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Interplay between cardiac function and heart development☆

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ABSTRACT

Mechanotransduction refers to the conversion of mechanical forces into biochemical or electrical signals that initiate structural and functional remodeling in cells and tissues. The heart is a kinetic organ whose form changes considerably during development and disease. This requires cardiomyocytes to be mechanically durable and able to mount coordinated responses to a variety of environmental signals on different time scales, including cardiac pressure loading and electrical and hemodynamic forces. During physiological growth, myocytes, endocardial and epicardial cells have to adaptively remodel to these mechanical forces. Here we review some of the recent advances in the understanding of how mechanical forces influence cardiac development, with a focus on fluid flow forces. This article is part of a Special Issue entitled: Cardiomyocyte Biology: Integration of Developmental and Environmental Cues in the Heart edited by Marcus Schaub and Hughes Abriel.

1. Introduction

Vertebrate organisms live with a beating heart from early in development to the end of their lives. The vertebrate heart is composed of the endocardium—an endothelial layer in contact with the blood flow, the myocardium—the muscle in charge of heart contractions, and the epicardium—the outermost mesothelial cell layer. The myocardium is formed mainly by cardiomyocytes, but also contains intracardiac fibroblasts and stromal cells, and is nourished by the coronary vasculature. Two myocardial layers can be distinguished: an outer compact layer and an internal trabecular layer. In mammals and birds, the heart is formed by two atrial and two ventricular chambers. The heart is surrounded by the pericardium wall. Between the epicardium and the pericardium is a cavity filled with pericardial fluid. At early developmental stages, the primitive heart consists of a straight tube of cardiac muscle (myocardium) lined by a single layer of endothelial cells (endocardium). Extracellular matrix accumulates between the endocardium and the myocardium, providing structural support to the developing heart through intracardiac pressure [105].

The role of mechanical forces in shaping the heart during cardiovascular development has been studied in a variety of animal models, including chicken, mice or zebrafish [94]. An early demonstration of the role of the heartbeat in cardiovascular development comes from studies with *Myosin light chain 2a* knockout (KO) mouse embryos, which have impaired cardiac contractility and as a consequence reduced blood flow. This cardiac defect leads to impaired yolk sac vessel remodeling [85]. With the advent of live imaging, knowledge in the field of mechanobiology is expanding rapidly. Improvements in technology, such as four-dimensional optical coherence tomography (OCT), have enabled observation of cardiac dynamics at high-speed acquisition rates and high resolution to reveal the complex interrelationship between cardiac layers and cardiac jelly during the heart cycle in chick and mouse models. Contractile activity can also be measured using other techniques such as brightfield time-lapse microscopy, confocal microscopy of calcium transients, and atomic force microcopy [20]. The development of light sheet microscopy [155], for *in vivo* imaging at low phototoxicity and cellular resolution is enabling detailed quantitative analysis of mechanical forces during cardiac development. In particular in combination with animal models such as the zebrafish, which has become a powerful model organism in cardiovascular research [7, 83]. The key advantages of the zebrafish are its amenability to *in vivo* cellular-resolution imaging of the heart and vasculature, genetic manipulation, chemical treatments, and quantitative force measurements [35]. The ability of zebrafish to survive without a beating heart for up to 7 days post-fertilization (dpf) by oxygen diffusion [136] makes it a good model for studying the influence of hemodynamic forces during cardiac development.

Mechanical forces are sensed by cells and induce downstream signaling cascades allowing them to react to environmental physical alterations. Mechanosensors known to operate in cells exposed to shear stress include cilia, integrin signaling components, cell membrane

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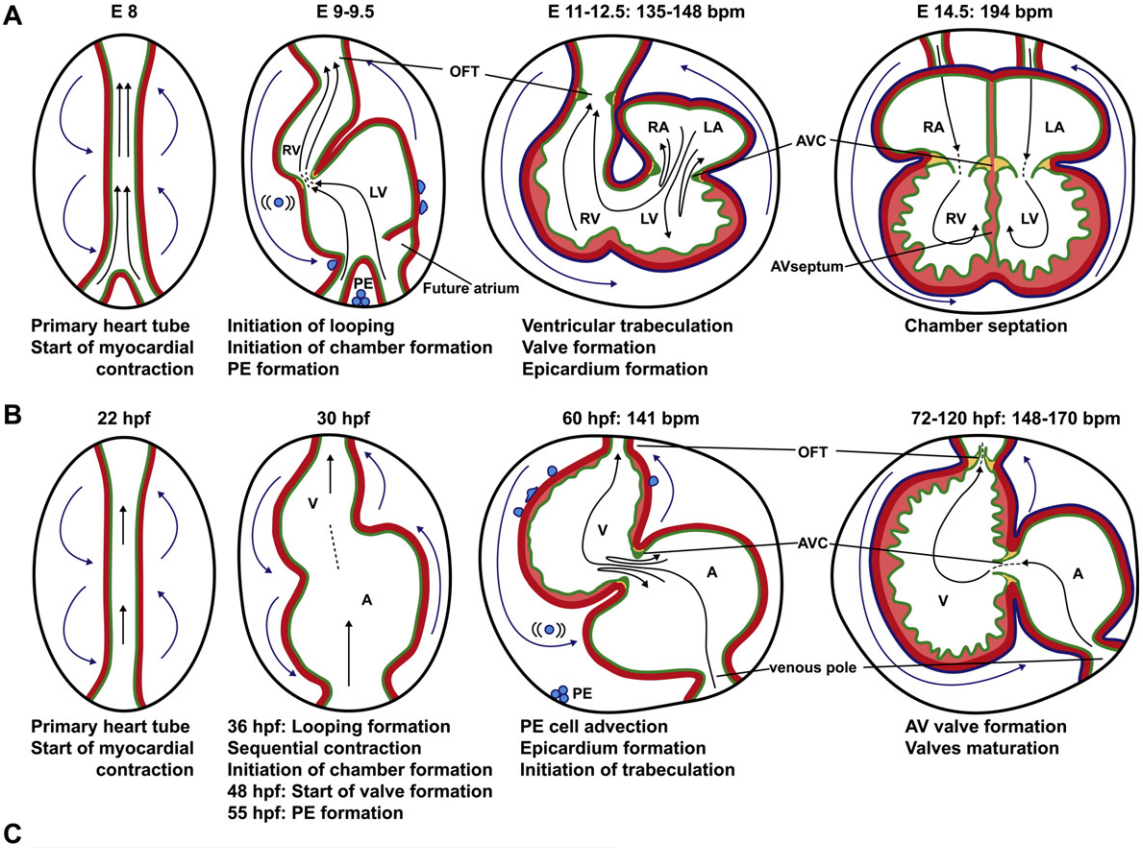
receptor kinases, stretch-sensitive ion channels, intercellular junction proteins and membrane lipids [86]. These mechanosensors can activate components of multiple mechanotransduction pathways such as Ras/RhoGTPases, MAPKs, phospholipase C, Ca^{2+} currents, nitric oxide (NO) signaling elements, and several microRNAs. This ultimately regulates the expression of signaling pathways involved in developmental processes, including the vascular endothelial growth factor (VEGF), bone morphogenetic protein (BMP) and the Notch signaling pathway [91]. This review provides an overview of the mechanical forces impacting cardiovascular development and describes some of the underlying mechanosensing and mechanotransduction pathways.

2. Types of mechanical forces influencing cardiovascular development

Cardiomyocytes start to beat in an autonomous manner before any external stimulus is present. In mice, beating typically starts between embryonic days E 8 and E 9 [21,82], when the heart field undergoes a morphological transition from a crescent to a heart tube [99] (Fig. 1A). In zebrafish, cardiomyocyte contraction begins at the venous pole of the linear heart tube at 22 hpf (hours post fertilization) (Fig. 1B). In chick, the heart begins beating at 42 h of incubation [2]. Initial cardiomyocyte contractions are irregular and uncoordinated, but in zebrafish show a more regular pattern around 28 hpf, with a frequency of 156 beats per minute (2.6 Hz) [13]. As the heart tube develops, the rate of contraction increases and the heart changes from a peristaltic-like pump to a beating heart [7,82].

From the onset of heart beating, blood flows through the cardiac lumen, and this physical stimulus influences chamber formation, trabeculation, cardiomyocyte proliferation and valve formation [43]. Blood flow induces shear stress parallel to the vessel wall (Fig. 2). In addition to shear stress, the changing blood flow during each cardiac contraction–relaxation cycle alters the strain to which cardiac cells are subjected. This force is known as mechanical loading and varies according to the blood viscosity, which is mainly determined by the concentration of red blood cells. In zebrafish, blood viscosity is altered by the silencing of *gata2*, leading to below-normal numbers of erythrocytes



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HUMAN			
Weeks	**14**	**20**	**26**
Right Ventricular Systolic pressure (mmHg)	30.996	44.28	57.564
Right VentricularDiastolic pressure (mmHg)	8.451	12.074	15.696
MOUSE			
Embryonic day	**10.5**	**12.5**	**14.5**
Systolic pressure (mmHg)	3.44	6.43	11.15
Diastolic pressure (mmHg)	0.52	0.90	0.88
ZEBRAFISH			
Body Weight (mg)	**0.5**	**2.25**	**3.75**
Ventricular Peak Systolic pressure (mmHg)	0.227	1.366	2.343
Ventricular End-Diastolic pressure (mmHg)	0.061	0.162	0.249

Fig. 1. Stages of cardiac development in the mouse and the zebrafish. The heart is subjected to the flow of blood (black arrows) and pericardial fluid (blue arrows) during most developmental stages and throughout adult life. Illustrations show ventral views of hearts. (A) Mouse heart development. The most important events taking place are listed. (B) Zebrafish heart development. In both animal models, laminar flow becomes turbulent at the site of valve formation. (C) Blood pressure across developmental stages in different animal models (modified from [81]). Endocardium is marked green, myocardium red, epicardium blue, and the developing valves yellow. Blue circles represent proepicardial cells. Dotted lines illustrate turbulent flow or oscillatory flow at valves. A, atrium; AVC, atrioventricular canal; bpm, beats per minute; LA, left atrium; LV, left ventricle; OFT, outflow tract; PE, proepicardium; RA, right atrium; RV, right ventricle; V, ventricle.

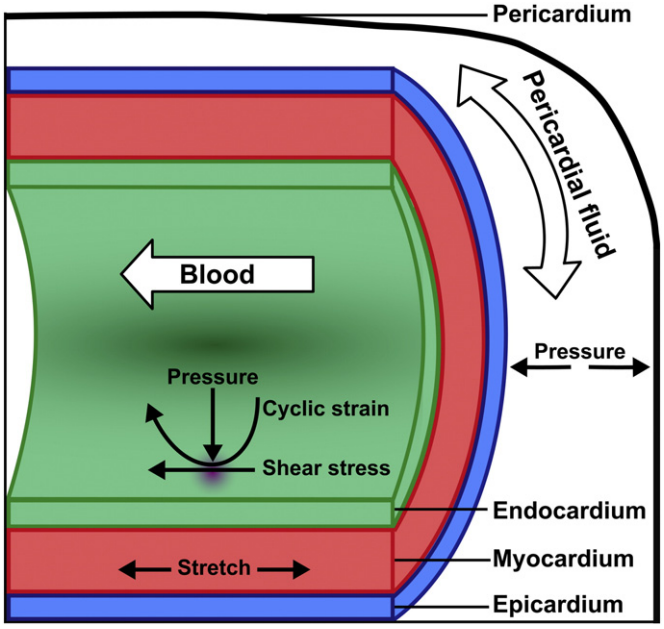

Fig. 2. Mechanical forces influencing cardiac development. Inside the heart, the blood is in direct contact with the endocardium and exerts shear force on the endocardium and cyclic strain on the three heart layers. Outside the heart, the pericardial fluid generates a shear stress force on the epicardium and pericardium as well as pressure on the whole heart. The illustration shows a section of the heart wall. Arrows indicate force vectors.

[150]. Blood flow in healthy situations is usually pulsatile and laminar, whereas disturbed and turbulent flow is often associated with pathological situations, such as atherosclerosis [44] or valvular heart disease [4]. However, disturbed flows also occur in the developing heart [74,161]. Moreover, tissue constraints generate a notable mechanical force transmitted through the myocardial wall by cell–cell attachments, and exerted on the wall by extracardiac pressures.

The ventricle pressure increases geometrically as the embryo develops (Fig. 1C), and the heart rate (beats per minute) rises with time in all animal models [53,82]. In the developing zebrafish heart, quantitative *in vivo* imaging of hemodynamics revealed high-shear flow forces during valvulogenesis [52]. The ventricular peak systole is consistently higher than the aortic systolic pressure, indicating a pressure gradient between the ventricle and dorsal aorta at each stage. The durations of diastole and systole shorten as cardiac development proceeds and the heart rate rises [53], and atrial systolic pressure during development is consistently higher than ventricular end-diastolic pressure, producing a pressure gradient across the atrium and the ventricle [54].

3. Mechanical forces in early heart development and chamber formation

After gastrulation, the heart field emerges bilaterally from the anterior mesoderm. In some species, such as teleost fish (zebrafish), the fields are completely separate, whereas in others, such as mice, they form a semilunar structure. The heart tube forms at the midline, through the migration of mesenchymal cardiac progenitors.

OCT live imaging of the mouse embryonic heart suggested that the organ's final position might be in part determined through direct interaction with its boundaries [38]. An early event in heart formation is the looping of the primitive heart tube [88,120,141,142]. In mouse and chicken this process occurs in two stages: c-looping, in which the straight heart tube bends ventrally and rotates to the right; and s-looping, when the primitive ventricle moves to its definitive caudal position and the distance between the outflow tract (OFT) and the atrium shortens [88,89]. In chicken, cardiac looping and early chamber formation occur between HH13 and HH18. During these stages, blood flow rate and stroke volume increase 2-fold. Wall shear rate and lumen diameter data suggest that changes in blood flow induce a shear-mediated vasodilation response in the developing OFT and influence cardiac looping [93]. A transient reduction in hemodynamic load after venous obstruction leads to impaired looping [16] and subsequent ventricular septal and valve defects. Further studies investigated whether c- and s-looping are equally dependent on mechanical forces. When cultured in isolation, chick embryo hearts spontaneously bend into c-shaped tubes [18,90], and this process is independent of blood flow [141]. In contrast, isolated c-looped hearts have no intrinsic potential to form an s-loop in culture. s-Looping might thus be dependent on external forces, likely those originating from the splanchnopleure [138,143,154]. Moreover, early s-looping depends on the accumulation of cardiac jelly and its swelling at the atrioventricular canal (AVC) [78,141]. These data are supported by computer models indicating that the later stages of cardiac looping requires forces external to the primitive heart tube [119]. Further support comes from physical simulation experiments based on deformation of a rubber tube, which suggest that the asymmetric position of the venous pole might be enough to determine the looping direction [10]. In the zebrafish model there is no evidence for a role of external forces on heart looping. Zebrafish hearts undergo a simplified, tissue-intrinsic looping event, even in the absence of left–right (LR) asymmetry. Zebrafish heart tubes loop, without influence of blood flow, after 24 h in isolated culture, forming a right-handed loop in 79% of cases [108]. More research is needed to identify the extent to which biophysical forces drive heart tube looping.

Cardiac looping transforms the shape of the primitive heart into a chambered heart with one atrium and one ventricle, separated by an AVC. Hemodynamic forces have been proposed to influence cardiomyocyte enlargement, myofibril content and coordination of cardiomyocyte contraction [5,76]. *In vitro* studies showed that the stretching of cultured rat neonatal cardiomyocytes induces robust hypertrophic growth [163]. Cardiomyocyte elongation plays a substantial role in creating the characteristic convex shape of the outer curvature (OC); on the opposing side of the chamber, maintenance of cuboidal cardiomyocyte morphology contributes to creating the concave inner curvature (IC). In zebrafish *weak atrium* (*wea*) mutants, with aberrant atrial sarcomeres, cardiomyocytes fail to expand normally at the OC of the embryonic ventricle, and the ventricle is consequently smaller, suggesting that blood flow and cardiac contraction influence chamber formation [11]. Further work showed that blood flow and contractility independently regulate cell shape changes in the emerging ventricle. Blood flow promotes ventricular cell enlargement and elongation, thereby promoting OC establishment, whereas contractility restricts OC formation.

Blocking cardiac contraction by inhibiting cardiomyocyte excitation with the myosin II inhibitor blebbistatin (BLEB) leads to chamber enlargement in 2 dpf zebrafish larvae. BLEB also leads to cardiomyocyte enlargement, the effects of which can be partially rescued by blood loss induced by tail amputation. However, BLEB-induced hypertrophy was not rescued in *cloche* mutant fish, which lack hematopoietic cells. This suggests that, cardiomyocyte hypertrophy and ventricular chamber enlargement are mediated not by endocardial shear stress but by transmural pressure. Although the direct mechanosensing pathways remain elusive, a chemical screening identified the phosphoinositide 3-Kinase (PI3K) signaling pathway as a downstream effector [162].

Cardiac contractility is also responsible for the spatially restricted expression of genes encoding signaling factors such as atrial natriuretic factor, which is restricted to the OC of the ventricle and atrium in zebrafish [5]. A second example of cardiac contractility-regulated genes is BMP4, whose expression is expanded in the absence of contractility [129].

4. Role of mechanical forces in endocardium development and valve formation

The relationship between contraction, sarcomere integrity and blood flow, and their impact on cardiac morphogenesis, is difficult to

dissect. Hove et al. performed pioneering experiments to test the role of shear forces during zebrafish valve formation *in vivo*, using beads placed at the cardiac inflow or outflow tract to stop blood flow. They obtained a heart with an abnormal third chamber, diminished looping, and impaired valve formation, demonstrating that shear stress is important for cushion formation [52]. Since these fish still have a heartbeat, these results raised the question of whether myocardial function is required for heart development. To test this hypothesis, the authors modulated myofibril contraction with different concentrations of 2,3-butanedionemonoxime (BDM). The pharmacological inhibition of myocardial function impaired the formation of endocardial cushions in zebrafish; but even in the absence of blood flow the early signs of cushion formation were visible in 58% of embryos, indicating that flow is not required for the early steps of valve development [9]. These results support the view that shear stress and myocardial function both play a role in valvulogenesis [95]. A recent article sheds some light on the independent functions of contraction and flow forces in valve formation [60]. The authors found that in *Situs Inversus* mutants, only animals with an unlooped heart had defective valves, suggesting that intracardiac flow dynamics regulate valve morphogenesis independently of myocardial contractility.

Reducing blood viscosity in zebrafish embryos led to cardiac valve abnormalities [150], pointing to a role for shear stress in valvulogenesis. Normal mature heart valves prevent intracardiac retrograde flow, but complete valve formation is preceded by regurgitation with oscillatory flow between chambers, and valve formation can be influenced by this change in flow direction [17].

Similar to the vascular endothelium, the endocardium lines the lumen of the heart and is thus exposed to blood flow. While several studies have identified the mechanosensory pathways through which flow forces act on the vascular endothelium, less is known about the mechanisms acting in endocardial cells. Primary cilia are found on endocardial cells. They are particularly visible at regions of low or disturbed flow and absent in areas of high flow [148]. There are two types of cilia, the primary cilia, which are immotile, and the motile cilia, which can exert mechanical force through their intrinsic movement. Motile cilia move at hundreds of microns per second [96], much slower than blood flow through the great arteries. During development, cilia-driven directional flows are involved in left–right (LR) symmetry breaking, which controls the asymmetric positioning of internal organs during development, including that of the heart [19,92,109,110,121]. Primary cilia protruding from the cell membrane sense flow through their bending.

The role of cilia as flow sensors has been studied *in vivo* in the zebrafish. In the vascular endothelium, primary cilia deflect at the onset of blood flow, and recent experiments revealed that the deflection angle correlates with modulation of intracellular Ca^{2+} signals (Fig. 3). Cilia bending also depend on blood viscosity. Results from *gata1/2*-morphants, which lack red blood cells but have normal mean blood velocity, show that low blood viscosity blocks cilia deflection and downstream intracellular Ca^{2+} signaling in cardinal-vein and aortic endothelial cells of 1 day old zebrafish [41]. While cilium bending correlates with intracellular Ca^{2+} influx in the vascular endothelium, such a correlation has not been reported for the endocardium. Several examples of null mutations in ciliary proteins suggest a role of cilia in endocardial maturation and valve formation [63], which could at least partially reflect their role in hemodynamic flow sensing in endocardial cells. Ciliation itself seems to be independent of cardiac contraction, since endocardial cells of non-contracting *troponin t2*-morphants do not lose their primary cilium [127]. The endocardial cushion area is the region of highest shear stress. This area lacks ciliated endocardial cells, presumably because high shear stress leads to cilia rupture. This cilia-free region coincides with the region in which endocardial cells undergo endothelial-to-mesenchymal transition (EndoMT) and migrate into the cardiac jelly to form the primordia of cardiac valves. EndoMT is characterized by the activation of the TGFβ pathway, loss of markers like CD31, and expression of mesenchymal markers including α-SMA and N-cadherin. This correlation might indicate that endothelial-cell cilia loss is a prerequisite for EndoMT [36].

One of the most studied endothelial mechanotransducing transcription factors is *Klf2*, whose expression is activated by shear stress [111]. During mouse development, *Klf2* ablation leads to myocardial thinning, high-output cardiac failure and death by E 14.5 [68]. Because *Klf2* is expressed not in the myocardium but in the endocardium, its effect on myocardial development must be indirect. *Klf2*$^{-/-}$ mice also have atrial septation and atrioventricular valve defects, due to aberrant EndoMT of endocardial cells [25]. In the mouse, shear-stress-induced upregulation of *Klf2* is mediated by Alk5/Erk5 phosphorylation. KLF2 in turn induces the expression of *Smad7*, forming a negative feedback loop that inhibits the TGFβ pathway. High shear stress can unbalance this regulatory pathway and activate TGFβ, leading to developmental abnormalities [37]. Recently it was shown that in zebrafish Klf2a controls endocardial cell morphology and size. *klf2a* morphants, which have normal contractility, have enlarged ventricular endocardial cells, whereas overexpression of *klf2a* significantly decreases endocardial cell size. Hence, blood flow-induced *klf2a* expression plays an important role in restricting endocardial cell size during cardiac ballooning stages [34]. Treating embryos with the VEGFR inhibitor Vatalanib during cardiac ballooning stages (24–54 hpf) does not affect endocardial or myocardial chamber morphology. However, chemical inhibition of Bmp signaling and genetic ablation of cardiac contraction impairs endocardium development. Thus, while in the vascular endothelium Vegf acts downstream of flow forces and Klf2 to drive angiogenesis [69], the growth of the endocardium is Vegf independent and is driven by blood flow and Bmp signaling [34]. This mechanism favors the endocardium's adjustment to myocardial chamber size during development, since the endocardium grows through proliferation, whereas the myocardium grows through the addition of cells at both heart tube poles [32,164]. Klf2a is also required for correct valve formation in the zebrafish, with *klf2a* knockdown impairing valve formation. *KLF2a* is expressed in the valve precursors in response to oscillatory flow, and is lost in the absence of this flow [150]. Oscillatory flow induces endocardial *klf2a* expression by activating Trpv4/Trpp2 Ca^{2+} channels [49]. *Klf2* can also be activated independently of blood flow, in a process mediated by β1-integrin signaling and cellular tension generated within endothelial cells. This regulation prevents angiogenic overgrowth and ensures the quiescence and differentiation of endothelial and endocardial cells [125].

Another important blood flow sensing pathway is the NO system [84]. In the mouse, the establishment of robust, unidirectional blood flow at E9.5 activates the expression of endothelial NO synthase (eNOS), also called NOS3, in endothelial cells. NO induces vasodilatation through inhibition of the vasoconstrictive peptide ET-1 [14]. *NOS3* expression is mainly restricted to endothelial cells in medium and large arteries and the endocardium, with minor expression in myocardium. NOS3 is pivotal to the morphogenesis of major coronary arteries and myocardial capillaries. *NOS3* KO mice have abnormal aortic valve development [70] and congenital atrial and ventricular septal defects [146]. An interesting study suggests that NOS3 function might play a key role in coordinating intrinsic genetic developmental programs with cardiac function. This hypothesis is based on a genetic interaction of *Tbx5* and *NOS3*. Septation in *Tbx5*$^{+/-}$ or *NOS3*$^{+/-}$ embryos is normal, but double heterozygous embryos have atrial septal defects [103]. Work in zebrafish suggests that correct endocardium formation is exquisitely sensitive to NO. Embryos exposed to high nitrite concentrations form a normal vascular network and chamber endocardium but develop specific defects in the valve leaflets [73].

Several reports indicate that miRNAs are important mechanotransducers in the cardiovascular system [97,107]. In the mouse, *miRNA-92a* mediates the flow-dependent regulation of *Klf2* [158]. In zebrafish, *miR-21* is expressed in the AVC and OFT and is crucial for valve formation, as shown by the absence of valves in *miR-21* morphants [8]. However, *miR-21* mutant mice are viable [22,113].

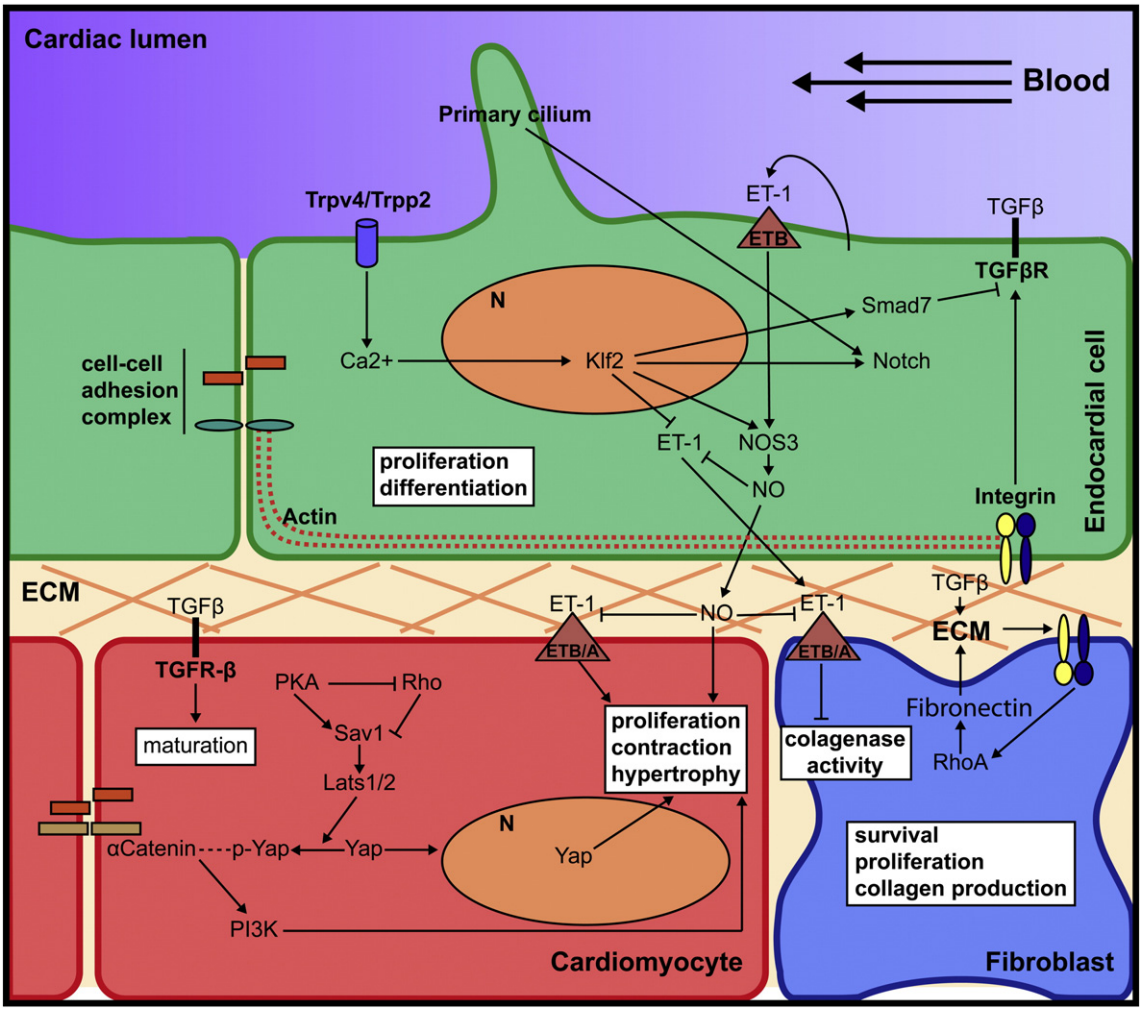

Fig. 3. Mechanosensors and mechanotransduction pathways involved in cardiac development. Transduction of blood forces to the cells forming the heart. White boxes indicate the final effect of mechanical forces in the different cell types. On the cell surface of endocardial cells, primary cilia, ion channels Trpv4/Trpp2 and integrins can act as mechanosensors. The lateral cell membrane contains the cell–cell adhesion complexes such the cadherin/catenin complex, which bind to their counterparts on adjacent cells. Tension is transmitted to the lateral borders and basal membrane, where adhesion receptors or integrins experience changes in tension. Within the cortical actin cytoskeleton, actin stress fibers mechanically connect different regions of the cell. Integrin-dependent complexes anchor the cells to the basement membrane. Klf2 plays a central role as a mechanotransducer. In cardiomyocytes, the α-catenin-YAP axis plays a major role in mechanotransduction. Nitric oxide and Endothelin signaling are important for propagation of the effect of mechanical forces between neighboring tissues *e.g.* from endothelial cells to cardiomyocytes and fibroblasts. Mechanical forces also control TGF-β activity. ECM, extracellular matrix; N, nucleus. Rest of abbreviations are explained in the main text.

As the heart grows it pumps more blood, and the increased driving pressure requires higher contractile capacity of cardiomyocytes and greater mechanical integrity of the extracellular matrix (ECM). The heightened biomechanical stability in the developing valves is largely transmitted by ECM proteins, which form a highly organized fibrous meshwork. AVC valves cultured under cyclical flow produce a disorganized fibrous ECM and develop morphologically elongated cushions. In the absence of flow, OFT cushions maintain a primitive phenotype, with dispersed mesenchymal cells surrounded by a disorganized ECM. In contrast, under physiological flow the cushions form compact cells and develop a fibrous ECM. Both the timing and magnitude of flow are implicated in correct OFT formation and the deposition of ECM proteins such as Tenascin C (TNC), Periostin, Elastin, and Collagen 6 [12], but it is not well understood how flow forces lead to ECM deposition. In the AVC, the activation of ECM production by flow forces is mediated through RhoA activity [12,145]. Blood flow direction might also influence the orientation of ECM components in the mature heart valves [80].

5. Role of mechanical forces in cardiac trabeculation and maturation

Blood flow and cardiac contractility regulate the shape of cardiomyocytes and the heart [23,135]. In the chick, changing the mechanical load alters development of the conduction system, impairs growth, and disrupts trabecula organization. Reduced mechanical load is mimicked by maintaining hearts at atmospheric pressure, while increased mechanical load is mimicked by injecting silicon oils to maximally fill the ventricles [130]. During development, the atrial muscle adopts a system for fast conduction in response to increased hemodynamic stress that augments conduction velocity. The atrial muscle, initially morphologically homogenous, segregates into at least two distinct regions: a slow conducting thin-walled myocardium and a muscle-bundle region enriched with the low resistance gap junction connexin 40 (Cx40) and the rapidly activating sodium channel Nav1.5. The large-diameter muscle conduits are coordinately stretched and induce proliferation and maturation in response to mechanical forces and the induction of marker expression [15]. Upon stretching, endothelial cells

produce proendothelin-1, which is then converted to mature ET-1 peptide by Endothelin converting enzyme 1 (ECE1). ET-1 is essential, and Endothelin expression is sufficient, for the transformation of cardiac myocytes into Purkinje cells during avian development, which coordinate the heartbeat through the transmission of electrical impulses [144]. In the developing chick embryo, pharmacological inhibition of stretch-responsive channels leads to decreased expression of ECE1 in endocardial cells and of Cx40 in Purkinje cells. Conversely, pressure overload by conotruncal banding increases Purkinje fiber formation [45].

The KO mouse embryos for ET receptors, *dnra*$^{-/-}$;*dnrb*$^{-/-}$, which have no endothelin signaling, were viable at birth. *dnra* expression within the heart was restricted to the myocardium while *dnrb* expression in the heart was restricted to the endocardium and coronary endothelium. In these null mice there are no alterations in the temporal or spatial expression pattern of the cardiac conduction system markers. Endothelin signaling in mice is thus not required for conduction system specification in the mouse [55]. Different studies support the idea that birds and mammals may have different requirements for endothelin signaling in cardiac conduction system development, but it could be that the apparent differences may simply reflect the differences between gain of- function and loss-of function approaches. ET-1 KO mice die of respiratory failure at birth and have craniofacial and cardiac abnormalities, whereas curiously ET-1$^{+/-}$ mice, which produce low levels of ET-1, develop elevated blood pressure [66]. ET-1 acts on the vascular smooth muscle ET_A receptor and has a role in the maintenance of basal vascular tone and blood pressure. However, $ET_A^{+/-}$ mice do not show a significant change in blood pressure [30]. Endothelium-specific ET-1 depletion using a Tie2:Cre line revealed low blood pressure [62]. These results suggest that ET-1 is essential for cardiac homeostasis.

The NO system also influences cardiomyocyte maturation. *In vitro* studies showed that it enhances the differentiation of embryoid bodies into cardiomyocytes. The addition of CysNO to culture medium had a pronounced effect on the spontaneous contractile activity of embryonic bodies, increasing the number of cardiomyocytes as well as the percentage of spontaneous contractions and their frequency [50]. Analysis of NOS3 KO mice confirmed that NO plays a significant role in regulating cardiac contractility [61].

The finding that the ET-1 and NO pathways influence the myocardium reveals that the mechanisms through which blood flow influences endocardium development also impact cardiac chamber maturation [128].

Optimal blood flow through the ventricle is important for the progress of trabeculation, as shown by its reduction in *wea* mutant zebrafish [116]. Cardiac contraction in zebrafish is required for chamber maturation/trabeculation through its role in regulating *notch1b*, *ephrin b2a* and *neuregulin 1* transcriptions in the ventricular endocardium. This endocardium-specific *notch1b* expression and activation is dependent on primary cilia and *klf2a* function. Hearts from non-ciliated *intraflagellar transport protein 88* (*ift88*) morphants have significantly below-normal *notch1b* and *neuregulin 1* expression [127]. In agreement with this finding, *Ift88* KO embryos reveal ventricular dilation, decreased myocardial trabeculation and abnormal outflow tract development [28]. Homozygous *cobblestone* mutant embryos, with a hypomorphic *Ift88* allele, also present a delay in ventricular trabeculation [139,156]. In addition, mice lacking primary cilia have impaired trabeculation and abnormal outflow tract development, suggesting that mechanosensing of shear stress by cilia drives these processes, similar to its action during outflow tract maturation [28,29]. However, alternative effects of cilia loss cannot be excluded.

Cardiac ECM composition changes significantly during cardiac development, and ECM incorporation into the myocardial wall leads to changes in myocardial elasticity and stiffness. Stiffness increases 2–3-fold between fetal stages and adulthood. ECM composition changes during fetal development, particularly the quantity of fibronectin and collagen, and this plays an important role in modulating the ability of the cells to generate traction stress against a substrate. The traction stress of cultured mesenchymal cells increases with increasing stiffness from 1 to 5 N/cm^2 when cells are cultured on a fetal-composition ECM gel, but not when cultured on adult ECM gels. Similarly, cell spreading increases with ECM stiffness [39]. The matrix component hyaluronic acid (HA) promotes firm adhesion to substrates. HA production is tightly regulated during development and is frequently upregulated in abnormal situations, suggesting that signaling between HA receptors and specific integrin ligands such as fibronectin alters mechanosensing by integrins [26]. This mechanotransduction role of ECM proteins is closely associated with the growth factor TGFβ. TGFβ contributes to endocardial cushion formation, EMT, ventricular development and myocardial maturation [17]. TGFβ binds to the ECM and is mechanically activated and released by cellular tension and fluid shear forces [1,6,87,147].

Cardiomyocyte proliferation also seems to be responsive to external mechanical forces. Recently, an interaction has been established between the cytoskeletal network and the Hippo pathway. Considerable evidence has revealed that the Hippo pathway plays a central role in controlling cardiomyocyte proliferation [48,102,153,157,160]. The activity of YAP, the downstream effector of the Hippo pathway, is regulated through its cellular localization. In its inactive form, YAP is anchored to adherent junctions by α-catenin, a member of the cadherin/catenin complex that binds to the actin cytoskeletal network [132]. Upon Hippo inhibition, YAP translocates to the nucleus, and enhanced YAP activity increases cardiomyocyte proliferation. α-catenin KO embryos have enhanced YAP activity in cardiomyocytes and enhanced cardiomyocyte proliferation, indicating that α-catenin restrains YAP protein and thereby inhibits cardiomyocyte proliferation [71].

6. Role of mechanical forces in epicardium formation

The epicardium is the last layer of the embryonic heart to form. This layer plays an essential role during cardiac maturation, providing growth factors and contributing intracardiac fibroblasts and cells of the coronary vasculature. The epicardium originates from the proepicardium (PE), a cluster of cells that forms close to the inflow tract of the looped heart tube and bulges out from the pericardium into the pericardial cavity that encloses the heart [133].

The mechanisms through which PE cells are transferred to the myocardium and form the epicardial layer have been extensively investigated. Classically this process has been analyzed on fixed samples. In chick and *Xenopus* a cellular bridge has been described, forming between the PE and the myocardium [58,104]. PE cells are then transferred through ECM bridges to the myocardium, and epicardial cells spread through these contact sites. In mammals [64,65,151] and some fish [56,101], release of PE cells into the pericardial cavity precedes their attachment to the myocardial surface. It is unclear whether these mechanisms are species-specific, and they have been proposed to work in parallel [115,126]. Recently, the process of PE formation has been analyzed *in vivo* in zebrafish, confirming that the release of PE cells into the pericardial cavity is the main mechanism leading to PE cell transfer to the myocardium [115]. When the heart beats, the myocardium touches the pericardium. As suggested by previous reports [126,134], this attachment and detachment between the PE and the myocardium might act as a "velcro-like" mechanism, leading to direct attachment of PE cells to the myocardium or the release of PE cells into the cavity. However, *in vivo* imaging in zebrafish shows that PE cells not in contact with the beating myocardium were also released into the cavity. When the heartbeat is absent, as a result of pharmacological treatments or silencing of genes required for cardiac contraction, the epicardial layer does not form [115,117]. The specification of PE cells is likely not affected, since expression of the PE markers *tcf21* and *wt1a* is maintained in *troponin-2a* morphants [137]. Importantly, without a heartbeat, PE cells are not released into the pericardial cavity and thus cannot reach the myocardium. If the heartbeat is stopped after PE cells have reached the myocardium, epicardium formation is less affected and epicardial cells proliferate during short periods of BDM treatment. However, migration of epicardial cells might be affected in the absence

of a heartbeat, as shown in *ex vivo* co-culture experiments of zebrafish hearts, in which epicardial cells can migrate onto control hearts but not onto non-contracting *troponin-2a* morphant hearts [117]. These results suggest that the heartbeat drives epicardium formation, mainly through the effect of pericardial fluid advections that allow the transfer of PE cells to the myocardial surface. Cardiac contraction might also be needed to promote complete epicardial layer formation through the promotion of cell adhesion or migration.

7. From development to homeostasis, disease and regeneration: mechanical forces in the adult heart

Shear stress within the chambers and in the OFT declines during the course of heart maturation; moreover, peak shear stress per cardiac cycle also decreases during maturation (from 50 to 25%) and the heart becomes exposed to a more uniform shear environment [59]. Signaling cascades triggered by blood flow, which lead to cardiac developmental growth and maturation, are switched off as a certain stage of maturation is reached. Stable hemodynamic forces are needed to preserve normal ventricular dimensions and functional parameters, and to avoid heart adaptation [114]. The pericardium fulfills an important role in stabilizing several mechanical parameters: it limits heart dilatation during diastole, reduces endomyocardial tension, prevents cardiac hypertrophy in pressure overload conditions, prevents ventricular-atrial blood retrogression under high end-diastolic ventricular pressure, preserves negative endothoracic pressure (crucial for atrial blood filling) and regulates cardiac frequency and arterial blood pressure [27,42,51]. In the adult, physiological levels of wall shear stress suppress proliferation of endothelial cells and promote their quiescence [112].

During cardiac disease, mechanical forces are disrupted and aberrant shear stress and strain can lead to fibrosis. Hemodynamic stress increases expression of the ECM protein TNC. While its role during development remains elusive, TNC may modulate the inflammatory response and contribute to tissue elasticity, protecting vascular tissue from destructive stress responses and controlling the cellular response to mechanical load during adaptation and pathological tissue remodeling [57,79]. Although *miR21* is induced by shear stress in the zebrafish embryo, stress-induced cardiac remodeling in mice proceeds in the absence of *miR-21* [22,113]. Other miRs, however, such as *miR-24* and *195*, are induced in mouse hearts subjected to pressure overload [149] and in mechanically stretched smooth muscle cells [98], suggesting a role for these miRNAs in cardiovascular pathology [106].

In the adult heart, cardiomyocyte proliferation is almost completely blocked. However, in species with a high regenerative capacity such as the zebrafish, cardiomyocytes can reenter the cell cycle upon injury. During a short window of their early postnatal life, mammals share this ability to regenerate their myocardium. As during embryonic development, initiation of cardiomyocyte proliferation in the adult requires inhibition of the Hippo pathway. Cardiac specific deletion of YAP ablates the neonatal regenerative capacity observed in wild-type mice following myocardial infarction (MI) [159]; conditional YAP KO mice had larger scar areas, suggesting that YAP is a critical mediator of heart regeneration shortly after birth. In contrast, overexpressing YAP in the adult myocardium improves cardiac function and survival and induces cardiomyocyte proliferation upon MI [77]. Moreover, myocardial specific deletion of the Hippo pathway components Salvador 1 (SAV1) or LATS1/2 in neonatal mice extends the regenerative potential after cardiac resection at postnatal day 8 [47]. *Sav1* conditional KO mice also had improved cardiac function and smaller scars after MI than wild-type controls. Even in unstressed *Sav1*-conditional KO mice, cardiomyocytes can reenter the cell cycle and undergo cytokinesis [47]. Targeting the Hippo pathway in human disease might therefore be beneficial in the treatment of heart disease.

While α-catenin KO adult mice show no evident abnormalities, lack of α-catenin has a protective effect after MI, associated with increased cardiomyocyte cell-cycle activity in the border and ischemic zones of the heart and with an increased fraction of cardiomyocytes with activated YAP [71,77]. α-catenin has other reported functions in the cell besides its anchoring function [152], but the α-catenin /YAP axis may be one mechanism through which extracellular forces influence cardiomyocyte proliferation during embryonic development and the adult injury response.

Work in zebrafish suggests that pericardial fluid flow has an important role during epicardium formation. In the adult, the pericardial fluid provides lubrication during heart beating and insulates the heart [24]. This fluid is a product of ultrafiltration through the epicardial capillaries and is thought to be drained mainly by the lymphatic capillary bed. Pharmacokinetic studies in humans suggest that pericardial fluid composition is the same in every position of the cavity [24]. Its concentrations of Na^+, Cl^-, Ca^{2+}, and Mg^{2+} are lower than in plasma, whereas K^+ concentration is higher [40,51]. The concentrations of plasma proteins are also lower, contributing to a lower osmolarity of pericardial fluid than blood plasma [40]. Due to the geometry of the pericardial cavity, most of the pericardial fluid is found at the atrioventricular and the intraventricular sulcus [31]. Pressure in the pericardial cavity increases during diastole (laminae approach) and decreases during systole [33,46,140]. Notably, the hydrostatic pressure depends on the position within the pericardial cavity, being higher close to the ventricular walls and lower at the sulcus. These pressure gradients lead to a constant movement of pericardial fluid during the cardiac cycle and allow a homogeneous distribution of the pericardial fluid within the cavity. In this way, during each heartbeat, the mesothelial cells of the pericardial wall are not only exposed to stretching but also to shear stress from the pericardial fluid. It will be interesting to study the effect of this shear stress on the mesothelial cells of the pericardial wall and epicardium. The pericardial fluid is a source of soluble factors involved in epicardial activation. Injection of human pericardial fluid from patients with acute myocardial ischemia into the pericardial cavity of mouse hearts enhances epicardial cell proliferation and expression of developmental genes [75].

8. Conclusions and Future Perspectives

During both cardiac development and homoeostatic adaptation, cardiac tissues sense physical forces generated by the heartbeat and respond with changes in gene expression. In this way, cardiac function, development and remodeling are strongly linked. During embryogenesis, the changing mechanical forces acting on the heart allow tight spatiotemporal regulation of signaling pathways involved in cardiac maturation. The role of hemodynamic forces has been extensively studied during the growth and maturation of the vasculature [3,123,131]. During development, fluid forces can influence the proliferation, migration and arterovenous fate of endothelial cells [67,100]. In the adult, physiological levels of wall shear stress suppress proliferation of endothelial cells and promote their quiescence [112]. Several pathways involved in mechanosensing have been studied, including the glycocalix and the calcium channel Piezo1 [72,118,122,124]. Given the similarities between vascular endothelial cells and endocardial cells, in particular their both being in direct contact with the blood flow, it will be interesting to study if similar mechanosensory pathways act in the endocardium.

While hemodynamic forces have been extensively studied, the pericardial fluid outside the heart tube also moves cyclically with the beating of the heart, and the flow generated contributes to cardiac morphogenesis. In line with findings in adults, the embryonic pericardial fluid might also include secreted proteins required for PE specification and epicardium development, and flow might control their availability to target cells. Moreover, it is also possible that the heartbeat might activate the expression of these secreted signaling molecules.

Congenital heart diseases (CHDs) are the main cause of fetal and neonatal mortality. Further insight into mechanical sensing during development will provide important information about how cardiac malfunction impacts cardiac development. For example, a better

understanding of the sensing role of cilia could be achieved through the analysis of conditional KO mice lacking primary cilia in different heart tissues [63]. Also, relatively little is known about the role of miRNAs and even less is known about other non-coding RNAs during mechanical sensing, and further research is warranted into their potential role as upstream integrators of force-sensing mechanisms.

In sum, a good understanding of how mechanical forces trigger genetic signaling cascades and ultimately influence morphogenetic events will provide a clearer picture of how alterations to cardiac function in the adult impact cardiac remodeling.

Conflict of interest

Nothing to declare.

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