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3 **Mechanisms influencing retrograde flow in the**
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5 **atrioventricular canal during early embryonic cardiogenesis**
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ABSTRACT

Normal development of the heart is regulated, in part, by mechanical influences associated with blood flow during early stages of embryogenesis. Specifically, the potential for retrograde flow at the atrioventricular canal (AVC) is particularly important in valve development. However, the mechanisms causing this retrograde flow have received little attention. In this study, a numerical analysis was performed on images of the embryonic zebrafish heart between 48 and 55 hpf. During these stages, normal retrograde flow is prevalent. To manipulate this flow, zebrafish were placed in a centrifuge and subjected to a hypergravity environment to alter the cardiac preload at various six-hour intervals between 24 and 48 hpf. Parameters of the pumping mechanics were then analyzed through the spatiotemporal analysis of processed image sequences. We find that the loss of retrograde flow in experimentally manipulated embryos occurs in part because of a greater resistance in the form of atrial and AVC contractile closure. Additionally, during retrograde flow, these embryos exhibit significantly greater pressure difference across the AVC based on calculations of expansive and contractile rates of the atrium and ventricle. These results elucidated that the developing heart is highly sensitive to small changes in pumping mechanics as it strives to maintain normal haemodynamic conditions necessary for later cardiac development.

INTRODUCTION

The mechanics of blood flow in the developing heart have a considerable influence on heart morphology and function, although the cellular responses are not fully understood (Hove et al., 2003a)(Knoll, 2015; Santhanakrishnan and Miller, 2011). To study the effects of these parameters on various aspects of cardiac development, zebrafish (*Danio rerio*) have been commonly used as a model for cardiogenesis (Ota and Kawahara, 2014; Tu and Chi, 2012). In this model, cardiac development is similar to humans and nearly all other vertebrates at the earliest stages of development (Dahme et al., 2009; Stainier et al., 1993). Zebrafish have many advantages as an animal model, including their transparency during early development (Driever et al., 1996; Ota and Kawahara, 2014; Tu and Chi, 2012). The use of zebrafish is also advantageous since there are many available mutant lines and morpholinos that can be used to manipulate and delineate specific signaling pathways involved in development (Driever et al., 1996).

Out of the cellular responses to blood flow, endothelial cells are known to be highly responsive to wall shear stress through mechanosensors (Li and Xu, 2007; Topper and Gimbrone, 1999). These sensors result in changes to intracellular signaling cascades that regulate gene expression and ultimately differentiation, proliferation, and organization of endothelial progenitor cells as well as endothelial cell morphology (Dietrich et al., 2014; Li and Xu, 2007). Though most stress-mediated signaling pathways controlling gene expression have yet to be discovered, several key genes in the embryonic heart have been determined to be flow and/or stress-responsive. One key gene, *Kruppel-like factor 2* (KLF-2), a transcription factor which is expressed strongly in regions of high shear, is necessary for formation of an atrioventricular valve and for erythropoiesis, or RBC generation (Dekker et al., 2002; Groenendijk et al., 2004; Groenendijk et al., 2005; Groenendijk et al., 2007)(Vermot et al., 2009; Zeller, 2016). Inhibition of retrograde flow results in the misexpression of the gene *klf2a*, impacting endocardial cushion differentiation and valve formation at the AVC (Vermot et al., 2009). An increase in normal retrograde

flow, such as that induced by ethanol, can cause impaired valve formation as well (Karunamuni et al., 2014).

Since multiple studies indicate that regular valve formation occurs poorly without retrograde flow, the objective of our present study is to understand the underlying mechanisms that may regulate retrograde flow in a zebrafish model. For this purpose, we evaluate the changes in pressure in the atrium and ventricle in relation to the direction of flow over the cardiac cycle. We further qualitatively characterize resistance to flow by evaluating the movement of the cardiac wall, and the extent and duration of contact arising between endocardial layers of the atrium and AVC over the cardiac cycle. To alter the retrograde flow, we centrifuge the embryo at various stages during development and image the embryos at 48 and 55 hpf. The time points were selected based on the sensitivity of later valve formation to altered flow during these stages (Vermot et al., 2009). Our results from analyzing these parameters provide us with new insights into the mechanisms regulating normal cardiogenesis.

METHODS

Zebrafish Preparation and Imaging:

Wild-type zebrafish (*Danio Rerio*) were raised and bred in accordance with Westerfield (Westerfield, 1995). Embryos were collected up to one hour post-fertilization and incubated at 28°C at all times except during imaging and preparation for centrifugation. A 0.5 g/L stock solution of methylene blue trihydrate (Acros Organics) was applied to the embryo water just after collection of the embryos to act as an antimicrobial agent (Kimmel et al., 1995). All embryos were dechorionated using forceps at 24±0.5 hpf. A Photron FASTCAM SA3 high-speed camera mounted to an Olympus SZX12 bright-field stereo microscope with SZX-AL20X lens attachment was used for imaging. Image resolutions ranged between 1050 and 1310 pixels per millimeter taken at 1500 frames per second. Optical resolution was estimated

between 0.88 and 1.5 microns. Some embryos were positioned in low-melting temperature agarose gel to more easily image the heart from a ventral-side view.

Centrifugation:

Centrifugation was performed using methods previously described (Johnson et al., 2015).

Centrifugation has the advantage of being non-invasive, and depending on conditions, may either decrease or increase retrograde flow. Briefly, embryos were loaded into polymer tubing (Cole-Parmer, FEP tubing) of 1/32" inner diameter, only slightly greater than the width of the embryo to limit movement. Embryos within the polymer tubing were loaded head-down into a microcentrifuge tube containing embryo water. Within the microcentrifuge tube, the polymer tubing was anchored into a pad of agarose (Sigma Aldrich, Type VII-A, A0701) to prevent the embryo from falling out of the inner polymer tube. Microcentrifuge tubes were mounted to a motor calibrated to 15 Hz (Johnson et al., 2015). Centrifugation was performed for 6-hour increments during four key developmental stages as determined by Kimmel, 24-30 hpf (beginning of circulation), 30-36 hpf (early cardiac looping), 36-42 hpf (mid-cardiac looping), and 42-48 hpf (late cardiac looping) (Johnson et al., 2015; Kimmel et al., 1995). Imaging was performed at 48 hpf for embryos centrifuged at intervals between 24-42 hpf and 55 hpf for embryos centrifuged at 42-48 hpf. Flow is allowed to recover from centrifugation for the time interval between centrifugation and imaging, which is at least 6 hours. The percentage of embryos centrifuged at each stage is provided in Table 1. Centrifugation was performed at incubation temperature (28°C).

Centrifugation was used to alter the overall flow through the heart for a six-hour interval at various points during development (Johnson et al., 2015). For this study, only centrifuged embryos that exhibited an absence of retrograde flow, but no other apparent morphological or functional defect were selected for comparison with most wildtype (WT) embryos, which exhibit retrograde flow during a short

period of the cardiac cycle. However, occasional WT embryos without normal retrograde flow can be observed (~5%). Since this study was only concerned with the underlying mechanisms of retrograde flow, these rare WT fish that sporadically lacked retrograde flow (N=8) were included as part of the experimental group of centrifuged embryos that similarly lacked retrograde flow (N=9).

Calculation of Pressure Contributions from Diameter Change with Axisymmetric Assumption:

The pressure associated with expansion and contraction of the atrium and ventricle was calculated through the balance of mass and momentum in a control volume within the chambers. By setting the control volume (Figure 1A) as a differential cylindrical volume with axial length Δz , set in the direction of flow, the momentum balance becomes:

$$\rho \frac{\partial u}{\partial t} \pi D^2 \Delta z + \rho (u^2 \pi D^2|_{z+\Delta z} - u^2 \pi D^2|_z) = -(P \pi D^2|_{z+\Delta z} - P \pi D^2|_z) - (4 \pi D \tau_{rz}) \Delta z + (\tau_{zz} \pi D^2|_{z+\Delta z} - \tau_{zz} \pi D^2|_z), \quad (1)$$

where ρ is density, u is velocity in the direction of flow, t is time, D is the diameter, P is pressure, and τ_{zz} and τ_{rz} are components of the viscous stress tensor. Cylindrical coordinates are used and are listed as subscripts where z is axial coordinate. This model neglects variations in the radius along the cross-section of the heart. Through the momentum balance (Eq. 1), we obtain the partial differential equation:

$$\rho \frac{\partial u}{\partial t} D^2 + \rho \frac{\partial u^2 D^2}{\partial z} = -\frac{\partial P D^2}{\partial z} + \mu \frac{\partial^2 u D^2}{\partial z^2} - \mu C u, \quad (2)$$

where D is diameter, μ is viscosity, and the variable C is a constant accounting for the velocity profile when calculating shear stress. Due to the small scale of the fluid, the terms on the left side of Eq. 2 can be canceled since the Reynolds and Womersley numbers are much less than one. We calculate both based on equations given in Table 2. After neglecting inertial terms, we are left with:

$$\frac{\partial P D^2}{\partial z} = \mu \frac{\partial^2 u D^2}{\partial z^2} - \mu C u. \quad (3)$$

Separately, the continuity of mass is also applied to the cylindrical control volume:

$$\frac{\partial}{\partial t}(\pi D^2 \Delta z) = -(u\pi D^2|_{z+\Delta z} - u\pi D^2|_z), \quad (4)$$

resulting in the partial differential equation:

$$\frac{\partial u D^2}{\partial z} = -\frac{\partial D^2}{\partial t}. \quad (5)$$

Eq. 6 is then substituted into Eq. 4 to yield:

$$\frac{\partial P D^2}{\partial z} = -\mu \frac{\partial}{\partial z} \left(\frac{\partial D^2}{\partial t} \right) - \mu C u. \quad (6)$$

This model assumes that the heart contracts axisymmetrically and that the rate of contraction (i.e., the 1st term on the right-hand side of Eq. 6) drives the pressure gradient within the heart. There is also a resistance to flow that develops due to viscous resistance (i.e., the 2nd term on the right-hand side of Eq. 6). Neglecting the viscous resistance term for a moment, we can estimate the pressure contribution from the contracting wall, independent of all other factors:

$$\Delta P = \Delta \left(-2 \frac{\mu}{D} \frac{dD}{dt} \right). \quad (7)$$

The viscosity of blood in the embryonic zebrafish has been estimated with considerable variability in previous studies (Hove et al., 2003b; Jamison et al., 2013; Santhanakrishnan and Miller, 2011).

Therefore, pressure in Eq. 7 was non-dimensionalized through viscosity and heart rate:

$$P_{norm} = \frac{P}{\mu * HR} = -\frac{2}{DHR} \frac{dD}{dt}, \quad (8)$$

where *HR* is heart rate. In order to account for variation in heart size associated with allometric scaling with the overall size of the embryo, diameters were normalized to the maximum atrial inlet diameter, as previously done (Johnson et al., 2015). Normalization is summarized in Table 2. Ensemble averages of the normalized diameters were calculated from six cardiac cycles. Since image sequences were 2-dimensional, the area of the interior heart normal to the flow was assumed to be of a circular profile. Advantages and limitations of this assumption have been described in detail by Johnson, et al. (Johnson et al., 2013a). Note that Eq. 8 is independent of fluid velocity.

Diameter Measurement:

Diameter changes at the center of the atrium and ventricle for Eq. 8 were analyzed using a method of spatiotemporal analysis developed by Johnson, et al. (Johnson et al., 2013a). These measurements are needed for pressure calculations described later. In short, the background was subtracted from registered image sequences. The heart rate was calculated to ensemble average further parameters across multiple cardiac cycles by creating a spatiotemporal (ST) plot from a reference line placed on the edge of the heart to determine the number of frames to repeat motion (Johnson et al., 2013a). To measure diameter, an additional ST plot was calculated from a reference line spanning the width of the heart at the region of interest, perpendicular to the direction of flow (Figure 1B).

Length of Closure Measurement:

Assuming axisymmetry, there is a viscous resistance to flow across the AVC. In relation to pressure, viscous resistance is inversely proportional to the diameter squared and is proportional to the length (dz) that the viscous resistance acts upon:

$$\Delta P \propto - \left(\frac{\mu C u}{D^2} \right) dz. \quad (9)$$

This term would be dominated by the smallest radius in the control volume, which occurs when the radius of the atrium and/or AVC is at a minimum. Alternatively, when the atrium and ventricle are fully open, this viscous resistance term would be small. Since we lack 3D information, we cannot calculate the viscous resistance term with reasonable accuracy, especially because the atrium is known to close with a dumbbell-shaped profile during systole, complicating the estimation of the orifice area. Furthermore, the lumen during atrial closure is very small and any error in measuring the lumen would create a large error in viscous resistance measurements to the relationship between pressure and the diameter squared (Eq. 9). Therefore, our analysis of flow resistance is limited to qualitative estimations of the

coaptation length, or “length of closure” along the contracting atrium and AVC, represented by dz in Eq. 9. The length of closure of endothelial cells on the interior of the atrium and AVC was calculated manually using imageJ software. Length was calculated via a segmented line drawn along the line of closure created by contact between endothelial layers as they meet during chamber contraction (Figure 1C). Closure lengths were determined at twelve evenly-spaced time points across each cardiac cycle. As with diameter measurements, allometric scaling was accounted for by normalizing closure lengths to the maximum atrial inlet diameter (Table 2).

RESULTS

According to Eq. 8, atrial expansion (atrial diastole) and ventricular contraction (ventricular systole) are expected to occur in conjunction with retrograde flow across the AVC. The percentage of embryos exhibiting either atrial expansion or ventricular contraction are compared with the presence of retrograde flow at 5% increments of the cardiac cycle (Fig. 2A). A small fraction of embryos experience retrograde flow as the atrium begins to expand and ventricle begins to contract at about 85% of the cycle. Between 15% and 45% of the cycle, the majority of embryos experience retrograde flow, which coincide with a majority of embryos experiencing atrial expansion and ventricular contraction (Fig. 2A, shaded region).

To further explore diameter changes, ensemble averages of normalized diameters are compared between embryos with and without retrograde flow (Fig. 2B), which reveals that the ventricular diameter change corresponds to retrograde flow. Specifically, the minimum diameter in the ventricle occurs at about 40% of the cardiac cycle for embryos with retrograde flow, compared to 30% of the cardiac cycle for embryos without retrograde flow. The maximum atrial diameter for all embryos occurs at approximately the same point in the cardiac cycle.

239
240 The contribution to the pressure gradient from rate of diameter changes in the atrium and ventricle is
241 presented in Fig. 3A. During the period of the cycle for which a majority of embryos experience
242 retrograde flow (15%-45%), pressure due to atrial expansion is relatively the same in embryos without
243 retrograde flow. During this same window, pressure due to change in diameter of the ventricle is
244 significantly less in embryos without retrograde flow, and even decreased below the pressure due to
245 atrial expansion at about 35% of the cycle. To determine the statistical significance of the pressure
246 difference due to change in diameters of the atrium and ventricle, p-values are calculated between the
247 groups of embryos with and without retrograde flow across the cardiac cycle (Fig. 3B). Pressure
248 differences are significantly less ($p < 0.01$) between about 90% and 50% of the cycle, except for a brief
249 period (16-20% of the cycle); when retrograde flow occurs in at least 50% of embryos, based on Fig. 2.
250 Thus, the timing of significant pressure difference fully overlaps the period when retrograde flow occurs
251 in the majority of embryos (15-45%), but it occurs other parts of the cardiac cycle as well (0-45% and 75-
252 100%).

253
254 Resistance to retrograde flow is also examined based on endothelial closure measured along the atrium.
255 Fig. 4 shows the length of closure of endothelia lining in embryos with and without retrograde flow
256 across a cardiac cycle. In all embryos with retrograde flow, there is a point of zero closure (at about 50%
257 of the cycle), when no contact is observed between the endothelial cells. Typically, the no-contact point
258 occurs just after the end of atrial diastole and just before ejection into the ventricle. In embryos without
259 retrograde flow, nearly all embryos experienced full contraction at the atrial inlet either slightly before,
260 or just as the atrium ejected through the open AVC. Therefore, some portion of the atrium remained
261 closed at all times. For the entire duration of the cardiac cycle that a majority of embryos experienced
262 retrograde flow, there was significantly less length of closure ($p < 0.0001$) in those embryos than in

embryos without retrograde flow. Furthermore, reverse flow only occurs when the normalized length of closure is less than 1. Resistance due to endothelial closure may be one inhibitory mechanism of retrograde flow, although more may exist.

DISCUSSION

Overall, the mechanisms driving reverse flow appear to occur through a combination of two mechanisms: 1) increased pressure drop associated with the ventricular contraction and atrial expansion rates, and 2) lowered resistance to flow due to reduced endothelial closure length. For the former, the maximum reduction in ventricular diameter occurs earlier in embryos lacking retrograde flow compared to those with normal retrograde flow. This inflection point changes the sign of the ventricular contribution to pressure drop in Eq. 8. The sign change causes an overall lower pressure drop for cases of no reverse flow relative to cases with reverse flow during a time interval when reverse flow is seen (15-45% of cycle), as shown in Fig. 3B. In summary, the driving pressure caused by contraction and expansion of the heart chambers is lowered in zebrafish lacking reverse flow relative to the driving pressure seen in embryos with reverse flow. Despite the fact that there exists a significantly greater pressure difference associated with diameter change in embryos with retrograde flow, this mechanism does not solely explain why retrograde flow occurs when it does. In particular, although much greater pressure differences exist earlier in the cardiac cycle, these are not sufficient to generate retrograde flow. Therefore, endocardial closure within the atrium appears to function as a second, inhibitory mechanism regulating retrograde flow. As the atrium contracts, the endocardial layer of cells appear fully closed, although red blood cells moving in reverse are still able to squeeze through the closure. As the atrium expands, the length of endothelial contact decreases, beginning with atrial expansion at the atrial inlet. By the time the atrium nears the end of expansion near the AVC, the length of closure offers only a limited resistance to the pressure difference across the AVC. This is supported by Fig. 4, which

shows that the closure length was significantly less in embryos with retrograde flow for most of the cardiac cycle except during atrial contraction. It can be seen that the difference between the closure length trends of embryos with and without retrograde flow undergoes a notably steady departure just before the period of the cardiac cycle in which a majority of embryos experience retrograde flow. This suggests that there is a maximum closure length at this time beyond which retrograde flow is unable to be generated. It is important to note that our resistance estimation is qualitative since we lack three-dimensional high resolution information of the orifice during closure.

A diagram illustrating the effect of the combination of pressure differences due to diameter change of the chambers and closure resistance on the generation of retrograde flow across the AVC is shown in Fig. 5. This diagram displays a simplified model of the system using two 'balloons' and a variable resistor. As one balloon (representing the atrium) expands and the other (representing the ventricle) contracts, fluid inside is pushed out of the contracting balloon towards the expanding balloon in the direction of retrograde flow, however flow is inhibited by the variable resistor until resistance decreases past a certain threshold. The ensemble average of the data supporting the above model predicts that retrograde flow is likely to begin when the normalized pressure differential reaches approximately 2.23 and when the normalized length of closure decreases past a factor of 1.07 of the maximum atrial inlet diameter. Retrograde flow should cease when closure length reaches a factor of 0.31 of the maximum atrial inlet diameter and when the pressure differential decreases to 0. These two mechanisms therefore work in tandem to confine retrograde flow to 15% to 45% of the cardiac cycle in a majority of normal embryos.

The primary limitation in this study is that image sequences were only obtained in two dimensions. Therefore, analysis of the flow using spatiotemporal plots assumed a circular profile in modeling the

lumen of the heart. When the atrium is contracted, closure of the endothelium seen on the focal plane in view causes the AVC to appear as if it has a zero diameter. However, red blood cells moving in retrograde could be seen behind the line of endothelial contact in a separate focal plane. Based on this, we predict that circular profile assumption would produce substantial errors in quantifying resistance, due inability to measure small diameters, which limits our analysis to a qualitative assessment. Additionally, pressures associated with diameter change assumed axisymmetric chambers, so the center diameter selected to represent ventricular and atrial diameter changes is not adequate to provide a highly accurate representation of the overall change in volume in either chamber. During contraction of the heart tube of embryonic chicks, contraction results in eccentric deformation of the tube, resulting in a significantly different shape than that of a circular profile, which is likely also the case in embryonic zebrafish as well (Manner et al., 2010). Integration of diameters on multiple focal planes and points along the heart into a more accurate volume would allow for resistance calculations and improve the accuracy of pressure calculations; however this would drastically increase the time of analysis. Though it would be beneficial to calculate highly accurate, non-normalized, pressure in further studies, it would likely not affect the overall outcome of this study as to the mechanistic influence of retrograde flow. The effect of centrifugation also provides additional limitations to this study. Centrifugation often results in impaired morphology and functional defects depending on the six-hour stage of development in which it is induced (Johnson et al., 2015). Therefore, when choosing embryos with a lack of retrograde flow to include in this study's experimental group, only a small selection of embryos with no other apparent morphological or functional defect were selected from a large batch of centrifuged embryos.

CONCLUSION

Since retrograde flow through the atrioventricular canal is an established biomechanical force regulating valve development, this study aimed to determine the influencing mechanisms that drive retrograde

flow. By comparing pumping mechanics between sets of embryonic zebrafish both with and without retrograde flow, two key parameters were determined to have the greatest effect. First, the pressure difference created by simultaneous expansion of the atrium and contraction of the ventricle proved to be significantly less in embryos without retrograde flow during the phase of the cardiac cycle that retrograde flow occurred. Secondly, the contracted region of endothelium closure resists retrograde flow as the atrium expands and the ventricle contracts. Retrograde flow initiates when the closure length decreases past a threshold such that it can no longer oppose the pressure difference driving flow in retrograde. Retrograde flow is then sustained until the pressure difference drops below zero, driving flow forward into the ventricle. This study illuminates the sensitivity of the haemodynamic conditions necessary for normal valvulogenesis to the pumping mechanics of the developing heart.

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447

FIGURES

Table 1. Percentage of Embryos Centrifuged at Various Key Developmental Stages

Group	Increment	Stage	% Embryos
1	24 – 30 hpf	Beginning of Circulation	33
2	30 – 36 hpf	Early Cardiac Looping	33
3	36 – 42 hpf	Mid-Cardiac Looping	22
4	42 – 48 hpf	Late Cardiac Looping	11

Table 2. Measured and Normalized Parameters of this Study

Parameter Name	Parameter Symbol	Normalized Parameter
Reynolds Number	Re	$\frac{\rho UD}{\mu}$
Womersley Number	α	$\frac{D}{2} \sqrt{\frac{2 \pi \rho H R}{\mu}}$

Figure 1. A: Diagram outlining the differential control volume within the heart used to estimate pressure associated with expansion and contraction of the chamber. **B:** Image of zebrafish heart at 48 hpf during atrial ejection. The reference line is shown across the atrioventricular canal, where retrograde flow is observed during atrial expansion and ventricular contraction. **C:** Image of zebrafish atrium during closure with segmented line drawn in ImageJ software to analyze closure length.

Figure 2. Timing of Retrograde Flow at the AVC Relative to Chamber Expansion and Contraction. **A:** The fraction of embryos with retrograde flow occurring across the AVC (solid black) relative to the fraction of embryos undergoing expansion of the atrium (dotted black) as well as contraction of the ventricle (solid grey) with respect to time across a cardiac cycle. Calculations were made at 5% increments. **B:** Ensemble average of normalized diameters at the center of the atrium (black) and ventricle (grey) in embryos with typical retrograde flow (Typ RF, solid line) and without retrograde flow (0 RF, dotted line) with respect to time across a cardiac cycle. Diameters are normalized to the maximum atrial inlet diameter. The shaded region corresponds to the period in which over 50% of embryos experience retrograde flow.

Figure 3. Pressure Differences due to Chamber Expansion and Contraction in Relation to Retrograde Flow. **A:** Ensemble average of the normalized pressure due to diameter change at the center of the atrium (black) and ventricle (grey) in embryos with retrograde flow (solid line) and without retrograde flow (dotted line) with respect to time across a cardiac cycle. Error bars indicate standard error. **B:** Ensemble average of the difference of the normalized pressure due to diameter change between the atrium and ventricle in embryos with retrograde flow (solid line) and without retrograde flow (dotted line). (* $p < 0.01$) The shaded region corresponds to the period in which over 50% of embryos experience retrograde flow.

Figure 4. Atrial/AVC Endothelial Closure in Relation to Retrograde Flow. Ensemble average of the normalized length of closure along the atrium and AVC in embryos with retrograde flow (solid line) and without retrograde flow (dotted line) with respect to time across a cardiac cycle. Closure lengths were normalized to the maximum atrial inlet diameter. Error bars indicate standard error. (* $p < 0.0001$) The shaded region corresponds to the period in which over 50% of embryos experience retrograde flow.

Figure 5. A model illustrating the dual mechanisms driving retrograde flow. “Balloons” are used to indicate the change in volume of the atrium and ventricle. A variable resistor is used to indicate the change in resistance due to a changing length of closure of the atrium and AVC.