Ischemia Is Not Required for Arteriogenesis in Zebrafish Embryos

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Objective—The role of ischemia in collateral vessel development (arteriogenesis) is a contentious issue that cannot be addressed using mammalian models. To investigate this, we developed models of arteriogenesis using the zebrafish embryo, which gains sufficient oxygenation via diffusion to prevent ischemia in response to arterial occlusion. Methods and Results—We studied gridlock mutant embryos that suffer a permanently occluded aorta and show that these restore aortic blood flow by collateral vessels. We phenocopied gridlock mutants by laser-induced proximal aortic occlusion in transgenic Fli1:eGFP/GATA1:dsRED embryos. Serial imaging showed these restore aortic blood flow via collateral vessels by recruitment of preexisting endothelium in a manner similar to gridlocks. Collateral aortic blood flow in gridlock mutants was dependent on both nitric oxide and myeloid cells. Confocal microscopy of transgenic gridlock/Fli1:eGFP mutants demonstrated no aberrant angiogenic response to the aortic occlusion. qPCR of HIF1α expression confirmed the absence of hypoxia in this model system. Conclusions—We conclude that NO and myeloid cell-dependent collateral vessel development is an evolutionarily ancient response to arterial occlusion and is able to proceed in the absence of ischemia. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: collateral circulation ■ angiogenesis ■ nitric oxide ■ blood flow ■ zebrafish

After arterial occlusion, “collateral vessels” can restore some blood flow to the occluded artery. These arise from preexisting endothelial communications by arteriogenesis, initiated by shear stress, and which subsequently remodel into mature collateral vessels. This remodeling is dependent on nitric oxide and certain leukocyte subtypes, notably the monocyte/macrophage.

In the 18th century, Hunter observed collateral vessels by injecting dye post mortem into the circulation of a stag, having previously ligated its carotid artery. Current models of arteriogenesis still closely resemble Hunter’s, using arterial ligation followed by detection of collateral vessels by some (usually invasive) technique. These models are intuitively relevant but suffer from significant disadvantages, particularly technical difficulty in visualizing arteriogenesis. Most use post mortem perfusion-fixed angiography in a state of maximum vasodilatation, which abolishes physiological regulation of flow and is impossible to perform serially.

Arterial ligation in mammals inevitably induces ischemia. This induces angiogenesis, inflammation, and necrosis. The contribution of these to arteriogenesis is unclear. Some studies suggest arteriogenesis proceeds independently of the downstream consequences of ischemia; others that molecules released in the hypoxic region induce local angiogenesis and upstream arteriogenesis. Using current models, it is impossible to separate angiogenesis from arteriogenesis, as both are consequent on arterial occlusion.

The zebrafish embryo possesses unique advantages for the study of vascular development. Its near-transparency and the availability of transgenics expressing fluorescent reporters allow serial vascular visualization. Specific gene knockdown by antisense morpholino oligonucleotides allows rapid assessment of gene function. Uniquely, the zebrafish embryo does not require a circulation for tissue oxygenation until several days old, gaining sufficient oxygenation via diffusion.

Here we determine mechanisms of blood flow restoration after aortic occlusion in zebrafish. We show that gridlock homozygote mutant embryos, which suffer a permanent occlusion of the proximal aorta attributable to a mutation in Hey2, develop collateral aortic blood flow despite a normally patterned vasculature. Aortic blood flow is restored in a manner identical to gridlock homozygotes after laser-induced occlusion of the proximal aorta in wild-type embryos, and serial imaging of Fli1:eGFP/GATA1:dsRED transgenics shows that this occurs via preexisting endothelial communications. Collateral aortic blood flow in gridlock homozygotes is, like mammalian arteriogenesis, dependent on both nitric oxide synthase (NOS) and myeloid cells. We show that the development of collateral aortic blood flow in zebrafish embryos is not dependent on ischemia.
our model occurs in the absence of hypoxia. We therefore suggest that arteriogenesis can occur independently of the ischemic consequences of arterial occlusion.

Materials and Methods

Zebrfish Care and Breeding
All studies conformed to Home Office requirements for use of animals in scientific experiments. Gridlock embryos were a gift of Dr Randall Peterson (Massachusetts Institute of Technology, Cambridge, Mass.). Fli1:eGFP transgenic zebrfish (expressing endothelial eGFP) were obtained from the Zebrfish International Resource Centre (Ore). Gridlock homozygotes expressing endothelial GFP were generated by crossing with Fli1:eGFP transgenics. GATA1:dsRED transgenic fish (expressing dsRED in the erythrocytes) were a gift of Dr Leonard Zon. These were incrossed with Fli1:eGFP/gridlock mutants to generate Fli1:eGFP/GATA1:dsRED/gridlock transgenics, or crossed with nacre (albinos) mutants to generate Nacre/Fli1:eGFP/GATA1:dsRED “wild-type” fish.

Detection of Collateral Aortic Flow
To detect collateral blood flow in the occluded aorta, embryos were lightly anesthetized using MS-222 (Sigma) and patterns of blood flow observed by stereomicroscope.

Digital Motion Analysis, Confocal Microscopy, and Microangiography
Vascular visualization was performed using confocal microscopy, microangiography,16 or digital motion analysis (DMA).17 Confocal movies of blood flow in GATA1:dsRED embryos were generated using the roundtrip mode to produce single Z slices with frame rates of 15 fps. The focal plane was adjusted by hand to visualize the vessels of interest. Movies were generated and annotated using ImageJ and the ArrowMaker plugin developed by Gilles Carpenter.18

Laser Occlusion of the Proximal Aorta
Five days postfertilization (dpf) wild-type or Fli1:eGFP/GATA1:dsRED/Nacre embryos (n = 27) were anesthetized and immobilized in 0.5% low-melt point agarose (Sigma). The proximal aorta just above the highest point of the yolk sac was occluded by laser injury delivered by a Micropoint laser mounted on a Zeiss Axiophot 2, repeated 3 hours later. Following laser occlusion, embryos were serially observed by stereomicroscope, digital motion analysis, or confocal imaging.

Drug Treatments
L-NAME or L-Arginine (Sigma) was dissolved in E3 medium at the concentrations indicated. 20 to 30 dechorionated gridlock homozygote embryos were placed in L-NAME, L-Arginine, or control at the times and doses indicated. 3 to 4 replicates were performed. Collateral aortic blood flow was assessed at the time points indicated.

Effect of L-NAME on Aortic Blood Velocity
We developed a novel method to measure blood velocity in GATA1:dsRED transgenic embryos which we term confocal kymography (for detailed methods please see the supplemental materials, available online at http://atvb.ahajournals.org). We measured blood velocity in the proximal, mid, and distal aorta in 5 days postfertilization (dpf) embryos incubated for 16 hours in [1 mmol/L] L-NAME or control (n = 9 per group).

Quantitative RT-PCR for HIF1α
To confirm upregulation of HIF1α expression by hypoxia, we incubated 4 dpf embryos in a hypoxic chamber at 5% O2, and 28°C for 4 hours in E3 medium which had been incubated in the chamber overnight to allow the dissolved oxygen concentration to equilibrate with the chamber. Controls were incubated in normally oxygenated medium within airtight containers inside the chamber. Total RNA was extracted and HIF1α expression was measured using an ABI 7900HT normalized to GAPDH expression. Primer/reporter sequences were: HIF1α, Forward CCATGAAGAGTTGAGAGAT-GCT, Reverse CTGCTGTTGTGTGCTCCCTTCTTT, Reporter TC- CAGAGAACAGCATCCA; GAPDH, Forward ACTTTGCAATGAT- GTTCATGCAA, Reverse CTGATACCCCACTTAATTGTG, Reporter CTGGGTCCTCCTCCCTATA.

To assess whether gridlock homozygote embryos experience hypoxia in response to aortic occlusion, heterozygous gridlock adults were incrossed and offspring sorted into phenotypically normal or gridlock homozygote embryos. Total RNA was extracted from 35 gridlock or phenotypically normal siblings at 3 to 4 dpf, and HIF1α expression measured as above.

Myeloid Depletion
One-cell stage embryos were injected with a morpholino antisense oligonucleotide against the transcription factor pu.1 or a control morpholino. The pu.1 morpholino has previously been shown to prevent myeloid development.19 Macrophages were visualized by Neutral Red staining as described.20 Collateral flow was assessed in 4 replicates of 30 to 40 gridlock pu.1 or controls injected with a 5-bp mismatched irrelevant MO.

Statistical Analysis
Data represents mean ± SEM. Statistical comparisons of more than 2 groups were by ANOVA, using Graphpad Prism 4.0 software.

Results

Endothelial Patterning of Gridlock/Fli1:eGFP Embryos
To determine whether the gridlock homozygote phenotype is accompanied by altered embryonic vessel formation, we performed confocal microscopy on 3, 4, and 5 dpf gridlock/Fli1:eGFP or phenotypically wild-type sibling embryos (n = 12 per group). Because of the absence of blood flow distal to the aortic occlusion, the aorta, subintestinal vessels, and intersegmental vessels of the gridlock homozygote mutants were collapsed and of smaller diameter. However, the actual patterning of the vasculature in gridlock homozygote mutants compared with phenotypically wild-type siblings was normal. We could detect no missing or aberrant vessels in gridlock homozygote mutants. In particular, we found no evidence of additional angiogenesis induced by the absence of distal blood flow. Supplemental Movies I-IV show representative confocal micrographs of 1 wild-type and 1 gridlock homozygote embryo at 4 dpf.

Development of Collateral Aortic Blood Flow in Gridlock Mutants
Gridlock mutant embryos recover blood flow distal to the occluded aorta over time. No gridlock homozygote mutant had distal aortic blood flow at 2 dpf. We occasionally observed distal aortic blood flow by 3dpf, but this increased to over 80% by 5dpf (Figure 1A). Digital motion angiograms from representative wild-type and gridlock homozygotes are shown in Figure 1B. The majority of gridlock homozygotes restore aortic flow via communications with the intestinal vasculature (88% of collaterals). Supplemental Movie V shows an example of a 5dpf gridlock Fli1:eGFP/GATA1:dsRED transgenic embryos. Some embryos also recover blood flow via reversal of flow in a proximal intersegmental artery (12% of collaterals), and as seen in the middle panel of Figure 1B these 2 patterns often coexist. Diagrams of these patterns are shown in supplemental Figure III.
When we performed confocal microangiography using 200-nm-diameter microspheres in 3dpf gridlock homozygote embryos we demonstrated microspheres passing into the aorta (Figure 2) via the dorsal longitudinal anastomotic vessel in embryos with no observable blood flow in these vessels by microscopy. This indicates that there are communications between the vasculature proximal and distal to the occlusion and that these are lumenised and carry flow of plasma, but that at some point in the vessel the lumen is not sufficiently large to allow erythrocyte passage until they are enlarged by vasodilatation or remodelled.

Laser-Induced Proximal Aortic Occlusion in 5 dpf Wild-Type Embryos

These observations did not exclude the possibility that collateral flow in gridlock homozygotes is a phenotypic manifestation of the mutation, rather than a compensatory response to aortic occlusion. We therefore sought to induce a gridlock-like aortic occlusion in wild-type 5 dpf embryos.

When we performed confocal microangiography using 200-nm-diameter microspheres in 3dpf gridlock homozygote embryos we demonstrated microspheres passing into the aorta (Figure 2) via the dorsal longitudinal anastomotic vessel in embryos with no observable blood flow in these vessels by microscopy. This indicates that there are communications between the vasculature proximal and distal to the occlusion and that these are lumenised and carry flow of plasma, but that at some point in the vessel the lumen is not sufficiently large to allow erythrocyte passage until they are enlarged by vasodilatation or remodelled.

Immediately after occlusion, we observed very small numbers of erythrocytes passing into the aorta from the intestinal vasculature in 2 of 27 embryos (7%), representing passive diversion of flow down a preexisting communication of sufficient lumen to allow passage of erythrocytes. Three hours later, however, collateral aortic blood flow was present in 51% of embryos, and 22 hours after occlusion 81% of embryos had observable blood flow between the intestinal vasculature and the distal aorta. The angiographic appearance of these embryos is strikingly similar to gridlock homozygotes (Figure 3). Laser occlusion reliably induced collateral flow via the intestinal vasculature (pattern A on supplemental Figure III). We seldom observed collateral flow via reversal of an intersegmental afferent vessel (pattern B), which we ascribe to the slightly more distal site of occlusion compared with gridlock (it is not possible to target the aortic bifurcation in 5 dpf embryos).

To determine whether these results were attributable to angiogenic remodeling or recruitment of preexisting endothelium, we generated double transgenic Fli1:eGFP/GATA1:dsRED embryos and serially visualized the response to aortic occlusion by confocal microscopy. Supplemental Movie VI shows the mid aorta of a 4 dpf embryo immediately prior to aortic occlusion. The area shown is indicated in...
supplemental Figure II. Supplemental Movie VII shows this embryo 15 minutes after laser-induced proximal aortic occlusion. Aortic flow is halted, though erythrocytes can be seen slowly moving toward the aorta in a dorsal branch of the intestinal vasculature. Before occlusion (supplemental Movie VI), this vessel carried a very small amount of flow in the opposite direction. In supplemental Movie VIII, 7 hours after occlusion aortic flow is reestablished from the dorsal branch of the intestinal vasculature via a communication visible on supplemental Movie VI (white arrow) before occlusion. To further demonstrate such communications before occlusion, we performed microangiography in 4 dpf wild-type nacre embryos. Supplemental Figure IV shows an example, with communications between the intestinal vasculature and dorsal aorta arrowed. Supplemental Movie IX is a confocal stack showing the same embryo at higher magnification showing these communications more clearly.

These data indicate that after aortic occlusion, there is a time-dependent recovery of aortic blood flow via preexisting communications with the intestinal vasculature. This is identical to the pattern seen in the majority of gridlock mutant.

The Role of Nitric Oxide in Development of Collateral Aortic Flow

Nitric oxide (NO) is the most consistent mediator of arteriogenesis in mammalian models.3–5 To determine whether zebrafish arteriogenesis shares this mechanism, we assessed the effect of nitric oxide synthase inhibition with L-NAME on vascular development, hemodynamic performance and arteriogenesis in our models.

When we incubated embryos in [0.0625–6.6 mmol/L] L-NAME from immediately postfertilization until 5 dpf, we found a concentration-dependent reduction in nitrite levels in the embryo medium, reaching 97% reduction at [1 mmol/L] (see supplemental Methods), suggesting that significant NOS inhibition is achieved at this dose.

To determine the effect of L-NAME on embryonic vascular development, we incubated Fli1:eGFP embryos in [1 mmol/L] L-NAME (n=5 to 7 embryos per group) from immediately postfertilization and imaged the vasculature at 24 and 48 hpf. By 24 hpf, both aorta and cardinal vein were present and aortic length was not different in the L-NAME group (97±5% of control length). Number of intersegmental vessels (ISV) was not different between groups (24 hpf, Control 13.6±0.7, L-NAME 12.6±1.6; 48 hpf Control 26±1, L-NAME 26±1) nor was there any difference in ISV length at 48 hpf (L-NAME 99.5±5.8% of control ISV length). No morphological defects were observed.

To determine the effect of L-NAME on hemodynamic performance, we measured erythrocyte velocity in the proximal, mid, and distal aorta in 5 dpf embryos treated with and without 16-hour treatment with [1 mmol/L] L-NAME by confocal kymography. We found no significant difference in aortic velocity between groups (Proximal aorta; Control 1270±58 μm/sec L-NAME 1179±131, Mid Aorta; Control 939±89 μm/sec, L-NAME 996±121, Distal Aorta; Control 544±61 μm/sec, L-NAME 565±50, n=9 per group).

To investigate the effect of L-NAME on arteriogenesis, gridlock mutant embryos were incubated from 1 dpf until 5 dpf in [1 mmol/L] L-NAME or L-Arginine or both. L-NAME reduced the percentage of gridlock mutant embryos which developed collateral aortic blood flow in a concentration-dependent manner (Figure 4A), reversed by L-Arginine coadministration.

We evaluated the effect of L-NAME on the recovery of blood flow in embryos with laser-induced occlusion of the mid aorta. Four-dpf embryos were pretreated with 16-hour [1 mmol/L] L-NAME or control, before occlusion of the aorta.
by focused laser. 22 hours following occlusion, collateral flow was present in the aorta of 85±8% of controls, compared with 30±8% of L-NAME–treated embryos (P<0.05, n=5 to 8 embryos per group, 3 replicates per group).

We next evaluated the timing of NO dependence of collateral aortic blood flow in gridlock mutants (Figure 4B). L-NAME treatment from 1 to 2 or 1 to 3 dpf did not significantly reduce arteriogenesis, compared with the significant reduction seen when treated from 1 to 5 dpf. This effect persisted even after a 3-hour washout. Later exposure to L-NAME (either 3 to 5 or 4 to 5 dpf) did not significantly reduce arteriogenesis.

The Effect of Myeloid Cell Depletion on Arteriogenesis

Zebrafish possess tissue resident macrophages which take up the histological dye Neutral Red. Previous work has shown that the entire myeloid lineage can be depleted by loss of function of the transcription factor pu.1. We therefore knocked down pu.1 by antisense morpholino injection into gridlock mutant embryos. At 2 dpf we were unable to detect any Neutral Red–positive macrophages in pu.1 morphants (0±0 versus 34±1.6 control, n=6, P<0.001, Figure 5A) though by 5 dpf macrophage number was not different between groups as the morpholino effect wore off. In parallel with macrophage numbers, significantly fewer pu.1 morphant gridlock mutants recovered aortic flow at 3 and 4 dpf compared with control morphants (Figure 5B). By 5 dpf, however, there was no difference between groups.

Absence of Hypoxia in Gridlock Mutants

It has been previously suggested that zebrafish embryos do not require a circulation for oxygenation for up to 14 days postfertilization, gaining sufficient oxygenation via diffusion from the water, accounting for the prolonged survival of mutants without functioning hearts or blood. However, the hypoxia-sensing mechanism is well developed even in early embryos. Previous work demonstrated HIF1α upregulation in 24 hpf zebrafish embryos incubated under hypoxic conditions for 24 hours. We repeated this experiment in 4 dpf embryos and found that even 4-hour incubation at 5% O2, significantly upregulated HIF1α (expression relative to GAPDH - Control 1.14±0.06, Hypoxic 1.72±0.04, n=3 groups of 25 embryos, P<0.002). However, we detected no HIF1α upregulation in 3 to 4 dpf gridlock embryos (wild-type 1.19±0.17, n=6 groups of 35 embryos; gridlock 0.95±0.20 n=4 groups of 35 embryos, P=0.36). This suggests that the development of collateral aortic blood flow in the gridlock mutant occurs in the absence of detectable hypoxia and ischemia.

Discussion

Our data show that proximal aortic occlusion in zebrafish embryos, either in gridlock mutants or after induced occlusion, results in collateral blood flow to the distal aorta. Though this becomes apparent in the majority of gridlock mutants at 4 to 5 dpf, the data from laser-occluded embryos show that when the vasculature is well-formed, collateral flow can develop in over 80% of embryos by 22 hours after occlusion. Using double transgenic Fli1:eGFP/GATA1:dsRED embryos, we have shown that the communications which are recruited to provide collateral flow following occlusion are present before occlusion.

In the zebrafish embryo, as in mammals, the ability to recruit collateral blood flow requires intact NOS. Even though L-NAME had no effect on aortic blood velocity, it was able to significantly impair the ability of gridlock fish to develop collateral blood flow. We used L-NAME because of the many mammalian studies which have shown this agent to reduce arteriogenesis. By varying the time and duration of L-NAME treatment, we found that to reduce collateral flow NOS had to be inhibited early, but once inhibited the effect persisted after L-NAME was removed. These data suggest a more profound role for NO than merely inducing vasodilatation. Ours is the first study to investigate the timing of NO-dependence in arteriogenesis, highlighting the utility of the zebrafish model for addressing such basic but important questions.

We also addressed the effect of myeloid cell depletion on the recovery of blood flow in our model, showing that reduction of myeloid cell number by morpholino knockdown of pu.1 impairs the ability to recover flow in the aorta.
Morphant embryos recovered the ability to restore aortic blood flow at the same time as macrophage numbers recovered. This suggests that arteriogenesis recovered as the effect of the morpholino wore off, though it is possible that myeloid cell depletion slows, rather than ultimately inhibits, arteriogenesis. This is supported by mammalian data. In a rabbit model, when MCP-1 was used to promote monocyte recruitment, this improved collateral flow at 1 week but not at 6 months. In mice, impairing monocyte infiltration by CCR2 knockout only affects arteriogenesis in certain strains, suggesting a less pivotal role than for NO.

We speculate that the presence of myeloid cells may accelerate arteriogenesis by matrix degradation to allow vessel expansion, or by delivery of vasoactive molecules such as VEGF. The clear suggestion of a contribution to arteriogenesis by myeloid cells in our model indicates another shared mechanism of arteriogenesis between mammals and zebrafish.

A unique feature of the zebrafish model is the absence of downstream angiogenesis, coupled with an apparent lack of hypoxia induced by arterial occlusion. This is the first time that arteriogenesis has been described without these concomitant processes. We used HIF1α expression as an assay of hypoxia, in keeping with previous mammalian studies. We also replicated previous work showing that HIF1α is upregulated in response to hypoxia, yet were unable to detect even a trend toward increased HIF1α expression in gridlock mutants. It is difficult to prove absolutely that gridlock embryos do not experience mild hypoxia below the ability of our assay to detect, though this would be contrary to other published studies. These data, therefore, strongly suggest that in zebrafish embryos arteriogenesis proceeds in the effective absence of hypoxia and ischemia. Although we cannot say that collateral flow is entirely independent of these processes, our data suggests that they are not required. Taken with previous mammalian studies, the weight of evidence suggests that collateral vessel development can occur in isolation to these downstream events.

An ability to provide collateral flow to an occluded artery in a fish embryo raises questions as to the basic function of such a response. It seems unlikely that this process has been fortuitously invoked by postnatal arterial occlusion. We hypothesize that the presence of myeloid cells may accelerate arteriogenesis by matrix degradation to allow vessel expansion, or by delivery of vasoactive molecules such as VEGF. The clear suggestion of a contribution to arteriogenesis by myeloid cells in our model indicates another shared mechanism of arteriogenesis between mammals and zebrafish.

Our study establishes the zebrafish as a useful model of collateral vessel development, allowing a novel approach to the study of arteriogenesis. For example, it lends itself well to screening small molecule libraries for drug discovery, and its ease of forward and reverse genetic manipulation will allow rapid identification of genes involved in collateral vessel development. Compared with mammalian models, the speed and economy of using the zebrafish allows less targeted hypothesis-generating studies to be performed which are likely to improve our understanding of the mechanism of arteriogenesis. We do not suggest that the zebrafish model will replace conventional mammalian models but that it will prove complementary in the dissection of the underlying mechanism of arteriogenesis. After over 200 years of reliance on the Hunterian model of arteriogenesis, the zebrafish represents an exciting tool to study one of the most clinically relevant mechanisms of vascular development.

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Disclosures

None.

References


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Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/09/20/ATVBAHA.107.143990.DC1
Supplementary Methods

The effect of L-NAME on Nitrite levels

To determine whether L-NAME incubation was associated with detectible changes in nitrite excretion, we used the Modified Griess Reaction to assess excreted nitrite in medium in 5dpf embryos incubated in various doses of L-NAME or control. Dechorionated embryos were treated with L-NAME from immediately post fertilisation until 5dpf (10 embryos per well in a 24well plate). At 5dpf, 100μl of medium was removed, mixed with 100μl Griess reagent (Sigma) placed in a 96 well plate for 15min and read in a spectrophotometer at 540nm (3-5 replicates per treatment).

The effect of L-NAME on normal vascular development

To determine whether L-NAME affected vasculogenesis or angiogenesis, we incubated Fli1:eGFP transgenic embryos in [1mM] L-NAME or control (n=12 per group) from immediately post-fertilisation for 48h. To assess vasculogenesis we determined the presence or absence of the axial vessels (aorta and vein) and measured the length of the aorta, expressed as percentage of control. To assess angiogenesis we counted the number of intersegmental vessels (ISV) arising from the aorta at 24 and 48hpf and measured the length of the longest ISV, expressed as percentage of control. We also recorded the presence of any aberrant vessel patterns.

Confocal Kymography

The fluorescent erythrocytes in GATA1:dsRED transgenic embryos move too rapidly to allow image capture by conventional fluorescent microscopy. However, using the line scanning function of a confocal microscope allows extremely rapid image
acquisition of an area of interest one pixel thick. When this area of interest is placed in the aorta or other vessel parallel to the scanning laser, repeated scanning can be used to generate a kymograph that shows movement of erythrocytes along the line of known length over time (Supplementary figure 1), from which velocity can be determined. We scanned 2000 times at 1.3 msec per line to allow averaging of velocity over several cardiac cycles.
Supplementary Figure Legends

Supplementary Movies are in Quicktime format (.mov) and can be viewed at http://www.cdbg.group.shef.ac.uk/research/chico/

Supplementary Figure I
Confocal kymography to measure erythrocyte velocity.

A. Z projection of 5dpf Fli1:GFP / GATA1:dsRED embryo (head is to the right). Region shown is of proximal trunk, showing aorta, cardinal vein (CV) and subintestinal vein (SIV). Endothelium is green, circulating erythrocytes are red, overlay is yellow. Dotted line in aorta shows region of line scanning to generate kymograph.

B. Confocal kymograph. Each “streak” is a red cell moving along the line of interest (horizontal dimension) over time (vertical dimension), allowing velocity measurement.

Supplementary Movies I-IV
3D confocal micrographs of Fli1:eGFP wildtype and gridlock mutant embryos. Movies show representative 4dpf Fli1:eGFP wildtype sibling (Movie I shows proximal and Movie II shows distal vasculature) and Fli1:eGFP/gridlock sibling (Movie III shows proximal and Movie IV distal vasculature). These movies are of high resolution and best viewed at double size.

Supplementary Figure II
Schematic diagram of 4-5 dpf zebrafish vasculature. Head is to the left. Area of Supplementary Movies V-IX highlighted.
**Supplementary Movie V**

Confocal movie of 5dpf Fli1:eGFP/GATA1:dsRED *gridlock* mutant. Area shown is indicated on Supplementary Figure II. Magenta arrow indicates aorta. Cyan arrow indicates intestinal vasculature, which is supplying aortic collateral flow via endothelial communication (white arrow).

**Supplementary Movies VI-VIII**

Timecourse of confocal movies of a 4dpf Fli1:eGFP/GATA1:dsRED transgenic after occlusion of the proximal aorta. Area shown is indicated on Supplementary Figure II. **Supplementary Movie VI** shows embryo prior to occlusion. **Supplementary Movie VII** shows same movie 15min after occlusion. **Supplementary Movie VIII** shows same embryo 7h after occlusion. Magenta arrow indicates aorta. Cyan arrow indicates intestinal vasculature, supplying aortic collateral flow via endothelial communication (white arrow).

**Supplementary Figure III**

Diagrams of the patterns of recovery of blood flow in *gridlock* mutants.

Upper panel shows flow patterns in wildtype embryos at 4-5 dpf. Afferent intersegmental vessels carry flow from aorta, efferent vessels into the cardinal vein. The intestinal vasculature carries blood in multiple directions, frequently reversing direction in the same vessel. There is little flow observed in the endothelial communications between the aorta and intestinal vasculature (asterisk), but is always from aorta to intestinal vasculature.
**Supplementary Figure IV**

Demonstration of pre-existing communications between aorta and intestinal vasculature in 4dpf wildtype embryo.

Figure shows mean intensity projection of confocal micrangiogram of trunk vessels of 4dpf embryo following micrangiography by injection of unconjugated quantum dots. Communications between intestinal vasculature and aorta arrowed. These are shown more clearly in Supplemental Movie IX.

**Supplementary Movie IX**

Demonstration of pre-existing communications between aorta and intestinal vasculature in 4dpf wildtype embryo.

Movie shows confocal Z stack of embryo in Supplemental Figure IV (area corresponds to area shown on Supplemental Figure II). As the scan moves through the Z plane, communications (arrowed) can be seen to connect with the intestinal vasculature and the aorta.
Supplementary Figure I
Supplementary Figure II
Supplementary Figure III
Supplementary Figure IV