Mechanosensitive Pathways Involved in Cardiovascular Development and Homeostasis in Zebrafish

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Abstract

Cardiovascular diseases such as coronary heart disease, myocardial infarction, and cardiac arrhythmia are the leading causes of morbidity and mortality in developed countries and are steadily increasing in developing countries. Fundamental mechanistic studies at the molecular, cellular, and animal model levels are critical for the diagnosis and treatment of these diseases. Despite being phylogenetically distant from humans, zebrafish share remarkable similarity in the genetics and electrophysiology of the cardiovascular system. In the last 2 decades, the development and deployment of innovative genetic manipulation techniques greatly facilitated the application of zebrafish as an animal model for studying basic biology and diseases. Hemodynamic shear stress is intimately involved in vascular development and homeostasis. The critical mechanosensitive signaling pathways in cardiovascular development and pathophysiology previously studied in mammals have been recapitulated in zebrafish. In this short article, we reviewed recent

knowledge about the role of mechanosensitive pathways such as Notch, PKC ϵ /PFKFB3, and Wnt/Ang2 in cardiovascular development and homeostasis from studies in the zebrafish model.

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Introduction

Cardiovascular diseases are the leading cause of death and increasingly becoming a worldwide burden [1]. While the advancement of biomedical research promises newer, faster, and cheaper diagnoses and therapies, the fundamentals of discovery remain deeply rooted in elucidating the pathogenic mechanisms at the molecular and cellular levels, especially with the advent of personalized medicine. Using animal models, gene functions and signaling pathways can be linked to normal development and pathophysiology, allowing for the identification and

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validation of pharmacological targets for novel therapeutics.

The zebrafish has been utilized as an important developmental model because it shares a largely conserved physiology and anatomy with that of mammals. Its unique transparency at the larval stage enables direct observation of organ development, including the cardiovascular system [2–5]. More recently, the role of zebrafish as human disease models has been expanded to include the adult zebrafish due to a genome that bears similarity to humans [4, 6, 7] and the relative ease of genetic manipulation utilizing the latest genomic engineering approaches [8–10]. Simple husbandry and rapid development enable a range of large-scale phenotypic screening [3, 4, 7]. Furthermore, the zebrafish demonstrates impressive regenerative capacity, such as vascular repair and heart regeneration, that scientists hope to unlock for therapeutic applications in humans.

Results and Discussion

Mechanosensors and Mechanotransduction

Hemodynamic shear stress, the frictional force from blood flow acting on vascular endothelial cells (ECs), plays a crucial role in vascular development and homeostasis. Many membrane-associated structures and molecules have been identified as sensors of shear stress to convert mechanical force into biochemical signals. These mechanosensors fall into 3 categories based on their localization on the cell surface: luminal, junctional, and basal [11]. On the luminal surface, plasma membrane microdomain structures such as cilia, glycocalyx, and caveolae are able to transduce mechanical stimuli [12]. Other luminal mechanosensors include G-protein-coupled receptors such as sphingosine 1-phosphate receptor 1 and the bradykinin B2 receptor, and ion channels [13–15]. Junctional molecules PECAM-1 and VE-cadherin, mainly located on the lateral surface, couple together with vascular endothelial growth factor (VEGF) receptors (VEGFRs) to form mechanosensory complexes, which may serve as the primary mechanosensors of shear force in EC [11, 16]. Integrins on the basal surface of EC connect extracellular matrix (ECM) and actin cytoskeleton, making them likely candidates of mechanosensors. Although it is still debatable whether integrins directly sense shear stress, a large body of studies support their role as integrators of shear-dependent signaling pathways [17–19]. The many mechanosensing molecules of ECs reported in the literature can sense shear forces either directly

or indirectly, with some having both sensing and adaptor functions. Cross talk among these mechanosensors may be important to translating physical force into biochemical signaling.

Downstream of mechanosensors, small GTPases act as an important set of signaling molecules that transduce shear stress-mediated signaling. Members of the Rho family of GTPases are highly sensitive to both spatial and temporal regulation by shear stress [20]. Shear stress transiently regulates the activities of RhoA and Rac, and the proper spatiotemporal activation of both Rho and Rac is required for aligning ECs in the direction of flow [17, 21]. In ECs subjected to shear stress, Cdc42 translocates to the EC membrane and directs the orientation of the microtubule organizing center (MTOC) [22, 23]. An increasing number of studies have demonstrated that distinct signaling pathways activated by shear stress can be coordinated via activation of small GTPases [19, 20, 24].

Multiple mechanosensitive signaling pathways downstream of mechanosensors and mechanotransducers act to modulate endothelial function [25]. Functional status of ECs is central to cardiovascular homeostasis and physiology. While many in vitro and in vivo models have been utilized to investigate mechanotransduction, the assignment of specific mechano-signaling pathways to cardiovascular physiology and pathology remains a challenge. Zebrafish is emerging as an excellent model to meet the challenge.

Mechanosensitive Pathways in Vascular Development and Regeneration in the Zebrafish

Hemodynamic fluid shear stress provides biomechanical cues for the differentiation of stem cells [26, 27] and mesenchymal progenitors [28] into vascular ECs [26, 29, 30]. Taking advantage of the zebrafish model system, our lab and others have demonstrated that shear stress activates mechanosensitive pathways such as Notch, protein kinase C isoform ϵ (PKC ϵ)/6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), and Wnt/angiopoietin-2 (Ang2) pathways that are implicated in vascular development and regeneration (Fig. 1).

Notch Signaling in Vascular Regeneration

It is well established that Notch signaling is essential for vascular morphogenesis. Ablation of Notch1 causes developmental retardation and results in embryonic lethality [31]. Missense mutation in the Notch3 gene causes the development of the degenerative vascular disease known as CADASIL (cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopa-

Fig. 1. Mechanosensitive signal pathways underlying zebrafish vascular development and regeneration. Hemodynamic shear stress activates multiple mechanical and metabolic pathways including Notch, Wnt/Ang2, and PKCε/PFKFB3 pathways to modulate vascular development and regeneration in zebrafish.

thy) [32]. Dysregulated Notch activity induces failure of vascular specification and results in abnormal endothelial proliferation, leading to the formation of a hyperplastic vascular network [31, 33]. The role of Notch signaling in vascular homeostasis was only recently elucidated. Mack et al. [34] reported that endothelial Notch1 is shear stress sensitive and necessary for the maintenance of junctional integrity, cell elongation, and suppression of proliferation. In embryonic stem cell-derived murine endothelial progenitor cells, unidirectional laminar shear stress activates the VEGF-Notch pathway to drive the expression of the arterial endothelial marker EphrinB2 (EFNB2) and to downregulate the venous endothelial marker EphrinB4 [35]. Use of embryonic zebrafish allows for genetic manipulations of blood viscosity to alter the

level of endothelial wall shear stress [36] and subsequently Notch signaling in vivo. Our observations in an embryonic zebrafish tail amputation model of vascular injury and repair support the notion that shear-sensitive Notch signaling plays an important role in vascular regeneration [37]. While control zebrafish developed vascular regeneration between the dorsal aorta and the dorsal longitudinal anastomotic vessel 3 days post-tail amputation (dpa), inhibition of Notch signaling such as using GI254023X, a pharmacological inhibitor of A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), to prevent proteolytic cleavage of Notch extracellular domain (NECD) and injecting dominant negative (*DN*)-*Notch1b* mRNA resulted in impaired vascular regeneration. As a corollary, injection of *NICD* mRNA to

promote Notch signaling restored vascular regeneration impaired by GI254023X and *DN-Notch1b* mRNAs [37].

PKC ϵ /PFKFB3 Pathway in Vascular Regeneration

PKC ϵ is a family of serine and threonine kinases involved in cell differentiation, proliferation, and migration [38–40]. PKC ϵ is abundantly expressed in EC and is closely associated with a series of pathways involved in angiogenesis and vascular formation [41, 42]. The flow-responsive VEGFR-endothelial nitric oxide synthase pathway drives the expression of PKC ϵ to maintain endothelial homeostasis and lumen formation [43–45]. Shear-mediated nitric oxide production [46, 47] further modulates PKC ϵ expression, which in turn attenuates mitochondrial reactive oxygen species following ischemia or reperfusion injury [48–52]. ECs are highly glycolytic [53], and endothelial glycolysis is mechanoresponsive [54]. When switching from quiescence to the proliferating state, ECs increase the level of glycolytic flux while glycolytic enzymes including PFKFB3 localize to lamellipodia [55]. As a rate-limiting enzyme and critical regulator of glycolysis, PFKFB3 regulates lamellipodia and filopodia extension for vessel formation. Our recent observation with embryonic zebrafish suggested that intravascular fluid shear stress modulates PKC ϵ -PFKFB3 to promote vascular regeneration after injury. In the transgenic *Tg(flk-1: GFP)* zebrafish tail amputation model, the control zebrafish developed robust vascular regeneration 3 dpa while shear stress reduction via *Gata1a* morpholino oligonucleotide (MO) injection delayed vascular regeneration from 3 to 5 dpa [56]. Knockdown of cardiac troponin T2 (*tnnt2a*) with MO as a means of arresting myocardial contractility and blood flow also resulted in impairment of vascular regeneration. Injection of the PKC ϵ mRNA overrides the effect of *Gata1a* MO, supporting the hypothesis that flow-driven vascular regeneration is at least partially mediated by PKC ϵ [56].

The Wnt/Ang2 Pathway in Vascular Development and Regeneration

Extensive studies have demonstrated the role of Ang1 and Ang2 in vascular development. Shear stress-mediated Ang2 in mature vascular endothelium was recently reported to play a role in tubulogenesis [57] and conferring atheroprotection [58]. While Ang1 is constitutively released by perivascular cells, Ang2 is expressed in ECs released from the Weibel-Palade bodies upon signal cues [59, 60]. Ang2, like Ang1, binds to endothelial-specific receptor tyrosine kinase 2 (TIE2) and acts as a negative regulator of Ang1/TIE2 signaling to promote angiogen-

esis [61]. Earlier studies demonstrated that Ang2 could be released by mechanical force such as endothelial stretch that occurs during hypertension [62]. Our recent work as well as work from Dr. Jo's lab both demonstrated Ang2 as a mechanosensitive gene involved in shear stress-mediated tubule formation and migration of ECs [57, 63]. In the mouse artery occlusion model, femoral artery ligation caused disturbed flow to stimulate Ang2 expression and arteriogenesis in mice [64].

Canonical Wnt/ β -catenin signaling is a pivotal pathway regulating development, cell proliferation, and migration [65]. We further demonstrated that shear stress-stimulated Ang2 expression is mediated by the canonical Wnt signaling pathway. While a Wnt agonist, Wnt3a, promoted Ang2 expression, inhibition of Wnt signaling with Dickkopf-1 (Dkk-1) or IWR-1 attenuated EC migration and tube formation. In the heat shock-inducible DKK-1 transgenic *Tg(hsp70l: Dkk1-GFP)* zebrafish embryos, Ang2b (zebrafish Ang2) expression was downregulated upon heat shock. Ang2 MO injection into transgenic *Tg(kdrl: GFP)* zebrafish to downregulate Ang2 resulted in impaired development of subintestinal vessels 72 h after fertilization. Inhibition of Wnt signaling also impaired subintestinal vessel development that is rescued with Ang2 mRNA. In the zebrafish tail amputation model, inhibition of Wnt signaling retarded and reduced the rate of vascular regeneration that is also rescued by Ang2 mRNA [63]. These findings support the notion that the mechanosensitive Wnt/Ang2 pathway modulates vascular development and regeneration.

Hemodynamic Fluid Force on Cardiac Development via Mechanosensitive Notch Signaling in Zebrafish

In addition to transcription factors involved in cardiogenic differentiation, hemodynamic fluid forces also play an essential role in cardiogenesis [36, 66–71]. Occlusion of flow at either the cardiac inflow or outflow tracts resulted in hearts with an abnormal third chamber, diminished looping, and impaired valve formation [36]. During heart development, the myocardium differentiates into 2 layers: an outer compact zone and an inner trabeculated zone. The trabeculae form a network of branching outgrowths from the myocardial wall [72]. Both trabeculation and compaction are essential for normal cardiac contractile function. The reduction in trabeculation is associated with deficiency in the ventricular compact zone (hypoplastic wall) that commonly leads to embryonic heart failure and early embryonic lethality. On the other hand, hypertrabeculation is closely associated with left ventricular noncompaction cardiomyopathy [73], the

third most common cardiomyopathy in the pediatric population after dilated and hypertrophic cardiomyopathies [74].

Cardiac trabeculation is a crucial morphogenetic process by which clusters of ventricular cardiomyocytes extrude and expand into the cardiac jelly to form sheet-like projections to enhance cardiac contractility and intraventricular conduction. Previous studies support the critical role of Notch signaling in heart development, including trabeculation [75, 76]. Notch1 has recently been reported as a mechanosensor in ECs [34], and Notch1 becomes sensitized to metalloprotease cleavage under shear stress [77]. Shear stress activates Notch signaling in both human and zebrafish endocardium [78]. In murine embryonic stem cell-derived vascular ECs, shear stress induces time-dependent Notch signaling [35]. To elucidate the role and mechanism of hemodynamic forces on trabeculation via notch signaling, Lee et al. [78] took advantage of genetic manipulation in zebrafish and lowered hemodynamic shear forces via: (1) microinjection of *gata1a* MO at cell stages 1–4 to reduce hematopoiesis and viscosity by 90% [79, 80], (2) microinjection of *tnnt2a* MO to arrest cardiomyocyte contraction in embryos [81, 82], and (3) genetic mutation of the *weak atrium*^{m58} (*wea*) mutant to inhibit atrial contraction [72, 83]. Lowered hemodynamic forces correlated with the inhibition of Notch signaling and trabeculation. Utilizing the *Tg(flk-1:mCherry, tp1:gfp)* fish line, shear stress-activated Notch signaling is confirmed to be localized to the endocardium. Endocardial activation of Notch signaling is required for trabeculation. The zebrafish *Cloche* (*clo*^{m39/lmo2-GF}) mutant, in which the endothelial line is abolished, developed small and thin ventricles [84, 85] along with reduced expression of cardiac Notch ligands, receptors, and target genes when compared to the wild type [78]. Notch activation in the endocardium results in the transcription of *EphrinB2*, which in turn upregulates neuregulin1 (*Nrg1*) [86]. As a secreted factor, *Nrg1* signals to the adjacent cardiomyocytes to promote trabeculation. Disruption of *Nrg1* expression after ischemic insult impairs cardiac contractility [87], whereas *Nrg1* preconditioning confers cardiac protection from ischemic injury [88]. In parallel, Notch activity in the endocardium activates bone morphogenic protein 10 (*BMP10*) expression in the adjacent myocytes to promote proliferation [86]. Unlike mouse development, *Nrg1/ErbB2* signaling contributes to both proliferation and differentiation of cardiomyocytes for trabeculation in zebrafish [89]. The advantages of the zebrafish model have enabled researchers to establish the essential role of mechanosen-

sitive Notch signaling in promoting cardiac trabeculation involving *EphrinB2*, *Nrg1*, and *ErbB2* (Fig. 2) [86, 89, 90].

Regulation of Metabolic Pathways by Mechanical Forces

Metabolomic analyses have led to the discovery of new metabolic biomarkers and therapeutic targets such as spermine for acute stroke, cinnamoylglycine, nicotinamide, and cysteine-glutathione disulfide for renal cancer, and 3-hydroxykynurenine and oxidized glutathione for Parkinson disease [91–93]. Hemodynamic forces have been shown to modulate mammalian metabolic pathways [94–96] to maintain vascular homeostasis. Elucidating flow-mediated metabolomic changes provides an entry point to uncovering metabolites involved in endothelial homeostasis [97, 98], migration [99], vascular development [53], and physical activity [100].

Endothelial glycolysis has been shown to be mechanoresponsive as well [54]. Rather than relying on oxidative metabolism for mitochondrial respiration, ECs generate over 80% of their adenosine triphosphate from the glycolytic pathway [101]. A recent study reports that laminar shear stress activates Krüppel-like factor 2 (KLF2) to modulate PFKFB3-mediated glycolysis, mitigating angiogenesis, and vessel sprouting [102]. On the other hand, flow-sensitive VEGFR signaling upregulates PFKFB3-driven glycolysis [53, 55]. We have recently demonstrated that shear stress promotes vascular regeneration and repair in zebrafish through VEGFR/PKCε/PFKFB3 signaling, and this effect is at least partially attributed to elevated glycolysis. Shear stress modulates a number of metabolites (Fig. 3a), including the elevation of the glycolytic metabolite dihydroxyacetone (DHA, C₃H₆O₃), which is dependent on flow-sensitive PKCε (Fig. 3b). DHA partially rescued tube formation in human aortic ECs and impaired vascular regeneration and repair in zebrafish upon PKCε knockdown (Fig. 3c, d). Hemodynamic shear stress also modulates stearoyl-CoA desaturase-1 (SCD1) expression via PPARγ in ECs, a major pathway regulating lipid metabolism [103]. Metabolomic analysis in combination with genetic manipulation of hemodynamic shear force in zebrafish would greatly improve the investigation of in-depth mechanisms of mechanotransduction in vascular development and pathophysiology.

Interaction and Synergy of Mechanosensitive Pathways

Multiple mechanosensitive pathways such as Notch, Wnt/Ang2, and PKCε/PFKFB3 signaling have been

Fig. 2. Shear stress activation of Notch signaling promotes trabeculation in zebrafish. Shear stress activates Notch signaling in endocardial cells to upregulate the expression of Nrg1 via EphrinB2. Nrg1 promotes cardiomyocyte proliferation and differentiation for trabeculation.

shown to play important roles in vascular development and regeneration in the zebrafish model. Nakajima et al. [104] recently developed a zebrafish line to model blood flow and monitor the spatiotemporal localization and transcriptional activity of yes-associated protein (YAP) in ECs of living zebrafish. They revealed that blood flow regulates localization and activity of YAP, demonstrating flow-sensitive endothelial YAP is essential in vessel maintenance. It has recently been reported that flow-sensitive SCD-1 generates palmitoleic acid. SCD1 activity modulates protein palmitoylation of TEA domain family (TEAD) members, the YAP/ transcriptional co-activator with PDZ-binding motif (TAZ) coactivator [105]. Furthermore, YAP regulates EC contact-mediated expression of Ang2 [106]. In cancer cells, SCD1 regulates YAP/TAZ activity via modulating Wnt signaling. Thus, mechanosensitive pathways are closely connected and

interact with each other. The ability for easy genetic manipulation in zebrafish makes it an excellent model to study the interactions of mechanical force-activated signal pathways in cardiovascular development and homeostasis.

Mechanosensitive Pathways of Different Mechanical Forces in Cardiac Morphogenesis

In response to hemodynamic shear forces, myocardial ridges and grooves develop in a wave-like trabecular network in alignment with the direction of the shear stress across the atrioventricular valves [107]. Both trabeculation and compaction are essential for contractile function during cardiac development [72]. We demonstrated that hemodynamic shear forces modulate the initiation of cardiac trabeculation in zebrafish via endocardial Notch-Nrg1 signaling, which activates myocardial *ErbB2* signal-

Fig. 3. The glycolytic metabolite dihydroxyacetone (DHA) promotes vascular regeneration/repair. **a** Pulsatile shear stress (PSS) and oscillatory shear stress (OSS) modulate a number of metabolites in human aortic endothelial cells (HAEC) including DHA. **b** The level of DHA is mechanosensitive dependent on PKC ϵ . **c** DHA is able to rescue siPKC ϵ -attenuated tube formation in HAEC. **d** DHA rescues PKC ϵ MO-impaired vascular regeneration/repair in zebrafish. Scr/scrambled, scrambled control siRNA; siPKC ϵ , PKC ϵ siRNA; MO, morpholino oligonucleotide. Reprinted with permission from *Antioxidants & Redox Signaling* [56].

Perspective and Conclusion

The advancement and application of a diverse array of reverse genetic manipulations in zebrafish allow targeted investigation of a gene of interest in a specific pathway by increasing, reducing, or silencing its expression. An important and helpful resource for reverse genetic investigation is the zebrafish mutant project that provides a growing list of fish lines with a defined mutation in a specific gene [111]. For example, Lee et al. [78] demonstrated the role of shear stress-mediated notch signaling in trabecu-

lation by taking advantage of the *wea* and *clo* mutants. Genome editing techniques such as ZFNs (zinc finger nucleases), TALENs (transcription activator-like effector nucleases), the CRISPR (clustered regulatory interspaced short palindromic repeats)/Cas9 system, and the Tol2 transposon system have made zebrafish transgenesis very efficient. In combination with the fluorescent protein technology, the large number of transgenic lines allow for labeling of various subcellular structures, cells, tissues, and organs as well as for spatiotemporally reporting of the activity of specific signaling pathways [56, 104]. The structural and functional visualization in vivo is very important to establish the correlation between genotype and phenotype. Their optical clarity and advantages in genetic engineering make zebrafish a model of choice when it comes to applying optical techniques involving fluorescent protein and optogenetic technologies. Technology advancements in this field allow new ways to visualize, quantify, and perturb developmental dynamics of the cardiovascular system. Recent application of light-sheet imaging enables the recapitulation and quantification of developmental cardiovascular mechanics [112, 113] that will further facilitate the study of mechanotransduction on cardiovascular development and homeostasis in the zebrafish model.

The general strengths of utilizing zebrafish for biomedical research are well known. Despite being phylogenetically distant from humans, zebrafish have remarkable similarities in genetics and electrophysiology. In the last 2 decades, a full array of genetic manipulation techniques have been developed and deployed in zebrafish. The critical mechanosensitive signaling pathways in cardiovascular development and physiology previously studied in mammals have been recapitulated in zebrafish. Studies in the zebrafish model further enhance our knowledge on the importance of mechanotransduction in cardiovascu-

lar development and homeostasis. The application of genomic, transcriptomic, proteomic, and metabolomic analyses in the zebrafish provides a well-characterized platform for the exploration of many biological processes. Furthermore, the zebrafish model allows high throughput genetic and chemogenomic screening based on critical pathways, making it particularly attractive for therapeutic applications in human diseases.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

R.L. conceived, wrote, and revised the manuscript. K.I.B., C.-C.C., and B.Z. wrote and revised the manuscript. T.K.H. conceived and revised the manuscript.

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