper understanding of the mechanisms behind cardiac fusion using the zebrafish as a model organism, which will hopefully provide insight into some aspects of the causes of congenital heart disease.

Cardiac fusion

In zebrafish, myocardial cells residing in bilateral portions of the anterior lateral plate mesoderm migrate medially and fuse to form a coherent cardiac ring

[5]. This migration is defined by two distinct types of directional movement, medial and angular [6]. Bilateral cardiomyocyte populations initially migrate in a medial direction (Figure 1A,B). Upon approaching the midline, the anterior and posterior portions of cardiomycytes on both sides switch to an angular direction in order to form a ring-like conformation of myocardium centered at the midline (Figure 1C,D)[6]. The formation of a cardiac ring of proper dimensions is a critical step in cardiac morphogenesis. An improperly designed cardiac ring can lead to defects in the morphology of the primitive heart tube and potentially affect later cardiac function [6].

During the process of cardiac fusion, the formation of the endocardium largely parallels that of the myocardium. Like the myocardium, the endocardium arises from the ALPM [7]. Endocardial cells rapidly migrate posteriorly and towards the midline, where they will be joined by the bilateral myocardial populations. The endocardium forms a sheet of cells dispersed uniformly across the dorsal midline of the embryo. At the same time, the myocardial cells, located dorsal to the endocardial sheet, begin their process of fusion into the cardiac ring. As the myocardial cells undergo fusion and subsequent extension, the endocardial sheet begins to migrate leftward and anteriorly to form the lining of the heart tube.

Analysis of zebrafish mutants has demonstrated two requirements for medial migration of cardiomyocytes during cardiac fusion. One group of mutations (including *casanova, bonnie and clyde, faust,* and *miles apart*) disrupt the formation of anterior endoderm and result in cardia bifida phenotypes [8-12]. In

mutants with cardia bifida, the bilateral cardiomyocyte populations never migrate to the midline and instead establish separate hearts in their respective bilateral positions. A second group of mutants (including *natter* and *hands off*) disrupt the extracellular matrix (ECM) around the cardiomyocytes, and these mutants also display cardia bifida phenotypes [13, 14]. Together, these phenotypes provide evidence for the roles of myocardium-endoderm and myocardium-ECM interactions in proper cardiac fusion.

Requirements for angular migration can be demonstrated by another zebrafish mutant known as *cloche* [6]. Analysis of *cloche* mutants reveals normal medial movement but a lack of angular movement, resulting in a dysmorphic cardiac ring. Because *cloche* mutants lack endocardium, they provide evidence for the role of myocardium-endocardium interaction to properly direct angular movements.

Other signaling pathways have been shown to influence migration of both endocardial cells and myocardial cells, as well as the subsequent formation of the heart tube. Experiments in morphants indicate that Slit2 and Robo1 are both required for proper cardiac migration, fusion, and extension [15]. Also, perturbing Vegf signaling through morpholino or small molecules prevents proper cardiac fusion. Since it seems that Slit/Robo signaling mediates the endocardial response to Vegf, these studies provide evidence for the role of a Slit/Robo/Vegf regulatory loop that influences cardiac fusion [15].

The transmembrane protein Tmem2 has also been shown to be important for proper myocardial and endocardial migration during cardiac fusion [16]. The

myocardial and endocardial cells of maternal-zygotic *tmem2* mutants do not migrate properly, resulting in cardia bifida. Interestingly, in zygotic *tmem2* mutants, atrioventricular valve formation is also disrupted, with ectopic atrioventricular canal characteristics in the ventricle, providing evidence for a role of Tmem2 in valve formation as well as in cardiac fusion [16, 17]. However, the relationship of Tmem2 with other regulatory influences on cardiac fusion is unknown because the molecular functions of Tmem2 have yet to be identified.

Small molecule screens in zebrafish

Although several regulatory influences have already been shown to affect cardiac fusion, it seems likely that additional signaling pathways could also play important roles. In order to gain novel insights into the pathways regulating this process, we utilized a small molecule screen to identify compounds that perturb the pathways regulating the morphology of the cardiac ring.

There are several benefits of conducting a small molecule screen in comparison to the classical forward genetics approach of screening a population of mutagenized embryos [18]. Mutant screens are time consuming and require the breeding of several generations in order to analyze individual point mutations. In vivo small molecule screens allow scientists the ability to assay the biological activity, toxicity, and off-target global effects of a compound from the time of the initial screen in a high-throughput fashion. Small molecules also allow the perturbation of a pathway during a specific time period, a strategy for elucidating

the mechanism of action and specificity of a pathway that is not available in mutant screens.

The zebrafish is a useful model organism that possesses molecular and physiological parallels to mammals and has the added benefits of external development of transparent embryos, high fecundity, and permeability to small molecules added to the embryo medium [19]. Thus, the zebrafish is ideally suited for small molecule screens. Small molecule screens in zebrafish have already led to the discovery of compounds that affect the developing heart [18]. For example, cardinogen-1 and cardinogen-3 were discovered via small molecule screen and have been shown to promote cardiogenesis by inhibiting Wnt/β-catenin dependent transcription [20]. We hypothesized that by screening for small molecules that perturb cardiac migration and fusion in zebrafish we might gain additional insight into the pathways regulating these processes. In this thesis, we focus on the identification of two compounds, ketanserin and scopoletin, that affect the fusion of the cardiac ring.

Ketanserin

Ketanserin was initially developed in 1981 by Jannsen Pharmaceutical as a selective 5HT-2A serotonin receptor antagonist [21-25]. As expected, the use of ketanserin to antagonize receptors of the neurotransmitter serotonin has been shown to have widespread neurological effects including roles in pain attenuation, reversal of sensitization to drug addiction, and modulation of impulse control [26, 27]. Ketanserin has also been shown to have anti-hypertensive effects

and is used in Europe to treat women with gestational hypertension [28-30]. Understanding the effects of ketanserin during cardiovascular development is therefore vital to protecting the unborn fetus from side effects of the drug.

Studies of the effects of ketanserin on cardiac development are limited. A recent study has demonstrated a correlation between the treatment of pregnant women with ketanserin and hypotension in the neonate [31]. It has also been shown that ketanserin blocks ATP-dependent potassium channels in isolated ventricular myocytes of rats and dogs in culture [32]. Only limited data on ketanserin's actions in fish are available. One study focused on behavioral swimming patterns in zebrafish and noted that ketanserin increased periods of inactivity in the juvenile fish [33]. Another study in the mangrove killifish *Rivulus marmoratus* demonstrated that ketanserin caused a dose-dependent decrease in the diurnal excretion of urea; however, no data regarding ketanserin's effect on cardiac function in fish has been published [34].

Our data suggest that ketanserin treatment expands the inner circumference of the cardiac ring and also affects the migration and formation of the endocardium. At later stages, ketanserin treatment causes blood regurgitation at the atrioventricular canal, possibly due to ectopic atrioventricular differentiation in the ventricle. Thus, our studies provide novel evidence for the influence of ketanserin treatment on cardiac development.

Scopoletin

Scopoletin is a coumarin derivative isolated from several species of plants, including *Eupatorium buniifolium, Angelica japonica,* and *Sinomonium acutum* [35-37]. Plants containing scopoletin have been widely used in Eastern medicine to treat a wide variety of ailments including arthritis and joint pain, gastrointestinal disorders, nausea, vomiting, and headaches. Recent studies have elucidated further the anti-inflammatory and antioxidant properties of the compound [36, 38]. Interestingly, scopoletin has also been shown to have a cytostatic and cytotoxic effect on hyperproliferative T lymphoma cells in vitro, and, in the same study, it was shown that scopoletin induced the proliferation of normal lymphocytes, evidence for the immunomodulatory and possible anti-tumor properties of this compound [39]. The influence of scopoletin treatment on normal T lymphocytes may be related to protein kinase C (PKC) activation, since the activation of resting T cells to enter the cell cycle requires PKC stimulation [40, 41]. Additionally, staurosporine, a potent PKC inhibitor, can interfere with the proliferative properties of scopoletin treatment [39]. To our knowledge, no previous studies of scopoletin's action in zebrafish have been reported, and comprehension of the effects of scopoletin on cardiovascular development is lacking.

We report that scopoletin affects the size and shape of the cardiac ring by stretching the ring along its anterior-posterior and that scopoletin also affects the proper formation of the endocardial sheet. Data at later stages suggests that scopoletin enhances the number of primitive erythrocytes. Thus, our studies

provide novel evidence for the influence of scopoletin on cardiac morphogenesis and hematopoiesis.

2.

RESULTS

In situ small molecule screen for perturbators of cardiac morphogenesis

The proper migration of bilateral populations of cardiomyocytes to the midline of the developing zebrafish embryo and the fusion of these populations to form a coherent, intact cardiac ring is a critical step in establishing proper cardiac morphology [6]. We hypothesized that screening for small molecules that perturb the formation of the cardiac ring might identify compounds with potent effects on cardiac migration and fusion.

Our screen for compounds influencing cardiac fusion was performed in collaboration with an additional project focused on identifying small molecules that influence chamber specification. Therefore, we chose to investigate the effects of these compounds on the expression patterns of the chamber-specific genes *atrial myosin heavy chain (amhc)* and *ventricular myosin heavy chain (vmhc)* [5]. By examining *amhc* and *vmhc* expression at 22 somites, we were able to simultaneously assay effects on cardiac fusion as well as effects on chamber specification.

We chose to conduct a small-scale pilot screen using compounds obtained from the lab of Dr. Randall Peterson (Massachusetts General Hospital). The Peterson Lab provided us with 276 known bioactive compounds selected from their larger collection. Based on the prior studies of the Peterson Lab, it seemed that none of these compounds were generally toxic to zebrafish embryos, that all of these compounds would permeate the embryo, and that many of these compounds had effects on circulation [42].

Ten wild-type embryos were added to each well of a 48-well plate. The embryos were then treated at 50% epiboly (5.3 hpf) with 8 μM of the test compounds (Figure 2A). The treated embryos were then fixed in 4% paraformaldehyde at 22 somites in preparation for whole-mount in situ hybridization. Each compound was classified into one of 11 categories based on its effect on the expression patterns of *amhc* and *vmhc* (Table 1). 203 compounds did not cause any changes in *amhc* or *vmhc* expression. Five of the compounds caused all of the embryos in the well to die. 32 compounds created severe developmental delays. Eight compounds caused an expansion of the *amhc*-expressing population of cells (Figure 2C). 12 compounds caused a decrease in the population of cells expressing *amhc* (Figure 2D). One compound, 3,7-dihydroxyflavone, caused severe cardia bifida (Figure 2E). Three compounds caused an increase in the *vmhc*-expressing population of cells (Figure 2G). Seven compounds caused a decrease in the *vmhc* expressing population of cells (Figure 2H). In one compound the anterior half of the cardiac ring did not fuse properly (data not shown). Four compounds -- sphondin, diethylstilbestrol, scopoletin, and ketanserin -- caused defects in the dimensions of the cardiac ring (Figure 2I and Figure 3).

Although the results of the small molecule screen encompassed many different phenotypes, all with potentially rewarding information regarding cardiac development, for the purposes of this thesis we chose to focus solely on the compounds that caused cardiac ring defects. Of the four compounds inducing defects in cardiac ring dimensions, sphondin was not readily commercially

available and diethylstilbestrol upon secondary screen proved to cause severe developmental delays to characterize further (data not shown). Therefore, we chose to focus our attention on the effects of ketanserin and scopoletin.

Ketanserin treatment causes expansion of the cardiac ring

Our initial findings with ketanserin revealed that ketanserin treatment appeared to expand the atrial and ventricular cardiac ring in all directions, retaining the original shape of the ring but increasing the overall diameter (Figure 3B,E). In order to confirm these results, additional experiments were performed. The goal of the additional experiments was to confirm the initial results found in the screen, compare the differences between different concentrations of the compounds on the cardiac ring, and to establish the effect of drug treatment across a broader timeframe by looking at heart function at 48 hpf.

We found that treatment with 20 μM and 40 μM concentrations of ketanserin caused expansion of the cardiac ring as assayed by *amhc* and *vmhc* expression (Figure 4A-F). These myocardial phenotypes suggest that ketanserin treatment perturbs the fusion and organization of myocardial cells into a cardiac ring of proper size. Cardiac ring expansion was also apparent when assayed using a gene expressed in both atrial and ventricular myocardium, *cardiac myosin light chain 2 (cmlc2)* (Figure 4G-H)[5]. Interestingly, a few of the ketanserin-treated embryos displayed additional defects in ring morphology. Some ketanserin-treated embryos displayed multiple cardiac rings (Figure 4H). Other embryos exhibited ectopic cells within the cardiac ring (Figure 4F and I).

These data suggest the possibility that signals that determine the inner boundary of the cardiac ring could be affected by ketanserin treatment.

Next, we wanted to investigate if ketanserin treatment also causes defects in the formation of the endocardium. Therefore, we chose to investigate the expression of *fli1a*, a marker of all endothelial cells that also marks the endocardium [43]. In this experiment we treated embryos at 50% epiboly with ketanserin and then fixed the embryos at 20, 22, 24, and 26 somites in order to visualize the impact of ketanserin treatment on the progression of endocardial morphogenesis.

In ketanserin-treated embryos, defects in the endocardial sheet can be seen as early as 20 somites. The sheet lacks the uniform integrity seen in control embryos; notably, patches of the endocardial sheet do not express *fli1a* (Figure 5E). Occasionally a portion of the endocardial sheet in control embryos will exhibit weak *fli1a* expression. However, the defects seen in the ketanserin-treated endocardial sheet seem more severe than those seen in controls, with as many as three or four areas within the sheet lacking *fli1a* expression in the ketanserin-treated embryos (arrows in Figure 5E), a defect never observed in control groups. Despite these defects, the endocardial sheet does migrate anteriorly and leftward (Figure 5F-G) similar to the control, but at 26 somites the endocardium still appears to lack a continuous integrity (arrow in Figure 4H), with weak *fli1a* expression and gaps in expression throughout the heart tube (circle in Figure 4H).

Our data suggest that ketanserin disrupts the regulation of the size and shape of the cardiac ring and also the proper migration and integrity of the endocardial sheet. However, it is not clear whether the endocardial defects observed in ketanserin-treated embryos could be responsible for the expanded cardiac ring circumference. Next, we wanted to investigate the effects of ketanserin on overall embryonic morphology and whether earlier phenotypes might correlate with any defects in cardiac function and circulation at later stages in development.

Ketanserin treatment results in ectopic atrioventricular canal characteristics in the ventricle

Analysis of embryos treated with ketanserin from 50% epiboly to 48 hpf revealed additional circulation and cardiac defects. Overall, the embryos looked normal with no significant morphological defects outside of the cardiac region (Figure 6B); however, treatment with ketanserin did cause pericardial edema and severe blood pooling in the pericardium along with poor circulation (arrow in Figure 6B).

Closer inspection of ketanserin-treated embryos revealed multiple defects in the appearance and function of the heart. Embryos treated with ketanserin exhibited linearized hearts, and ventricles that appeared skinny and dysmorphic, which was particularly evident in the ventricular endocardium (Figure 7B). Embryos also displayed a contracting atrium that successfully pushed blood into

the ventricle; however, the ventricle did not contract normally (Figure 6D) and blood seemed to toggle back and forth between the atrium and ventricle.

We hypothesized that the blood toggling between the atrium and the ventricle in ketanserin-treated embryos could be due to defects in improper atrioventricular canal (AVC) specification. Specialized differentiation of the AVC in the developing zebrafish begins between 30 and 36 hours post-fertilization and is characterized by morphological changes in endocardial and myocardial cells [44, 45]. Endocardial cells located at the border between the atrium and ventricle begin to change shape from squamous to cuboidal and express the cell surface adhesion molecule Dm-grasp on their lateral surfaces. Dm-grasp is localized to the AVC in endocardial cells and is weakly expressed throughout the myocardium, including the myocardium of the AVC [16, 45, 46]. Morphological changes are also accompanied by the restriction of *bmp4* and *notch1b* expression to the AVC [6, 16, 47, 48]. Initially, *bmp4* is expressed throughout the cells of the myocardium, but its expression gradually becomes restricted to the myocardial cells of the AVC. Similarly, *notch1b* is widely expressed by endocardial cells at first but then its expression becomes restricted to the endocardial cells of the AVC.

Expression of *bmp4* and *notch1b* in ketanserin-treated embryos revealed that these markers were ectopically expressed in the ventricle (Figure 8C,D), compared to controls where *bmp4* and *notch1b* were properly restricted to the AVC (Figure 8A,B). This result suggests that ketanserin treatment may result in ectopic AVC differentiation. To test this hypothesis, we are currently

investigating the localization of the adhesion molecule Dm-grasp. Our preliminary data suggest that Dm-grasp is found ectopically in the ventricular endocardium of ketanserin-treated embryos. However, further experiments are required to confirm this observation.

Together, our data suggest that ketanserin affects both early cardiac fusion and endocardial development as well the later process of AVC differentiation. It will be interesting to determine how these phenotypes are related to one another in future studies.

Scopoletin treatment disrupts the regulation of cardiac ring size and endocardial sheet formation

Results from our small molecule screen suggested that scopoletin treatment expanded the ventricular cardiac ring in the anterior-posterior direction (Figure 3F). Scopoletin treatment also appeared to reduce the level of *amhc* expression (Figure 3C). However, additional experiments demonstrated that this initial *amhc* result was most likely an artifact of the in situ process, and we were able to confirm that scopoletin treatment causes changes in the morphology of the cardiac ring in both chambers. Scopoletin treatment consistently caused formation of a cardiac ring that appears stretched in the anterior-posterior direction as assayed by *amhc* and *vmhc* (Figure 3F and Figure 9B,C,E and F). Thus, it appears that scopoletin treatment can disrupt the normal pathways that regulate the proper size and shape of the cardiac ring. We hypothesize that

these defects may be due to improper cardiomyocyte migration to the midline, possibly due to a lack of angular migration to form a cardiac ring of proper shape.

Additional studies demonstrated that scopoletin treatment causes endocardial sheet defects similar to those resulting from ketanserin treatment, with patches of absent or reduced *fli1a* expression within the endocardial sheet (Figure 9H and I). Interestingly, analysis of additional timepoints in endocardial development revealed that the endocardial tube at 24 hpf appeared normal in scopoletin-treated embryos, and that endocardial cells did appear to properly line the heart tube (data not shown). Thus, scopoletin does limit the number of cells expressing *fli1a* at 20 somites, but it appears that the treated endocardial cells are able to recover from this problem in time to properly line the nascent heart tube. Therefore, we hypothesize that scopoletin treatment disrupts endocardial integrity such that the endocardial sheet lacks uniformity but is capable of extending throughout the heart tube normally.

Scopoletin treatment enhances the primitive erythrocyte population in the developing embryo

Next, we sought to understand any global defects caused by scopoletin treatment as well as to investigate cardiac function in scopoletin-treated embryos at later stages in development. To accomplish this, we analyzed the effects of scopoletin on embryos treated with the compound from 50% epiboly to 48 hpf. At 48 hpf, scopoletin-treated embryos appeared grossly normal but observation of cardiac function revealed blood toggling at the venous pole accompanied by

blood pooling (arrow in Figure 10B). Circulation defects were also present in scopoletin-treated embryos. The dorsal aorta, caudal artery, caudal vein, and posterior cardinal vein appeared abnormally packed with erythrocytes (arrow in Figure 10D). The blood circulation also appeared slow and sluggish.

Our observations of more red blood cells in the large vessels of scopoletin-treated embryos suggested the possibility that scopoletin treatment causes formation of an excess number of primitive erythrocytes in the early embryo. Alternatively, our observations could result from an abnormal distribution of erythrocytes throughout the vasculature. However, we have not found any obvious defects in vasculogenesis or angiogenesis in scopo letin-treated embryos. For example, formation of intersegmental vessels (ISVs) appears to proceed normally in scopoletin-treated embryos (data not shown).

To test the hypothesis that scopoletin treatment increases the number of embryonic erythrocytes, we used FACS to quantify the number of erythrocytes at two different timepoints. First, we utilized a transgenic line expressing dsRed under the control of the *gata1* promoter to quantitate the number of erythrocytes at 48 hpf [49]. We conducted two experiments at this timepoint, but the data were inconclusive (data not shown). One experiment did show a significant increase in the percent of live cells expressing dsRed in scopoletin-treated embryos; however, a second experiment did not confirm these results. The differences between these two experiments might be the result of inconsistent drug treatment or inconsistent amounts of blood clotting between embryos.

In our second set of experiments, we used a different transgenic line, *Tg(hbae1:GFP)* (unpublished, courtesy of Traver Lab – University of California, San Diego). α-globin is a critical component of hemoglobin and is therefore a useful marker of primitive erythroid cells [50]. We also decided to investigate the effects of scopoletin at an earlier timepoint (24 hpf) because we thought that any blood clotting taking place in the scopoletin-treated embryos would not be as prominent at earlier stages. Using this strategy, we hoped to eliminate the variability observed in the 48 hpf experiments.

FACS was performed on 24 hpf *Tg(hbae1:GFP)* embryos treated with DMSO or scopoletin. We observed a significant increase in the percentage of live cells expressing GFP in embryos treated with scopoletin compared to embryos treated with DMSO ($p < 0.005$). In the control, an average of 10.2% of live cells expressed GFP, and in embryos treated with scopoletin, an average of 16.2% of live cells expressed GFP (Figure 11). This evidence suggests that scopoletin may be affecting pathways regulating primitive hematopoeisis either by increasing the proliferation of primitive erythrocytes or by triggering the differentiation of hematopoietic progenitors into erythrocytes.

In conclusion, scopoletin treatment causes defects in the migration of the cardiomyocytes to the midline and their fusion into a coherent ring. It is possible that the seeming anterior-posterior stretch in the scopoletin-treated embryos is due to a lack of angular migration in the anterior and posterior regions of migrating cardiomyocytes. This may be due to the defects seen in the endocardial sheet of scopoletin treated embryos; however, further experiments

are required to elucidate the mechanisms behind this finding. Interestingly, scopoletin does appear to enhance the number of erythrocytes at 24 hpf and possibly at later stages of development. It will be interesting to investigate the molecular mechanisms underlying this result and also the relationship between the effects of scopoletin on erythrocyte number and cardiac phenotypes in future studies.

3.

DISCUSSION

Small molecule screening as an effective means of interrogating cardiac development

Our in situ based small molecule screen was successful in isolating four compounds that affect cardiac fusion, two of which we focused on in this thesis. The experiment served as a pilot screen for the lab to help understand the benefits and drawbacks of a screen performed under these conditions. One advantage of an in situ hybridization based assay, in contrast to a transgene based assay, is that wild-type embryos can be used for the screen and are generally readily available for use, making the availability of embryos less of a hindrance. However, a drawback of using an in situ hybridization based assay is that the in situ process is time consuming and can sometimes result in variable levels of staining and resolution based on slight changes to the hybridization time, probe concentration, antibody concentration or time spent developing the embryos.

If this pilot study were scaled up to screen thousands of compounds for their effects on fusion, using in situ hybridization as an assay may become burdensome. Future screens may benefit from the use of transgenic zebrafish that express cardiac reporters such as *Tg(cmlc2:egfp)*. This line could be screened for phenotypes quickly and without the necessity for a labor intensive process like in situ hybridization. However, some transgenic lines often exhibit variable levels of fluorescence. Also, accurate detection and imaging of fluorescent cells via dissecting scope may be difficult, particularly over background created by the yolk. However, if a transgenic line with adequate

fluorescence was available, then this type of assay could be effectively utilized for a high-throughput screen involving thousands of chemicals.

Lastly, it may be beneficial in future screens to adjust the timeframe during which the embryos are treated with the compounds. Our pilot screen was designed to accommodate interests in progenitor specification, and therefore the window of treatment was broader than needed to focus on aspects of fusion. Adjusting the length of time that the embryos are exposed the compound would avoid dismissing compounds that interfere with gastrulation, and thus are never screened for later effects on heart development.

Ketanserin is a novel small molecule perturbator of cardiac fusion and valve specification

Analysis of cardiac migration and fusion in ketanserin-treated embryos demonstrated that bilateral cardiomyocyte populations successfully migrate to the midline and fuse to form a cardiac ring, although the dimensions and morphology of the cardiac ring are abnormal. It also appears that the inner circumference of the cardiac ring lacks proper organization and integrity. These phenotypes could be due to numerous causes. One possibility is that the cardiomyocytes in ketanserin-treated embryos might lack the correct migrational velocity, and therefore might not be able to reach the midline within the normal timeframe. Therefore, these migrating cells fail to form a compact ring and are less tightly associated prior to extension. Another possibility is that the signals that instruct the migrating cardiomyocytes to conduct proper angular movement

could be affected by ketanserin treatment. Errors in regulating the direction or attenuation of angular movement could result in abnormal continuation of cardiomyocyte migration towards the center of the ring, forming either multiple cardiac rings (Figure 4H) or cells that appear to invade the inner perimeter of the cardiac ring (Figure 4F and I). Previous studies have utilized the transgene *Tg(cmlc2:egfp)* to track the movement of cardiomyocytes during ring formation [6, 15, 51]. This type of experiment would be helpful in elucidating the specific defects in migrational patterns associated with ketanserin treatment.

Ketanserin treatment also results in defects in endocardial morphogenesis during cardiac fusion. The endocardial sheet of ketanserin treated embryos appears to lack the level of cellular organization seen in the control embryos. Although ketanserin treatment does not seem to cause a developmental delay in endocardial morphogenesis, we do observe abnormal expression of *fli1a* throughout the endocardial sheet. This could result from abnormally paced or improperly coordinated migration of endocardial cells, resulting in a non-uniform endocardial sheet (Figure 4E). It is also possible that the defects seen in the endocardial sheet of ketanserin-treated embryos are due to problems with the formation of endocardial intercellular junctions, resulting in cells that do not properly adhere and therefore behave independently instead of in a coordinated fashion. Timelapse analysis of endocardial morphogenesis using a transgenic line such as *Tg(fli1a:neGFP)y7* could provide further insight into how ketanserin treatment affects the development of the endocardium.

The defects seen in the myocardial ring could potentially be a consequence of the defects in endocardial morphogenesis. Previous studies have shown that endocardium-myocardium interaction is important for regulating angular movement of cardiomyocytes during fusion [6], but it is unclear whether these relatively subtle defects in endocardial sheet formation are substantial enough to influence myocardial migration. In future experiments, it would be interesting to compare the myocardial and endocardial cell behavior defects in ketanserin-treated embryos and to investigate how these closely related processes influence each other during ring formation and subsequent extension of the heart tube.

The nature of the myocardial and endocardial defects observed in ketanserin-treated embryos is reminiscent of the phenotypes observed in knockdown experiments involving components of the Vegf/Slit/Robo pathway [15]. It has been demonstrated that Vegf signaling is required for the proper migration of bilateral heart fields to the midline, and that Slit/Robo signaling is important to mediate the endocardial response to Vegf [15]. Morpholinos against *slit2* cause hypermigration of endocardial cells to the midline that exhibit loss of directionality and cause formation of multiple cardiac rings, phenotypes resembling what we see in ketanserin-treated embryos. It was also shown that Vegfr inhibition through the small molecule Vatalanib, and a morpholino against *vegfa* can cause defects in the migration of both endocardial and myocardial progenitors to the midline, resulting in malformed cardiac rings reminiscent of the effects of ketanserin treatment [15]. The similarities between ketanserin treatment and these

phenotypes suggest that ketanserin may be acting, either directly or indirectly, on this pathway to inhibit proper migration and ring formation. However, Vegfr inhibition also results in patterning defects of the intersegmental vessel and head vasculature that were never observed with ketanserin treatment (data not shown), so it is unlikely that ketanserin is acting directly on Vegf signaling to produce these phenotypes [15]. The existence of a Slit2 receptor(s) that mediates the negative regulation of Vegf signaling was also hypothesized. Ketanserin may be acting on this receptor to negatively regulate Vegf signaling.

The relationship between the early cardiac fusion defects and the later AVC patterning defects in ketanserin-treated embryos remains to be determined, but it is intriguing that the same correlation is seen in zebrafish *tmem2* mutants. The maternal-zygotic *tmem2* mutant displays migration and fusion defects in both the endocardium and myocardium [16]. In zygotic *tmem2* mutants, AVC markers are not properly restricted and ectopic AVC differentiation occurs in the ventricular endocardium [16, 17]. However, the molecular connection between fusion defects and valve defects in the *tmem2* mutants are still under investigation.

The *tmem2* mutant phenotype is also similar to the phenotype of the zebrafish *apc* mutant, in which the Wnt pathway is constitutively active [48]. To understand the localization of Wnt signaling in the zebrafish heart, a transgenic line, *Tg(TOP:GFP),* was utilized to show that Wnt signaling activity is localized to the AVC in the wild-type heart at 48 hpf [48]. In *apc* mutants, ectopic *Tg(TOP:GFP)* reporter activity is apparent in the ventricle [48]. Although it would be exciting to

examine *Tg(TOP:GFP)* reporter activity in ketanserin-treated embryos, we have not yet been able to reproduce the localization of *Tg(TOP:GFP)* activity at the AVC in wild-type embryos. Another recent paper has also reported difficulty in reproducing the results demonstrating *Tg(TOP:GFP)* activity localized to the AVC, so it remains to be clarified how Wnt signaling activity is localized in wild-type embryos [46].

Historically, ketanserin has been used as an antagonist of the serotonin receptor 5-HT_{2A}. The precise role of 5-HT receptors in cardiac development and disease is unclear; however, certain studies have implicated serotonin signaling in cardiac pathology and disease. Intriguingly, ketanserin can prevent cardiac fibroblast migration, suggesting that 5-HT signaling might be able to modulate cardiomyocyte movement and behavior [52]. In patients with carcinoid syndrome, enterochromaffin tumors increase delivery of 5-HT to the bloodstream. Interestingly, approximately one-third of patients with carcinoid tumors also develop cardiac symptoms such as thickened tricuspid valve leaflets or related valvulopathy, suggesting that 5-HT signaling can modulate valve morphogenesis [53].

Based on this previous context, it would be interesting to investigate whether ketanserin is acting through the zebrafish equivalent of 5HT-2A to influence cardiac fusion and/or valve formation. Genomic analysis in zebrafish reveals a predicted gene orthologue, *hrt2a*, which is 63% similar to its human counterpart. No mutants or morpholino knockdowns of *hrt2a* have been reported, and information on the expression pattern of this gene are limited, with

no detailed examination of whether there is expression in the heart. Previous studies in mouse using monoclonal antibodies against 5-HT_{2A} receptors revealed expression in the myocardium and endocardial cushions [54]. It would therefore be interesting to determine if *hrt2a* exhibits conserved cardiac expression in zebrafish. In addition, investigating the role of *hrt2a* in cardiac fusion and valve development through morpholino knockdown or treatment with additional 5-HT specific antagonists would provide more insight into ketanserin's mechanism of action. It is possible that in ketanserin-treated embryos, the response to serotonin is limited, resulting in defects in the proper migration and differentiation of heart tissue and the heart valves; therefore, we hypothesize that *hrt2a* morphants would present a phenotype similar to treatment with ketanserin.

Scopoletin is a novel small molecule perturbator of cardiac fusion and hematopoiesis

Embryos treated with scopoletin displayed defects in the size and shape of the cardiac ring. The cardiac ring appeared to be stretched in an anterior-posterior direction and compressed medially (Figure 9B,C,E,F). This phenotype suggests a lack of angular movement by the bilateral heart fields. If the two bilateral populations failed to undergo angular movement, but instead continued to migrate in a medial direction, then the cardiac ring would appear more elongated along the anterior-posterior axis. This hypothesized lack of angular movement would be distinct from the apparent effects of in ketanserin-treated embryos, where we hypothesize that the cells initiate the proper medial

movements but lack the correct migrational velocity to form a compact ring. The hypothesized lack of angular movement in scopoletin-treated embryos may be due to endocardial defects; since in mutant embryos that lack endocardium, such as *cloche*, cardiomyocytes fail to undergo angular movements [6].

Endocardial defects are also seen with scopoletin treatment with patches of the endocardial sheet lacking expression of *fli1a* (Figure 9H,I). We suspect that this phenotype is caused by endocardial cells migrating to the midline in an uncoordinated or disorganized fashion. Some embryos appear to have endocardial cells localized to the site of the future arterial pole but lacking in other areas of the sheet (Figure 9H). This suggests that scopoletin causes defects in the migrational velocity and directionality of the migrating endocardial cells, resulting in cells that migrate too quickly to the center of the sheet, and thus do not distribute evenly across the midline. Other embryos appear to have endocardial cells organized in regions sporadically through the sheet (Figure 9I). This may be due to a perturbation of signals that instruct the cells to distribute uniformly across the sheet. Time-lapse analysis would be helpful to further elucidate the exact migrational patterns of myocardial and endocardial cells in scopoletin-treated embryos, and may also help to correlate defects in the myocardium with the endocardial defects.

Scopoletin treatment also resulted in an increase in the number of primitive erythroid cells as early as 24 hpf based on our FACS analysis. However, the mechanism of scopoletin's action remains a mystery. It is possible that scopoletin treatment could increase the number of early blood progenitors.

Examining the expression pattern of a primitive blood marker such as *gata1* at early stages would be a first step toward testing this hypothesis. A second possibility is that scopoletin regulates the proliferation of these progenitors rather than their specification. Interestingly, the number of *gata1*+ cells normally increases as the bilateral populations migrate towards the midline [50]. Performing a proliferation assay with BrdU in *gata1+* cells will test the proliferative potential of scopoletin during primitive hematopoiesis.

The molecular mechanism underlying scopoletin's effect will also be an important subject for future research. One study showed that scopoletin induced cell proliferation of normal T lymphocytes due to the interaction with protein kinase C [39]. Since activation of PKC activity can enhance cell proliferation and differentiation of other hematopoietic lineages, we hypothesize that scopoletin may be acting through protein kinase C to increase the proliferation of erythrocytes [55]. If this is true, measuring the localization of PKC to the plasma membrane as a read-out of PKC activity in scopoletin-treated erythrocytes would test this possibility.

Notch signaling has been implicated to mediate a cell fate switch between the endothelial and hematopoietic lineages [56]. Embryos overexpressing an activated form of the Notch receptor have been shown to exhibit a significant increase in the number of erythrocytes and a significant decrease in the number of endothelial cells. The similarities in phenotypes between increased Notch activity and scopoletin treatment suggest that scopoletin might increase erythrocyte number through enhanced Notch activity. To test this possibility, we

will examine scopoletin's effect on Notch activity using a transgenic GFP reporter line.

Future studies are necessary to uncover the molecular targets of both ketanserin and scopoletin. In each case, we will pursue investigation of the known molecular targets of the compound. In addition, we will evaluate whether the compound affects pathways that are known to cause similar phenotypes upon disruption. Finally, if these approaches are not fruitful, we may need to search more broadly for novel targets of each compound that are responsible for their effects during cardiovascular development.

4.

MATERIALS AND METHODS

Zebrafish strains

Embryos and adult fish were maintained and raised under standard laboratory conditions [57]. In addition to wild-type zebrafish strains, we used the following transgenic lines: *Tg(fli1a:neGFP)*^{y7}[58]*, and Tg(hbae1:GFP)* (unpublished – generously provided by Dr. David Traver, Traver Lab – University of California, San Diego).

Small molecule screen

Four 96-well plates containing 276 compounds were obtained from the Peterson lab (Massachusetts General Hospital) and stored at -80°C. Plates were placed in a dessicator for one hour to eliminate condensation and centrifuged at 300 rpm for 10 min before removing the foil seal. A 48-well plate was prepared by adding 300 µl of 1x E3 buffer + 0.3% dimethylsulfoxide (DMSO). Ten embryos were added to each well of the 48-well plate. Compounds were added to a concentration of 8 µM (1 µl of compound for this screen) when the embryos reached 50% epiboly. Embryos were incubated at 28.5°C until fixation in 4% paraformaldehyde at 22 somites. Five embryos from each well were processed for *vmhc* expression and five embryos were processed for *amhc* expression. Compounds were considered for follow-up experiments if three of the five embryos processed displayed the phenotype. Compounds that resulted in either death or developmental delay were not processed for in situ analysis. Follow-up experiments were performed according to the same protocol as the initial screen. Compounds for additional experiments were obtained from Sigma and

resuspended in DMSO to a concentration of 10 mM. Compounds were diluted to final desired concentration upon treatment and an equivalent volume of DMSO was added to control groups.

In situ hybridization

Whole-mount in situ hybridization was carried out as previously described [5]. *amhc, vmhc, cmlc2, and fli1a* antisense probes were used [5, 43, 59]. In situs were cleared in a 2:1 benzyl benzoate/benzyl acetate solution and imaged on a Zeiss M2 Bio dissecting microscope using a Zeiss AxioCam. Images were processed using Axiovision software and Adobe Photoshop.

Confocal microscopy

Confocal images were captured using a 20X dry objective on a Leica SP5 confocal microscope. Embryos were anethesized in 0.5-1% tricaine and mounted on their side. Images were generally comprised of 100 optical slices that were 1.5 microns thick. 3D projections were generated using Imaris software.

Fluorescence activated cell sorting

Whole zebrafish embryos treated with scopoletin or DMSO were dissociated in 1x HPBS containing Mg^{2+}, Ca^{2+}, and liberase at 37°C. Dissociated embryos were passed through a 40 μM filter. Cells were resuspended in 1x PBS and spun down at 300 g for 10 min in a 4°C centrifuge. Sytox red (Sigma) was

added to a concentration of 1µg/ml to exclude dead cells. Flow cytometry quantification was based on Sytox red exclusion, forward scatter, side scatter, and GFP fluorescence with a FACSVantage flow cytometer (Beckton Dickinson).

5.

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6.

TABLES AND FIGURES

Category	**# of compounds**
Normal	203
Dead	5
Developmental delay	32
Increased *amhc*	8
Decreased *amhc*	12
Increased *vmhc*	3
Decreased *vmhc*	7
Increased *amhc* and *vmhc*	1
Cardia bifida	1
Cardiac ring defects	**4**
Anterior fusion defect	1
Total compounds	**276**

Table 1. Results of small molecule screen.
276 compounds were screened for their effects on *amhc* and *vmhc* expression at 22 somites. Embryos were treated with compound at 50% epiboly and left in the drug until fixation. Compounds were grouped into categories based on their effects on *amhc*-expressing and *vmhc*-expressing cells. Four compounds caused defects in cardiac ring dimensions.

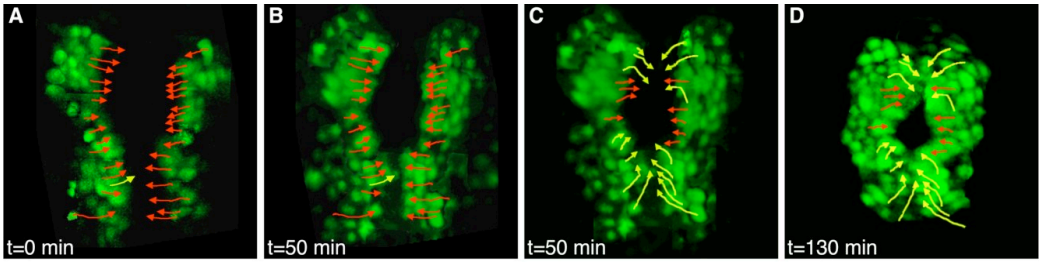

Figure 1. Cardiomyocyte cell behavior during cardiac fusion.
Adapted from Holtzman et al., 2007. Selected images from a time-lapse of cardiac fusion in a typical wild-type embryo expressing *Tg(cmlc2:egfp)* from 16-somite to 20-somite stages. Dorsal views, anterior to the top. Red arrows, medial movement. Yellow arrows, angular movement. Each arrow extends from the cell's starting position (in A or C) to its ending position (in B or D). Cardiac fusion begins with coherent medial movement (A,B) followed by a transition to angular movement in the anterior and posterior regions (C,D).

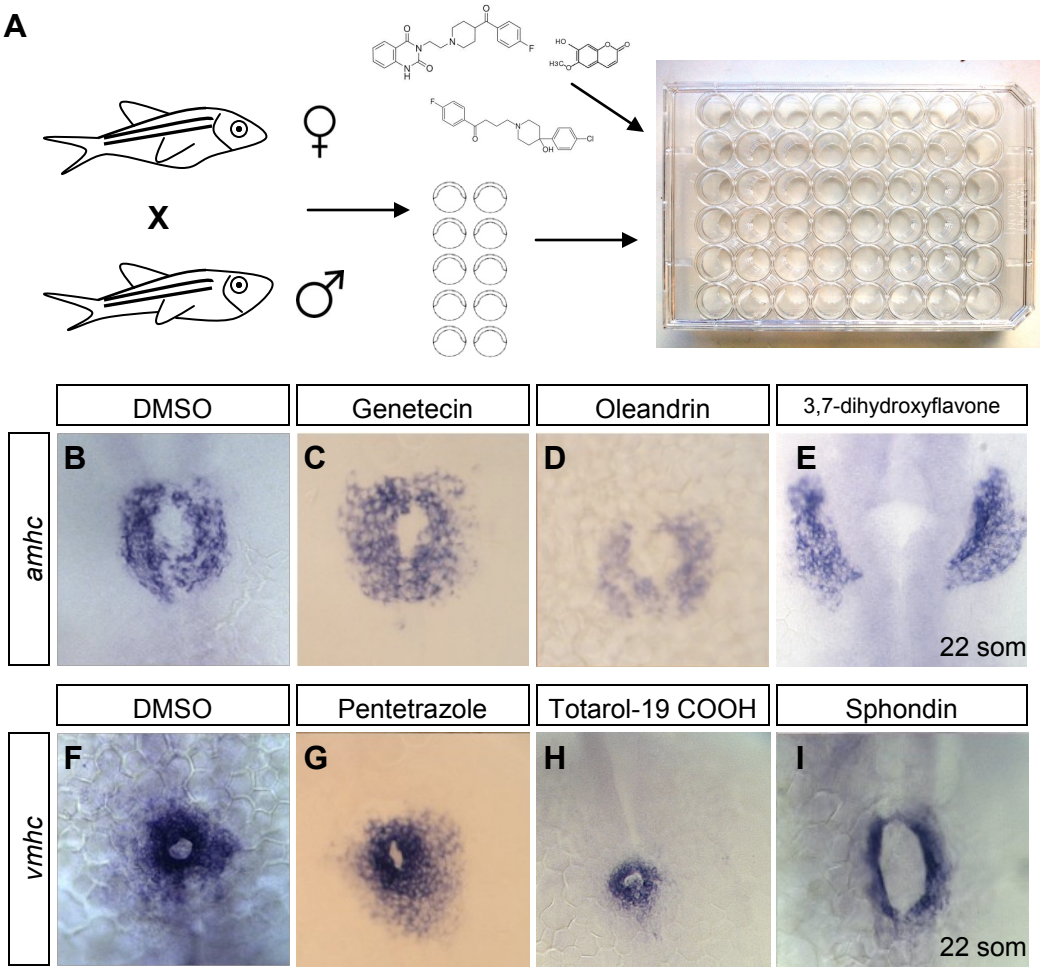

Figure 2. In situ small molecule screen identified compounds that perturb cardiac morphogenesis at 22 somites.
(A) Schematic of small molecule screen design. Ten wild-type embryos were added to each well of a 48-well plate containing 8uM of a series of compounds. (B-I) Dorsal views, anterior to the top, of expression patterns of *amhc* (B-E) and *vmhc* (F-I). Compounds were categorized based on their effects on the expression patterns of *amhc* and *vmhc*. For example, categories include increased *amhc* expression (C), decreased *amhc* expression (D), cardia bifida (E), increased *vmhc* expression (G), decreased *vmhc* expression (H), and cardiac ring defects (I).

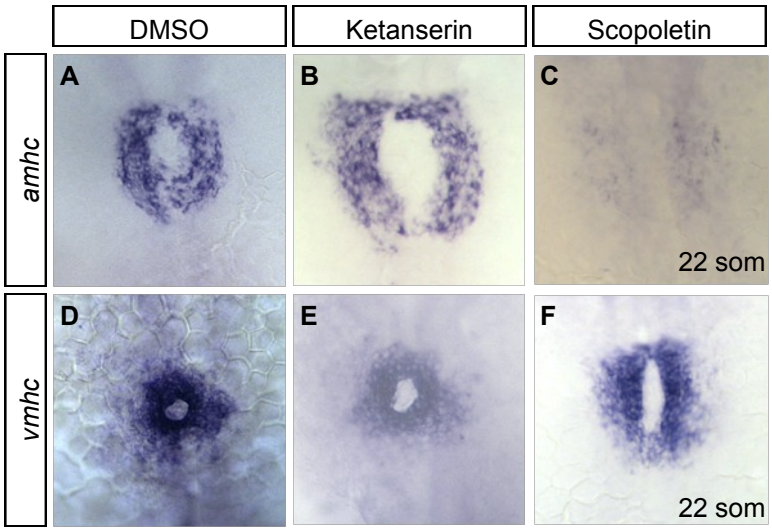

Figure 3. Ketanserin and scopoletin cause cardiac ring defects.
(A,D) Wild-type expression of *amhc* and *vmhc*. Initial results revealed an expanded atrial ring in ketanserin-treated embryos (B), a slightly expanded ventricular ring in ketanserin-treated embryos, an expanded ventricular ring in scopoletin-treated embryos (F), and an apparent loss of *amhc* expression in scopoletin-treated embryos (C). Dorsal views, anterior to the top.

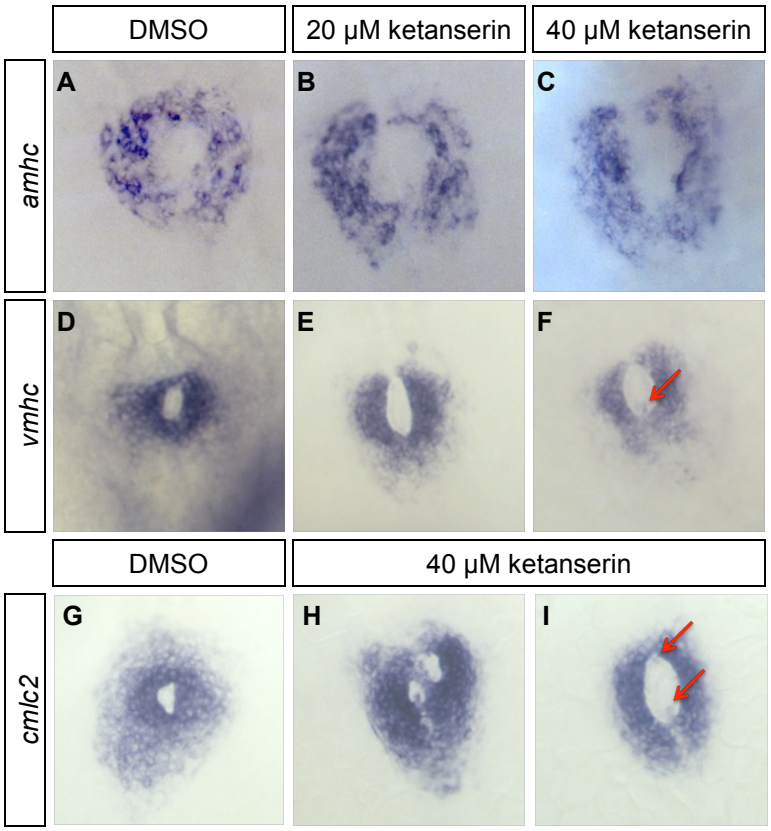

Figure 4. Ketanserin disrupts regulation of cardiac ring dimensions.
Wild-type embryos treated with DMSO exhibit normal expression of *amhc* (A), *vmhc* (D) and *cmlc2* (G). Embryos treated with ketanserin exhibit an expansion of the atrial ring (B,C) and ventricular ring (E,F) at both 20 μM and 40 μM concentrations. A few ketanserin-treated embryos exhibited other ring defects including multiple cardiac rings (H) and cardiomyocytes entering the center of the ring (red arrows in F and I). Dorsal views, anterior to the top.

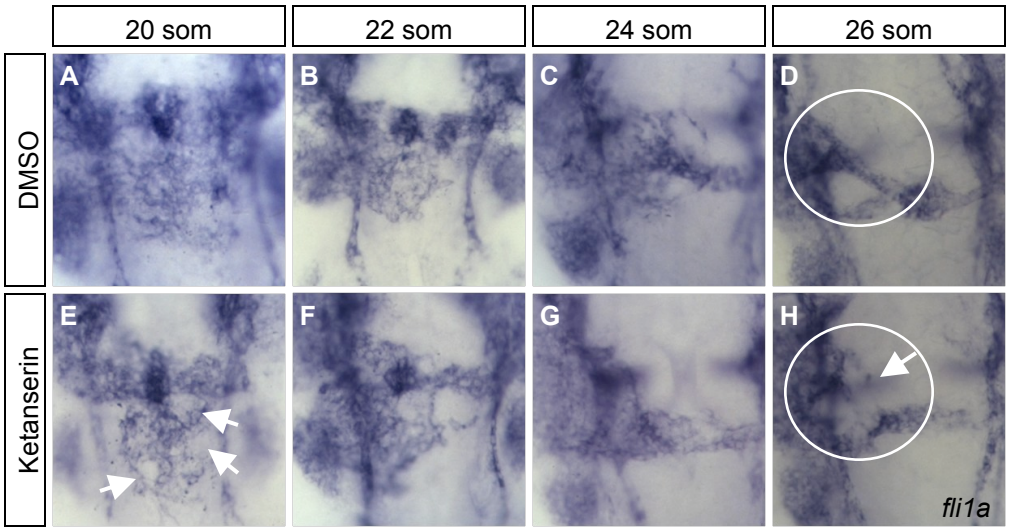

Figure 5. Endocardial defects in ketanserin-treated embryos from 20 to 26 somites. In wild-type embryos, the endocardial sheet is complete and evenly distributed across the mid-line at 20 somites (A). The sheet then migrates anteriorly and leftward (B), followed by further migration to form a linear tube that will line the primitive heart (C). By 26 somites, the linear tube is complete (circle in D). Ketanserin treatment causes defects in the integrity of the endocardial sheet at 20 somites as visualized by *fli1a* expression (arrows in E). These defects persist throughout the development of the endocardium (F,G, arrow in H). Dorsal views, anterior to the top.

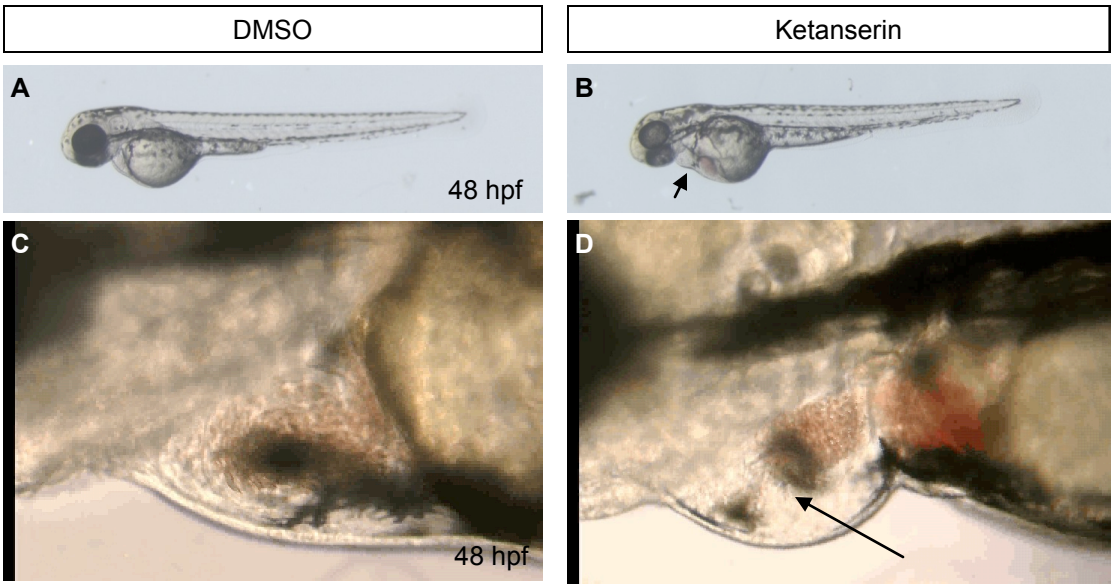

Figure 6. Ketanserin causes blood regurgitation at the AVC.
Embryos were treated with 40 µM ketanserin from 50% epiboly to 48hpf. (A,B) Brightfield images of whole embryo at 48hpf. Ketanserin treatment causes slight pericardial edema and blood pooling in the pericardium (arrow in B). (C,D) Close-up images of DMSO and ketanserin-treated embryos. Ketanserin treatment causes blood toggling at the AVC (arrow in D). Lateral views, dorsal to the top.

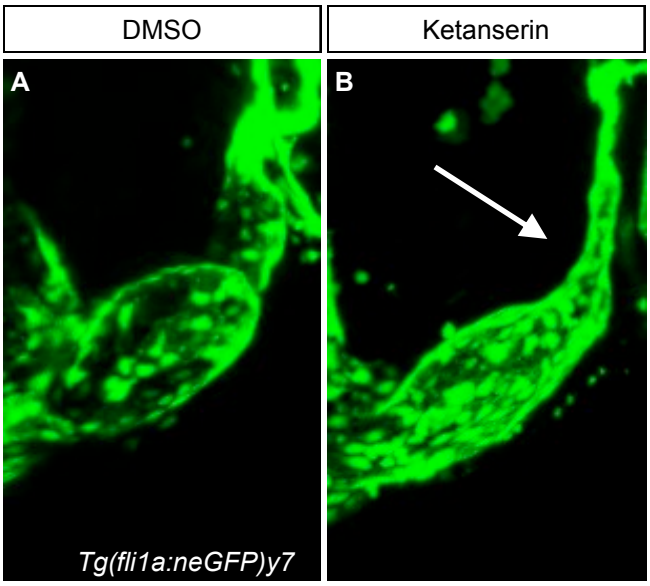

Figure 7. Ketanserin treatment results in a linearized heart with defects in ventricular endocardium.
(A,B) Confocal reconstructions of the endocardium of 48 hpf embryos expressing GFP in endocardial cells. Treatment of embryos with ketanserin results in a linear heart with a misshapen and skinny ventricular endocardium (arrow in B). Lateral views, dorsal to the top.

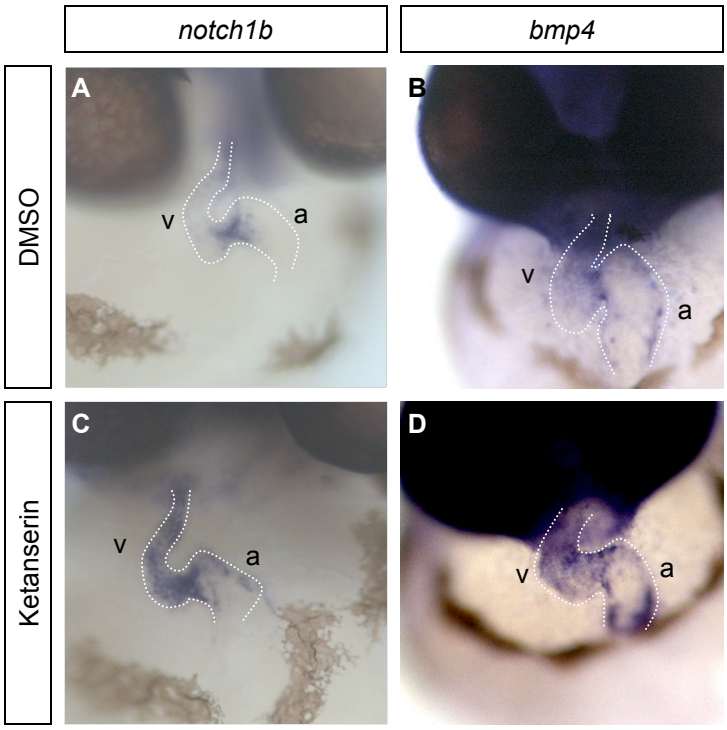

Figure 8. Ketanserin treatment causes ectopic expression of AVC markers in the ventricle.
Whole-mount in situ hybridizations of *notch1b* and *bmp4* at 48hpf. In control embryos the expression of *notch1b* and *bmp4* is properly restricted to the AVC (A,B). Ketanserin treatment results in the ectopic expression of *notch1b* and *bmp4* in the ventricle (C,D). Dotted white lines demarcate the atrium and ventricle (designated a and v respectively). Dorsal views, anterior to the top.

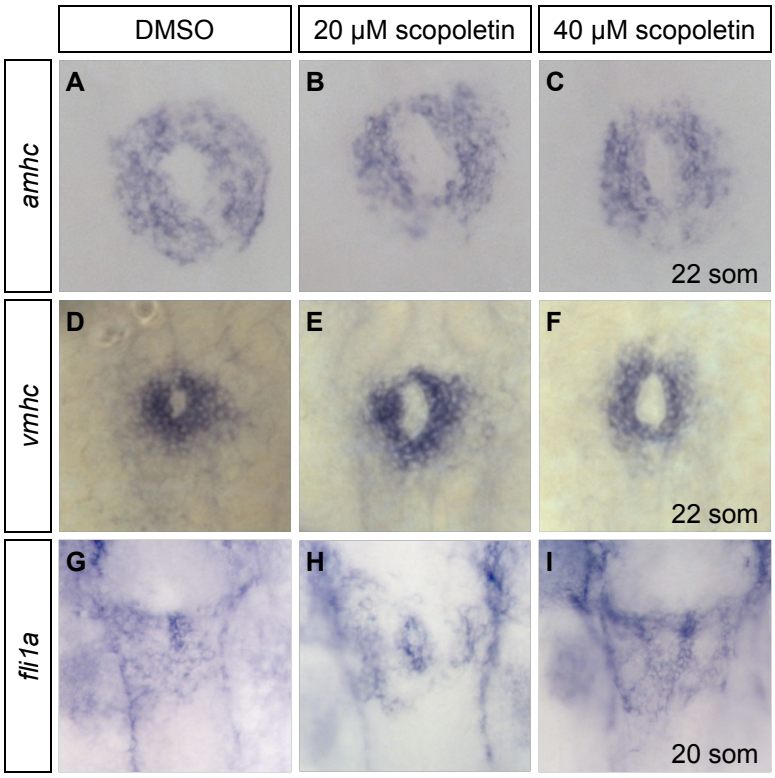

Figure 9. Scopoletin disrupts regulation of cardiac ring dimensions and endocardial sheet formation.
Wild-type embryos treated with DMSO exhibit normal expression of *amhc* (A) and *vmhc* (D). Embryos treated with 20 μM or 40μM scopoletin exhibit an expansion of the atrial cardiac ring (B,C) and ventricular cardiac ring (E,F). Scopoletin-treated embryos exhibit an anterior-posterior stretching of the atrial ring (B,C), and the ventricular ring (E,F). Analysis of the endothelial marker *fli1a* indicates a disruption of the endocardial sheet in scopoletin-treated embryos with portions of the sheet lacking *fli1a* expression. Dorsal views, anterior to the top.

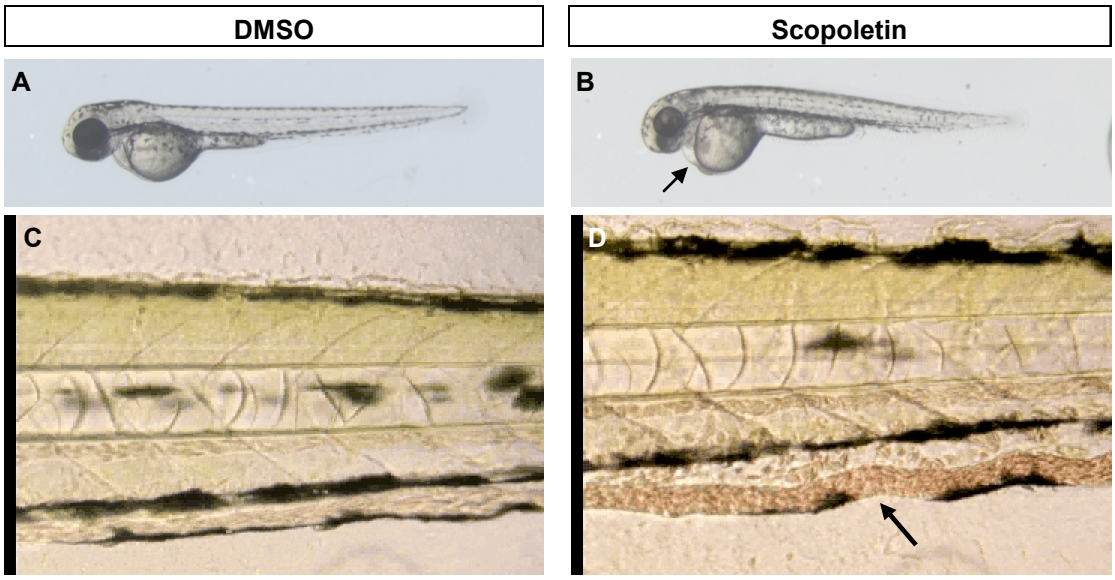

Figure 10. Scopoletin treatment causes pericardial edema and circulation defects at 48 hpf.
Wild-type embryos were treated with 80 μM scopoletin from 50% epiboly to 48 hpf. (A,B) Bright-field images of whole embryos at 48 hpf. Scopoletin treatment causes blood pooling at the venous pole (arrow in B). (C,D) Close-up images of blood flow in dorsal aorta and posterior cardinal vein. Scopoletin treatment results in a tightly packed, slow moving population of erythrocytes in the posterior caudal vein (arrow in D).

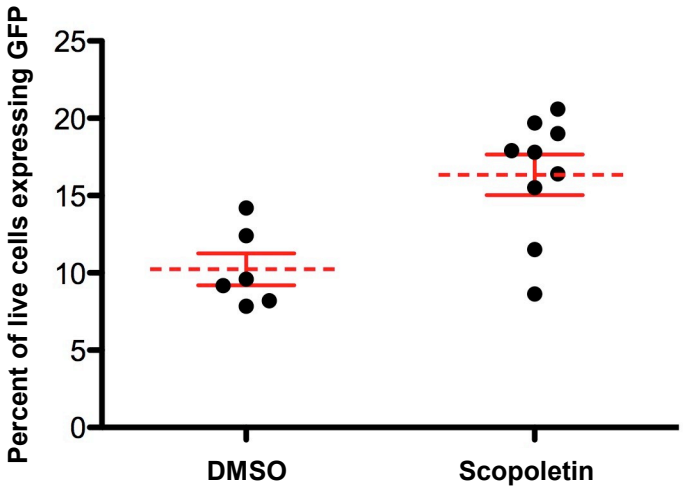

Figure 11. Scopoletin enhances the number of erythrocytes at 24 hpf.
FACS analysis was performed on either DMSO or scopoletin treated embryos expressing *Tg (hbae1:GFP)*, a marker of erythrocytes. Quantification was based on Sytox red exclusion, forward scatter, side scatter, and GFP fluorescence. Scopoletin treatment results in an increase in the percentage of GFP+ cells. Each data point represents a group of five embryos. Dashed red line represents the mean. Solid red lines represent the standard error of the mean. DMSO mean - 10.2%, Scopoletin mean - 16.2%. Results of students t-test between two populations returns a significant p-value < 0.005.