DEVELOPMENTAL DYNAMICS 238:1796–1802, 2009

Analysis of Heart Valve Development in Larval Zebrafish

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Malformations of the cardiac endocardial cushions (ECs) and valves are common congenital dysmorphisms in newborn infants. Many regulators of EC development have been identified, but the process of valve maturation is less well understood. Zebrafish have been used to understand cardiogenesis through 6 days postfertilization, yet mature heart valves are not present at this stage. By analyzing valve development in larval zebrafish, we identify that valve development proceeds in two phases. Valve elongation occurs through 16 dpf independently of localized cell division. Valve maturation then ensues, resulting from deposition of extracellular matrix and thickening of the valves. Whereas elongation is consistent between larvae, maturation varies based on larval size, suggesting that maturation occurs in response to mechanical forces. Taken together, our studies indicate that zebrafish valve morphogenesis occurs in the larval period, and that zebrafish may provide a unique opportunity to study epigenetic mechanisms leading to human congenital valvular disease, when studied at the appropriate developmental stages. ***Developmental Dynamics 238:1796–1802, 2009.*** **© 2009 Wiley-Liss, Inc.**

Key words: heart valve development; extracellular matrix; zebrafish; congenital heart disease; biomechanical forces; epigenetics; epithelial mesenchymal transformation

Accepted 11 April 2009

INTRODUCTION

The heart is one of the organs most commonly affected by congenital birth defects, with cardiac malformations occurring in approximately 1% of all births (Hoffman et al., 2004). Therefore, understanding the genetic, molecular, cellular, and epigenetic factors regulating heart morphogenesis may provide significant medical benefits to newborns and infants. Errors in very early heart development tend to lead to embryonic lethality; thus, congenital disease is predominately caused by errors in late steps of heart development, specifically malformations of the endocardial cushions (ECs) and their derivatives: the septae and valves. A body of studies in multiple in vivo and in vitro systems has focused on elucidating the many regulators of EC development (reviewed in Person et al., 2005), but less is known about the development of the true valve leaflets.

Among in vivo model systems, unique opportunities for understanding morphogenesis are provided by zebrafish embryos which allow for continuous observation of the developing organism and in which perturbation of development can readily be achieved through several means (e.g., forward genetics screens, chemical treatment). Recently the process of EC morphogenesis in zebrafish has been extensively described through the first 6 days of development (Hove et al., 2003; Bartman et al., 2004; Beis et al., 2005). These studies have verified that genes implicated in EC morphogenesis in other species also play a role in zebrafish, have identified novel genes required for EC development, and have implicated physical forces as a major epigenetic factor regulating EC morphogenesis. Yet, surprisingly, neither the mechanisms by which early structures give rise to true valve leaflets during the larval stage, nor the regulators of valve development,

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DOI 10.1002/dvdy.21976
Published online 14 May 2009 in Wiley InterScience (www.interscience.wiley.com).

have been identified in the powerful zebrafish model.

Some work in other species has sought to understand the process of valve development from ECs. The expression patterns of cardiac regulatory genes and extracellular matrix molecules has been described in both developing mouse and chick valve leaflets (Lincoln et al., 2004). Other studies have shown that molecular changes occur in the murine heart after birth, presumably in response to the altered hemodynamic conditions seen by the heart after transition from intrauterine to extrauterine life (Kruithof et al., 2007). Understanding the process of valve leaflet development in the larval zebrafish will allow for comparison and contrast between these model systems, and will guide future studies of valvulogenesis to the organism with the strengths to answer any particular hypothesis.

Recently, it has been reported that zebrafish may form heart valves from a process of invagination, rather than from mesenchymal ECs (Scherz et al., 2008). While this study does indeed demonstrate a linear structure protruding into the lumen of the AV boundary before 60 hpf, the putative valve described exists only on the superior side of the AV boundary, and functions to occlude the lumen of the AV boundary through compression of the sides of the boundary rather than through a more typical parachute-like function seen later in development in higher species. How this simplified structure, which appears markedly different from mature zebrafish valves, transfigures beyond 4 dpf was not explored in their study.

Therefore, comparing all available images of zebrafish ECs or "valves" before 6 dpf with images of fully mature valves in adult zebrafish (Hu et al., 2000), we recognized that significant morphogenetic events occur between 4 and 6 dpf and adulthood which have never been analyzed in this model system. We have chosen to perform a systematic analysis of AV valve development in the zebrafish, beginning at 6 dpf and continuing through 28 dpf, to provide a reference which will allow the zebrafish to be used for the study of normal and abnormal valvulogenesis. Our data demonstrate that the primitive structure present at 6 dpf does undergo significant changes before resembling a mature leaflet or cusp. Furthermore, we discovered that valve development through this period, much like earlier steps of heart morphogenesis, appears to be affected by epigenetic factors.

RESULTS

To study the process by which zebrafish atrioventricular (AV) valves arise from the formed ECs, zebrafish larvae from 6 days postfertilization (dpf) up to 28 dpf were prepared for histological analysis. Previous studies have detailed the molecular and cellular events of zebrafish endocardial cushion formation leading up to 6 dpf (Beis et al., 2005), but provided scant details regarding the process by which mature valves arise. We have focused our work on AV (as opposed to outflow tract) valve development to allow our work to build upon the literature of EMT/EC development which is typically studied in the AV region.

Significant Leaflet Elongation Occurs from 6 to 16 dpf

In zebrafish, endocardial cushions or very primitive leaflets have formed at the AV boundary of the heart by 6 dpf through previously described mechanisms (Beis et al., 2005; Scherz et al., 2008). We made extensive use of the *flk1::GFP* transgenic line (Beis et al., 2005; GFL, green fluorescent protein) and immunohistochemisty to identify the appearance and timing of valvulogenesis from these early structures. On sagittal sections at 6 dpf, the ECs tend to appear globular, and are generally aimed toward each other across the lumen of the AV boundary (Fig. 1A, representative sample shown). Proceeding through 8, 12, and 16 dpf, the valve leaflets become progressively longer and thinner, and have their distal tips pointed into the ventricle, as would be expected if they are to provide resistance to regurgitant flow from the ventricle to atrium (Fig. 1B–D, representative samples shown). To measure valve length in a sample population, hematoxylin and eosin staining was performed on serial 5 μm sagittal sections of multiple embryos/larvae and the section with the longest valve length from each embryo/larva was measured. At 8 days, the average maximal length of either AV valve leaflet seen on serial sagittal sections was 17.7 μm with a standard deviation of 0.27 μm. By 12 dpf, the length of either valve was 26.4 μm with a standard deviation of 0.26 μm. By 16 dpf, average valve length increased further to 28.4 μm but the length of the valves varied more widely between individual larvae, with a standard deviation of 7.55 μm. Through the first 16 days, the valve leaflets appear to be highly cellular, with little extracellular matrix separating the cell nuclei (Fig. 1A–D). Both flk1::GFP-positive and flk1::GFP-negative cells are seen in the valve during this developmental period. Beyond 16 dpf, and through 3 mpf, the length of the valves seen on sagittal sections continued to increase, although less dramatically than before 16 dpf and still with wide variation between individuals (data not shown).

Minimal Cell Proliferation Occurs in the Valve Leaflet From 6 to 16 dpf

To determine whether lengthening of the valve leaflet is accompanied by localized proliferation of EC/leaflet cells, bromodeoxyuridine (BrdU) staining was performed on serial sections from at least five embryos/larvae at times from 6 through 16 dpf in embryos/larvae carrying a *flk1::GFP* transgene. At 6 dpf, rare BrdU-positive cells were seen in the ECs (Fig. 2A). However, at 8 and 12 dpf, no BrdU-positive cells were seen on any section from any larva in the developing leaflet (Fig. 2B,C). By 16 dpf, proliferative cells again appeared in some sections of some larvae in the valve leaflet, most commonly at the distal tip (Fig. 2D). At all stages, BrdU staining was seen in the villi of the intestine or the heart chamber myocardium as a positive control (not shown). Lengthening of the nascent valve leaflet between 6 and 16 dpf despite the absence of any evidence of cell proliferation suggests that in the zebrafish, valve development from the globular ECs or primitive valve occurs through a process of cell migration and/or reorganization.

Epithelial–Mesenchymal Transformation Occurs in the Leaflet From 6 to 16 dpf

At the earliest stages of development, the atrioventricular ECs contain cells which are predominantly GFP-positive in a flk1::GFP transgenic line (Beis et al., 2005). However, as seen in Figure 1D, by 16 dpf a significant number of cells in the leaflet are flk1::GFP-negative, suggesting that some AV cells are no longer endothelial as flk1 expression is a hallmark of endothelial cells (Liao et al., 1997; Sumoy et al., 1997; Kamei et al., 2004; Jin et al., 2005). To determine the identity of these cells, immunohistochemistry was performed using antibodies that are specific for either epithelial or mesenchymal cells and which identify cells transitioning between these identities. Epithelial cells are typically identified by an organized structure, the presence of tight junctions which contain ZO-1, and cytokeratins (Lee et al., 2006). Mesenchymal cells are expected to stain for vimentin, be surrounded by ECM components such as Versican, and have increased focal adhesions and focal adhesion kinase. An intermediate cell type also exists which shows markers of both epithelial and mesenchymal cells along with a loss of tight junctions (Lee et al., 2006). Our data show that at 12 dpf (Fig. 3A–C), the developing AV valves are positive for ZO-1–containing junctions (Fig. 3A), punctate focal adhesion kinase staining (Fig. 3B), are pancytokeratin positive (Fig. 3G), and lack significant Vimentin (Fig. 3H) indicating the epithelial identity of these cells. However, by 16 dpf, cells are found in the developing leaflet which have lost their ZO-1–containing tight junctions (Fig. 3D), stain for both pancytokeratin (Fig. 3J) and vimentin (Fig. 3K), and which have large depositions of focal adhesion kinase (Fig. 3E), which suggest that AV valve cells are transitioning between an epithelial and mesenchymal phenotype, which is indicative of a intermediate cell type. Taken together, these data suggest that the flk1::GFP-negative cells at 16 dpf are intermediate—demonstrating both epithelial and mesenchymal traits. These data also suggest that the developing valve leaflets are initially composed primarily of sheets of epithelial endocardium, but that expansion and organization of the valves takes place as endocardial cells enter the valve mesenchyme.

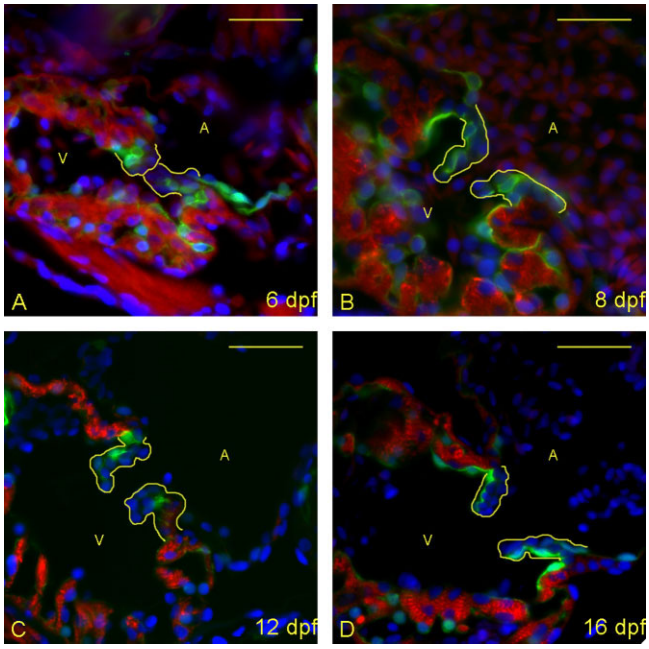
Deposition of Extracellular Matrix and Valve Maturation Occurs Beyond 16 dpf, but to Differing Extent in Larvae of Various Size

Although some variability in the appearance of the developing AV valve leaflets is apparent at 16 dpf, in general the leaflets remain thin and cellular, with little supporting substance between cell bodies. At 16 dpf, juvenile fish are freely swimming and feeding, and are generally a consistent size and weight, although very significant growth will still be required to reach adult size. Because previous images of adult zebrafish AV valves (Beis et al., 2005) indicates that the appearance of the valve leaflets at 16 dpf is still not mature, we examined the morphology of the AV valves at 28 dpf. We found that the majority of larvae examined at 28 dpf had valve leaflets with significantly more deposition of extracellular matrix components such as collagens and Versican when compared with the majority of larvae examined at 16 dpf (Fig. 4). The maturation of the valves from 16 to 28 dpf also leads to a change in morphology from mostly linear protrusions into the lumen of the heart toward a more cusp-like shape (apparent in Fig. 4B).

As mentioned above, after examining multiple larvae at various ages of development, we realized that despite raising embryos/juveniles in a consistent manner, all larvae at a particular age did not necessarily have valve leaflets of identical appearance. Furthermore, our assessments indicated that while larvae/juveniles tended to grow fairly consistently up to 16 dpf, beyond 16 dpf variances in juvenile size would often occur even in the same tank. To determine whether larval size affects the morphology of the developing valves, multiple animals at time-points from 8 dpf to 3 mpf were weighed and measured, and the thickness and length of the valves were determined by serial sagittal section as described previously. We discovered that larval age was a poor predictor of valve thickness or length, especially beyond 28 dpf (Fig. 5A). However, using larval weight as the independent variable led to a much better correlation with valve length and thickness over the entire range of ages examined (Fig. 5B). To confirm that valves would be at different stages of morphology at the same age dependent on size, small and large larvae at 28 dpf were selected for immunohistochemical analysis. At 28 dpf, smaller larvae (Fig. 6A,C) did show the presence of Versican, Collagen I, and Collagen II which are not present in 16 dpf larvae (Fig. 4A); however, the expansion of the ECM in small larvae occurred to a much lesser extent than what is seen in larger 28 dpf larvae (Fig. 6B,D). These data suggest that later steps of valve morphogenesis are influenced by the size of the developing animal, possibly in re-

Fig. 1. Zebrafish heart valves elongate but remain highly cellular between 6 and 16 days postfertilization (dpf). **A–D:** Five micrometer paraffin sections of flk1::GFP (GFL, green fluorescent protein) transgene-carrying zebrafish embryos/larvae were imaged to identify the morphology of the atrioventricular (AV) boundary and intraluminal structure. Atria (A) and ventricles (V) are labeled (yellow letters), and yellow lines outline the extent of the AV structure (cushion and/or valve). Staining identifies cell nuclei (DAPI, 4′,6-diamidine-2-phenylidole-dihydrochloride; blue), myocardium (anti-MF-20: red), and endothelial cells (anti-GFP; green). Through this time period, the valves progressively lengthen and thin, and little to no extracellular matrix is seen within the valve leaflets. The most significant change is in cellular organization from 12 (C) to 16 (D) dpf, with the leaflets developing a linear, two-cell-layer thick form. Scale bar = 20 μm.

Fig. 2. Proliferative cells are seen in the atrioventricular (AV) boundary at 6 and 16 days postfertilization (dpf), but not between these times. **A–D:** Five micrometer paraffin sections of flk1::GFP transgene-carrying zebrafish embryos/larvae were imaged to identify proliferating cells in the AV boundary and intra-luminal structure. Atria (A) and ventricles (V) are labeled (yellow letters), and yellow lines outline the extent of the AV structure (cushion and/or valve). Staining identifies cell nuclei (DAPI; blue), proliferative cells (anti-bromodeoxyuridine [BrdU]; red), and endothelial cells (anti-GFP; green). (DAPI not shown in panel D to emphasize BrdU-positive cells). Proliferative cells are seen in the developing valves at 6 dpf (A) and 16 dpf (D), but not 8 and 12 dpf (B,C). Scale bar = 20 μm.



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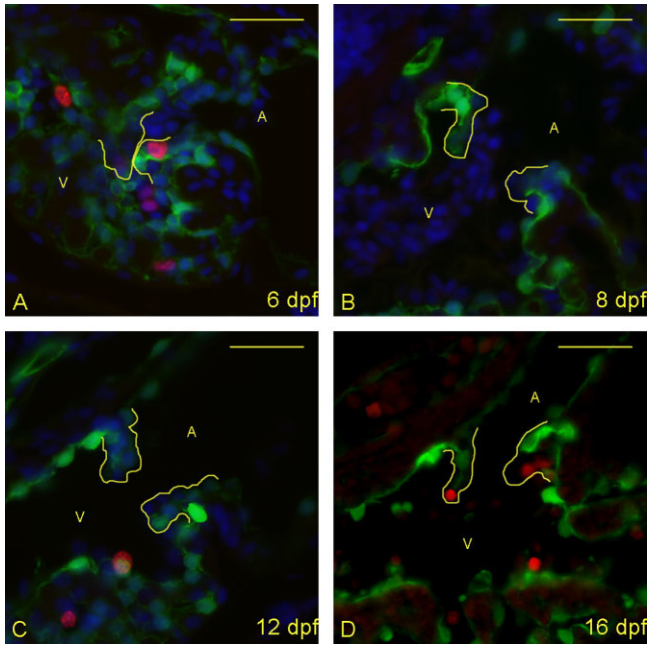

Fig. 2.

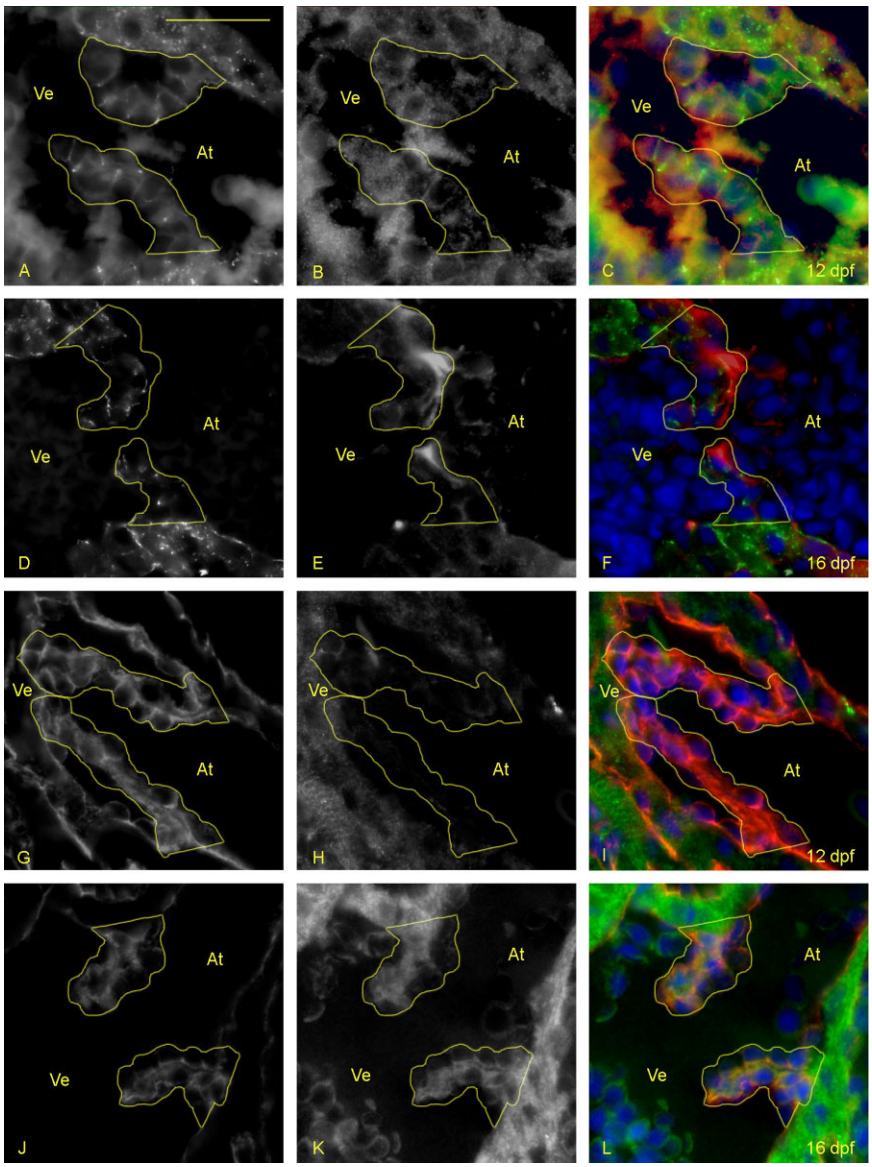

Fig. 3.

Fig. 3. Valve leaflet cells become intermediate or mesenchymal between 12 and 16 days post-fertilization (dpf). A–L: Five-micrometer paraffin sections of flk1::GFP (GFL, green fluorescent protein) transgene-carrying zebrafish embryos/larvae were immunostained to identify epithelial and mesenchymal cell phenotypes in the developing leaflets. Atria (At) and ventricles (Ve) are labeled (yellow letters), and yellow lines outline the extent of the atrioventricular (AV) valves. A–F: Staining identifies ZO-1 (A, D; green in C and F), focal adhesion kinase (B, E; red in C and F), and cell nuclei (DAPI: blue in C and F). The presence of ZO-1 reveals tight junctions that form to make cell to cell connections, which is indicative of an epithelial phenotype. FAK reveals whether there are transient (punctate) or long lived (large) cell:matrix adhesions occurring between these cells. **A–C:** At day 12, AV valve cells are organized, have ZO-1–containing tight junctions, and punctate FAK staining. **D–F:** By day 16, cells are less organized and some lack ZO-1–containing junctions. G–L: Staining identifies pancytokeratin (G, J; red in I and L), Vimentin (H, K; green in I and L), and cell nuclei (DAPI, 4′,6-diamidine-2-phenylidole-dihydrochloride; blue in I and L). **G–I:** At 12 dpf, the valves have a high level of pancytokeratin staining indicating that surrounding cells have a predominantly epithelial phenotype. J–L: By 16 dpf, a large increase in Vimentin is seen, indicating a shift toward mesenchymal cell phenotypes. Scale bar = 20 μm in A.

sponse to the forces exerted on the valves by the cardiovascular system.

DISCUSSION

This study provides the first detailed analysis of zebrafish valve elongation covering the period from the end of EC formation (at approximately 6 dpf) through the development of cusp-like multilayered valves (at approximately 28 dpf). Our analysis indicates that the process of AV valve development in the zebrafish proceeds through two distinct stages. First, the valves elongate from the ECs, thereby changing from the appearance of clusters of cells at the AV boundary to linear features which protrude into the lumen of the heart, angled into the ventricular chamber. This process occurs relatively consistently between embryos/larvae over the period from 6 to 16 dpf, and does not depend on localized cell division. After valve elongation a process of valve maturation ensues, during which the valve leaflets thicken with depositions of extracellular matrix molecules such as Collagens and Versican (Fig. 7). Our data suggest that endothelial cells of the nascent valve leaflet undergo an EMT event to populate the mesenchyme of the developing structure. Notably, maturation of the valves does not occur at a set time in development, but seems responsive to larval size which suggests that the pressure or flow experienced by developing valves can affect their structure.

Our data, combined with studies describing the timing of endocardial cushion development in zebrafish (Beis et al., 2005), demonstrate that heart valve development occurs in the zebrafish during larval stages, and has implications for work which seeks to use the zebrafish to understand the regulators of EC and valve development. Since the first forward genetic screens in zebrafish (Stainier et al., 1996), regurgitation of blood from the ventricle to the atrium of the zebrafish heart at approximately 48 hpf has been assumed to be an easily observable marker of dysmorphogenesis of the AV valves. However, more recent studies suggest that development of a significant intraluminal structure at the AV boundary has not yet occurred by 48 hpf (Beis et al., 2005). Our data clearly indicate that valve leaflets or cusps are not significantly developed at such an early stage. This suggests that ECs and/or valves can not be required for forward blood flow at 48 hpf, nor is the presence of regurgitant blood flow at this age an indicator of true valve malformation. Biomechanical studies in zebrafish indicate that between the stages of peristalsis and valve-limited flow, a phase occurs during which the heart acts as a suction pump, generating unidirectional flow without the need for valves (Forouhar et al., 2006), while other studies have demonstrated that proper myocardial function as well as intracardiac blood flow may be required for formation of the ECs (Hove et al., 2003; Bartman et al., 2004). Therefore, our data suggest that studies in zebrafish seeking to indentify the roles of genetic and epigenetic factors in valve leaflet development should be performed during the larval stages of development and that phenotypes seen before 4–6 dpf should not be attributed to valve leaflet dysmorphogenesis.

The specific mechanism by which the developing zebrafish heart valves sense the physiologic environment of the larval/juvenile zebrafish and adapt in response will require further study. The various ECM components of the valve leaflets have been shown experimentally to contribute differing structural and biomechanical properties to the valve (Butcher et al., 2007). Therefore, it is possible that both developing and mature valves may have mechanisms for adjusting the relative levels of proteoglycans, collagens, and cell bodies to alter valve flexibility and strength, and that disturbance of this process may lead to both congenital and acquired valvular diseases. Due to the ease of perturbing the environment in which larvae are raised combined with their optical clarity, further exploration of the elongation and maturation of heart valves in zebrafish may provide in vivo data to complement in vitro studies aimed at dissecting the forces required for valve development (Hildebrand et al., 2004; Goodwin et al., 2005; Morsi et al., 2007). Taken together, these studies indicate that regurgitant flow and EC malformations may occur together because proper flow and forces are required to regulate EC development in a manner similar to that demonstrated with other aspects of heart morphogenesis (Auman et al., 2007), and our current data extend the role of epigenetic factors in controlling heart development to include the much later process of valve maturation.

EXPERIMENTAL PROCEDURES

Zebrafish

Zebrafish were maintained as described in Westerfield (2000). Timed matings were used to guarantee that developmental age could be assessed within one hour. The genetic backgrounds used were either wild-type AB strain homozygotes or AB fish carrying a *flk1::EGFP* transgene (Beis et al., 2005). Fluorescent microscopy was used to select GFP-positive embryos before fixation. Embryos/larvae were raised at 28°C in egg water for the first 6 days and in system water at 28°C beyond 6 days. Animal protocols were approved by the IACUC of Cincinnati Children's Hospital Medical Center.

Immunohistochemistry and Imaging

Time-staged zebrafish were fixed overnight at 4°C in 4% paraformaldehyde diluted in phosphate-buffered saline (PBS). The fixed zebrafish were then dehydrated in series to xylene. Once in xylene, the zebrafish were incubated in hot paraffin and embedded on their sides. Tissue was sectioned at 5μm and melted on Thermo Polysine Microscope Slides (Anatomical Pathology International) overnight at 60°C. After baking, the tissue was run through 3 changes of xylene and rehydrated to PBS. After hydration, the tissue was then blocked for 2 hr in a 4% serum (Jackson Laboratories) corresponding to the source of the secondary antibody. The primary antibodies were added and allowed to incubate overnight at 4°C. The tissue was washed 5 times in PBS-triton between each addition of the secondary antibody. Tissue was then coverslipped with mounting medium containing DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride; Vector Labs) and imaged 12 hr later. Antibodies used and

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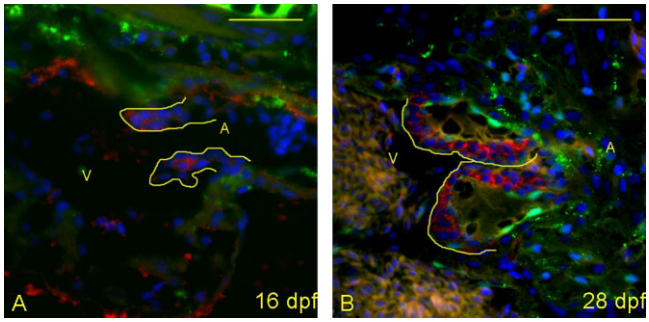

Fig. 4. Maturation of the valve leaflets initiates between 16 and 28 days postfertilization (dpf). A,B: Five-micrometer paraffin sections of zebrafish larvae were imaged to identify the structural appearance of the AV boundary and intraluminal structure. Atria (A) and ventricles (V) are labeled (yellow letters), and yellow lines outline the extent of the atrioventricular (AV) structure (cushion and/or valve). Cells were stained with DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride; blue), anti-collagen II (green) and anti-versican (red). **A:** At 16 dpf, the valve leaflets are highly cellular, with little collagen or versican present. **B:** By 28 dpf, the leaflets start to thicken with deposition of significant collagen fibers and versican.

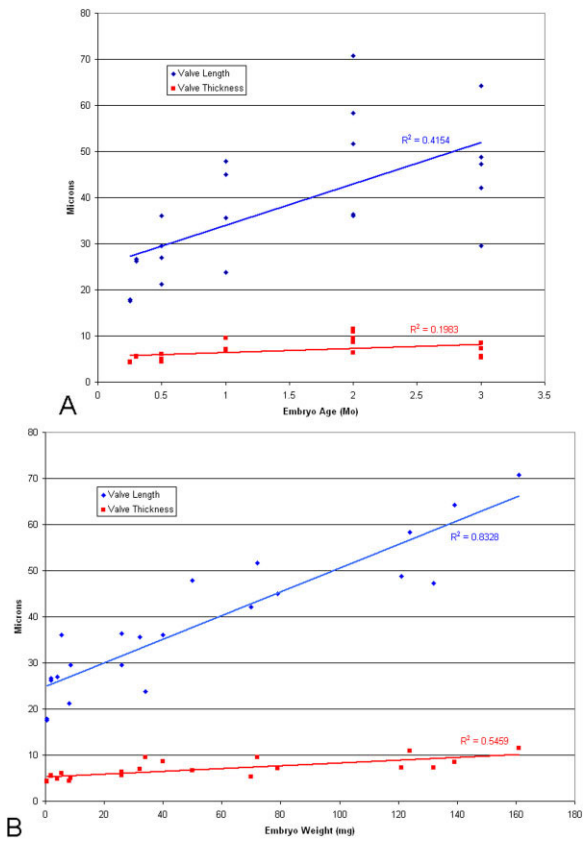

Fig. 5. Valve length and thickness correlate with larval size and not larval age. **A:** Plot of atrioventricular (AV) valve length (blue) or valve thickness (red) shows poor linear correlation against larval age from 8 days postfertilization (dpf) to 3 months postfertilization (mpf). R^2 values did not appreciably improve with nonlinear regression models. **B:** Plot of AV valve length (blue) or valve thickness (red) shows much better correlation with larval weight, suggesting that valve lengthening and maturation are not regulated exclusively by temporal factors, but also by animal-specific physiologic parameters.

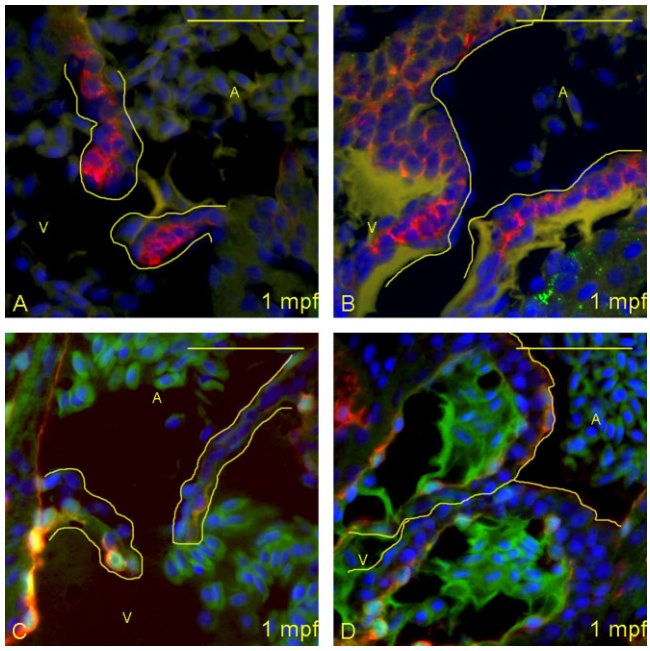

Fig. 6. Larger larvae have more pronounced maturation of the valves at 28 days postfertilization (dpf). A–D: Five-micrometer paraffin sections of flk1::GFP (GFL, green fluorescent protein) transgene-carrying zebrafish larvae were imaged to identify the structural appearance of the atrioventricular (AV) boundary and intra-luminal structure. Atria (A) and ventricles (V) are labeled (yellow letters), and yellow lines outline the extent of the AV structure (cushion and/or valve). **A,B:** Sections were immunostained with DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride; blue), anti-Versican (red) and anti-Collagen I (green). Whereas the smaller larva (A) has started deposition of Versican in the leaflet, the larger larva (B) has thickened considerably more, with increased deposition of Versican and Collagen I. **C,D:** From separate larvae than shown in A,B, sections were stained with DAPI (blue), anti-GFP (red), and anti-Collagen II (green). Again, the larger larva (D) has significantly more deposition of collagen than the smaller larva (C), despite being at the same age. Scale bar = 20 μm.

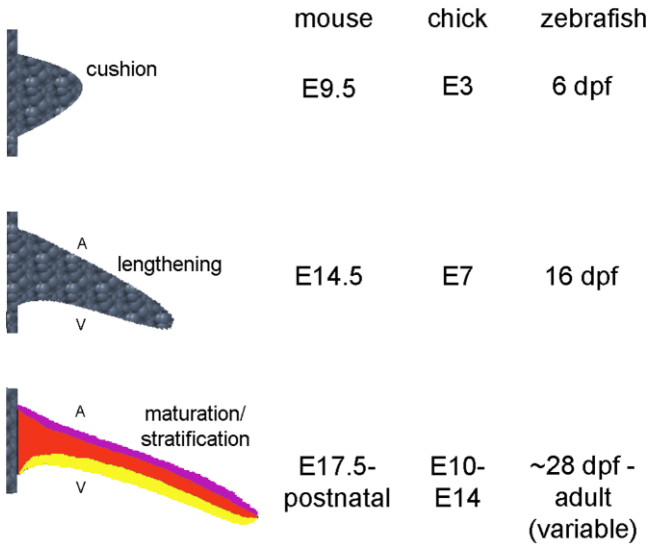

Fig. 7. Schematic of valve development in mouse, chick, and zebrafish. Illustrations of comparable stages of mouse (Lincoln et al., 2004; Kruithof et al., 2007), chick (Lincoln et al., 2004), and zebrafish atrioventricular (AV) valve development are shown. During the cushion and elongation stages, valve primordia are highly cellular, but become less so during maturation and stratification. The mature valves are layered with collagens on the ventricular side (yellow), elastin on the atrial side (purple), and are glycosaminoglycan-rich in between (red).

appropriate concentrations are as follows: mouse anti-MF20 (1:500, Iowa Hybridoma Bank), rabbit anti-Focal Adhesion Kinase (1:100, Santa Cruz Biotechnology), goat anti-Vinculin (1:100, Santa Cruz Biotechnology), rabbit anti-GFP (1:400, Molecular Probes), mouse anti-ZO-1 (1:300, Zymed), goat anti-Vimentin (1:200, Santa Cruz), mouse anti-pancytokeratin (1:300, Sigma), mouse anti-BrdU (1:200, Molecular Probes), mouse anti-collagen I (1:200, Iowa Hybridoma Bank), mouse anti-versican (1:400, Iowa Hybridoma Bank), and goat anti-collagen II (1:200, Santa Cruz Biotechnology). The appropriate secondary antibodies were used with Alexa Fluor 488 fluorescein isothiocyante or 594 Texas Red conjugates (1:200, Molecular Probes). All imaging was performed using a Zeiss Axioplan 2 microscope with an Axiocam MR camera. The imagery was captured with Axiovision Release 4.6 software. Images shown are representative samples of at least five embryos/larvae examined.

BrdU Uptake Assay

Our protocol was derived from (Poss et al., 2002). Time-staged zebrafish were incubated with BrdU (Aldrich) in system water at 6, 8, 12, and 16 dpf. Each time stage was subjected to 100 μM/ml BrdU uptake for 6 hr at room temperature, washed, and incubated for 2 more hr at room temperature. The embryos/larvae were then fixed overnight at 4°C in 4% paraformaldehyde and then subjected to immunohistochemical analysis.

Zebrafish Body Weight/Age/Valve Length and Width

Body length and weight of staged zebrafish of the AB strain were measured before fixation in 4% paraformaldehyde. Once fixed, the zebrafish were processed from PBS through 70% ethanol, embedded in paraffin, and sectioned at 6 μm. The paraffin sections were placed on Thermo Polysine microscope slides (Anatomical Pathology International) and baked overnight at 60°C. The slides were then subjected to hematoxylin and eosinophil staining, coverslipped, and viewed using a Zeiss light microscope. Photographs were taken and the length and width of the AV valves was measured using Metamorph software.

ACKNOWLEDGMENTS

T.B. was funded by the American Heart Association and the March of Dimes Foundation.

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