Klf2 Is an Essential Regulator of Vascular Hemodynamic Forces In Vivo

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Summary

Hemodynamic responses that control blood pressure and the distribution of blood flow to different organs are essential for survival. Shear forces generated by blood flow regulate hemodynamic responses, but the molecular and genetic basis for such regulation is not known. The transcription factor KLF2 is activated by fluid shear stress in cultured endothelial cells, where it regulates a large number of vasoactive endothelial genes. Here, we show that Klf2 expression during development mirrors the rise of fluid shear forces, and that endothelial loss of Klf2 results in lethal embryonic heart failure due to a high-cardiac-output state. Klf2 deficiency does not result in anemia or structural vascular defects, and it can be rescued by administration of phenylephrine, a catecholamine that raises vessel tone. These findings identify Klf2 as an essential hemodynamic regulator in vivo and suggest that hemodynamic regulation in response to fluid shear stress is required for cardiovascular development and function.

Introduction

During embryonic development, a complex genetic differentiation program guides formation of the heart and blood vessels from cardiovascular progenitor cells (Carmeliet, 2000). As the embryo establishes a regular heart-beat, blood begins to flow and vascular hemodynamic forces arise (Jones et al., 2004; Phoon et al., 2000). Later in life, hemodynamic forces regulate virtually all aspects of cardiovascular function, including maintenance of blood pressure (Gabe et al., 1969), oxygen delivery (Sarnoff et al., 1963), and even vascular inflammatory responses associated with common diseases such as atherosclerosis (Gimbrone et al., 2000). Since vascular endothelium is directly exposed to fluid mechanical forces, it is believed to play a critical role in dynamically transducing intravascular hemodynamic forces such as shear stress into vascular signaling responses. Recently, a number of candidate molecular mediators of endothelial fluid shear forces have been identified (reviewed in Orr et al., 2006), but those required by endothelial cells to transduce hemodynamic forces into genetic responses in vivo are not known.

Recent studies of endothelial cell responses to fluid shear stress ex vivo have demonstrated that the transcription factor Kruppel-like factor 2 (KLF2) is rapidly and stably upregulated by pulsatile fluid shear forces that mimic those found in arterial blood vessels (Dekker et al., 2002; Huddleson et al., 2004). KLF2 function in cultured endothelial cells is both necessary and sufficient for the expression of many flow-regulated endothelial genes (Dekker et al., 2005; Parmar et al., 2006), suggesting that KLF2 may be an important molecular transducer of fluid shear forces in endothelial cells. In the present study, we have used a combination of genetic and physiologic studies to investigate the role of mouse Klf2 and its zebrafish ortholog, klf2a, in vivo during cardiovascular development. We find that the site and level of Klf2 expression closely follow the predicted pattern of elevated intravascular shear forces in the developing vasculature, and that embryos lacking Klf2 die by embryonic day 14.5 (E14.5) of heart failure. Surprisingly, this heart failure is caused by a high-cardiac-output state that occurs in the absence of anemia or structural vascular defects, indicating a profound loss of peripheral vascular resistance in Klf2-deficient embryos. These physiologic abnormalities are also observed in zebrafish embryos after morpholino inhibition of the Klf2 ortholog klf2a. Lethal high-output heart failure in both species can be rescued by phenylephrine, a catecholamine that directly increases vessel tone. Conditional deletion studies in the mouse demonstrate that embryonic heart failure in Klf2-deficient mouse embryos is due to defective endothelial rather than smooth muscle, myocardial, or hematopoietic cell function.

These studies support a model in which fluid shear forces drive expression of endothelial Klf2 that, in turn, regulates smooth muscle tone in the developing embryo. They highlight the importance of complementary genetic and physiologic approaches and suggest that Klf2 links hemodynamic and genetic responses in an evolutionarily conserved molecular pathway required for cardiovascular development and function.

Results

Endothelial Expression of Klf2 Follows the Development of Fluid Shear Forces in Mouse Embryos

To determine if Klf2 expression in the developing mouse embryo correlates with hemodynamic shear forces, in situ hybridization studies of Klf2 expression were performed on wild-type mouse embryos. The first
detectable expression of Klf2 occurs at E8.5 (Figures 1A–1C), a time point at which blood flow is first detectable in the developing mouse embryo but is weak and relatively nonpulsatile because the heart is still a linear tube without endocardial cushions to function as valves (Jones et al., 2004). Between E8.5 and E10.5, endothelial and endocardial expression of Klf2 rises sharply as the heart tube loops and endocardial cushions form (Figures 1D–1F). At E11.5, endothelial Klf2 expression is higher in arteries than in veins (e.g., Figure 1G), and very high levels are evident in the endothelium overlying the endocardial cushions and the common ventricular outflow tract, sites predicted to experience particularly high fluid shear forces (Figures 1G–1I). At E14.5, Klf2 expression becomes restricted to the flow side of the developing valves as the cushions mature (Figures 1K and 1L), a pattern consistent with exposure to fluid shear stress. By E18.5, Klf2 expression is also detectable in the endocardium lining the intraventricular papillary muscles that are exposed to fluid forces as blood is ejected from the heart (data not shown). Endothelial Klf2 expression therefore correlates closely with exposure to pulsatile fluid flow throughout cardiovascular development.

Conditional Deletion of Klf2 by Tie2-Cre in Endothelial and Hematopoietic Cells, but Not by SM22-Cre in Smooth and Cardiac Muscle Cells, Confers Embryonic Lethality

To identify the cell type(s) in which Klf2 is required during cardiovascular development, conditional Klf2 animals were generated by using gene targeting as outlined in Figure S1A (see the Supplemental Data available with this article online). Progeny homozygous for the conditional Klf2 allele (Klf2fl/fl) were born in expected numbers after heterozygous crosses and exhibited no phenotypic abnormalities (Figure S1B and data not shown). Homozygous conditional Klf2 mice were crossed to mice heterozygous for a null Klf2 allele that also carried either the Tie2-Cre or SM22-Cre transgene. The Tie2-Cre transgene drives Cre expression throughout the early embryonic endothelium as well as in some hematopoietic stem cells (see Figure 5 and Constien et al., 2001), but not in smooth or cardiac muscle cells. Conversely, the SM22-Cre transgene drives Cre expression in cardiac and smooth muscle cells, but not in endothelial or hematopoietic cells (Lepore et al., 2005). Neither endothelial nor endocardial Klf2 expression was detectable by in situ hybridization in E12.5 Tie2-Cre; Klf2fl/fl ("Tie2-Cre
KO) embryos, although expression in developing bone was unchanged (Figure S1C), and cardiac KO was reduced by >95% relative to control (Klf2fl/fl) litters (Figure S1D). Analysis of liveborn progeny revealed embryonic loss of Tie2-Cre KO animals, while SM22-Cre KO animals grew to adulthood without phenotypic abnormality, despite the complete loss of Klf2 in the genomic DNA of smooth muscle cells cultured from those animals (Tables 1 and 2 and Figure S2A).

Klf2 KO and Tie2-Cre KO Embryos Die after E11.5 without Primary Structural Vascular Defects or Hemorrhage
To compare the phenotypes of KO and Tie2-Cre KO ano-
imals, embryos were analyzed between E10.5 and E14.5 of gestation. All KO and Tie2-Cre KO embryos were viable, as determined by the presence of a visible heart-
beat, as determined by the presence of a visible heart-
beat, and grossly normal through E11.5 (Table 1 and data not shown). At E12.5, 25% of Tie2-Cre KO embryos lacked a heartbeat, and by E14.5 all were dead, a result virtually identical to that previously reported for KO an-
imals (Kuo et al., 1997a). Thus, the timing of embryonic lethality of Tie2-Cre KO embryos precisely matches that of embryos lacking Klf2 entirely.

Klf2-deficient embryonic lethality has previously been attributed to loss of tunica media stability and lethal hemorrhage (Kuo et al., 1997a). We therefore examined KO and Tie2-Cre KO embryos of different gestational ages for similar findings. At E11.5, yolk-sac vessels of Tie2-Cre KO embryos were blood-filled, and all embryos exhibited a normal heartbeat with no evidence of hemorrhage (data not shown). At E12.5, some Tie2-Cre KO embryos exhibited a pseudoaneurysm in the umbilical artery of a late-surviving E14.5 KO embryo (data not shown), by either light or transmission electron microscopy (Figures 2I–2L). Significantly, previous observations of smooth muscle defects and hemorrhage in Klf2-deficient embryos were also restricted to late-stage embryos (>E13; C. Kuo, personal communication). These findings indicate that lethal hemorrhage secondary to vascular smooth muscle defects is not the primary cause of death in KO and Tie2-Cre KO embryos.

Klf2 KO and Tie2-Cre KO Embryos Exhibit Cardiac Defects and Histologic Signs of Heart Failure after E11.5
At E11.5, KO and Tie2-Cre KO embryo hearts were noted to have a slightly thinner compact zone of ventricular myocytes than littermate controls, although changes at this time point were subtle and were not accompanied by signs of heart failure (data not shown). By E12.5, the compact myocardium of deficient embryos was severely hypoplastic, often only 1–2 cell layers thick compared to a thickness of >4–5 cell layers in littermate control hearts, and the epicardium was detached from the myocardial surface (Figures 3A–3F). Staining for proliferating cells with phosphohistone H3 antibody and for apoptotic cells with TUNEL revealed no significant changes in cardiomyocyte proliferation of apoptosis (Figure S3). Coincident with the development of these cardiac abnormalities was the appearance of large pericardial and peritoneal effusions, as well as marked hepatic venous congestion (Figures 3G–3O). These findings suggest that Klf2 KO and Tie2-Cre KO embryos die of cardiac failure after E11.5.

Klf2-Deficient Mouse Embryos Die of High-Output Heart Failure
Since Klf2 is expressed in the endocardium, peripheral vascular endothelium, and hematopoietic cells, heart failure in Tie2-Cre KO embryos could arise due to loss of Klf2 in any or all of these cell types. A clue to the basis of the heart failure was the observation that the cardiac abnormalities observed in E12.5 KO and Tie2-Cre KO embryos are virtually identical to those previously reported in embryos of the same age lacking erythropoietin.
EpoR-deficient embryos die at E13.5–E14.5 and exhibit thinning of the compact zone of cardiomyocytes and detachment of the epicardium, abnormalities that arise due to anemia, as they are rescued by transgenic expression of EpoR in erythroid cells (Suzuki et al., 2002). However, genetic studies and analysis of embryonic blood indicated that Klf2 Tie2-Cre KO embryos are not anemic (Figure 5 and discussed further below), suggesting that embryos lacking Klf2 and EpoR might share an abnormality in cardiovascular physiology, rather than a common defective cell type or genetic pathway.

Klf2 regulates endothelial genes known to control vascular smooth muscle tone, while studies of anemic fetal hydrops in humans reveal that heart failure arises due to a sustained high-cardiac-output state (Machin, 1989), a condition that is also observed with loss of peripheral vascular tone in clinical settings such as sepsis (Vincent and Van der Linden, 1990). We therefore hypothesized that loss of Klf2 might result in high-output heart failure secondary to a defect in vessel tone. Elevated cardiac output in anemic fetal hydrops is identified clinically by a rise in embryonic arterial flow velocity by using Doppler ultrasound (Cosmi et al., 2002). In contrast, fetal hydrops due to primary cardiac failure is associated with a depressed arterial flow velocity consistent with low cardiac output (Rodriguez et al., 2005). To distinguish between primary cardiac failure with low cardiac output due to endocardial loss of Klf2 and secondary cardiac failure with high cardiac output due to peripheral vascular endothelial loss of Klf2, we performed serial embryonic ultrasound studies between E11.5 and E14.5 of gestation.

At E11.5, two-dimensional (2D) ultrasound revealed structurally normal placentas and yolk sacs and beating hearts with normal heart rates in Tie2-Cre KO embryos (Table 3 and Tables S1 and S2). Doppler flow studies, however, demonstrated markedly elevated peak systolic blood flow velocities in the descending aorta of Tie2-Cre KO embryos that were nearly double those of control littermates (Figure 4A, Table 3, and Table S1). The average left-ventricular stroke volume of E11.5-deficient embryos was more than 2.5-fold higher than that of control littermates, and cardiac outputs were nearly 3-fold higher than controls (Figure 4B, Table 3, and Table S1). Similar increases in blood flow velocity, stroke volume, and total blood flow were found in the umbilical arteries of the same embryos (Table S2). Using 2D ultrasonography to measure cardiac chamber size before and after cardiac contraction, E11.5 Tie2-Cre KO embryos had significantly higher ventricular ejection fractions compared to littermate controls (60% versus 52%, Table 3). Significantly, no evidence of valvular regurgitation was detected, indicating that excess flow was in the forward direction.
2D and Doppler flow studies performed 24 hr later (at E12.5) in the same embryos revealed a dramatic reversal in the hemodynamic and cardiac performance of surviving KO embryos. By E12.5, the average ejection fraction of Tie2-Cre KO embryos had declined from 60% to 43%, a level significantly below that of control embryos (54%, Table 3 and Table S1). The cardiac output had dropped to less than half that observed 24 hr previously and was
less than that of littermate control embryos (Figure 4B, Table 3, and Table S1). Finally, the heart rate of KO embryos declined dramatically from a mean of 128 bpm at E11.5 to 86 bpm at E12.5, and 2D ultrasonography revealed that most E12.5 embryos developed pericardial and peritoneal effusions, clinical signs of congestive heart failure (Figure 4C and Movies S1 and S2). By E13.5, most mutant embryos were dead (71.4%), and survivors had persistent cardiac systolic dysfunction (Table 3); by E14.5, all mutant embryos were dead (data not shown). Significantly, umbilical venous Doppler flow studies revealed no differences between mutant and control embryos between E11.5 and E14.5 other than total blood flow (Table S2), indicating that heart failure in Tie2-Cre KO embryos is not due to an abnormality in the placental vasculature.

These findings demonstrate that the first cardiovascular phenotype observed in Tie2-Cre KO embryos is an elevated cardiac output state and that the histologic cardiac defects observed, like those in Epor-deficient embryos, arise secondarily and not as a result of endocardial loss of Klf2. This conclusion is supported by in situ hybridization studies and qRT-PCR demonstrating normal expression of known endocardial growth factor genes in Tie2-Cre KO hearts (Figure S4 and data not shown).

High-Output Heart Failure in Tie2-Cre KO Embryos Is Not Associated with Anemia or Arteriovenous Malformation

A sustained high-cardiac-output state is a response to reduced peripheral vascular resistance, a condition with three major underlying causes: (1) anemia with vasodilatation secondary to tissue hypoxia, (2) arteriovenous malformation with abnormal vessels that shunt arterial blood back to the venous system, and (3) primary vasodilatation due to reduced vascular smooth muscle tone (Guyton and Hall, 2000). To investigate the cause of the lethal high-cardiac-output state in Klf2-deficient embryos, we next addressed these three possibilities.

To determine if hematopoietic loss of Klf2 results in anemia, we generated Vav-Cre KO animals in which Klf2 is excised in hematopoietic cells, but not in endothelial and endocardial cells (Stadtfeld and Graf, 2005). Vav-Cre KO mice survive to adulthood without embryonic loss, have normal hematocrit and hemoglobin levels, and do not exhibit cardiovascular abnormalities (Table 2, Table S3, and data not shown). qRT-PCR revealed that Klf2 RNA levels in bone marrow and thymus were decreased by >200-fold and >1400-fold, respectively, in Vav-Cre KO animals (Figure S2), and, consistent with reports of radiation chimeras generated by using Klf2-deficient fetal liver (Carlson et al., 2006; Kuo et al., 1997b), Vav-Cre KO mice lack T cells in the peripheral blood (Table S3 and data not shown). To address genetic deletion of Klf2 during embryonic development, we next compared hematopoietic excision by Tie2-Cre and Vav-Cre at E14.5 by using Cre reporter transgenes. Consistent with previously published studies (Constien et al., 2001; Stadtfeld and Graf, 2005), Vav-Cre drove complete excision in CD45+ fetal liver hematopoietic cells, while Tie2-Cre drove excision in only 40%–70% of such cells (Figures 5A and 5B). Finally, since Tie2-Cre KO embryos develop high cardiac outputs by E11.5, a time point that may precede Klf2 excision in Vav-Cre KO embryos, we directly measured circulating erythrocyte numbers in E11.5 Tie2-Cre KO embryos and control littersmates. E11.5 Tie2-Cre KO embryos had circulating erythrocyte numbers indistinguishable from those of control littersmates (Figure 5C), a result consistent with the genetic studies described above as well as histologic studies demonstrating abundant normal-appearing erythrocytes and a lack of embryo pallor. These studies exclude anemia due to hematopoietic loss of Klf2 as the cause of high cardiac output in Tie2-Cre KO embryos.

A rare cause of fetal high-output heart failure is an arteriovenous malformation that allows arterial blood to return to the heart through direct connections to the venous system with low vascular resistance (Rodriguez et al., 2002). Arteriovenous malformations have been reported in mutant embryos lacking essential vascular signaling pathways such as transforming growth factor β and Notch. To identify a vascular malformation extensive enough to confer a 3-fold increase in cardiac output, we performed whole-mount immunostaining for PECAM, a receptor expressed at high levels on endothelial cells. PECAM immunostaining of E10.5 and E11.5 Tie2-Cre KO embryos revealed a normal pattern and

<table>
<thead>
<tr>
<th>Day</th>
<th>Klf2</th>
<th>HR (bpm)</th>
<th>DA vel, s (mm/s)</th>
<th>DA VTI (mm)</th>
<th>DA SV (μl)</th>
<th>DA CO (μl/min)</th>
<th>EF (%)</th>
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<tr>
<td>11.5</td>
<td>WT/Het</td>
<td>125 ± 26 (n = 16)</td>
<td>48.8 ± 11.5 (n = 13)</td>
<td>6.8 ± 1.5 (n = 13)</td>
<td>0.19 ± 0.04 (n = 13)</td>
<td>23.7 ± 5.3 (n = 13)</td>
<td>52 ± 5 (n = 15)</td>
</tr>
<tr>
<td>KO</td>
<td>125 ± 25 (n = 7)</td>
<td>95.0 ± 31.0 (n = 7)</td>
<td>13.4 ± 4.1 (n = 7)</td>
<td>0.51 ± 0.12 (n = 7)</td>
<td>64.6 ± 18.7 (n = 7)</td>
<td>60 ± 3 (n = 7)</td>
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<tr>
<td>12.5</td>
<td>WT/Het</td>
<td>127 ± 23 (n = 17)</td>
<td>72.9 ± 21.0 (n = 14)</td>
<td>9.4 ± 2.5 (n = 14)</td>
<td>0.30 ± 0.06 (n = 14)</td>
<td>40.2 ± 6.7 (n = 14)</td>
<td>54 ± 4 (n = 16)</td>
</tr>
<tr>
<td>KO</td>
<td>86 ± 41 (n = 7)</td>
<td>109.2 ± 65.1 (n = 6)</td>
<td>11.9 ± 5.5 (n = 6)</td>
<td>0.32 ± 0.11 (n = 6)</td>
<td>33.2 ± 23.0 (n = 6)</td>
<td>43 ± 10 (n = 7)</td>
<td></td>
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<tr>
<td>13.5</td>
<td>WT/Het</td>
<td>123 ± 13 (n = 16)</td>
<td>92.7 ± 26.5 (n = 12)</td>
<td>13.2 ± 4.0 (n = 12)</td>
<td>0.56 ± 0.14 (n = 12)</td>
<td>66.6 ± 17.2 (n = 12)</td>
<td>66 ± 4 (n = 14)</td>
</tr>
<tr>
<td>KO</td>
<td>105 ± 25 (n = 3)</td>
<td>133.7 ± 24.2 (n = 3)</td>
<td>19.6 ± 5.8 (n = 3)</td>
<td>0.74 ± 0.03 (n = 3)</td>
<td>79.7 ± 21.5 (n = 3)</td>
<td>31 ± 7 (n = 3)</td>
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WT, wild-type; Het, heterozygous Klf2 KO mice; KO, homozygous Klf2 mice; HR, fetal heart rate; DA, descending aorta; vel, velocity; s, systole; SV, stroke volume; CO, cardiac output; EF, ejection fraction.

p < 0.001 when measurements from WT/Het embryos are compared to each other by using ANOVA.

Those WT/Het measurements that are not significantly different from each other by multiple comparisons.

p < 0.01 when KO measurements are compared to the WT/Het group by using t test.

Those WT/Het measurements that are not significantly different from each other by multiple comparisons.

p < 0.05 when KO measurements are compared to the WT/Het group by using t test.

Those WT/Het measurements that are not significantly different from each other by multiple comparisons.
number of vessels throughout the embryo (Figure 5D and data not shown). These studies and the lack of any vascular abnormality detected on histologic sections suggest that the large increase in cardiac output in Tie2-Cre KO embryos does not arise from arteriovenous shunting or a vascular malformation.

High-Output Heart Failure in Klf2-Deficient Mouse and Zebrafish Embryos Is Rescued by the Direct Smooth Muscle Agonist Phenylephrine

Exclusion of anemia and a vascular malformation suggested that the high-cardiac-output state of Tie2-Cre KO embryos might arise due to a loss of smooth muscle tone. Smooth muscle tone is regulated by the endothelium, and ex vivo studies suggest that Klf2 may participate in this regulation (Dekker et al., 2002, 2005; Parmar et al., 2006). Since there is no means of directly measuring vessel tone in embryos, we adopted a functional strategy to test this hypothesis and administered maternal phenylephrine to pharmacologically raise embryonic vascular smooth muscle tone and rescue lethality, thereby circumventing the requirement for endothelial Klf2. Maternal catecholamine administration has been successfully used to rescue lethality due to embryonic catecholamine deficiency (Thomas et al., 1995), indicating that these small molecules can successfully cross the placenta. Phenylephrine is a highly selective agonist for the \( \alpha_1 \) adrenergic receptor subtype found on human and mouse vascular smooth muscle (Cavalli et al., 1997) and does not activate \( \beta \) adrenergic receptors on cardiac
Figure 5. Tie2-Cre KO Embryos Are Not Anemic and Do Not Develop Vascular Malformations

(A) Hematopoietic excision is complete in Vav-Cre embryos. Vav-Cre transgenic animals were crossed to the ROSA26R Cre reporter strain, and excision in CD45+ fetal liver cells was measured by flow cytometry to detect the fluorescent β-galactosidase substrate FDG. Note the complete population shift observed, which indicates uniform Cre excision (n = 3).

(B) Hematopoietic excision is partial in Tie2-Cre embryos. Tie2-Cre transgenic animals were crossed to the Z/EG Cre reporter strain, and excision in CD45+ fetal liver cells was measured by flow cytometry to detect GFP. Note the presence of two distinct populations of GFP+ (excised) and GFP− (unexcised) cells (n = 5). The lower-right panel represents a negative control animal.

(C) E11.5 Tie2-Cre KO embryos have normal numbers of circulating erythrocytes. Shown are the absolute numbers of Ter119+ erythrocytes released from the circulation of individual live E11.5 Tie2-Cre KO ("KO") and control ("CTRL") littermates. The mean number and p value obtained with Student’s t test are shown.

(D) Tie2-Cre KO embryos exhibit normal vessel patterning and density. Whole-mount anti-PECAM staining of E11.5 Tie2-Cre KO ("KO") and a wild-type ("WT") littermate embryo is shown. Boxes indicate regions shown at higher magnification. No abnormal vessels or increased vessel density is observed in Tie2-Cre KO embryos. Images shown are representative of n = 4 Tie2-Cre KO embryos and n = 3 controls.
muscle cells (Hardman et al., 2001). Phenylephrine is therefore predicted to rescue high-output heart failure by increasing vessel tone, not by improving myocardial performance. Maternal phenylephrine administration rescued ~50% of Tie2-Cre KO embryos at E14.5 (Figure 6A), indicating that direct activation of vascular smooth muscle tone can compensate for endothelial loss of Klf2. Histologic examination of the hearts of E14.5 Tie2-Cre KO embryos rescued by maternal phenylephrine revealed normal myocardial thickening and no evidence of epicardial detachment or hepatic congestion (Figure 6B and data not shown).
Administration of maternal phenylephrine is an indirect approach to pharmacologic rescue of Klf2-deficient mouse embryos, and rescue could, in theory, be mediated by maternal responses to phenylephrine rather than by direct vasoconstrictive effects in embryos. To more directly assess the ability of phenylephrine-induced vasoconstriction to reverse the high-output state of Klf2-deficient embryos and to determine if the role of Klf2 in cardiovascular development is conserved across species, we next investigated loss of klf2a in zebrafish. Cardiovascular development in zebrafish is genetically similar to that in mice (MacRae and Fishman, 2002), but zebrafish embryos develop externally and do not require blood flow for survival during cardiovascular development, an advantage for pharmacologic rescue of cardiovascular phenotypes. Zebrafish express three genes related to mouse Klf2: klf2a, klf2b, and klf4, of which only klf2a is expressed in the developing heart and blood vessels in a manner analogous to mouse Klf2 (Oates et al., 2001; Parmar et al., 2006; data not shown). Vascular klf2a expression is reduced in silent heart mutant fish that lack blood flow (Parmar et al., 2006), suggesting that klf2a expression, like that of mouse Klf2, is flow regulated. Injection of morpholino oligonucleotides (MOs) designed to block translation of klf2a into 1- to 2-cell-stage embryos resulted in pericardial edema and venous pooling of blood around the yolk sac in 71% of embryos at 72 hr postfertilization (hpf) (Figure 6C), a phenotype consistent with heart failure. Injection of a control MO at the same concentration caused no phenotypic defects (Figure 6C). To determine if loss of klf2a in fish embryos results in high-output heart failure like that observed with loss of Klf2 in mouse embryos, we measured the instantaneous erythrocyte flow velocity in the dorsal aorta of fish at 54 hpf, a measure analogous to the measurement of blood flow in the mouse embryo with Doppler ultrasound. Treatment with klf2a morpholino increased peak flow velocity by 57% compared to control MO-treated embryos (2302 ± 1465 μl/s versus 1465 ± 1465 μl/s, p = 0.025) (Figure 6E). In contrast, treatment with a morpholino directed against the cardiomyocyte transcriptional coactivator eya4 results in reduced flow velocity consistent with primary, low-output heart failure (Schonberger et al., 2005). Thus, loss of klf2a in fish embryos results in high-output heart failure in a manner analogous to loss of Klf2 in mouse embryos. To address the role of vessel tone in the development of high-output heart failure, embryos injected with anti-klf2a morpholino were exposed to varying concentrations of phenylephrine and scored for pericardial edema. Rescue of pericardial edema was observed with concentrations of phenylephrine as low as 10 μM, and at 100 μM there was a 38% decrease in the fraction of embryos exhibiting edema (Figures 6C and 6E). These studies demonstrate that Klf2 plays a preserved role in the regulation of embryo hemodynamics across species, and that raising vessel tone can rescue high-output heart failure due to loss of Klf2 in both mouse and fish embryos.

Endothelial Target Gene Expression in Klf2 Tie2-Cre KO Embryos

Gene expression studies with cultured endothelial cells have identified a large number of putative downstream Klf2 target genes that are both positive and negative regulators of vessel tone (Dekker et al., 2005; Parmar et al., 2006). We therefore measured the expression level of candidate Klf2 target genes in E11.5 Tie2-Cre KO embryos to identify the endothelial Klf2 target gene(s) required for hemodynamic regulation during embryonic development. In situ hybridization studies of endothelial gene expression in the major embryonic vessels demonstrated normal expression of genes known to be required for smooth muscle cell recruitment and arterial specification (Figure S4 and data not shown). A more extensive analysis of the expression of candidate Klf2 target genes identified by ex vivo studies that have been associated with smooth muscle or cardiomyocyte cell function was performed by using qRT-PCR of E11.5 total embryo, umbilical cord, yolk sac, and heart tissue. This analysis failed to reveal significant changes in the expression level of these candidate genes, including the Edg1 receptor, a Klf2 target gene in T cells (Carlson et al., 2006) (Figure S4 and data not shown). These studies suggest that the hemodynamic abnormalities in embryos lacking endothelial Klf2 arise either due to loss of an unidentified target gene(s) or as a result of small changes in a large number of known target genes.

Discussion

Changes in vessel size in response to changes in blood flow were first noted in blood vessels of chick embryos in the 19th century (Thoma, 1893). More recently, ex vivo studies have associated exposure to fluid flow with complex endothelial transcriptional responses proposed to regulate vascular tone, inflammation, and thrombosis (Berk et al., 2001; Garcia-Cardena et al., 2001). An understanding of the role of blood flow in modulating endothelial and vascular function in vivo has been slowed, however, by the difficulty in identifying the molecular pathways that underlie flow-mediated vascular responses as well as by the difficulty of measuring embryonic hemodynamics. Recent gene expression studies by several laboratories have identified the transcription factor KLF2 as a key component of the endothelial transcriptional response to flow ex vivo (Dekker et al., 2002; Groenendijk et al., 2005; Huddleson et al., 2004), and studies in fish, chick, mouse, and humans suggest that endothelial expression of KLF2 is regulated by blood flow in vivo (Dekker et al., 2005; Groenendijk et al., 2005; Parmar et al., 2006). Here, we use a combination of genetic and physiologic methods to demonstrate that endothelial Klf2 deficiency causes high-output heart failure and fetal death in association with reduced vessel tone. These findings support a model in which endothelial transduction of fluid flow forces to transcriptional responses by Klf2 regulates cardiovascular hemodynamics soon after formation of the heart and blood vessels.

The regulation of hemodynamics during embryonic cardiovascular development has not been well investigated because it requires the combined use of genetic and physiologic approaches. Studies of silent heart mutant zebradfish that develop without a beating heart reveal that primary vascular patterning and development is “hardwired” and not reliant upon responses to blood flow (Isogai et al., 2003). However, blockade of fluid flow forces interrupts looping and valve formation during
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zebrafish heart development (Hove et al., 2003) and can alter arteriovenous vascular differentiation in the chick yolk sac (le Noble et al., 2004), suggesting that responses to fluid flow regulate later stages of cardiovascular development. In the mouse, where live embryos are not easily accessed and loss of the beating heart results in early embryonic death, analysis of embryo hemodynamics has required the development of highly sensitive transuterine imaging. This has recently been accomplished by using optical microscopy and high-frequency ultrasound (Jones et al., 2004; Phoon et al., 2000), and these studies have shown a rapid rise in pulsatile fluid shear forces between E8.5 and E10.5 as the heart loops and endocardial cushions form (Jones et al., 2004). In the present study, genetic approaches using Tie2-Cre, Vav-Cre, and SM22-Cre transgenics to conditionally delete Kit2 indicated that Kit2 is required in endothelial and/or endocardial cells during midgestation, while histologic studies of deficient embryos revealed cardiac abnormalities and evidence of heart failure. A straightforward explanation for these findings might be that endocardial Kit2 is required for myocardial growth, and that loss of Kit2 results in primary heart failure. Instead, physiologic studies revealed that heart failure in Tie2-Cre KO embryos arises after the development of a high-cardiac-output state, indicating a primary peripheral vascular defect and hemodynamic load that cannot be supported by the developing heart.

There exist three possible explanations for reduced vascular resistance in E11.5 Tie2-Cre KO embryos: anemia that secondarily results in vasorelaxation as hypoxic tissues release vasodilating agents, an arteriovenous shunt that allows arterial blood to flow directly back to the venous system without passing through the smaller vessels that confer vascular resistance, and a primary loss of vascular smooth muscle tone. Our studies of Vav-Cre KO animals and direct measurement of circulating erythrocyte numbers exclude anemia, and a combination of histologic analysis and PECAM whole-mount immunostaining did not reveal evidence of a vascular malformation that could mediate a shunt large enough to confer a nearly 3-fold increase in cardiac output. In contrast, ex vivo studies of Kit2 in endothelial cells suggest that it may regulate the expression of endothelial genes that control vessel tone (Dekker et al., 2005; Parmar et al., 2006), and phenylephrine, a direct stimulant of vessel tone, is able to rescue lethality of Tie2-Cre KO embryos. Importantly, reduction of the orthologous gene klf2a in fish also results in embryonic high-output heart failure that can be rescued by phenylephrine. These findings support failure of endothelial cells to regulate smooth muscle tone as the cause of lethal high-output heart failure in Kit2-deficient embryos. Our Kit2 expression studies in the mouse and the reduction of klf2a expression in silent heart mutant fish (Parmar et al., 2006) further suggest that Kit2 regulates vessel tone in an evolutionarily conserved response to rising fluid shear forces in the developing embryo. The relatively late lethality of Kit2-deficient mouse embryos may indicate that Kit2-mediated regulation does not become necessary until after the development of a threshold level of shear forces.

Our studies suggest that endothelial Kit2 is expressed in response to fluid shear forces and is required for vascular hemodynamic regulation during development, but they do not identify the upstream and downstream pathways in which Kit2 participates to perform this critical function. Although gene expression studies with cultured endothelial cells have identified numerous downstream Kit2 target genes believed to affect vascular smooth muscle tone (Dekker et al., 2005; Parmar et al., 2006), we do not find significant changes in the expression level of individual candidate genes. These findings are unexpected and may indicate that the genes regulated by hemodynamic forces in developing embryos differ from those in the mature vasculature. This hypothesis is consistent with the fact that mice lacking genes deemed critical for such regulation in mature vessels, e.g., the nitric oxide pathway, do not experience embryonic lethality (Nath and Madri, 2006). Alternatively, given the large number of potential Kit2 target genes identified by transcriptional profiling studies, it is possible that lethality in Kit2-deficient embryos results from small changes in the expression of a large number of genes. Parallel studies in adult animals made conditionally deficient for Kit2 should determine whether this transcription factor controls distinct sets of genes in developing and mature vessels and should provide further insight into the divide between ex vivo and in vivo Kit2 target genes.

A more general finding in these studies is that the cardiac defects observed in Kit2-deficient embryos arise secondarily to the high-output state imposed by the peripheral vascular abnormality. The close phenotypic resemblance between Kit2-deficient and EpoR-deficient hearts suggests that these cardiac defects represent a common response to hemodynamic stress. A large number of other mouse mutants exhibit a similar phenotype of midgestation myocardial thinning and heart failure, including mice unable to synthesize catecholamines (Zhou et al., 1995), and mice lacking the vasoactive peptide adrenomedullin or its receptor (Caron and Smithies, 2001; Dackor et al., 2006). Thus, genetic defects that give rise to primary physiologic abnormalities ultimately exhibit secondary structural and histologic abnormalities that can be easily interpreted as primary events. These findings explain the previous report of vascular defects in late-stage, Kit2-deficient embryos (Kuo et al., 1997a) and emphasize the importance of investigating cardiovascular development and function by using both genetic and physiologic tools.

Experimental Procedures

Generation of Conditional Kit2 Mice

SV129 ES cells heterozygous for the Kit2 conditional allele were generated by using standard gene-targeting techniques. Kit2"mice were kindly provided by Dr. Jerry Lingrel, University of Cincinnati, and were generated by deletion of exons 2 and 3. Tie2-Cre transgenic animals were purchased from Jackson Research Laboratories (Bar Harbor, ME). SM22-Cre and Vav-Cre transgenic animals have been described previously (Lepore et al., 2005; Stadtfeld and Graf, 2005).

In Situ Hybridization and Histology

Tissues were prepared and studied as described at: http://www.uphs.upenn.edu/mrcr/histology/histologyhome.html. Whole-mount staining for PECAM was performed with the MEC13.3 anti-PECAM antibody (Pharmingen).
Electron Microscopy

Tissues were fixed in 2% glutaraldehyde with 0.1 M sodium cacodylate (pH 7.4) for 72 hr at 4°C. Samples were further incubated with 2% osmium tetroxide and 0.1 M sodium cacodylate (pH 7.4) for 1 hr at 4°C. Ultrathin sections were stained with lead citrate and uranyl acetate and were viewed on a JEM 1010 microscope.

Hemodynamic Analysis with Serial High-Frequency Embryonic Ultrasound

Prior to genotyping of embryos, all littersmates were analyzed serially by noninvasive in utero fetal ultrasound by using a 23 MHz spectral pulsed-wave (PW) Doppler. Embryos were identified by numbering individuals from the uterine midline, identified by the maternal bladder. For 2D imaging, blood column widths were used to determine vessel diameters, and three or four chamber views of the heart were used to assess LV ejection fraction (EF) and fractional area change (FAC) by planimetry of epicardial borders as described previously (Yu et al., 2004). For Doppler-derived blood flow parameters, heart rate, blood flow velocities, and blood flow, volumes were determined as described previously (MacLennan and Keller, 1999; Phoon et al., 2004). All studies were performed in triplicate, and all Doppler incident angles were less than 30°. For statistical analyses, control embryos included wild-type and heterozygotes. During scanning, maternal body temperature and heart rate were maintained within normal limits.

Measurement of Circulating Erythrocyte Number in E11.5 Embryos

Pregnant female mice harboring Klf2 KO and control embryos were sacrificed at E11.5, and uteri were removed. Intact embryo-yolk sac-placenta tissue blocks were dissected, rinsed with PBS, and examined by microscopy for beating hearts and absence of hemorrhage. Viable, nonbleeding embryos and yolk sacs were cut from placentae directly into 1 ml heparinized Tyrode’s buffer at 38°C and were allowed to hemorrhage until embryo was exsanguinated. The suspension of whole blood in buffer was incubated for 2 hr with a directly conjugated anti-Ter119 antibody. Flow cytometric analysis was used to count absolute numbers of Ter119+ erythrocytes on all embryos. Small pieces of embryo or yolk sac harvested after exsanguination were used for genotyping.

Maternal Phenylephrine Treatment

Pregnant female mice harboring Tie2-Cre KO and control embryos at E8.5 were administered 500 μM phenylephrine continuously in egg water and diluted to achieve the final concentration. At 48 hpf, egg water was removed and replaced with fresh egg water and phenylephrine. Statistical significance was assessed with chi-square analysis.

Zebrafish Measurement of Flow Velocity

Embryos were placed on glass depression slides, video microscopy was performed on an Axioplan (Zeiss) upright microscope, and serial images were obtained (FastCam PCI, Photron) at 250 frames per second (fps) of the dorsal aorta in the region around the cloaca. The transit of single erythrocytes was tracked manually across multiple cardiac cycles in individual frames, the distance traveled between serial frames was measured, and the instantaneous flow velocity was calculated. Average data are expressed as the arithmetic mean ± SEM; statistical significance was assessed with Student’s t test.

Supplemental Data

Supplemental Data describe the generation and characterization of conditional Klf2 mice; the efficiency of Tie2-Cre, SM22-Cre, and Vav-Cre transgenes; analysis of E11.5 cardiomyocyte proliferation and apoptosis; Klf2 target gene analysis; movies showing ultrasound images of E12.5 heart function; and tables of hemodynamic measurements in individual embryos, hemodynamic measurements in umbilical vessels, and blood counts in Vav-Cre KO and control animals. These data are available at http://www.developmentalcell.com/cgi/content/full/11/6/845/DC1/.

Acknowledgments

We thank Drs. Ed Morrisey and Alvin Chin for valuable discussions, Dr. Michael Parmacek for help with smooth muscle cell culture, and Dr. MinMin Lu for histology expertise. This work was supported by National Heart, Lung, and Blood grants HL081084 (J.S.L.) and HL081654 (M.L.K.).

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