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Mechanotransduction in embryonic vascular development

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Abstract A plethora of biochemical signals provides spatial and temporal cues that carefully orchestrate the complex process of vertebrate embryonic development. The embryonic vasculature develops not only in the context of these biochemical cues, but also in the context of the biomechanical forces imparted by blood flow. In the mature vasculature, different blood flow regimes induce distinct genetic programs, and significant progress has been made toward understanding how these forces are perceived by endothelial cells and transduced into biochemical signals. However, it cannot be assumed that paradigms that govern the mature vasculature are pertinent to the developing embryonic vasculature. The embryonic vasculature can respond to the mechanical forces of blood flow, and these responses are critical in vascular remodeling, certain aspects of sprouting angiogenesis, and maintenance of arterial–venous identity. Here, we review data regarding mechanistic aspects of endothelial cell mechanotransduction, with a focus on the response to shear stress, and elaborate upon the multifarious effects of shear stress on the embryonic vasculature. In addition, we discuss emerging predictive vascular growth models and highlight the prospect of combining signaling pathway information with computational modeling. We assert that correlation of precise measurements of hemodynamic parameters with effects on endothelial cell gene expression and cell behavior is required for fully understanding how blood flow-induced loading governs normal vascular development and shapes congenital cardiovascular abnormalities.

Keywords Mechanotransduction · Embryonic vasculature · Arterial–venous identity · Wall shear stress · Cardiovascular biology · Growth models

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Abbreviations

Alk1	Activin receptor-like kinase 1
AP-1	Activator protein-1
ASS	Argininosuccinate synthase
ATF-2	Activating transcription factor-2
CBP	CREB-binding protein
DLAV	Dorsal longitudinal anastamotic vessel
DLL4	Delta-like 4
EDHF	Endothelial-derived hyperpolarizing factor
EDN	Endothelin
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-related kinase
HDAC	Histone deacetylase
HH	Hamburger–Hamilton
HMOX-1	Heme oxygenase-1
ICAM-1	Intracellular adhesion molecule
IκB	Inhibitor of κB
IKK	IκB kinase
ISV	Intersegmental vessel
Jag	Jagged
JNK	Jun kinase
KLF	Krüppel-like factor
MAPK	Mitogen-activated protein kinase
MCP-1	Macrophage chemotactic protein-1

MEF	Myocyte enhancer factor
MEK	Mitogen-activated protein kinase kinase
miR	MicroRNA
NF-κB	Nuclear factor kappa B
NIK	NF-κB-inducing kinase
NO	Nitric oxide
NRF-2	Nuclear factor erythroid 2-related factor 2
NRP	Neuropilin
PECAM-1	Platelet endothelial cell adhesion molecule
PI3K	Phosphatidyl inositol-3-kinase
PPAR	Peroxisome proliferator-activated receptor
PTGDS	Prostaglandin D2 synthase
RB	Retinoblastoma protein
RISC	RNA-induced silencing complex
RPSI	Relative pulse slope index
RUNX1	Runt-related transcription factor 1
SELE	E-selectin
TNNT2	Cardiac troponin T2
VCAM-1	Vascular cell adhesion molecule-1
VECAD	Vascular endothelial cadherin
VEGFR2	Vascular endothelial growth factor receptor 2
βTRC	Beta-transducin repeat-containing protein
WSS	Wall shear stress

1 Introduction

Endothelial cells are exposed not only to paracrine factors and circulating factors within the bloodstream, but also to the mechanical forces imparted by blood flow. Specifically, endothelial cells are directly exposed to shear stress, which is associated with the tangential, frictional force that acts on the apical surface of the endothelium, more or less parallel to the direction of flow. Shear stress is dependent upon the velocity profile at the vessel wall and is proportional to blood viscosity and inversely proportional to the cubed vessel radius. In idealized thin-walled vessels, circumferential stress is directly proportional to blood pressure and vessel radius and inversely proportional to the vessel wall thickness. The vasculature readily adapts to and attempts to "normalize" these hemodynamic forces, as first postulated by Thoma (1893). Based on observations of chick embryonic vasculature, Thoma suggested that the relationship between blood flow and vascular architecture is governed by three basic mechanical principles: (1) In the face of increased or decreased flow velocity, blood vessel caliber increases or decreases, respectively, to normalize shear stress; (2) In the face of increased or decreased blood pressure, the vessel wall thickens or thins, respectively, primarily by the modulation of vascular smooth muscle cell investment, to counterbalance this force and thereby normalize circumferential stress; and (3) Increases in capillary pressure can lead to new capillary growth.

Thoma's postulates have subsequently been validated in immature and mature vessels in diverse model systems, including dogs, rabbits, and sheep, and in clinical applications. For example, surgically induced arteriovenous shunts, arterial anastomoses, or ligations lead to altered flow velocity, which results in adaptive changes in vessel caliber: arteries that experience increased flow increase their caliber, whereas arteries that experience decreased flow decrease their caliber and in extreme cases, completely regress (Brownlee and Langille 1991; Di Stefano et al. 1998; Kamiya and Togawa 1980; Langille et al. 1989; Langille and O'Donnell 1986). Notably, with velocities up to four times normal flow rates, a compensatory change in vessel caliber can bring shear stress to normal levels (Di Stefano et al. 1998; Kamiya and Togawa 1980). Changes in vessel caliber require an intact endothelial layer and are initially mediated by changes in the contractile state of vascular smooth muscle cells, suggesting that shear stress is transduced by the endothelium into a signal that affects smooth muscle cell contraction. However, changes in vessel caliber quickly become smooth muscle-independent and are associated with changes in endothelial cell number, suggesting a permanent remodeling of the arterial wall (Langille et al. 1989; Langille and O'Donnell 1986). One can also see Thoma's principles at work in the process of arteriogenesis, a hypoxia-independent vascular remodeling event in which pre-existing collateral arterioles are recruited to bypass an arterial blockage (Ito et al. 1997). In response to increased shear stress and blood pressure, these low-flow arterioles mount a proliferative response in both the endothelial and smooth muscle cell compartments, resulting in increased vessel caliber and wall thickness. These morphological changes counterbalance hemodynamic forces while restoring circulation to sites distal to the blockage (Cai and Schaper 2008).

Although the effects of blood flow on vascular architecture have been well documented over the past 120 years, it is only in the past few decades that we have begun to understand how the mechanical forces of blood flow are transduced into biochemical signals that regulate these remodeling events, and we are just beginning to appreciate the role of blood flow in shaping the embryonic vasculature in normal and pathological states. Approximately 1 in 100 children are born with clinically significant congenital heart defects, representing 30,000 children each year in the United States and 1,300,000 children worldwide (van der Linde et al. 2011). Neonatal great vessel abnormalities are frequently associated with heart defects: for example, conotruncal malformations can result in pulmonary atresia with no pulmonary trunk, whereas hypoplastic left heart syndrome can result in a tiny aorta just sufficient to feed the coronary circulation. Understanding the alterations in fluid dynamics associated with aberrant cardiovascular development is critical for defining the mechanisms responsible for these

clinically prevalent defects and for optimizing their in utero and postnatal management (Matsui and Gardiner 2007; Makikallio et al. 2006; Tulzer et al. 2002). In this review, we summarize effects of shear stress on endothelial cell gene expression and discuss current theories of mechanotransduction; relate these biochemical responses to critical events in vascular development; and highlight emerging engineering modeling techniques and supportive imaging modalities that will allow us to better measure mechanical forces and correlate changes in mechanical loading to endothelial cell gene expression and behavior in developing vertebrate embryos.

2 Molecular signature of shear stress associated with laminar versus disturbed flow

In straight vessels, blood flow is laminar and shear stress is net forward-directed and sustained at a high magnitude. In contrast, at branches and curvatures, blood flow is disturbed, resulting in reciprocating and/or low-magnitude shear stress at the vessel wall. Remarkably, laminar and disturbed flow patterns exert different effects on endothelial cell gene expression and cell behavior. Continuous exposure to strong, net forward-directed laminar shear stress generally favors endothelial cell quiescence, characterized by growth arrest, nitric oxide (NO) production (which promotes vascular smooth muscle cell relaxation and thus vasodilation), and cytoskeletal alignment in the direction of flow. Conversely, endothelial cells generally respond to disturbed flow and associated reciprocating and low shear stress by upregulating cytokines and leukocyte adhesion molecules, thereby priming the vasculature for an inflammatory response (Chen et al. 2001; Chu and Peters 2008; Garcia-Cardena et al. 2001; Kondapalli et al. 2004; Mun et al. 2009; Ohura et al. 2003; Yoshisue et al. 2002). While steady or pulsatile laminar shear stress is considered to be atheroprotective, sites within the arterial tree that experience disturbed flow are atheroprone (Cecchi et al. 2011). At the heart of these flow responses are transcription factors and microRNAs (miRs), which coordinately control expression levels of multiple genes (Fig. 1).

2.1 KLF2 expression and vascular quiescence

Endothelial cells exposed to high levels of laminar shear stress activate a transcriptional program that favors a quiescent, anti-inflammatory, anti-thrombotic, and atheroprotective state, which in large part can be accounted for by activation of the zinc finger transcription factor, Krüppel-like factor 2 (KLF2). In the human, chick, and mouse vasculature, *KLF2* is expressed at sites of laminar, atheroprotective flow (Dekker et al. 2002, 2005; Groenendijk et al. 2004), and this transcription factor is upregulated by laminar shear stress in cultured endothelial cells (Dekker et al. 2002). The orchestration of shear stress-induced upregulation of KLF2 is remarkable, with regulation at the transcriptional and post-transcriptional levels. At the transcriptional level, a 160-bp proximal promoter element that binds the shear stress-inducible transcription factors, myocyte enhancer factor 2a (MEF2a) and MEF2c, is required for shear stress-induced transcriptional activation of *KLF2* (Huddleson et al. 2004; Parmar et al. 2006). Furthermore, under static or disturbed flow conditions, histone deacetylase 5 (HDAC5) binds to MEF2c at the *KLF2* promoter and inhibits transcription by enhancing histone-DNA interactions, whereas steady laminar shear stress results in phosphorylation and nuclear export of HDAC5, derepressing *KLF2* expression (Wang et al. 2010b). The mitogen-activated protein kinases MEK5 (also known as MAP2K5) and ERK5 (also known as MAPK7) act upstream of MEF2c in transcriptional regulation of *KLF2*, but the mechanism by which shear stress activates MEK5 is not understood (Parmar et al. 2006). Post-transcriptionally, *KLF2* mRNA is destabilized by miR-92a (Fang and Davies 2012; Wu et al. 2011). Expression of miR-92a dominates in atheroprone regions of disturbed shear in the porcine aorta and is downregulated by laminar shear stress in cultured endothelial cells. Therefore, in atheroprotected regions of high laminar flow, low levels of miR-92a allow stabilization of its mRNA targets, including *KLF2* (Fang and Davies 2012; Wu et al. 2011).

KLF2 coordinates expression of numerous genes (Fig. 1), generally favoring vasodilation and opposing inflammatory, thrombotic, and oxidative stress responses (Dekker et al. 2006; Parmar et al. 2006). Vascular tone is controlled by a balance of vasodilators and vasoconstrictors. NO, which is generated by endothelial nitric oxide synthase (eNOS, encoded by *NOS3*), and prostaglandins, which are arachidonic acid metabolites, are critical endogenous vasodilators, whereas endothelin-1 (EDN1) is a potent vasoconstrictor. Remarkably, KLF2 upregulates expression of genes involved in generating or utilizing NO and prostaglandins, such as *NOS3*, argininosuccinate synthase (ASS), prostaglandin D2 synthase (PTGDS), and prostaglandin transporter, while downregulating *EDN1* (Dekker et al. 2006; Parmar et al. 2006). Furthermore, anti-thrombotic and antioxidant genes such as thrombomodulin and heme oxygenase-1 (*HMOX-1*) are induced by KLF2, whereas pro-inflammatory genes such as vascular cell adhesion molecule-1 (*VCAM-1*), intracellular adhesion molecule-1 (*ICAM-1*), monocyte chemotactic protein-1 (*MCP-1*) and numerous interleukins and chemokines are repressed by KLF2 (Dekker et al. 2006; Parmar et al. 2006; Fledderus et al. 2008). This gene expression program leads to vascular quiescence and atheroprotection.

Among the genes upregulated by KLF2, the most widely studied is *NOS3*. Laminar shear stress-induced KLF2 binds to the *NOS3* promoter and upregulates gene expression, and KLF2 and eNOS colocalize in atheroprotected regions of

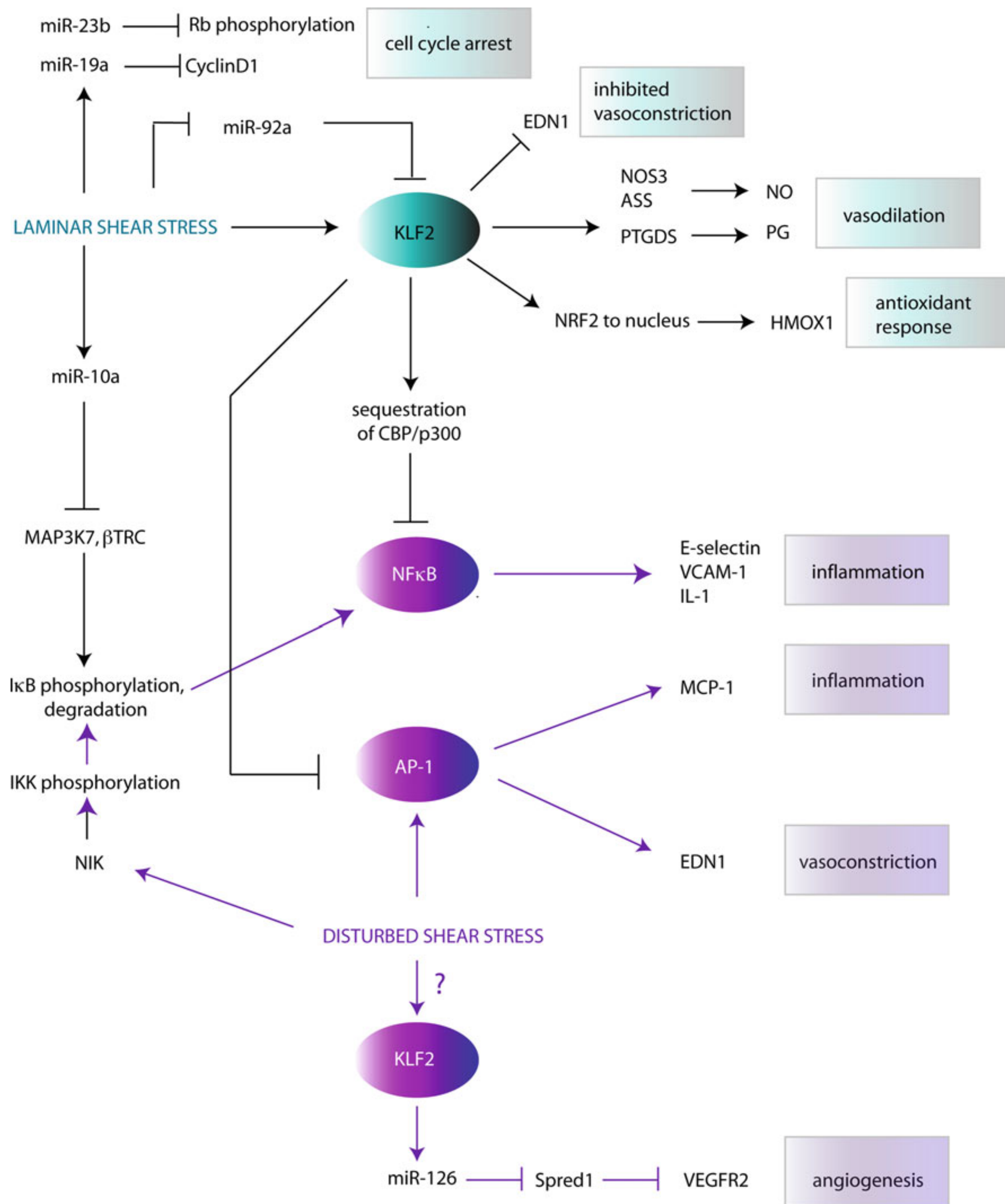

Fig. 1 Integration of molecular responses to shear stress in laminar and disturbed flow regimes. Transcription factors and pathways affected by laminar flow are shown in *blue*, whereas transcription factors and pathways affected by disturbed flow are shown in *purple*. The relationship between disturbed flow and KLF2 (marked by *question mark*) has been demonstrated only in embryonic development. Please see text for details

high laminar shear stress in the chick vasculature (Dekker et al. 2006, 2005; Groenendijk et al. 2004; SenBanerjee et al. 2004). While many laminar shear stress- and KLF2-inducible genes may be regulated in this straightforward manner, KLF2 can also promote nuclear localization of the antioxidant transcription factor, nuclear factor erythroid 2-related factor 2 (NRF2), which in turn directly upregulates genes such as *HMOX-1* via binding to an antioxidant response element (Fledderus et al. 2008). Interestingly, genes induced by either KLF2 or NRF2 overexpression include 76 % of genes induced by laminar shear stress, demonstrating that these two transcription factors play pivotal roles in the

atheroprotective response. Finally, it should also be noted that *KLF4* is highly upregulated by laminar shear stress in a MEK5/ERK5/MEF2-dependent manner, similar to *KLF2*, and that 42 % of genes regulated by KLF2 are also regulated by KLF4, suggesting some redundancy (Villarreal et al. 2010; Clark et al. 2011; Ohnesorge et al. 2010). The mechanism by which KLF2 downregulates gene expression is discussed in Sect. 2.3 below.

2.2 NF-κB expression and inflammation

Although prolonged laminar shear stress evokes quiescence in cultured endothelial cells, acute laminar shear stress elicits an inflammatory, atheroprone response that we now understand to be characteristic of the response to disturbed flow. This finding suggests that the gene expression program elicited by disturbed flow reflects a repeated acute response to shear stress that is subsequently downregulated in continuous steady or pulsatile laminar flow conditions (Garcia-Cardena et al. 2001). The transcription factor nuclear factor kappa B (NF-κB) is a key player in coordinating this disturbed flow response (Fig. 1). NF-κB is a heterodimer consisting of p50 and p65 that is sequestered in the cytoplasm by Inhibitor of κB (IκB). Upon acute onset of laminar shear stress or in the face of continuous low or reciprocating shear stress, NF-κB-inducing kinase (NIK) phosphorylates IκB kinase (IKK), which phosphorylates IκB to promote its ubiquitinylation and degradation. Release of NF-κB unmasks a nuclear localization signal and thus promotes its nuclear translocation (Bhullar et al. 1998; Hay et al. 2003). Within the nucleus, NF-κB binds to a shear stress-responsive element, at the core of which is GAGACC, in a variety of genes, including *E-selectin* (*SELE*), *VCAM-1*, and several interleukins, which are hallmarks of an inflammatory response (Bhullar et al. 1998; Khachigian et al. 1995; Lan et al. 1994; Resnick et al. 1993). As would be expected, nuclear localized NF-κB and NF-κB-dependent mRNAs are highest in vivo in endothelial cells at sites of disturbed flow (Mondy et al. 1997; Nagel et al. 1999). Additional transcription factors have also been implicated in the disturbed shear stress response, including activator protein-1 (AP-1), which mediates disturbed shear-induced expression of *MCP-1* and *EDN1* (Fledderus et al. 2007; Shyy et al. 1995). AP-1 is a heterodimer composed of c-Jun and a binding partner, one of which is activating transcription factor-2 (ATF-2). High levels of phosphorylated, nuclear localized ATF-2 are found at sites of disturbed shear stress in human atherosclerotic tissue (Fledderus et al. 2007).

2.3 Antagonism between KLF2 and NF-κB, AP-1

From the above discussion, it should be clear that continuous laminar shear stress and disturbed shear stress have very different effects on endothelial gene expression, with the former seemingly inhibiting the latter (Fig. 1). KLF2 inhibits expression of NF-κB-responsive genes including *VCAM-1* and *SELE*, thereby inhibiting white blood cell attachment and rolling, which are key components of the inflammatory response (SenBanerjee et al. 2004). The effect of KLF2 on NF-κB-mediated gene expression neither requires DNA binding of KLF2 nor diminishes DNA binding of NF-κB and may be explained by the observation that KLF2 can titrate out the transcriptional co-activators, CREB-binding protein (CBP) and p300, which are required for NF-κB activity (SenBanerjee et al. 2004; Das et al. 2006). KLF2 also inhibits AP-1-dependent gene expression by decreasing levels of nuclear phosphorylated c-Jun and ATF-2 (Fledderus et al. 2007; Boon et al. 2007). The mechanism by which KLF2 inhibits AP-1 activation may involve KLF2-dependent cytoskeletal changes that inhibit activation of Jun kinase (JNK), which is the kinase responsible for both c-Jun and ATF-2 phosphorylation (Boon et al. 2010).

2.4 microRNA control of endothelial cell transcripts

Small non-coding RNAs known as miRs play a critical role in regulation of mRNA transcript stability and translation (Suzuki and Miyazono 2011). miRs are generated as primary transcripts containing one or more stem-loop regions. These stem-loops are excised from the primary transcript by a nuclear RNase III, Drosha; the resulting pre-microRNA stem-loops are exported from the nucleus; and the cytoplasmic RNase III, Dicer, subsequently cleaves the loop to generate the active duplex microRNA. One strand of this ∼22 nucleotide duplex is loaded into the RNA-induced silencing complex (RISC) and brings RISC to mRNAs containing complementary sequence within their 3′ untranslated regions. As RISC contains an mRNA-directed RNase H, this pairing results in mRNA destabilization or degradation.

As mentioned previously, *KLF2* is a miR-92a target and miR-92a is downregulated by laminar shear stress; therefore, low levels of miR-92a in atheroprotected regions help to stabilize *KLF2* (Wu et al. 2011; Fang and Davies 2012). Most of the other laminar shear stress-regulated miRs identified to date are upregulated in this context and serve to downregulate endothelial cell proliferation and inflammation. For example, miR-23b and miR-19a are both upregulated by steady or pulsatile laminar shear stress and are required for cell cycle arrest (Wang et al. 2010a; Qin et al. 2010). miR-23b expression is required for laminar shear stress-induced dephosphorylation of retinoblastoma protein (RB) and consequent blockage of the G1/S transition, although the direct target of miR-23b responsible for this effect has not been determined (Wang et al. 2010a). miR-19a targets *Cyclin D1*, which is required for RB phosphorylation and cell cycle progression (Qin et al. 2010). miR-10a is also induced by laminar shear

stress and serves to dampen the inflammatory response (Fang et al. 2010). miR-10a is predominantly expressed in atheroprotected regions and targets the MAP kinase, *MAP3K7*, as well as β-transducin repeat-containing protein (βTRC). Because MAP3K7 and βTRC enhance IκB phosphorylation and thus transcriptional activity of NF-κB, miR-10a inhibits the NF-κB-mediated inflammatory response (Fang et al. 2010).

miR-126 is upregulated by blood flow in zebrafish embryos, although the type of mechanical force required for this upregulation has not been described (Nicoli et al. 2010). Expression of *klf2a* (the zebrafish functional homolog of mammalian *KLF2*) is required for blood flow-induced miR-126 expression, and miR-126 targets *spred1*, which encodes a negative regulator of vascular endothelial growth factor receptor 2 (VEGFR2) activity (Fish et al. 2008; Nicoli et al. 2010). Therefore, KLF2-induced miR-126 enhances VEGFR2 activity and thus opposes endothelial cell quiescence, a conclusion reflected in the requirement for miR-126 in directed sprouting of select embryonic arteries (Nicoli et al. 2010). These results are somewhat surprising given the established role of KLF2 in vascular quiescence in the adult vasculature.

microRNAs have been implicated not only in mediating the response to laminar shear stress but also in mediating the response to disturbed flow (Zhou et al. 2011). Reciprocating flow induces AP-1 activation, which transcriptionally activates expression of miR-21. miR-21, in turn, targets peroxisome proliferator-activated receptor α (*PPAR*α) for degradation. Because PPARα inhibits AP-1 activity (Delerive et al. 1999), miR-21 facilitates a positive feedback loop to reinforce AP-1-mediated expression of inflammatory genes such as *VCAM-1* and *MCP-1* (Zhou et al. 2011). Notably, miR-21 is enriched at sites of disturbed flow in vivo (Fang et al. 2010; Shyy et al. 1994).

3 Mechanosensation and mechanotransduction of shear stress

Although the effects of shear stress on endothelial cell gene expression have been well studied, the mechanism by which force applied to the apical surface of the endothelial cell is transduced into a biochemical signal is poorly understood. Just as effects of shear stress on gene expression differ with different flow regimes, so too may mechanisms of mechanosensation and mechanotransduction. Primary cilia, the endothelial cell glycocalyx, ion channels, and cell–cell adhesions have all been implicated in mechanosensation, and it is widely accepted that the cytoskeleton and perhaps nuclear A-type lamin reorganization play some role in transducing this signal into a biochemical response (Hoffman et al. 2011; Philip and Dahl 2008).

3.1 Primary Cilia

Primary cilia are microtubule-based apical mechanosensory appendages that are found on most cell types and play critical roles in embryonic left/right patterning and renal tubule development, among numerous other processes (Sharma et al. 2008). In cultured endothelial cells, moderate levels of laminar shear stress lead to cilial bending and opening of the associated transient receptor potential (TRP) calcium channel, Polycystin-2, resulting in an immediate but fleeting increase in intracellular calcium. Intracellular calcium binds calmodulin, which activates the serine/threonine kinase, Akt. Akt in turn phosphorylates and thereby activates eNOS, resulting in increased NO production (AbouAlaiwi et al. 2009). Although it has been reported that endothelial cilia are required for shear stress-induced NO production in cultured endothelial cells (AbouAlaiwi et al. 2009), this may not be the case in vivo because cilia thus far have been found only on select endothelial cells in regions of disturbed, atherogenic flow (Van der Heiden et al. 2006, 2008). In fact, the presence of cilia is inversely correlated with expression of atheroprotective genes such as *KLF2* (Van der Heiden et al. 2006), and in cultured endothelial cells, physiological levels of laminar shear stress result in either dismantling of cilia or cleavage of Polycystin-1, which abrogates Polycystin-2 function (Hierck et al. 2008; Iomini et al. 2004; Nauli et al. 2008). These observations suggest that primary cilia may not be required for sensing physiological levels of steady or pulsatile laminar shear stress, but may play a functional role in regions of disturbed flow.

3.2 Glycocalyx

The endothelial apical membrane is covered in a gel-like layer of sulfated proteoglycans, hyaluronan, and glycoproteins that is known as the glycocalyx (Weinbaum et al. 2007). This layer can be up to 500 nm in height and plays an important role in the regulation of vascular permeability. In fact, there is almost no fluid flow through the glycocalyx. Therefore, it has been suggested that shear stress must be perceived first by the glycocalyx, which bends in the direction of flow and transduces this force via the cortical actin skeleton to adherens junctions (Thi et al. 2004). At physiological levels of laminar shear stress, this force is strong enough to favor disjoining of adherens junctions; to prevent this disjoining, it is hypothesized that cells realign their actin cytoskeleton in the direction of flow. But is the glycocalyx a somewhat passive transducer of mechanical force, or is it required for mechanosensation? Treatment of cultured endothelial cells or arteries ex vivo with heparinase (to degrade heparan sulfate), hyaluronidase (to degrade hyaluronan), and/or neuraminidase (to degrade sialic acid) impairs NO production and vasodilation but not prostacyclin production in response

to laminar shear stress (Florian et al. 2003; Mochizuki et al. 2003; Pahakis et al. 2007). Similarly, treatment of embryonic stem cell-derived endothelial cells with heparinase impairs expression of only a subset of laminar shear stress-responsive genes (Nikmanesh et al. 2012). Moreover, a more recent study suggests that only hyaluron may be required for force transduction, because although degradation of hyaluronan, heparan sulfate, or sialic acid inhibits laminar shear stress-induced NO production, the latter two treatments release membrane-associated superoxide dismutase from the cell membrane. Loss of superoxide dismutase allows superoxide to accumulate and scavenge NO, thereby limiting availability of free NO (Kumagai et al. 2009). Together, these studies suggest that there are multiple mechanisms of mechanotransduction and that there are limitations inherent in this enzymatic approach to determining the necessity of the glycocalyx in this process.

3.3 Ion Channels

Endothelial cells respond to shear stress with an inwardly rectifying potassium current that is detectable on a time scale of a few hundred milliseconds post-stimulation (Olesen et al. 1988). This potassium current leads to endothelial cell hyperpolarization that is postulated to contribute, in the guise of endothelial-derived hyperpolarizing factor (EDHF), to vasodilation. This shear stress-induced potassium current may be attributed in part to rapid opening of the inwardly rectifying potassium channel, Kir2.1 (Hoger et al. 2002). In addition, the opening of the mechanosensitive calcium channel, TRP vanilloid 4 (TRPV4), leads to the opening of calcium-operated potassium channels, further contributing to the potassium current. TRPV4-mediated calcium influx has also been shown to be important in shear stress-induced NO production, likely via calcium/calmodulin/Akt activation and eNOS phosphorylation, in small resistance arteries but not in the carotid artery (Loot et al. 2008; Mendoza et al. 2010). Notably, shear stress induces translocation of TRPV4 to the endothelial cell membrane (Loot et al. 2008) and induces production of cytochrome P450-derived epoxyeicosatrienoic acids that are required to activate TRPV4 (Huang et al. 2005; Loot et al. 2008). These data suggest that TRPV4 does not open in direct response to shear stress and is thus not a direct mechanosensor.

3.4 The PECAM/VEGFR2/VECAD complex

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is an endothelial cell-specific cell adhesion molecule that localizes to adherens junctions and is rapidly tyrosine phosphorylated in response to shear stress (Osawa et al. 1997, 2002). Notably, application of force to PECAM-1 antibody-coated magnetic beads bound to cultured endothelial cells also results in rapid PECAM-1 tyrosine phosphorylation, suggesting that PECAM-1 itself is a mechanosensor (Osawa et al. 2002; Tzima et al. 2005). The importance of PECAM-1 in mechanosensation was further confirmed in vivo: in *Pecam-1* null mice, flow-induced vascular remodeling is impaired (Chen and Tzima 2009; Tzima et al. 2005). However, PECAM-1 alone cannot confer responsiveness to mechanical stimuli (Osawa et al. 2002), suggesting that additional, potentially endothelial cell-specific factors may also be required. In fact, two additional endothelial-specific proteins are required for mechanosensation: VEGFR2 and vascular endothelial cadherin (VECAD) (Tzima et al. 2005). VEGFR2 is a tyrosine kinase receptor that plays a pivotal role in nearly all aspects of vascular development and its myriad functions have been extensively described (Koch et al. 2011). While VEGFA ligand can bind to and activate autophosphorylation of VEGFR2, this receptor can also be activated by shear stress in a ligand-independent manner (Jin et al. 2003). Furthermore, shear stress results in accumulation of both VEGFR2 and VECAD at adherens junctions, and a requirement for VEGFR2 has been demonstrated in multiple shear-mediated responses (Jin et al. 2003; Shay-Salit et al. 2002). Building upon these observations, a series of elegant epistasis experiments revealed a somewhat unifying pathway whereby shear stress induces PECAM-1 phosphorylation, which leads in turn to activation of Src tyrosine kinase and VEGFR2, with VECAD serving as an adaptor or scaffolding protein required for VEGFR2-mediated activation of phosphatidylinositol-3-OH kinase (PI3K) (Tzima et al. 2005). Active PI3K then phosphorylates Akt, which can lead to numerous molecular events. For example, Akt activity can cause a conformational change in basally located integrins that leads to increased binding of these adhesion molecules to the extracellular matrix at sites known as focal adhesions (Tzima et al. 2005). This strengthening of focal adhesions is associated with activation of small G-proteins, which are required for actin filament stress fiber alignment in the direction of flow and positioning of the microtubule organizing center downstream of the nucleus (Tzima et al. 2001, 2002, 2003). Integrin activation is also required to activate NF-κB and its associated inflammatory response (Tzima et al. 2002). Akt can also phosphorylate and activate eNOS, leading to enhanced NO production and vasodilation (Jin et al. 2003; Dimmeler et al. 1999). Importantly, ectopic expression of *PECAM-1*, *VECAD*, and *VEGFR2* in non-endothelial cells confers typical endothelial cell-like flow responses, including Akt activation and cytoskeletal alignment with flow (Tzima et al. 2005). Taken together, these experiments demonstrate that PECAM-1 is a mechanosensitive adhesion molecule that, when complexed with VEGFR2 and VECAD, can transduce shear stress into a biochemical response.

4 Hemodynamic forces in vascular development

In the developing vertebrate embryo, endothelial cells differentiate from the mesoderm and coalesce into solid cords, a process known as vasculogenesis. These cords interconnect to form a seamless primitive vascular network and subsequently form lumens, either by a cell-hollowing or by a cord-hollowing mechanism (Blum et al. 2008; Jin et al. 2005; Kamei et al. 2006; Strilic et al. 2009). These early vasculogenic events occur before the onset of circulation, and blood flow is generally not required for lumen formation during vasculogenesis or the subsequent process of angiogenesis (Isogai et al. 2003; Wang et al. 2010c), during which new vessels sprout from existing vessels. Because in many cases embryonic blood flow is established well before the requirement for convective oxygen and nutrient transport, it has been postulated that the sole purpose of early embryonic blood flow is to mold the nascent vasculature via hemodynamic forces (Burggren 2004). In support of this hypothesis, zebrafish circulation is established by approximately 24 hours post-fertilization, with a primitive vascular network perfusing the trunk and tail (Isogai et al. 2001). However, convective oxygen transport is not required in developing zebrafish until approximately 14 days post-fertilization, at which point the dissolved oxygen gradient between external water and tissues is no longer sufficient to drive diffusional oxygen transport to all cells (Pelster and Burrgen 1996; Jacob et al. 2002). A similar lack of requirement for convective oxygen transport in very early, small embryos with established circulations has been demonstrated in chick and frogs (Cirotto and Arangi 1989; Territo and Burggren 1998). In fact, the hemodynamic forces imparted by blood flow do play a critical role in several aspects of normal vertebrate vascular development, including remodeling and extension of existing networks, stabilization of nascent vessels, arterial–venous specification, and definitive hematopoietic stem cell development. Furthermore, embryonic blood vessels, like those in the adult, can adapt to changes in hemodynamic parameters by co-opting collateral vessels via the process of arteriogenesis.

4.1 Hemodynamic forces are required for embryonic vascular remodeling

Thoma's principles withstand scrutiny not only when assessing surgically manipulated and pathological changes in blood flow, but also when assessing the relationship between blood flow and vascular architecture in normal vertebrate development. For example, in the 2–4-day chick embryo, vessel diameters at bifurcations adhere remarkably well to Murray's Law [$(D_{d1}^3 + D2_{d2}^3)/D_p^3 = 1$, where D_p is the diameter of the parent vessel, D_{d1} and D_{d2} are diameters of daughter vessels] (Taber et al. 2001). This relationship serves to standardize shear stress at a set level—which varies with development stage—regardless of vessel caliber (Kamiya et al. 1984). Note that at these early time points, chick endothelial tubes are not supported by contractile smooth muscle cells (Hungerford and Little 1999), demonstrating that endothelial cells themselves can effect flow-based changes in vessel caliber. Changes in the caliber of the lamb postnatal abdominal aorta also nicely support Thoma's principles: at birth, flow velocity dramatically decreases and pressure increases in this vessel, and these changes correlate with a compensatory decrease in caliber and increase in wall thickness, suggesting normalization of shear stress and circumferential stress, respectively (Langille et al. 1990). Developmental adaptation to flow pattern has also been beautifully demonstrated in the branchial aortic arches, which are initially present in all vertebrate embryos in a gill-like configuration comprising six bilaterally symmetric pairs of vessels. While these vessels retain their basic embryonic pattern in gill-bearing animals, they remodel extensively to serve the needs of air-breathing animals. In mouse embryos, the left sixth aortic arch persists and gives rise to the ductus arteriosus, whereas the right sixth aortic arch regresses. This pattern favors persistence of the left fourth aortic arch and consequently, development of a mature left-sided aortic arch. Mechanical forces play a critical role in this remodeling process. During normal development, outflow tract rotation results in increased flow toward the left versus the right sixth aortic arch, correlating with left sixth retention and right sixth regression, whereas genetically induced inhibition of outflow tract rotation directs flow equally to both left and right sixth aortic arches and randomizes aortic arch sidedness (Yashiro et al. 2007; Wang et al. 2009). Chick embryonic aortic arches similarly remodel in response to surgically induced changes in flow patterns (Hogers et al. 1997), and we have demonstrated that high shear forces in the 3–4-day chick right fourth aortic arch correlate with increased vessel caliber and persistence of this vessel (Wang et al. 2009). Once again, this remodeling process in the chick occurs in the absence of contractile smooth muscle cells, which are not present until day 18 (Hungerford and Little 1999). Together, these examples demonstrate that embryonic and early postnatal endothelial cells must sense and respond to blood flow to properly shape the developing vasculature.

The mouse yolk sac vasculature develops at embryonic day (E) 8.0–8.5 as a honeycomb-like plexus of uniformly sized vessels that remodels extensively by E9.5 into a hierarchical vascular tree. There are numerous examples of mouse mutants that fail to remodel their yolk sac vasculature; although some of the genes that cause remodeling defects when mutated are expressed in endothelium [for example, *Activin receptor-like kinase 1 (Alk1)*, *Endoglin*], other genes are expressed exclusively in myocardium (for example, *Myosin light chain 2a, Titin*) (Huang et al. 2003; May et al. 2004; Oh et al. 2000; Urness et al. 2000; Li et al. 1999). The latter

category results in decreased or absent blood flow, which could lead to defects in vascular remodeling due to hypoxia, the absence of circulating signaling factors, and/or the modification of hemodynamic force. Remarkably, among these three factors, only shear stress is required for yolk sac plexus remodeling (Lucitti et al. 2007). Decreasing hematocrit by chemical sequestration of red blood cells in the yolk sac blood islands results in similar defects in yolk sac vascular remodeling as impaired heartbeat; because this treatment does not interfere with plasma flow, the absence of a circulating signal is not likely to be the cause of impaired remodeling. Furthermore, yolk sac vascular remodeling in embryos with sequestered red blood cells can be rescued by intravascular injection of a polymer that raises the viscosity of the blood and therefore increases shear stress without increasing convective oxygen transport, pointing to a role for shear stress in this remodeling process.

Given the dependence of embryonic vascular remodeling on shear stress, an obvious question to ask is whether these processes rely on the mechanotransduction pathways described earlier that have been defined primarily in cultured cells or in the mature vasculature. In the mouse yolk sac vascular plexus remodeling studies, low hematocrit and impaired remodeling correlated with decreased expression of PECAM-1 and eNOS, both of which were rescued, along with vascular remodeling, when viscosity was restored (Lucitti et al. 2007). However, neither *Pecam-1* nor *Nos3* null mice exhibit embryonic vascular defects, therefore neither PECAM-1 nor eNOS is necessary for yolk sac vascular remodeling (Duncan et al. 1999; Shesely et al. 1996). The lack of a requirement for eNOS does not reflect redundancy because mice deficient in all three NOS isoforms (*Nos1, Nos2, and Nos3*) develop a normal embryonic vasculature; however, in adulthood, these mice exhibit deficits in endothelium-dependent vasodilation and are susceptible to atherosclerosis and spontaneous myocardial infarction (Takaki et al. 2008; Tsutsui et al. 2006). And although global deletion of *Klf2* in mice is embryonic lethal by E12.5–14.5 due to insufficient vascular smooth muscle coverage and consequent hemorrhage, these mice develop a normal yolk sac vasculature (Kuo et al. 1997). Whether KLF4 can compensate for KLF2 in vascular remodeling remains to be determined.

4.2 Effects of blood flow on embryonic angiogenesis

The zebrafish embryo is an excellent model for the study of the effects of hemodynamic force on vascular development because diffusional oxygen transport can sustain these embryos for up to two weeks (Jacob et al. 2002; Pelster and Burrgen 1996). Therefore, stopping heartbeat will reveal effects of either hemodynamic forces or circulating factors on embryonic vascular development in the absence of the confounding issue of hypoxia. In the zebrafish trunk, the dorsal aorta and posterior cardinal vein develop via vasculogenesis. Angiogenic primary intersegmental vessels (ISVs) sprout from the dorsal aorta and merge dorsally to form the dorsal longitudinal anastamotic vessel, or DLAV (Isogai et al. 2003), whereas later emerging posterior cardinal vein-derived secondary ISVs either seed the thoracic duct (Yaniv et al. 2006) or merge basally with primary ISVs, which subsequently lose their connection to the dorsal aorta and are transformed into veins (Isogai et al. 2003). In cardiac troponin t2a (*tnnt2a*) mutants, which never initiate heartbeat (Sehnert et al. 2002), the dorsal aorta, posterior cardinal vein, primary and secondary ISVs, and DLAV form in a normal spatiotemporal manner. However, secondary ISVs fail to connect to primary ISVs, and endothelial cells remain highly active in terms of filopodial projections, which normally quiet at the onset of flow (Isogai et al. 2003). Blood flow similarly dampens endothelial cell filopodial projections in central arteries, which penetrate the hindbrain, and this quiescent phenotype depends on flow-induced downregulation of the chemokine receptor, *cxcr4a* (Bussmann et al. 2011). Because the mammalian *cxcr4a* functional homolog, *CXCR4*, is downregulated by laminar shear stress and KLF2 (Melchionna et al. 2005; Uchida et al. 2009), these data suggest that hemodynamic force, as opposed to a circulating factor, may be responsible for flow-induced dampening of protrusive behavior and consequent nascent vessel stabilization.

Using zebrafish embryos, we have demonstrated a requirement for blood flow in setting normal caliber of select angiogenic cranial arteries (Corti et al. 2011). Blood flow is required for the expression of *alk1,* which encodes an endothelial-localized TGF-β family type I serine/threonine kinase receptor. In the absence of *alk1*, the cranial arteries proximal to the heart—including the first aortic arch, the internal carotid artery and its caudal division, and basal communicating artery—enlarge greatly, and analysis of enlarged arteries reveals supernumerary endothelial cells that have a larger surface area than their wild-type counterparts (Fig. 2). Notably, *alk1* is expressed predominantly in these same arteries, suggesting that this transmembrane receptor might lie in a mechanosensitive pathway that limits arterial caliber at the onset of flow. Epistasis experiments reveal that this pathway does not involve *klf2a*, though a possible redundant role for *klf4* has not been investigated. Intriguingly, *edn1* is expressed in cranial vessels in the same restricted arterial pattern as *alk1*, and *edn1* expression is lost in *alk1* mutants. Given that the vascular architecture in these *alk1*- and *edn1*-positive vessels comprises many sharp turns and that *EDN1* is repressed by laminar shear stress but induced by disturbed shear stress (Dekker et al. 2006; Parmar et al. 2006; Yoshisue et al. 2002), it seems possible that Alk1 may play a role in a disturbed flow response. However, micro-particle image velocimetry-based measurement of flow velocities in one *alk1*- and *edn1*- positive vessel, the first aortic arch, revealed pulsatile, laminar

flow (Chen et al. 2011; Corti et al. 2011). Thus, the relationship between flow and *alk1* expression remains to be determined.

Although the above examples demonstrate a requirement for blood flow in stabilizing and quieting nascent angiogenic vessels, there is also evidence that blood flow can have pro-angiogenic effects. In zebrafish embryos, four bilateral clusters of angioblasts differentiate in a rostral-to-caudal sequence from the lateral pharyngeal mesoderm, and from each cluster, two angiogenic sprouts emerge, with one migrating ventrally and one migrating dorsally. These sprouts give rise to the branchial aortic arches (aortic arches 3–6). Ventrally, the branchial aortic arches merge at the midline to form the ventral aorta, whereas dorsally, they connect to the lateral dorsal aortae, thereby forming a vascular cage around the pharynx (Anderson et al. 2008). While aortic arches 3 and 4 connect independently to the lateral dorsal aorta, aortic arches 5 and 6 fuse at the level of the primary head sinus (a major primitive cranial drainage vein). Aortic arch 5 then lumenizes and, as blood pulsates in this blind-ending vessel, it extends its trajectory to connect to the lateral dorsal aorta (Anderson et al. 2008). In the absence of blood flow, extension of aortic arch 5 toward the lateral dorsal aorta does not occur. This flow-dependent process requires Klf2a-mediated expression of miR-126, which facilitates degradation of *spred1* to derepress VEGFR2 activity (Nicoli et al. 2010). In mature vessels, *KLF2* expression is associated with laminar shear stress and vascular quiescence (Dekker et al. 2006; Parmar et al. 2006), whereas in this particular embryonic vessel, *klf2a* is associated with an oscillatory flow pattern and angiogenic activation. Notably, zebrafish *klf2a* expression is also associated with oscillatory flow at the level of the atrioventricular canal, where it is required for normal cardiac valve development (Vermot et al. 2009). These findings demonstrate that one cannot assume that paradigms that explain flow responses in mature vessels will pertain to the embryonic vasculature.

4.3 Arterial flow patterns promote arterial gene expression

Arterial and venous endothelial cells both in the embryo proper and in the yolk sac can be distinguished by differential gene expression patterns even before the onset of blood flow, suggesting that arterial and venous differentiation is genetically hard-wired (Wang et al. 1998; Othman-Hassan et al. 2001). Arterial specification in zebrafish trunk vessels involves a well-orchestrated cascade of events in which sonic hedgehog (Shh) produced by the notochord signals to somites to induce *vegfa* expression, which in turn activates *notch3* expression in *vegfr2*-positive presumptive arterial endothelial cells. With Notch signaling active, these cells express arterial genes, whereas expression of venous genes is repressed (Lawson et al. 2002). Arterial endothelial cells express *Ephrinb2 (Efnb2); Neuropilin-1 (Nrp1)*, which is a co-receptor for VEGF and Semaphorins; Notch ligands *Delta-like 4 (Dll4)*, *Jagged1 (Jag1)*, and *Jagged2 (Jag2)*; *Notch1, 3*, and/or 4 (depending on species); Notch targets such as *Hey1* and *Hey2*; *Alk1*; and the connexin, *Gja5*. In contrast, venous endothelial cells do not express any of these genes but instead express *Ephb4*, which encodes the receptor tyrosine kinase that is activated by EphrinB2; *Neuropilin-2 (Nrp2)*; and the transcription factor, *Coup-TFII* [reviewed in Ref. (le Noble et al. 2008)].

Although it is clear that arterial and venous specification is initially hard-wired in the vertebrate embryo, there is considerable evidence that blood flow plays an important role in maintaining these identities and, if appropriate, unmasking plasticity. Cultured endothelial progenitor cells exposed to laminar shear stress differentiate into arterial endothelial cells, as defined by expression of *Efnb2, Notch1/3, Hey1/2,* and *Alk1* and repression of *Ephb4* and *Nrp2* (Obi et al. 2009). Curiously, although the effect on arterial gene expression correlated linearly with shear stress magnitude, the range of shear stress used in this experiment (0.1–5 dynes/cm^2) corresponded to venous rather than arterial shear stress. Embryonic stem cells subjected to arterial-like laminar shear stress (5–20 dynes/cm^2) also differentiate into arterial endothelial cells, exhibiting VEGFR2-dependent upregulation of expression of *Notch1/4, Dll4, Jag1, Jag2,* and *Efnb2* (Masumura et al. 2009). Thus, it is tempting to speculate that both genetic and flow-dependent mechanisms of arterial specification rely on VEGFR2 signaling, with Shh-induced VEGF activating VEGFR2 in genetic specification and shear stress activating VEGFR2 in a ligand-independent manner in flow-dependent specification.

Transplantation of isolated arterial or venous endothelial cells from quail to chick embryos demonstrates that these differentiated cells retain plasticity (Moyon et al. 2001; Othman-Hassan et al. 2001). Both cell types contribute in a relatively unbiased fashion to host arteries and veins, and donor venous endothelial cells that populate host arteries turn on *Efnb2*, whereas donor arterial endothelial cells that populate host veins turn off *Efnb2*. These results suggest that differentiated endothelial cells can sense and respond to cues in their new environment. Given the different flow types in arteries versus veins, one obvious candidate for this cue is shear stress. In the chick, mechanical obstruction of the right vitelline artery results in reversal of blood flow through distal portions of this vessel; in other words, a portion of this artery is now perfused by venous, retrograde flow. Notably, arterial segments that are subjected to venous flow quickly lose expression of *Efnb2* and *Nrp1* and, on a slower time scale, gain expression of *Nrp2*. This effect is fully reversible upon removal of the mechanical obstruction and reestablishment of normally-directed blood flow (le Noble et al. 2004). These observations strongly suggest that mechanical

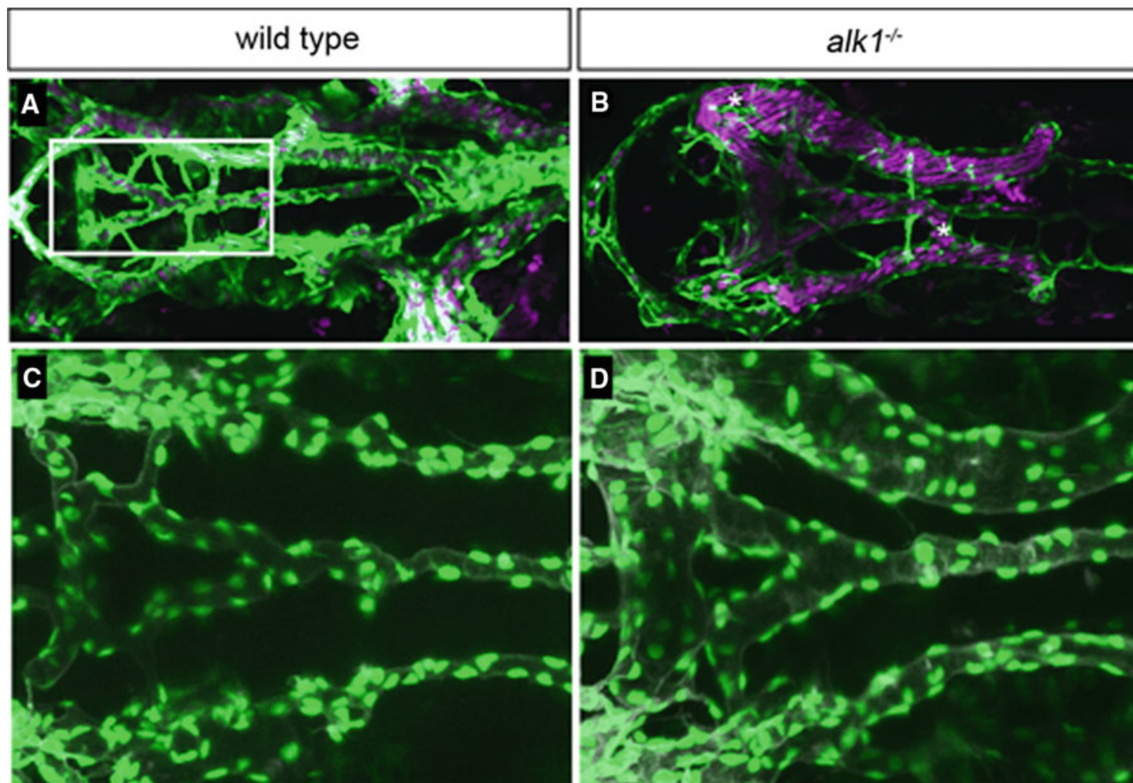

Fig. 2 Loss of *alk1* function in zebrafish embryos results in enlarged arteries and arteriovenous shunts. Alk1 expression is dependent on blood flow and plays an important role in limiting arterial caliber; in its absence, *alk1*-dependent arteries exhibit increased endothelial cell number and decreased endothelial cell density (compare **d**, *alk1* mutant to **c**, wild type). Arterial enlargement results in flow-dependent development of arteriovenous malformations (*asterisks* in **b**). **a**, **b** endothelial cell cytoplasm is *green*, red blood cells are magenta. **c**, **d** endothelial cell nuclei are *green*, endothelial cell membranes are grayscale. The *boxed region* in **a** is similar to the region shown at higher magnification in independent embryos in **c**, **d**. 48 h post-fertilization, dorsal views, anterior *left*

forces associated with arterial flow are required to maintain an arterial gene expression program. But exactly how are the forces in arteries different from the forces in veins? Assessment of flow parameters in chick yolk sac vessels reveals that the most distinguishing parameter is the ratio of maximal red blood cell acceleration rate to mean velocity, or relative pulse slope index (RPSI), which can be used to robustly categorize arterial versus venous flow (Buschmann et al. 2010). The difference in RPSI is due primarily to the high pulsatility of arterial flow and in fact, pulsatile but not steady laminar flow can enhance arterial gene expression in cultured human arterial endothelial cells (Buschmann et al. 2010). Chick yolk sac arterial vessels that, after ligation, are categorized by RPSI as veins lose expression of the arterial marker, *Gja5*, and gain expression of the venous marker, *Coup-TFII* (Buschmann et al. 2010). Together, these observations elegantly demonstrate that arterial-type hemodynamic forces are transduced to biochemical signals that serve to maintain arterial identity and that these signals can trump genetic specification and actually instruct arterial identity. These findings are important from a clinical standpoint because a primary cause of failure of vein grafts, which are used to treat occluded arteries and to generate arteriovenous fistulas in dialysis patients, is neointimal hyperplasia, which may be due in part to incomplete arterialization (Cahill 1987; Henderson et al. 1986; Wallitt et al. 2007). Understanding the mechanotransduction pathway that allows facile venous-to-arterial reprogramming in the embryo may lead to increased vein graft success.

The concept that arterial flow patterns maintain arterial identity is further supported by evidence that blood flow is required for maintenance of dorsal aorta-derived definitive hematopoietic stem cells (North et al. 2009; Wang et al. 2011). These cells transdifferentiate from hemogenic endothelium located in the ventral wall of the dorsal aorta in a process that depends on runt-related transcription factor 1 (Runx1). After emergence from the endothelium and release into the circulation, definitive hematopoietic stem cells subsequently home to and seed hematopoietic organs (Bertrand et al. 2010; Chen et al. 2009; Zovein et al. 2008). In zebrafish embryos, blood flow is required to maintain arterial gene expression (*efnb2*, *dll4*) and hematopoietic stem cell differentiation (*runx1*) in the dorsal aorta. Blood flow-induced

arterial gene expression and *runx1* expression require Klf2a, Notch, and NO, although the mechanism by which NO mediates expression of these genes is not understood (North et al. 2009; Wang et al. 2011).

4.4 Alterations in hemodynamic forces can trigger embryonic arteriogenesis

Patients with peripheral arterial obstructive disease or coronary artery disease can develop collateral vessels that bypass arterial obstruction, and efficiency of collateral network formation is positively correlated with clinical outcome. Arteriogenesis—the process of generating this collateral network—is elicited by local changes in shear stress that result in co-option and enlargement of pre-existing arterioles. This process involves NO-mediated vessel dilation; PECAM-1/VECAD/VEGFR2-dependent induction of MCP-1 and cell surface receptors that promote monocyte adhesion; and enhanced proliferation within both the endothelial cell and smooth muscle cell compartments [reviewed in (Cai and Schaper 2008).] Remarkably, arteriogenesis can also occur in the developing embryo in response to changes in hemodynamic forces. Zebrafish embryos harboring mutations in the Notch target, *hey2* (also known as *gridlock*), fail to connect the anterior lateral dorsal aortae to the trunk midline dorsal aorta, preventing blood flow to the trunk and tail (Weinstein et al. 1995). However, many *hey2* mutants bypass this blockage by recruitment of pre-existing intestinal vessels (Gray et al. 2007). Arteriogenesis in this model requires NO and the chemokine receptor, *cxcr4a* (which is associated with angiogenic, protrusive behavior) and is at least partially dependent on the presence of monocytes (Gray et al. 2007; Packham et al. 2009). In zebrafish *alk1* mutants, we also observe co-option of existing vasculature in response to changes in hemodynamic forces. As mentioned earlier, zebrafish *alk1* mutants develop enlarged cranial arteries. Downstream of these enlarged arteries, normally transient connections between receiving arteries and neighboring veins are aberrantly retained and enlarge into high-flow arteriovenous shunts [Fig. 2, and (Corti et al. 2011; Roman et al. 2002)]. Notably, these connections are only retained in the presence of blood flow, suggesting that enhanced shear stress not only prevents regression but also elicits a compensatory response that increases vessel caliber (Corti et al. 2011). Enhanced expression of *cxcr4a* is noted in *alk1* mutant arteries, although in contrast to collateral formation in *hey2* mutants, *cxcr4a* is not necessary for arteriovenous shunt formation in *alk1* mutants (Corti et al. 2011). It remains to be determined whether other molecular mechanisms defined in adult arteriogenesis are at play in this process in *alk1* mutants. Given that heterozygous mutations in *ALK1* result in Hereditary Hemorrhagic Telangiectasia type 2, characterized by susceptibility to development of arteriovenous malformations, it is tempting to speculate that these malformations represent an arteriogenesis-like response to altered hemodynamics caused by upstream arterial enlargement.

5 Predictive computational engineering studies

In mature vessels, absolute mechanical loading can be correlated with gene expression, but similar studies in developing systems are complicated by strong regulatory coupling between molecular signaling, vessel morphology, and mechanical environment over developmental time. For example, a mechanosensitive gene expressed at a particular instant during embryonic development can result in a cascade of morphological changes that in turn will alter loading state and therefore subsequent gene expression. Furthermore, endocrine and paracrine signaling pathways can result in non-localized morphomechanical responses, which can further contribute to the phase lag between mechanical load and molecular changes. Further quantitative understanding can be obtained through next-generation predictive numerical models that incorporate vessel growth and mechanosensitive gene expression pathways. Here, we highlight these predictive models, speculate on possible computational principles, and briefly review imaging modalities required to support these models.

5.1 Computational frameworks

Numerical prediction of multi-scale vascular growth, three-dimensional vessel shape changes, and selection of vessel network configuration (for example, aortic arch selection) are crucial problems in cardiovascular science and engineering as they impact a broad range of clinical applications including pediatric pre-surgical cardiovascular planning, in which vascular growth is a key consideration. Several pioneering numerical growth models with varying degrees of complexity have been applied to predict diseased cerebral aneurysm growth (Li and Robertson 2009; Watton et al. 2009) and angiogenesis (McDougall et al. 2002; Sun et al. 2005). These predictive models are founded on traditional continuum solid mechanics formulation, as well as on lattice- or cell-based modeling frameworks (Checa and Prendergast 2010). In both approaches, arbitrary and unknown "empirical" growth stimulus laws are specified as a direct function of stress and fluid shear (Olivares et al. 2009). Compared with continuum models, multi-scale models and cellular level frameworks can more practically simulate cell–cell communication, cell differentiation, cell migration, and signaling pathways that govern vascular growth (Egginton 2011; Guidolin et al. 2009; Travasso et al. 2011). Detailed three-dimensional growth kinematic analysis has also been employed in finite-element models as well as in the quantitative analysis of in vivo growth (Goktepe et al. 2010; Tsamis

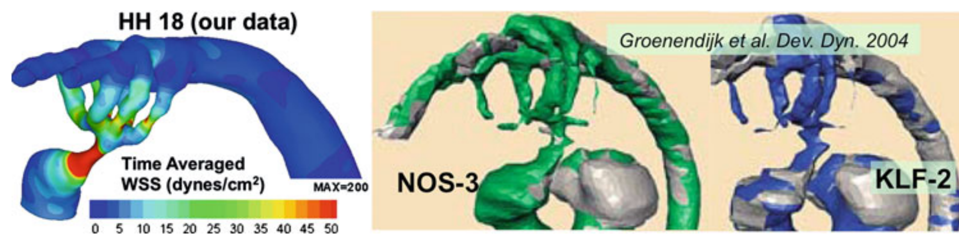

Fig. 3 Correlation of 3D high-resolution wall shear stress mapping to gene expression data in chick aortic arches. Subject-specific 3D high-resolution wall shear stress mapping at HH18 (*left*) is consistent with published data on expression of the laminar shear stress-responsive genes, *NOS3* and *KLF2*, at HH18 [*right*; reprinted with permission from (Groenendijk et al. 2004)]

et al. 2012). However, none of these models has yet attempted to integrate multiple scales, organ- and cell-level functional requirements, and three-dimensional tissue level structural growth organization.

We recently introduced a predictive optimization-based growth numerical framework (Kowalski et al. 2012) that employs vascular objective functions (including global energy budget and structural vessel constituents), expanding the earlier one-dimensional studies (Kassab and Fung 1995; Zhou et al. 1999; Zhang and Kassab 2008). The main advantage of the optimization-based growth paradigm is that it circumvents the need for commonly applied growth rate laws (Rodriguez et al. 1994; Rachev 1997; Taber 1998a) or micro-structurally motivated phenomenological models (Humphrey et al. 1999; Gleason and Humphrey 2004), because the comprehensive embryonic in vivo data set required by these models is not usually available. In our study, effects of outflow tract rotation were investigated using a two-dimensional optimization-based growth model, founded on a computational fluid dynamics-coupled shape optimization, of the chick Hamburger–Hamilton stage 18 (HH18) right lateral aortic arch (Dur et al. 2011). The individual aortic arch diameters were free to alter during the course of simulation in response to a global objective function that minimizes the total energy expenditure (Taber 1998b) while maximizing diffusive capacity (Kamiya et al. 1990). This model predicted HH18 aortic arch diameters within one standard deviation (25 % error) of the published mean diameter measurements (Wang et al. 2009). Further, minimizing energy alone converged to the single mature arch of aorta, derived from the right fourth aortic arch (Kowalski et al. 2012). Achieving in vivo validated numerical simulations of “normal” growth response is the first major step toward predictive models that can be used to answer biological and clinical questions, as the underlying cardiovascular biology that drives topological, structural, and genetic alterations is significantly different for disease, normal embryonic growth and development, and acute and chronic adaptations.

To our knowledge, no published growth modeling framework has incorporated a genetic component or mechanogenetic feedback loop, starting from the first principles and ultimately relating mechanical forces to complex signaling pathways, cell behavior (apoptosis, proliferation, migration), and vessel structure (for example, spatial synthesis of extracellular matrix constituents). Subject-specific microscopic image-based computational fluid dynamics analysis of the chick aortic arches (Wang et al. 2009) provides encouraging correlations with expression patterns of major wall shear stress (WSS)-sensitive genes at a single developmental time point (Fig. 3). To obtain these data, both commercial and in-house Cartesian grid computational fluid dynamics (Pekkan et al. 2005; Gilmanov et al. 2003) and solid mechanics solvers were employed to compute spatial WSS and mechanical loading distribution. Flow and shear stress fields at different biological time points were computed under unsteady conditions and averaged over the cardiac cycle. In addition to the well-established WSS indices, WSS gradients, WSS angle deviation, oscillating shear index, and flow field-derived parameters such as residence time, secondary flows, and flow invariants can also be computed and correlated with gene expression patterns.

Next-generation predictive models that combine mechanical loading data with spatiotemporal molecular signaling data are essential for understanding the effects of flow dynamics on vascular biology. Once the governing cardiovascular biology is specified, a computational framework to predict fluid–tissue interaction can quantify the relative degree of mechanical contribution to the observed modes of vascular shape and structural change. Ideally, a computational optimization framework could be integrated with gene expression and proteomic studies, enabling computation of, for example, spatiotemporal synthesis rates of constituents that build the vessel wall. We anticipate continued expansion and refinement of computational approaches aimed at understanding the role of biomechanical factors in key events in cardiovascular development.

5.2 Quantitative imaging modalities supporting computational models

It is worth emphasizing that computational models need to be validated through a comprehensive set of in vivo multi-modal

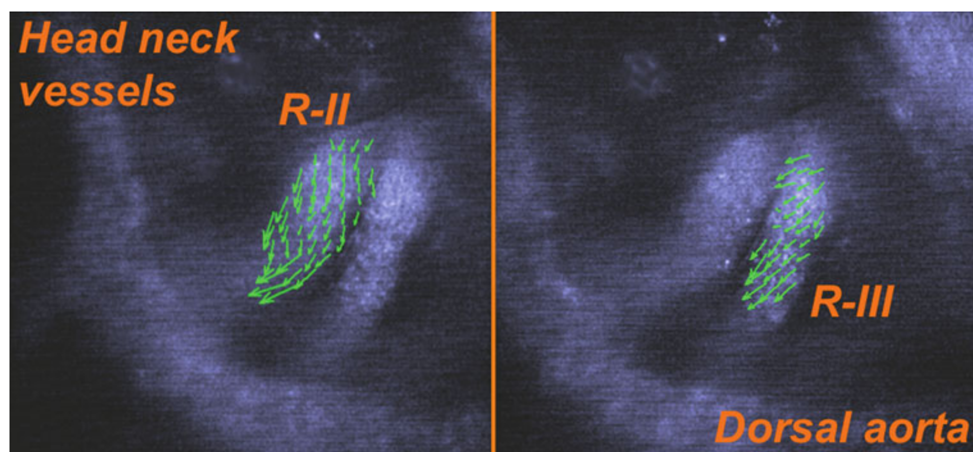

Fig. 4 Demonstration of micro-PIV analysis of the chick embryonic aortic arches. Aortic arch-specific flow profile data can be used for numerical model validation and wall shear stress estimate. Using 0.5 µm fluorescent particles injected into the single ventricle, *right* aortic arch 2 and 3 velocity vectors (*green*) are computed at HH21. Embryo is oriented *right* side up. *R-II* labels the right second arch and *R-III* labels the *right* third arch. Data were collected with a standard 15 Hz PIV system. Arch vessels ~100 µm in diameter

experiments performed in live embryos at multiple time points. Flow imaging modalities including pulsed-Doppler ultrasound (Oosterbaan et al. 2009), confocal micro-particle image velocimetry (Chen et al. 2011; Patrick et al. 2011), non-invasive phase contrast optical coherence tomography (Jenkins et al. 2010), and standard micro-particle image velocimetry (Vennemann et al. 2006) provide detailed flow velocity profiles (Fig. 4), time-resolved intracardiac and vascular velocity waveforms (Srinivasan et al. 1998), and cellular level flow measurements. Morphometric data (three-dimensional anatomical reconstructions; two-dimensional diameter measurements of heart and vessels; growth rates) are acquired through micro-computed tomography (Johnson et al. 2006; Kim et al. 2011), two-dimensional micro-ultrasound, and high-resolution optical coherence tomography (Yelbuz et al. 2002; Rugonyi et al. 2008; Liu et al. 2009). These data will generate baseline geometry for the predictive model. Furthermore, expansion of recently emerging advanced microscopy protocols, such as multi-photon second harmonic autofluorescence collagen/elastin in vivo imaging (Robertson et al. 2011) as well as micro-structural and micro-hemodynamic measurements provide a strong empirical foundation to validate and refine predictive numerical models in vivo. Biological data such as transcript expression and protein expression can then be incorporated into the computational models of vascular morphogenesis as additional growth laws that govern final arterial structure and function.

6 Concluding remarks

When considering factors that shape the developing vasculature, one cannot ignore hemodynamic forces. And although application of mechanical force to cultured endothelial cells has shed considerable light on the mechanisms by which force is perceived and transduced into biochemical signals, this method has inherent limitations because the intricate architecture of developing vessels that dictates flow fields in vivo cannot easily be recapitulated in cultured cells. Using accessible vertebrate embryo models like zebrafish and chick, one can combine in vivo imaging-derived hemodynamic parameters, computational models, and gene expression data to derive robust relationships between mechanical loading, intracellular signaling, cellular behavior, and vascular architecture. Developing models that incorporate this powerful combination of methodologies will be critical for our understanding of normal and pathological vascular development. Most children born with congenital heart defects are born to families with no history of this disease and represent either new genetic mutations or other insults that alter cardiac morphogenesis in utero. In humans, fetal surgical intervention that normalizes biomechanical loading can improve the growth and remodeling of left heart structures (mitral valve, aortic valve, aorta), though recovery of fetal myocardial growth after fetal intervention remains suboptimal (McElhinney et al. 2010; Pekkan et al. 2008; Wang et al. 2009). Due to the inextricable relationship between mechanical loading, gene expression, and endothelial cell behavior, improving therapies for these defects will require development of interdisciplinary approaches and predictive tools that consider both vascular biology and biomechanics.

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