ASWAN HEART CENTRE
SCIENCE & PRACTICE SERIES
A Qatar Foundation Academic Journal

OPEN ACCESS

Review article

The cycle of form and function in cardiac valvulogenesis

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ABSTRACT

The formation and remodeling of the embryonic valves is a complex and dynamic process that occurs within a constantly changing hemodynamic environment. Defects in embryonic and fetal valve remodeling are the leading cause of congenital heart defects, yet very little is known about how fibrous leaflet tissue is created from amorphous gelatinous masses called cushions. Microenvironmental cues such as mechanical forces and extracellular matrix composition play major roles in cell differentiation, but almost all research efforts in valvulogenesis center around genetics and molecular approaches. This review summarizes what is known about the dynamic mechanical and extracellular matrix microenvironment of the atrioventricular and semilunar valves during embryonic development and their possible guidance roles. A variety of new computational tools and sophisticated experimental techniques are progressing that enable precise microenvironmental alterations that are critical to complement genetic gain and loss of function approaches. Studies at the interface of mechanical and genetic signaling in embryonic valvulogenesis will likely pay significant dividends, not only in terms of increasing our mechanistic understanding, but also lead to the development of novel therapeutic strategies for patients with congenital valve abnormalities.

Keywords: Morphogenesis, hemodynamics, congenital heart defects, animal models, blood pressure, extracellular matrix, remodeling, human, photoablation, microsurgery

DOI: http://dx.doi.org/10.5339/ahcsps.2011.10

Published: 30 December 2011

QSCIENCE.com
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Cite this article as: Lindsey SE & Butcher JT. The cycle of form and function in cardiac valvulogenesis, *Aswan Heart Centre Science & Practice Series* 2011:10
doi: http://dx.doi.org/10.5339/ahcsps.2011.10

INTRODUCTION

Congenital heart defects are among the most severe congenital abnormalities [1], accounting for over 29 percent of deaths from developmental abnormalities and 1 percent of infant mortality [2]. Advances in diagnostic technology and perinatal/neonatal intervention techniques facilitated a 33.3 percent decline in deaths from congenital heart defects between 1999 and 2006. If all patients with congenital heart defects born in the United States in the year 2000 underwent full treatment, the American Heart Association estimated 750,000 survivors for those suffering from simple lesions, 400,000 for those suffering for moderate lesions and 180,000 for those suffering from complex lesions. Without treatment these numbers would decline to 400,000, 220,000, and 30,000 respectively [2]. While technology and access to health care facilities are prevalent in the United States, most developing countries are not afforded the same luxuries. In impoverished countries, where 80 percent of all cardiovascular disease occurs, there is a lack of research and applicable therapies for cardiovascular disease [3]. Overshadowed by the trials of infectious disease, the large burden of cardiovascular disease is often overlooked in these countries [4].

Complementing advancements in surgical techniques has been a steady increase in our understanding of how the heart and valves develop and malform [5–7]. The classical approach was to observe morphological changes developed as a result of experimental manipulations in animal models, but over the past thirty years tools from the genetic revolution have dominated learning in this field [8–11]. Very recently, however, the joining of classical experimental and genetic approaches has revealed vast potential for understanding that can be applied directly to clinical experience [12,13]. It is becoming increasingly clear that the proper formation of the heart is intimately tied to its proper function at each stage of development. While these responses at a cellular level are driven by gene expression changes, it is networks of genes rather than single genes that are coordinated to bring about cardiac tissue assembly and remodeling [14]. Therefore, the role of the cellular microenvironment, particularly mechanical forces [15] and the heterogeneous extracellular matrix [16,17], have moved center stage in this pursuit. Advancing this frontier does not require the latest in first world research technology, but rather a focused synergy of biological and engineering disciplines. In this review, we summarize what is known about the morphogenesis of heart valves from the perspective of microenvironmental signaling, animal models and experimental techniques. We then conclude with some comments regarding microenvironmental regulation of key congenital heart defects.

Morphogenesis of valves

Prevalvular Cushions. The early embryonic heart originates from bilaterally symmetric fields of mesoendodermal cells that migrate and fuse medially to form a linear tube with an outer sheath of myocardial progenitor cells and an inner layer of endocardial cells. Separating these two layers is a gelatinous acellular hyaline matrix called the cardiac jelly. Initially, the cardiac jelly is present throughout the heart tube, but becomes restricted to the atrioventricular (AV) canal and outflow tract (OT) segments [18]. Recent studies suggest that TBX3 [19] and Notch1 [20] act in these regions to localize the myocardial-endocardial signals that initiate valve formation. In the first stage of this process, a subset of endocardial cells lining these two zones transform into a mesenchymal phenotype and invade the cardiac jelly [21,22]. The molecular process of endocardial to mesenchymal transformation (EMT) has been studied for over thirty years, with over 100 regulatory genes identified [23–29]. This invasive, proliferating mesenchyme progressively remodels the hyaluronan matrix, replacing it with proteoglycans, matricellular proteins, and eventually structural proteins such as collagen I [16,30,31]. These amorphous, compliant, cellularized masses, now dubbed cushions, continue to grow and extend into the lumen space [32]. Two cushions (superior and inferior) form initially in the AV canal at HH16 (E9.5 in mouse), followed by the appearance of two mural/lateral cushions on the left and right side of the AV canal at HH19 [33] (Fig. 1A). The superior and inferior AV cushions fuse together by HH26 (E12 in mouse) forming a septation of the AV canal that joins with the ventricular septum and the protruding atrial cap. The lateral portions of this fused mass undergo continued remodeling to valves, as do the left and right mural cushions.

Outflow tract. The outflow tract is somewhat different in that endocardial cells along nearly the whole tubular lumen undergo EMT. Paired bulges emanating in proximal (just outside the right ventricle) and distal zones (just after a 'dogleg' bend in the OT) become cushions around HH22/E10, while the rest

of the cardiac jelly regresses. A third distal cushion ridge forms later (HH25/E11). The proximal/conal cushions are alternatively referred to as the septal/sinistroventral and parietal/dorsodextral ridges [34,35]. These growing cushions also fuse in the midline, creating two tortuous lumens. Between HH26/E11.5 and HH30/E13, the distal dorsal cushion of the OT aligns with the proximal left cushion along the inner heart curvature, continuous with the superior cushion of the AV canal. Simultaneously, a wishbone shaped ridge of mesenchyme invades the outflow tract in a spiraling pattern, separating it into left and right portions and dividing the outflow cushions into two groups of three. While the fused proximal cushions myocardialize and form a muscular infundibulum separating the right and left ventricular outlets, the distal cushions become the rudiments of the pulmonary and aortic outlet valves [35] (Fig. 1C). Comparative animal staging is presented in Table 1.

Remodeling into fibrous leaflets

Atrioventricular valves. The AV myocardium forms a fold at its junction with the ventricular myocardium creating a substrate on which the AV cushions can extend. The cushions extend along their substrate through the expansion of a proliferation zone in the subepithelial portion of the AV cushions [36]. Fenestrations develop as a result of the elongating cushions and the ventricular tissue underneath the cushion tissue delaminates, resulting in primitive leaflets that are continuous with developing papillary muscles [37] and the simultaneous expansion of the ventricular OT [38]. The myocardial tissue of the AV valves disappears and they condense into fibrous leaflets (Fig. 2) [39]. Thin strands of elongated muscle remain tethered to the valve tissue with thickened trabecular aspects on the ventricular myocardial wall. These structures become the tendinous chords and papillary muscles of the mature valve [40].

Outflow tract. Unlike the AV valves, which formed through delamination from the muscular walls, valves of the OT form through a process of excavation or hollowing of the cushion's aortic side. Cushion excavation begins at HH29/ED13 with a small depression in the arterial face of the cusps. The endothelium lining the aortic surface of the valves becomes thickened with rounded cells that

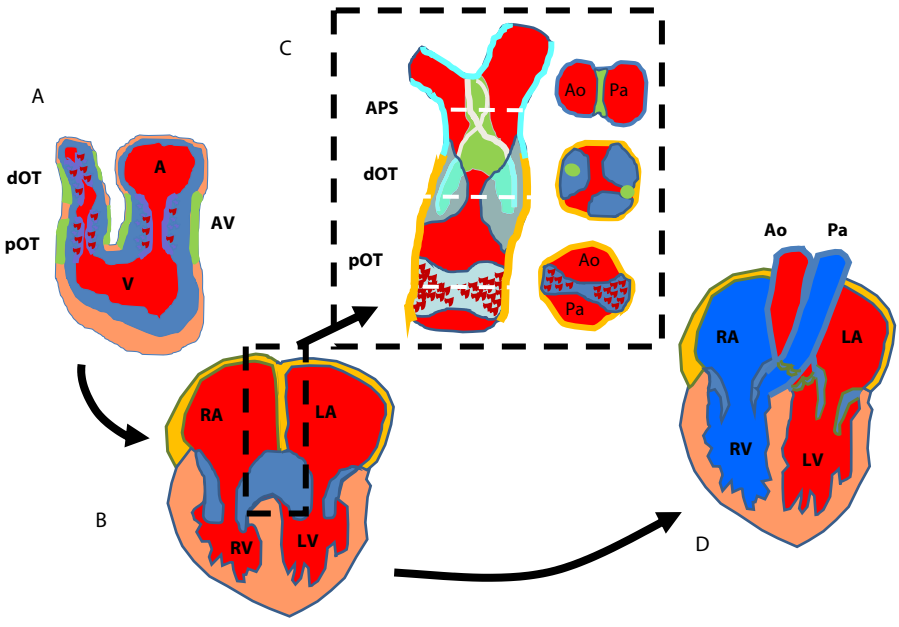


Figure. 1 **Cardiac and valvular morphogenesis. After the heart loops, endocardially derived mesenchyme (blue) invades the cardiac jelly (blue) in the atrioventricular (AV) junction and distal and proximal outflow tract (dOT and pOT respectively) segments (red cells) (A). These primitive structures, dubbed cushions, grow into the early atrioventricular (B) and semilunar valves (C) of the now septated four chambered fetal heart. Note the invading aorticopulmonary septum (APS, green) that spirals through the outflow tract to create separate pulmonic (Pa) and aortic (Ao) outlet arteries, each with their own valve primordia at the dOT site, while at the pOT site the cushions become myocardialized to form the pulmonary infundibulum. The valves become fully condensed into thin fibrous tissues in the mature heart (D). A, atrium; V, ventricle; R, right; L, left.**

flake and undergo apoptosis, while the ventricular epithelium remains flat and elongated [41]. The deepening furrow condenses the fibrous matrix around it [42], creating thin cusps of tissue that are attached in an arc pattern called the commissures (Fig. 2).

Valve tissue maturation

Extracellular matrix striation. Throughout the formation of the prevalvular complexes, endocardial cushions undergo a transition from more pliable structures (HH 17) to rigid structures (HH21) capable of successfully fusing and opposing flow [17]. This increase in rigidity is accompanied by a change from a hyaluronan rich cushion to a more collagen filled cushion. As the valves mature the extracellular matrix (ECM) transforms into three overlapping layers known as the fibrosa, spongiosa and ventricularis. The fibrosa, or arterial aspect of the cusp, is composed primarily of collagen fibers aligned in the parallel orientation providing stiffness and strength to the valve [43,44]. The central spongiosa zone is composed of loosely arranged proteoglycans, presenting a compressible matrix that allows for shape change during the cardiac cycle [44]. The ventral side of the ventricularis contains elastin fibers interspersed with short radially aligned collagen fibers [45]. In the human

Table 1. Comparative cardiovascular development across animal models. Adapted from [109–111].

Human (days) [weeks]	Mouse (E)	Chick (HH)	zebrafish (hpf)	Major events in heart development
22 [3 wks]	7–8	7–10		Fusion of paired heart tubes
22 [3 wks]	7.5–8.5	10	24–36	| First appearance of myofibrils in myocytes | First myocardial contractions | Cardiac looping (mouse E 8.5)
24 [3.5 wks]	8–8.5	9–12+	22	First blood flow through heart
26 [3.5+wks]	9–11	11–12		First ventricular trabeculations
28 [4 wks]	10–12	13–22	60	First definable endocardial cushions (chick 28)
29 [4 wks]	11–13.5	15–23		First appearance atrial septum prium
31 [4.5 wks]	12	24–28		First appearance primordia semilunar valves, start AV septation
33 [4.5+wks]	12–13	25–28	96	| Completion AP septum
35 [5 wks]	13–15	26–31		| completion intraventricular septation
37–43 [5+wks to 6wks]		27–34	105	| maturation semilunar valves

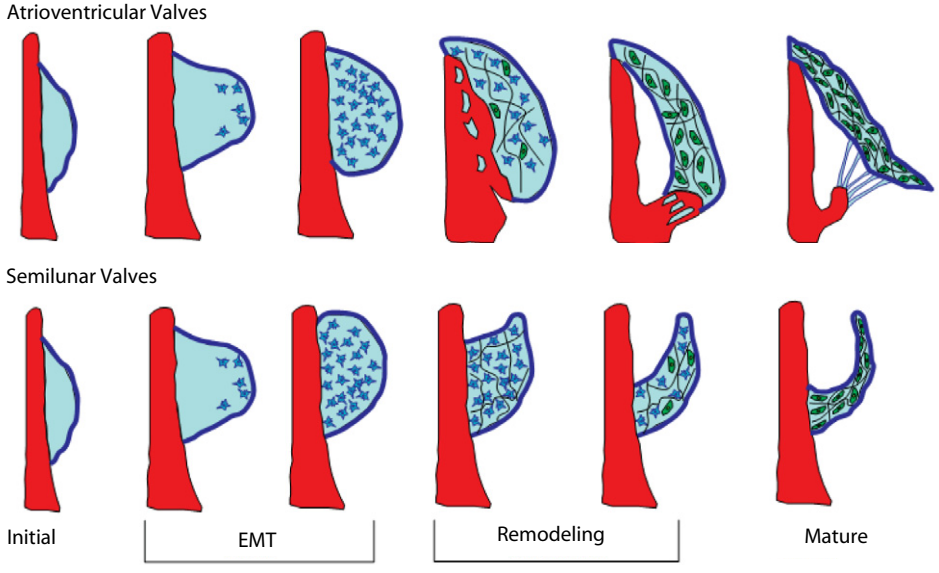


Figure. 2 Schematic of the morphogenic transition from globular cardiac jelly to fibrous cusps/leaflets of the semilunar and atrioventricular valves. Taken from [42].

fetus, the homogenous structure of 14 week post gestation valves becomes a bilaminar structure with sparse, loose, unorganized collagen by week 20. This period of ECM stratification corresponds with valvular interstitial cell (VIC) compartmentalization. Between the 14 and 20 week gestation period, VICs showed significantly higher proliferation indices and expressed the α-smooth muscle actin (α-SMA) positive phenotype attributed to myofibroblasts [46]. Trilaminar structure doesn't become apparent until 36 weeks of gestation and still differs from the normal adult valve structure. During tissue elongation both aggregan, a chondroitin sulfate proteoglycan, and collagen III, a collagen found abundantly in cartilage, are expressed. Aggregan and collagen can also be seen in the fibrosa during cusp remodeling, with aggregan appearing in the spongiosa as well. Tenascin, a large elastic glycoprotein is localized to the annulus, on which the valves anchor, during cushion elongation and extends into the fibrosa and ventricular endothelium during cusp remodeling. It has been shown to mediate cell adhesion and migration functions [47]. Elastin, a vascular matrix protein, is present in the proximal ventricularis during cusp remodeling and exhibits increased expression throughout development.

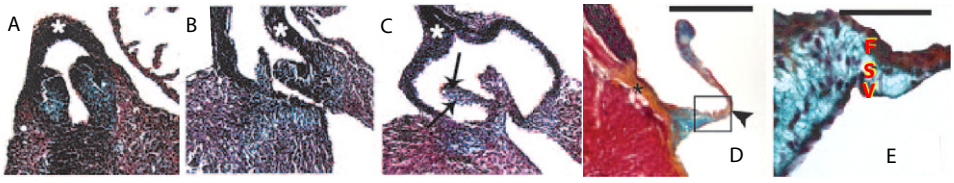
Versican is a chondroitin sulfate proteoglycan with a high affinity for binding to hyaluronan [48]. It is encoded by the versican (PG-M) gene and can be found in the crest of the developing atrial and ventricular septa throughout cushion and valve development. Versican expression is detected in the AV canal as cells undergo EMT and migrate into the cardiac jelly. At E10.5 versican is highly expressed in both the AV cushions and the newly developing cushions of the OT. Versican protein expression in the AV and OT cushions increases as the cushions transition into more rigid structures and continues to be highly expressed throughout development [49]. Gaussin et al. suggest that the late embryonic and early neonatal valves undergo a process of condensation, elongation, formation of nodular thickenings, and ECM remodeling. Condensation begins at the atrial cusp side (mouse E15.5) and expands toward the ventricular side (Mouse E18.5), resulting in a 1.3 fold increase in cellular density. Increased expression of α-SMA, fibronectin, N-cadherin, and proliferating cell nuclear antigen indicate underlying mechanisms of interstitial collagen bridging, cellular adhesion and proliferation. At E18.5, the length of the papillary muscle side of the leaflets elongates past its once continuous position with the free edge. Both edges continue to increase in length postnatally, with the papillary muscle level remaining significantly longer than the free edge. Proliferation is shown to be restricted to the distal tip of the leaflet and the point anchoring the papillary muscle [50]. A second phase of elongation occurs after neonatal day (N) 4.5, when rapid growth of the heart is thought to pull the leaflets at the papillary level. Elongation is accompanied by decreased cell density and decreased interstitial collagen cell bridging. This is followed by postnatal ECM remodeling. At E15.5 and 18.5 both hyaluronan and versican are present in the mitral mural leaflet except in the area of condensation. These proteins become restricted to the arterial side of the leaflet beginning at N6.5. Similarly, collagen I expression is seen throughout the entire leaflet between E15.5 and E18.5 before being restricted to the ventricular side after birth. The triscupid leaflet also exhibits these patterns of homologous expression followed by restriction (Fig. 3). Hyaluronan and versican become restricted to the atrial side of the tricuspid leaflet at 8 weeks of age; collagen I is restricted to the ventricular side of the leaflet at 8 weeks of age [50]. Nodular thickenings, marked by collagen IV expression, develop at the closure points of the AV valve [51].

Cellularity and differentiation. Many of the structural changes that occur throughout development are facilitated by phenotypical changes at the cellular level. Along with ECM stratification, valve maturation is accompanied by a valvular interstitial cell transition from an activated myofibroblastic-like phenotype to a quiescent fibroblast phenotype. VICs exhibit an activated myofibroblast-like phenotype abundant in matrix metalloproteinase-collagenases thoughout development [46]. These cells later regress into a quiescent state for much of adulthood. Aikawa et al. hypothesize that changes are a result of the valvular tissue adapting to its environmental conditions. When VICs are stimulated by mechanical loading they become activated to mediate connective tissue remodeling. The cells then return to quiescence after equilibrium is restored [46]. Endocardial cell activation is accompanied by a change from a polygonal quiescent epithelial phenotype to spindle shaped migratory cells capable of invading the hyaluronan-rich cardiac jelly matrix. Endocardial cell transformation into a mesenchymal phenotype is characterized by expression of α-SMA [52]. α-SMA-positive cells are thought to play an important role in initiating or enforcing fusion of the OT cushions, as their expression persists until valve leaflets formation [34]. Cellular remodeling into a differentiated fibroblastic phenotype is responsible for the cushion condensation seen in the

remodeling of the AV valves [53]. Throughout the whole process of valve maturation and remodeling, a progressive decrease in cell density is observed and continues throughout life; the total number of cells decreases substantially [54,55]. While it has already been established that cardiac neural crest cells play a critical role in septation [56], recent studies have suggested that neural crest cells also orchestrate changes in ECM and apoptosis during valve remodeling [57]. Cardiac neural crest cells greatly contribute to the mesenchyme of the outflow tract [58]. Depleting the heart and aortic arch arteries of neural crest derived cells can result in hemodynamic changes that precede structural defects [59]. Semilunar valve leaflet remodeling is thought to be dependent upon interactions of the second heart field, neural crest and valve mesenchyme [57].

Hemodynamic changes during valvulogenesis

Growth and morphogenesis of the early embryonic heart are accompanied by changing hemodynamic function. Tables 2 and 3 evaluate changes in heart rate and blood pressure in humans and common animal models of cardiovascular development. In general, the embryonic heart rate



Early	Late	Postnatal
Hyaluronan +++	Hyaluronan ++	Hyaluronan + (S)
Versican +	Versican ++	Versican +
Fibrillin 2 +	Tenascin ++	Tenascin ++
Fibronectin +	Collagen I +	Collagen I +++
Collagen I –	Collagen II, V, VI ++	Collagen III ++ (F)
Elastin –	Elastin +/ –	Elastin ++ (V)

Figure. 3 Protein expression in extracellular matrix remodeling. (+) and (–) indicate degrees of expression. S, Spongia; V, ventricularius; F, Fibrosa. Pictures examine Movat's pentachrome stain of extracellular matrix (ECM) organization and valvular interstitial cell (VIC) compartmentalization within mouse semilunar valves as seen in the (A) cushion, (B) elongation, and (C) remodeling period. The white asterisk indicates the aorta; arrows highlight cusp and leaflet remodeling. (D) Showa mature valve cusps, the black asterisk marks the annulus, the arrow marks the leading edge ventricularis. The boxed area is magnified in (E). Elastin fibers are black, collagen yellow, proteogylcans blue, cell nuclei purple, and muscle red. Adapted from [46,50,54,55].

Table 2. Heart rate across developmental animal models. Adapted from [62,110,112,113].

Human:	Days or Weeks	bpm	Zebrafish:	dpf	bpm
	37 days	101–109		2	141
	41 days	120–134		3	147.2
	45 days	130–158		4	165.9
	50–52 days	120–175		5	171.5
	8+wks	150–176			
	9+wks	150–172			
	10+wks	140			
Chick:	Stage (HH)	HR, bpm	Mouse:	Emb day	HR mean
	16	110		10.5	124.7
	18	147.5		11.5	135.6
	21	145		12.5	147.3
	24	155		13.5	173.6
	27	155		14.5	194.3
	29	194			209
				15.5	
	31	221			
	35	230			

increases as development progresses, though there are variations in species. Systolic and diastolic function follow a similar pattern, increasing as the embryo grows to accommodate increasing needs. As early as 43 hours after incubation, two blood streams are apparent in the heart. Incongruities in the size of the streams leads to spiraling, as the force of the larger stream pulls the smaller stream around it [60]. Movement of the spiraling site plays a large role in heart formation. The rapid growth of the endothelial tube inside the primitive heart is a result of the increase in blood pressure [61]. It is after this proliferation that cardiac jelly swellings appear in the AV canal and OT. In this manner, blood flow guides cardiac morphogenesis, sculpting tissue by promoting growth in response to increased demands. As the heart continues to grow, the energy extended in pulsatile flow increases from one-third to two-thirds of total energy between HH 18 and 29 [62]. Theoretical models of growth suggest the duration of vessel growth and their morphological characteristics are related to blood flow, shear stress and stretching forces [63]. Along with tissue sculpting, shear stress has been shown to regulate gene expression [13]. The magnitude of wall shear stress (WSS) is dependent on flow, pressure gradient and vessel wall motion [64]; WSS regulates events from cushion formation to leaflet maturation. As the heart forms, shear stress is greatest in the inner curvature and sites of lumen constrictions, corresponding to the AV canal and OT where the endocardial cushions form and develop into functioning valves. The lowest values of shear stress are found in the outer curvature and intertrabecular sinuses [65]. Shear stress patterns in the transvalvular region are summarized in Fig. 4C. Differences in wall shear stress are thought to affect valve formation. The formation of the muscular flap of a chick embryo develops in the presence of peak wall shear stress, while it's left mural counterpart differentiates into a fibrous leaflet under low shear stress [15]. High unidirectional shear stress has been suggested to promote cushion extension in the direction of flow in contrast to low recirculating flows, which encourage a prevalvular sculpting process [15]. Blood viscosity is defined by shear rate, the relationship of shear stress to the gradient of velocity [66]. The shear rate is determined both by the velocity of flow and the size of the vessel [67].

As shown in Fig. 4A, the velocity profile of flow through the valves begins as a laminar parabola (HH 17), before resembling plug flow; wall shear stress follows a similar pattern (Fig. 4B). Cushions form in areas of turbulent vortices, which arise shortly after peak inflow velocity and extend perpendicularly to the direction of flow [15]. The presence of cardiac cushions changes the geometry of the OT lumen cross-section, which plays a role in reducing blood flow [64]. Retrograde flow in these vortices creates a downward lip in the cushions at HH23 which become more pronounced throughout development [15]. Flow patterns as described in this section are necessary for normal development. Changes in blood flow, and subsequent WSS patterns, are associated with many diseased conditions. While arterial flow may be altered to compensate for changing environmental conditions, maintenance of circulatory energy efficiency and pressure are critical for development [68].

Table 3. Blood Pressure across developmental animal models. Adapted from [114–117].

Human: weeks	**Right Ventricular Systolic Pressure (mmHg)**	**Right Ventricular Diastolic Pressure (mmHg)**	**Zebrafish: Body mass (mg)**	**Systolic Pressure (mmHg)**	**Diastolic Pressure (mmHg)**
14	30.996	8.4518	.5	0.061	0.3575
16	35.424	9.6592	1.25	0.1045	0.84575
18	39.852	10.8666	2.25	0.1625	1.49675
20	44.28	12.074	3.25	0.2205	2.14775
22	48.708	13.2814	3.75	0.2495	2.47325
24	53.136	14.4888			
26	57.564	15.6962			

Chick: Stage (HH)	Ventricular Systolic Pressure (mmHg)	Vantricular Diastolic Pressure (mmHg)	Mouse: Emb Day	Systolic Pressure (mmHg)	Diastolic Pressure (mmHg)
16	1.15	0.25	10.5	3.44	.52
18	1.31	0.33	11.5	5.01	.50
21	1.61	0.34	12.5	6.43	.90
24	1.96	0.4	13.5	9.0	.86
27	2.35	0.56	14.5	11.15	.88
29	3.45	0.82			

Hemodynamics and gene regulation

Shear stress and shear stress-induced or repressed gene expression are important factors in remodeling of the cardiovascular system [13,65]. While it is well known that looping of the primitive heart can progress in the absence of hemodynamic forces [69], the role of hemodynamic signaling in valve formation is less understood and somewhat controversial. While many highlight shear stress development of cardiac malformations [5,6,12,70], other studies claim myocardial function trumps shear stress as a major epigenetic factor [9]. The close association of changes in flow pattern with genetic knockout models strongly supports the notion of shear stress as a major epigenetic factor. Hove et al. found the collapse of inflow and outflow tracts after blockage of flow to the atrium or from the heart to the aorta closely resembles that of jekyll mutatants, who exhibit abnormal flow due to a missing AV valve [5]. Hogers et al. show the divergence of blood from the yolk sac region to a more ventral course along the outer curvature of the conotruncus results in a host of cardiac malformations paralleling that of endothelin-1 knockout mice and neural crest ablated embryos [70]. While the exact mechanism is unknown, endocardial cells lining the luminal surface of cushions may play a role in regulating cushion and valvular morphogenesis through the integration of hemodynamic stimuli and signaling of the underlying mesenchyme [17].

A number of studies have demonstrated shear sensitivity of valvular morphogens *in vivo*. Endothelin-1 (ET-1) and endothelial nitric oxide synthase (NOS-3) are shear stress response genes thought to be involved in cardiovascular development, as knockout mice for these genes display a spectrum of cardiovascular defects [10,11]. Groenendijk et al. investigated their expression throughout cardiovascular development in conjunction with kruppel-like factor-2 (KLF2), which has been shown to produce AV valve dysgenesis in zebrafish knockouts [65]. Results were consistent with the hypothesis that changes in blood flow result in morphologic changes by way of shear stress induced alterations in gene expression. Periods of intense cardiovascular remodeling (HH20–HH30) were marked by ET-1 and KLF2/NOS-3 restriction to narrow sites [65]. ET-1 was negatively correlated to shear stress, while KLF2 and NOS-3 were positively correlated to shear stress. Changes in flow patterns due to vitelline vein obstruction resulted in altered shear stress and gene expression which, in turn, lead to cardiovascular malformations [13]. Following venous clipping and the induction of increased shear stress, KLF2 and NOS-3 levels were augmented, while ET-1 was downregulated.

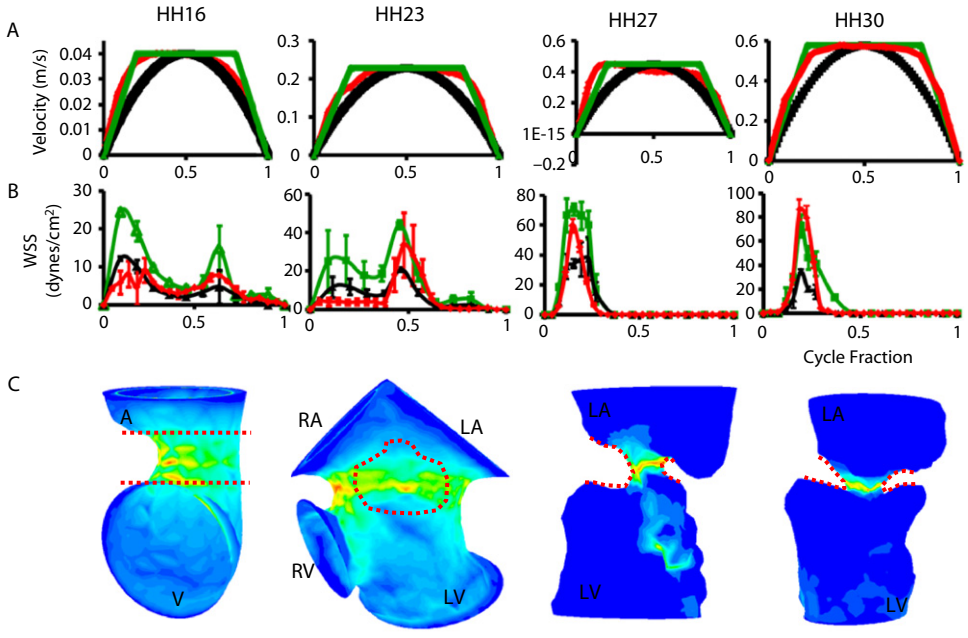


Figure. 4 Hemodynamic changes in valvulogenesis. (A) peak velocity profiles (B) spatially averaged wall shear stress profiles. Red profiles correspond to flow and wall shear stress as calculated by computational fluid dynamics, black refers to the poiseuille profile and green plug flow (boundary layer is taken to be one-fifth diameter). (C) transvalvular shear stress quantifications calculated by computational fluid dynamics. High magnitudes of shear stress correspond to red; low magnitudes, blue. Adapted from [108].

Areas of high shear stress in normal development, such as the upstream slope of outflow cushions, exhibited prominent changes in gene expression [13]. Both the magnitude and the retrograde nature of flow have been shown to influence valve patterning [15,71]. During valvulogenesis, retrograde flow is thought to trigger flow responsive genes in the AV canal and initiate valve formation. Expression of Notch1b, KLF2a, and BMP4 become restricted to a region of high reversing flow in the AV canal [71].

In addition to the above genes, the expression patterns of potent vavular morphogens transforming growth factor-β (TGFβ), bone morphogenetic protein (BMP) and vascular endothelial growth factor (VEGF) are spatially and temporally restricted in a manner that suggests hemodynamic regulation [14,72]. In situ hybridization shows TGFβ appearing in the endocardium of the valve forming regions around HH20, as flow transitions from laminar Poiseuille flow to more plug-like flow and rapidly increases in velocity [15]. Low levels of VEGFA gene expression are also found in the endocardium of the AV cushions at this time. By HH25 retrograde flow vortices are present throughout the cushion region, sculpting a downward pointing lip in the cushions [15]. TGFβ can be seen across cushion endocardium and mesenchyme and BMP is rapidly restricted to the boundary between the AV cushion and junctional myocardium. Following cushion fusion (HH28–29), a period coincident with increased flow velocity and increased peak vorticity of the transvalvular region [15], TGFβ expression becomes restricted to remodeling regions of the valves, while VEGFA expression remains endocardial-specific [14]. The pronounced curvature of the septal leaflets causes inflowing blood to strike the left free wall of the left ventricle and spiral back towards the top of the ventricle. After peak velocity, spiraling flow along the outflow surface of the cushions results in a negative shear stress with respect to the inflow direction [15]. From HH33–36, TGFβ expression increases in the endocardium of the AV valves with greater expression on the atrial inflow side [14] (Fig. 5). By this time, BMP2 expression is no longer detectable in the AV region.

Animal models of valve formation and maturation

The heart is already four chambered by the time human hearts are visible via ultrasound (8 weeks). Therefore, almost all of what we know about valve development has been obtained through animal models, including zebrafish, chick and mouse. Each model provides genetic or experimental advantages, with varying degrees of anatomical and morphogenetic similarities to that of humans (Table 4). The zebrafish embryo is virtually transparent, which permits the imaging of its internal structure using standard light microscopy [73]. The zebrafish's heart is the first organ to develop,

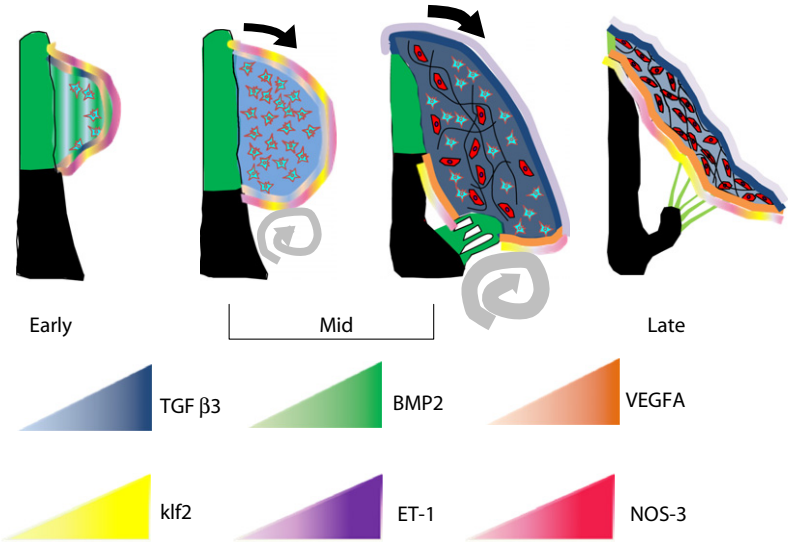


Figure. 5 Summary of flow and gene expression in early, late and mid valvular cusps. ET-1 is the only gene still expressed on both sides of the leaflet at late valvulogenesis. The increasing force of unidirectional flow in the atrial, inflow, surface corresponds with TGFβ and ET-1 expression, while the increase in magnitude of retrograde flow vortices on the ventricular side corresponds with VEGFA, NOS-3, KLF2, and ET-1 expression. BMP is restricted to the boundary between the cushions and junctional myocardium. Black arrows indicate inflow, gray spiral retrograde flow vortices. Adapted from [14,65].

resembling a three-week old human heart at 24 hours post fertilization (hpf) [74]. A large advantage of the zebrafish is that the embryo itself is not dependent on the cardiovascular system for at least 5 days post fertilization (dpf); sufficient oxygen is obtained by diffusion [75]. This allows for easy genetic manipulation of the heart. Large numbers of genetically identical offspring can be generated in the zebrafish. While heart formation of the zebrafish does parallel that of the human, the zebrafish only possesses a two-chambered heart. In addition, the zebrafish atrioventricular endocardial cells do not form valves through EMT, but rather fold inward to create a thin, bilayerd tissue without mesenchyme until much later [76].

The chick model is traditionally the most used model in the study of cardiac morphogenesis. The embryos are large, sustainable and develop externally to the mother. The chick embryo has been extensively study and its course of development universally staged. The anatomy of the chick heart closely parallels that of the human heart. A small difference is that, while the initial development of the AV leaflet resembles that of the human heart, the mesenchymal tissue is replaced by myocardium tissue resulting in the formation of a muscular flap valve in the right atrioventricular junction (AVJ) [77]. Constructs cannot be routinely injected into the chick embryo, as the cells are too small for the direct injection of constructs [78]. Genetic mutation is therefore performed through the use of electropolaration, where an electric field induces the direct opening of pores in the cell membrane, lipofection, microparticle bombardment, and viral vector transmission [79–81]. Transient or long-term gene expression or repression is possible, as well as the tracing of affected cell lineages [80]. Recent advances have even allowed electroporation of expression vectors and morpholino oligonucleotides soon after laying with precise spatial and temporal control [82].

The mouse is the standard model for genetic manipulation as it is easy to genetically manipulate and provides a stable mammalian model of development. The mammalian cardiovascular system differs from that of the vertebrates and amphibians in that it couples to both yolk sac and placental circulations. Mouse embryos are harder to access as they develop inside their mother. While the mouse is a mammal, its heart still contains structural differences from that of the human heart. The mouse chordate are far less prominent than the pronounced tendinous chords of the human heart valves [77]. The arrangement of the atrioventricular septal structures is different in mice. The membraneous septum found in the human heart is seen as a less extensive thick structure in the fetal mouse, possibly as a result of incomplete delamination of the septal leaflet of the tricuspid valve [83].

Experimental techniques

Quantification of hemodynamic performance throughout development is a challenging task as the vessels are still forming and are very delicate. The use of imaging provides a noninvasive way to evaluate cardiac performance in normal and abnormal development. Jones et al. used confocal laser-scanning microscopy, a technique in which a laser is used to repetitively scan along a single line positioned perpendicularly to the targeted vessel [84]. The erythroblasts are tagged with green fluorescent protein (GFP) and a measurement of velocity is calculated based on the number of times a cell is seen crossing the same line. The minimum velocity detected can be adjusted through the number of line scans performed, while the maximum velocity is a limit of instrumentation speed. Jones et al. were able to calculate speeds well above those detected in the embryonic mouse, highlighting confocal microscopy as a valuable tool. The method is limited in that it cannot determine the level of oscillatory stress. Unlike laminar stress, continuous particle tracking is needed to measure forces exerted by particles with circular or irregular trajectories [84]. Hove et al. used high speed

Table 4. Summary of advantages and disadvantages of animal models in the study of cardiac development.

Organism:	Easy to Manipulate genetically?	Easy access to embryos?	Extensive staging system?	Easily facilitated observation of organogenesis?	Major anatomical/formation
Chick	yes	yes	yes	no	Muscular flap valve in AVJ
Mouse	yes	no	no	no	Less extensive striation Less pronounced chorda tendinae
Zebrafish	yes	yes	no	yes	No EMT

fluorescent confocal imaging to visualize flow patterns inside the heart chambers of zebrafish [5]. Their study was limited in that digital particle velocimetry requires 3D movements be projected into a 2D plane, thereby collapsing and underestimating distances traveled by a given cell. Despite dealing with the underestimation of distance traveled, Hove et al. were able to quantify relative changes between a normal and developmentally perturbed heart. Beads were implanted into GFP embryos either in front of the sinus venous, to block blood influx into the atrium, or in the back of the ventricle to block blood efflux from heart to aorta. They noted an accumulation of erythrocytes in front of the atrium and inside the heart chamber respectively. In both cases the embryos showed severe regurgitation of blood inside the heart and reduced blood flows, resulting in dramatically reduced shear forces [5].

India ink is another method researchers use to visualize flow patterns in the developing embryo. Hogers et al. used India ink to study flow patterns in the normal and abnormal chick heart [70]. In their 1997 study, abnormal development was induced through the ligation of vitelline veins. Following unilateral ligation of the vein, blood immediately re-routed itself [70] producing a range of cardiac defects. Observed malformations included subaortic ventricular septal defects, semilunar valve anomalities, atrioventricular anomalies, and paryngeal arch artery malformations [70]. Ligations of the right and left vitelline veins produced a similar array of abnormalities. Irrespective of the ligated veins, the shift in blood flow was predominantly to the ventral side of the outflow tract. This shift in force is thought to play a role in improper looping [6]. Perturbed embryos did not form a third chamber, lacked heart looping and possessed weak inflow and outflow tract walls which were collapsed and fused. Similar to vitelline vein ligation, venous clippings in which a microclip is inserted into the right lateral vein, induce a spectrum of outflow abnormalities. In the venous clip model, the load on the embryonic myocardium is temporarily reduced resulting in less developed ventricles and delayed cardiac looping [70]. In addition to ligating veins, left atrial ligations and conotruncal banding, in which a suture is passed over the conotruncus but not pulled tightly enough to arrest blood flow, have been performed [85]. In conotruncal banding, ventricular dilation was observed along with changes in the trabeculae. Interestingly, the right AV valve no longer consisted of a muscular flap but rather resembled a bicuspid structure. Left atrial ligations resulted in hypoplasia of the left ventricle. In all the above studies, surgically manipulated embryos developed abnormal cardiovascular phenotypes. The problem with ligations and clippings is that remodeling may be the result of mechanical interferences in addition to disruption of hemodynamic properties. There exists a need for more clinically relevant models.

Photoablation within an embryo is a powerful tool that can be used to study the underlying mechanisms of morphogenesis and as well as aid in the restoration of properties required for normal morphogenesis. Männer et al. demonstrated the use of photoablation as an alternative to ligation in their study of photoablation of the proepicardium (PE) in chick embryos [86]. Loss of PE function was induced by photoablation of the PE which led to long lasting loss of PE function. Previous experiments involving the blocking of normal PE behavior by means of physical barriers did not permanently prevent the colonization of the developing heart with PE derived cells, instead it caused a delay in the formation of some PE derived tissues. Photoablation facilitated the complete elimination of the PE without damaging adjacent structures [86]. The authors were able to conclude that a subpopulation of PE-derived cells invading the mesenchyme of the AV cushions does not contribute any substantial number of cells to the mature AV valves. Yalcin et al. used photoablation as a way to disrupt AV cushion mechanics, instigating cardiac remodeling in a way similar to mechanical ligations. Results suggested a promising new minimally invasive technique for the study of disease formation in the embryonic heart [7].

Hemodynamics and congenital heart defects

The embryonic heart grows and develops to adapt ventricular geometry and function to optimize mechanical efficiency [87]. It is therefore unsurprising that many defects arise from alterations in the normal embryonic environment. Goerttlet suggested that deviations in normal heart development resulting from genetic or exogenous factors lead to alterations in embryonic tissue, while abnormal cardiac structure is the result of displaced blood-streams [88]. Distinctions between gene and hemodynamic-related abnormalities are not that well defined. Chromosomes linked to particular defects have been identified but do not provide the full picture, with roughly 10–15 percent of left ventricular outflow malformations linked to a chromosomal abnormality [89]. While underlying

genetic mutations are associated with congenital heart defects, one cannot simply mutate a gene to induce a congenital heart defect. Alternatively, the mechanical perturbation of blood flow can induce diseased phenotypes, as altered flow patterns are the mark of many congenital heart defects. In this section the pathology of three major defects associated with valvular abnormalities are explored. These are bicuspid aortic valve, hypoplastic left heart syndrome, and tetralogy of fallot. Abnormal hemodynamic patterning is associated with the development of all three defects.

Bicuspid aortic valve. Bicuspid aortic valve (BAV) disease is the most frequent congenital anomaly of the heart. It is commonly associated with aortic valve stenosis, regurgitation and endocarditis, though these symptoms develop much later despite the formation of irregular valves during development. In bicuspid aortic valve, the patient possesses a valve of limited mobility, as the free edges of the bicuspid valve are more straight than rounded. The leaflets are usually of unequal size with a raphe, or seam-like union, apparent in the larger leaflet [90]. Excessive length of one or both cusps results in abnormal contact which in turn leads to fibrous thickening that will later become diffuse and calcified. The strong correlation between a deficiency in the shear stress induced NOS gene and the development of BAV supports a role for hemodynamics in disease formation [8].

Stenosis usually develops in bicuspid valves containing no redundant cusp tissue, while valve incompetence is associated with redundancy and endocarditis. The valve likely becomes stenotic as its cusps become fibrotic and calcified. The large calcific deposits associated with BAV are unusual before the age of thirty and very prevalent thereafter [91]. In a 2003 study of 44 bicuspid aortic valves, BAV patients without significant stenosis or regurgitation were found to have a larger aortic annulus, aortic sinus and proximal ascending aorta when compared to normal tricuspid valves. The peak aortic velocity [92] and peak systolic wall velocity in the anterolateral region of the ascending aorta [93] were higher in BAV patients than controls, implying these regions are subjected to great levels of stress.

Hypoplastic left heart. Hypoplastic left heart syndrome (HLHS) is marked by severe underdevelopment of the left ventricle. Patients born with HLHS continue to have some of the highest mortality rates within the first year of life among all infants with congenital heart defects [94]. No strong genetic correlation exists. In a study of 83 HLHS patients, nine had underlying chromosomal abnormalities, four had single gene defects, ten had one or more extracardiac anomaly and two were patients of insulin-dependent mothers [95]. Cardiac defects associated with HLHS include mitral valve hypoplasia or mitral stenosis coincident with a left heart obstruction of a hypoplastic left ventricle and aortic atresia, hypoplastic aorta, or coarctation of the aorta. The ascending aorta and aortic arch in patients with HLHS are thought to become hypoplastic as a result of diminished flow to the left ventricle and aortic outflow tract throughout development. Retrograde aortic flow may be responsible for impaired development of the aortic root and ascending aorta [96]. Out of 96 HLHS patients, 37.5 percent were found to have malfunctioning aortic and mitral valves, 50 percent had malformed AV valves and 12.5 percent exhibited dysplastic aortic valvular stenosis [97].

Harh et al. were among the first to investigate the hypothesis that alterations in the site of the primordial mitral valve may induce the HLHS phenotype. They accomplished this by placing a nylon device in the left AV canal. This resulted in greatly reduced or eliminated ejection of blood flow from the left ventricle into the ascending aorta, which limited the range of rhythmic aortic expansion. Failure of the cushion differentiation into a thin fibrous tissue resulted in a thickened hypoplastic valve and a subsequent hemodynamically induced hypoplasia. Narrowing or closure of the mitral valve resulted in an obligatory reversed atrial shunt [98]. Other mechanically induced HLHS embryos have been shown to display altered ventricular filling patterns and altered epicardial strain patterns [99], though variations in heart rate and AV inflow velocity were acute to non-existent [100]. All of these studies add credence to the paradigm that altered ventricular filling in development results in altered ventricular function and geometry.

Tetralogy of fallot. Tetralogy of fallot (TOF) is a combination of a large ventricular septal defect, pulmonary stenosis, right ventricular hypertrophy, and an overriding aorta. As a result of anterocephaled deviation of a malaligned oulet septum, in combination with hypertrophy of septoparietal trabeculations, there is narrowing of the subpulmonary infundibulum [101]. This narrowing of the infundibulum facilitates greater right to left shunting via the malaligned ventricular septal defect and overriding aorta [102]. Aortic dilation has been shown to vary inversely with the degree of right ventricular outflow tract dilation [103]. In addition, there is a positive correlation

between histological changes and the degrees of aortic dilation. Histological abnormalities are present in the media of the aortic root and ascending aorta. These abnormalities include focal loss of smooth muscle cells, fibrosis, elastic fragmentation, and disruption of elastic lamellae [104]. Among TOF patients, aortic dilation is greatest when coupled with pulmonary atresia, or malformation of the pulmonary valve, as aortic volume overloading is maximal. Hemodynamic stress from volume overloading is thought to play an important role in the initiation of aortic dilation, as aortic root dilation and regurgitation are commonly seen in congenital defects where there is volume overloading of the aortic root and ascending aorta [103]. Aortic regurgitation may occur as a direct result of dilation of the aortic annulus, which results in incomplete coaption of the aortic cusps or as a result of infective endocarditis [102].

CONCLUSION

In 2008 the infant mortality rate was 49 per 1000 live births worldwide, 6 per 1000 in developed nations and 85 per 1000 in severely underdeveloped countries [105]. Congenital heart defects account for 5 to 31 percent of these deaths [106]. Undoubtedly, much remains to be done to guarantee the appropriate medical care to children born across the world with cardiac abnormalities. The surgical treatment of congenital heart defects requires complex infrastructures and highly skilled professionals. Many developing countries are just now starting to build structures capable of delivering the appropriate care [106]; others lack the appropriate resources. In Africa, the ratio of congenital heart surgeons to individuals is 1:38,000,000 compared to the 1:3,500,000 seen in North American and Europe [107]. More accessible strategies are required to address the needs of children with congenital heart defects in developing countries. Researchers have a responsibility to focus on creating new solutions to tackle the clinical problems affecting children of developing countries using economically attainable strategies. These problems can begin to be addressed in animal models of clinically relevant congenital heart defects which are used to elucidate mechanisms of impaired remodeling. Understanding the underlying mechanisms of hemodynamics is essential to understanding valve development and congenital heart defect formation. The sequelae of genetic defects associated with congenital heart defects are suspected to be the result of upstream hemodynamic changes that are either poorly adapted to or caused by mechanics itself. New experimental and computational techniques can further this understanding and enable direct prediction of mechanical environmental consequences on heart and valve development. Regenerative strategies should utilize developmental signaling paradigms to accelerate and control tissue remodeling in valve disease.

ACKNOWLEDGEMENTS

This research was supported by funding from the American Heart Association, the National Science Foundation, the LeDucq Foundation, and The Hartwell Foundation. The authors have no financial interests to disclose.

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