Microarray profiling reveals CXCR4a is downregulated by blood flow in vivo and mediates collateral formation in zebrafish embryos

Ian M. Packham,1* Caroline Gray,1,2* Paul R. Heath,3 Paul G. Hellewell,3 Philip W. Ingham,1 David C. Crossman,2 Marta Milo,2,4 and Timothy J. A. Chico1,2

1Medical Research Council Centre for Developmental and Biomedical Genetics, 2National Institute of Health Research Cardiovascular Biomedical Research Unit, Sheffield Teaching Hospitals National Health Service Trust, 3School of Medicine, University of Sheffield, and 4Department of Biomedical Sciences, University of Sheffield, United Kingdom

First published June 9, 2009; doi:10.1152/physiolgenomics.00049.2009.

ARTERIAL OCCLUSION INDUCES a vascular response that attempts to compensate for impairment of blood flow. This comprises two separate phenomena: collateral vessel development and angiogenesis. Collateral development [also called arteriogenesis (3)] refers to recruitment of pre-existing communications between the occluded and neighboring arteries that enlarge to restore blood flow (13). Angiogenesis refers to capillary proliferation forming a microvasculature (13). Collateral vessel development and angiogenesis are triggered by separate events after arterial occlusion. Collateral formation is initiated by hemodynamic force exerted upon interarterial communications that normally experience little or no blood flow, while angiogenesis is initiated by hypoxia driving endothelial proliferation (13).

In keeping with the role of hemodynamic force in initiating collateral vessel development, many genes that promote collateral formation are upregulated by blood flow, notably endothelial nitric oxide synthase (10) and monocyte chemoattractant protein-1 (5). The transcriptional response of endothelial cells to altered hemodynamic force has been assessed in isolation in several in vitro studies (4, 6, 14, 22, 23), but these data may not fully reflect the complex intercellular interactions in vivo, since several nonendothelial cells, most particularly leukocytes (31), are involved in collateral formation. Performing in vivo studies of the transcriptional response to hemodynamic force in mammals is technically challenging; altering blood flow almost inevitably induces confounding effects such as inflammation, tissue necrosis, and ischemia.

Zebrafish embryos possess unique advantages for cardiovascular biologists, including an ability to oxygenate via diffusion (17, 28), which permits cessation of cardiac output without induction of ischemia or hypoxia. In the current study we exploited this characteristic to determine the transcriptional response to hemodynamic force in vivo. We compared gene expression after onset of embryonic cardiac contraction with expression in embryos that never develop circulation of blood due to morpholino antisense-mediated knock-down of cardiac troponin T2 (tnnt2). We identified 308 differentially expressed genes between tnnt2 and control morphants. One such (CXCR4a) was significantly more highly expressed in tnnt2 morphants at 48 and 60 hpf than controls. In situ hybridization localized CXCR4a upregulation to endothelium of both tnnt2 morphants and gridlock mutants (which have an occluded aorta preventing distal blood flow). This upregulation appears to be of functional significance as either CXCR4a knock-down or pharmacologic inhibition impaired the ability of gridlock mutants to recover blood flow via collateral vessels. We conclude absence of hemodynamic force induces endothelial CXCR4a upregulation that promotes recovery of blood flow.

* I. M. Packham and C. Gray contributed equally to this work.

Address for reprint requests and other correspondence: T. J. A. Chico, Lab D38, Univ. of Sheffield, Firth Court, Sheffield S10 2TN, UK (e-mail: t.j.chico@sheffield.ac.uk).

MATERIALS AND METHODS

Zebrafish care and breeding. Studies conformed to Home Office requirements for use of animals in scientific experiments. Transgenic lines were described previously (12).

Microarray sample preparation. Wild-type AB zebrafish were injected with morpholinos against tnnt2 (12, 32) or Gene-Tools’ stock control. Groups contained equal numbers of embryos from each parent pair.

At 36, 48, and 60 h postfertilization (hpf), whole embryo RNA was extracted from 3 groups of tnnt2 or control morphants (n = 100–130 embryos/group) using TRIzol and cleaned using Qiagen RNeasy minikits. Gene expression was determined using Affymetrix Zebrafish
chips (1 chip per group, 18 chips in total) following standard protocols (http://www.Affymetrix.com).

**Microarray preparation.** RNA was quantified using a NanoDrop ND100 spectrophotometer and quality assessed using an Agilent Bioanalyser 1000 Nanochip. We used 5 μg of total RNA for production of cDNA by introduction of an oligo d(T) molecule with an attached T7 polymerase binding site. T7 polymerase was then used to drive the production of antisense RNA with biotinylated nucleotides incorporated. The RNA was fragmented by heating and included in the hybridization solution injected into a GeneChip Zebrafish Genome array. Hybridization was performed at 45°C with rotation at 60 rpm in a rotisserie oven. Posthybridization washing and staining were carried out using the Fluidics station 400 (Affymetrix) following manufacturer’s instructions; unincorporated material was removed by stringency washing and biotinylated nucleotides were labeled using streptavidin-phycocerythin. The chip was then scanned using the GeneChip Scanner 3000 (Affymetrix) and the resultant .DAT image converted to a .CEL file by the Gene Chip Operating Software. Solutions and materials for sample labeling, hybridization, and staining were all purchased from Affymetrix. RNA integrity was monitored using the NanoDrop and Agilent Bioanalyser systems to ensure full quality control of the materials.

**Microarray analysis.** Microarray data were analyzed using a published probabilistic model, mgMOS and related family, for analysis of oligonucleotide gene expression data (2, 25). This performs probe-level analysis of the data and associates to each gene expression level a measure of uncertainty. This quantified low-level variance is then used in downstream analysis of the data to improve accuracy and increase the robustness of the statistical analysis (29).

The software is open source and is available via Bioconductor (www.bioconductor.org) as package puma, or www.bioinf.man.ac.uk/resources/puma. Gene expression estimates were evaluated using mgMOS for each time point independently and subsequently normalized for comparison. To identify candidate genes differentially expressed (DE) between groups we used pplr (probability of positive log-ratio), which detects differential gene expression using a Bayesian hierarchical model to combine probe-level measurement error and variance between replicates. It calculates the probability of upregulated genes (positive log-ratio) in a Bayesian fashion instead of P values. Downregulated genes can also be found by calculating the probability of negative log-ratio (21).

The log-ratios were calculated for each gene at each time point. We set a cut-off of a twofold change in expression, coupled with a probability of >80% for upregulated genes and <20% for downregulated genes to generate a robust list of candidates.

Once candidate genes were identified, we performed hierarchical clustering using Eisen software: Cluster and Treeview (27) (http://rana.lbl.gov/EisenSoftware.htm). Gene Ontology analysis of differentially expressed genes was performed using PANTHER (http://www.pantherdb.org) (35, 36). DE genes were assigned to biological processes to determine which biological processes were over- or underrepresented. The “fractional difference” is derived from the percentage of differentially expressed genes in a particular biological process observed in tnt2 morphants (%DE) and the percentage expected by chance (%Exp): Fractional difference = %DE genes − %DE/% Exp.

If the percentage of differentially expressed genes in a particular biological process is the same as expected by chance, fractional difference is 0, whereas a fractional difference of 1 indicates a biological process is overrepresented by a 100% increase compared with the expected number of genes. A negative fractional difference indicates an underrepresentation of a biological process in tnt2 morphants.

Data have been deposited in the Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/ (accession GSE16740).

**RESULTS**

**Tnt2 knock-down prevents cardiac contraction without affecting embryonic development or vascular patterning.** Consistent with a previous report (16), tnt2 morphants never developed cardiac contraction or blood circulation. Control morphants developed a weak heartbeat by 28 hpf, developing brisk blood flow through the cerebral vessels, aorta, cardinal vein, and intersegmental vessels by 48 hpf. Tnt2 morphants hatched and swam but developed pericardial edema by 60 hpf. Figure 1 shows representative examples of the trunk vasculature in tnt2 and control morphant Fli1:GFP transgenics that express green fluorescent protein (GFP) in endothelium. The absence of blood flow did not alter embryonic vasculogenesis (which forms the axial aorta and cardinal vein) or angiogenesis (which patterns the intersegmental vessels) (16), even though these never experienced blood flow. This supports our hypothesis that transcriptional differences between tnt2 and control morphants reflect the response to hemodynamic force rather than indirect effects on embryonic development.

**Absence of blood flow induces significant transcriptional differences during embryonic development.** We used microarrays to serially profile expression of >14,900 transcripts in control and tnt2 morphants at 36, 48 and 60 hpf. Total gene expression rose from 36 to 48 hpf and fell by 60 hpf (Fig. 2) in both control and tnt2 morphant groups, suggesting expression differences were due to the absence of circulation rather than tissue death or arrested development.

Using cutoffs of ≥2-fold difference in expression combined with ≥80% probability we found 181 genes more highly expressed in control morphants (Supplemental Table S1, 1.1% of transcripts represented on the chip) and 127 (Supplemental Table S2, 0.84% of transcripts represented) more highly expressed in tnt2 morphants in at least one time point. We used gene ontology (GO) to define biological

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1 The online version of this article contains supplemental material.
processes altered between groups. Figure 3 shows processes overrepresented in control or \textit{tnnt2} morphants at each time point. We observed very few biological processes overrepresented in either group at 36 hpf, but there was a significant overrepresentation of several processes in \textit{tnnt2} morphants at 48 hpf, particularly cholesterol metabolism and protein phosphorylation, but also protein modification, cell adhesion-mediated signaling, cell cycle control, steroid metabolism, cell proliferation, and differentiation and cell structure and motility. By 60 hpf this pattern had reversed with few processes overrepresented in \textit{tnnt2} morphants but several (different) processes overrepresented in control morphants including fatty acid metabolism, skeletal development, sensory perception, vision, G protein-mediated signaling, and muscle contraction. This suggests that at 48 hpf absence of blood flow upregulates a number of processes, and since these were not overrepresented in controls at 36 hpf, these are likely to reflect a specific response to the absence of blood flow rather than developmental delay. Conversely, by 60 hpf, controls upregulate a number of processes, including skeletal and neurological development, which is retarded in \textit{tnnt2} morphants, suggesting these processes may be in part dependent upon circulation even though tissue oxygenation is not impaired. It is noteworthy that we did not observe an increase in \textit{tnnt2} morphants of processes such as angiogenesis or the response to hypoxia that would have been expected in mammals if circulation had been interrupted.

Supporting this, there was no overlap between genes differentially expressed in our study and those detected by Ton et al. (37) in zebrafish embryos exposed to hypoxia, demonstrating the absence of hypoxia in zebrafish embryos deprived of circulation.

Next we compared our gene set with known expression patterns. Zfin (http://zfin.org/cgi-bin/webdriver?Mival=ZDB_home.apg) curates a large number of gene expression patterns identified by in situ hybridization. Data on embryonic expression at one or more developmental stage were available for 139 of the 181 genes upregulated in controls and 100 of the 127 genes upregulated in \textit{tnnt2} morphants. Supplemental Tables S1 and S2 show the anatomic terms identified for each expression pattern. Although this identified few genes specifically expressed in the vasculature, in situ hybridization is a relatively insensitive method compared with other approaches. We therefore compared our gene set with data from Covassin et al. (7). In this study, Fli1:GFP transgenic zebrafish embryos were dissociated and GFP-expressing cells transcriptionally profiled to identify genes differentially expressed in hematopoietic and vascular endothelial cells. Although Covassin et al. used embryos younger than those in our study, 96% of differentially expressed genes in our study were either more highly expressed in GFP+/ve cells or expressed at equivalent level in GFP+/ve and –ve cells (Supplemental Tables S1 and S2), indicating these are expressed in zebrafish endothelial or hematopoietic cells. Since these are the major cell types responsive to alterations in hemodynamic force, this suggests the majority of transcriptional changes detected in our microarray are likely to arise from the vasculature.

To determine whether the genes we identified are expressed in human endothelial cells, we next compared our data with an ex vivo study by Andersson et al. (1) that subjected human umbilical veins to shear stress or pressure and assessed endothelial gene expression using Affymetrix microarrays (Gene Expression Omnibus accession number GDS1317). We identified unambiguous orthologs (sharing a common gene name between human and zebrafish) for 159 of our 308 differentially expressed genes represented on the human Affymetrix chip used. From 79 genes upregulated in control embryos in our study, 39 (49%) were expressed at sufficiently high level in human umbilical vein endothelial cells (HUVEC) to be deemed present on at least one chip,
whereas from 80 genes upregulated in tnt2 morphants 61 (76%) were deemed present. Although absence from this study by Andersson et al. does not indicate these genes are never expressed in HUVEC or other endothelial cells (as the majority of these genes can be found expressed in human endothelial cells under other conditions within the GEO database), their presence indicates endothelial expression under physiological conditions in human tissue.

Supplemental Tables S1 and S2 contain the mean log2 expression level for each differentially expressed gene at each time point. Figure 4 shows differentially expressed genes more highly expressed in control morphants at 36 hpf are highlighted in box A. These included three receptors for vasoactive peptides: alpha 2D (adra2d) and 2C adrenergic receptors (adra2da) and endothelin receptor B (ednrb1), suggesting initiation of blood flow induces expression of receptors for vasoregulation. Genes upregulated in control morphants at 48 hpf represented the largest node (box B). Fifty-six genes showed an extremely specific pattern of expression, with little expression at 36 or 60 hpf in controls and little expression at any time point in tnt2 morphants. Remaining genes in this cluster were expressed detectably in tnt2 morphants at 48 hpf but significantly less than in controls.

Figure 5 shows genes more highly expressed in tnt2 morphants. Cluster A contains genes more highly expressed in tnt2 morphants at 36 hpf and that remained upregulated at 48 and 60 hpf. Cluster B shows genes more highly expressed in tnt2 morphants than controls at 48 hpf. These included CXCR4a, one of two zebrafish homologs of CXCR4, the receptor for the chemokine SDF1. CXCR4 is known to mediate collateral formation in mice by promoting recruitment of circulating mesenchymal cells (19).

CXCR4a is upregulated in response to absent blood flow. CXCR4a has been shown previously to be expressed in Fli1+ve cells (7) and human endothelium (1). Our microarray data showed that CXCR4a expression was very similar in control and tnt2 morphants at 36 hpf but subsequently declined rapidly in control morphants. However, in tnt2 morphants, CXCR4a remained expressed, suggesting onset of circulation downregulates CXCR4a (Fig. 6). Quantitative PCR of RNA from replicate control and tnt2 morphants at 48 hpf confirmed even higher differential expression of CXCR4a when corrected by GAPDH expression: control 3.5 ± 1.2, tnt2 28 ± 11 (arbitrary units).

To ascertain the anatomic location of CXCR4a expression, we performed in situ hybridization in 48 hpf control or tnt2 morphant embryos. Though we could detect very little CXCR4a expression in control morphants, there was clear expression in the distal vasculature of tnt2 morphants (Fig. 7), suggesting lack of blood flow upregulates endothelial CXCR4a expression. To prove this effect was mediated by lack of blood flow rather than nonspecific effects of tnt2 knock-down, we performed in situ hybridization for CXCR4a in 48 hpf homozygous gridlock mutants, which have an occluded proximal aorta. Although we have shown distal aortic blood flow is subsequently restored in these mutants by collateral vessels this does not occur until later than 48 hpf (12). Heterozygous gridlock mutants (which are phenotypically normal) demonstrate similar CXCR4a expression to controls, whereas homozygous gridlock mutants with occluded aortas showed upregulation of CXCR4a expression in the vasculature distal to the occlusion (Fig. 7). To prove this expression was truly vascular, we performed fluorescent dual in situ hybridization for CXCR4a with immunohistochemistry for GFP in 48 hpf Fli1:GFP/homozygous gridlock transgenic embryos that express endothelial GFP. Confocal microscopy showed colocalization of CXCR4a with GFP (Fig. 8), proving that absent blood flow upregulates CXCR4a in endothelial cells.

CXCR4a knock-down does not affect vascular development but significantly impairs zebrafish collateral vessel development. Next, we determined the effect of morpholino antisense knock-down of CXCR4a on embryonic vascular development in Fli1:GFP/GATA1:dsRED transgenics. Figure 9 shows confocal micrographs of representative CXCR4a or control morphant embryos at 48 hpf. Three-dimensional reconstructions of these stacks are included as Supplemental Movies S1–S3. In keeping with its rapid downregulation during normal development, CXCR4a knock-down had no effect on formation of the
Fig. 4. Hierarchical cluster analysis of genes significantly more highly expressed in control compared with mnt2 morphants at at least one time point. Genes that were differentially expressed by >2-fold (with >80% probability) are clustered for similar expression patterns.
axial vasculature, cardinal vein, intersegmental vessel number, or blood flow. We therefore determined whether endothelial upregulation of CXCR4a seen in gridlock mutants enhances their subsequent ability to restore blood flow to the occluded aorta via collateral vessels. As we previously described (12), gridlock mutants develop collateral vessels via communications with the intestinal vasculature, such that by 5 dpf, >80% of embryos recover distal aortic blood flow (12). However, CXCR4a knock-down significantly reduce the percentage of gridlock embryos that restored blood flow to the occluded aorta via such collateral vessels (52 ± 16% of control, \( P < 0.05 \)). Treating gridlock mutants from 3 to 5 dpf with [25 \( \mu \text{M} \)] AMD3100, a CXCR4 inhibitor also significantly impaired collateral formation (61 ± 7% of vehicle treated, \( P < 0.01 \)), supporting the importance of CXCR4a in the compensatory response to arterial occlusion.

**DISCUSSION**

Collateral vessel development is believed to be initiated by increased hemodynamic force exerted upon interarterial communications in response to arterial occlusion (13). Our aim in this study was therefore to determine the transcriptional response to hemodynamic force in vivo in an attempt to identify genes that might modulate collateral vessel development. To this end we determined the transcriptional effect of preventing cardiac contraction by \( tnt2 \) knock-down during embryonic development in zebrafish embryos. These have previously been shown not to experience hypoxia or ischemia if convective circulation is abolished (17, 28). We showed that, although control and \( tnt2 \) morphants displayed very similar patterns of gene expression overall, 308 genes were differentially expressed between groups (Supplemental Tables S1 and S2). Among these were several previously implicated in collateral vessel development in mammals, including metallothionein (20) and CXCR4a (19). We did not detect differential expression of the ligand for CXCR4a (SDF-1).

We then evaluated CXCR4a in further studies of expression, its role in vascular development, and in the response to arterial occlusion. Expression of CXCR4a in controls declined rapidly after 36 hpf, whereas absence of circulation prevented this decline. In situ hybridization revealed that this persistent CXCR4a expression is localized to endothelium deprived of blood flow, either in \( tnt2 \) morphants or in response to proximal aortic occlusion induced by gridlock mutation. CXCR4 expression has been shown to be inhibited by laminar shear stress in cultured endothelial cells in vitro.
(24), but ours is the first study to confirm this relationship in vivo.

We found that although CXCR4a gene knock-down had no effect on normal embryonic vascular development, both CXCR4a knock-down and pharmacologic inhibition inhibited recovery of blood flow in response to aortic occlusion. The relationship between CXCR4 and the compensatory response to arterial occlusion is complex, illustrated by the fact that mammalian studies have shown that both CXCR4 inhibition (33) and stimulation (15) enhance the angiogenic or collateral response to arterial ligation. In mammals, CXCR4 is expressed on both endothelial and hematopoietic progenitor cells (26) and systemic CXCR4 inhibition mobilizes bone marrow-derived progenitor cells that enhance angiogenesis after ischemia (18). However, stimulating endothelial progenitors with the CXCR4 ligand SDF-1 enhances their adhesion and resistance to shear (38), and gene transfer of SDF-1 improves collateral flow following arterial ligation (15). The absence of ischemia and concurrent angiogenesis in gridlock mutants (12) means that hemodynamic force rather than ischemia-induced progenitor cell mobilization is the driving force behind collateral vessel development. Our data suggest that when endothelium is deprived of hemodynamic force it upregulates CXCR4 and that this favorably influences collateral vessel development. This is the first suggestion that endothelium downstream of an arterial obstruction initiates a program to restore blood flow and adds a novel facet to the current paradigm of collateral development.

Our use of whole embryo RNA for the microarray means that we cannot determine the cell types responsible for the transcriptional changes we detected, and we are unable to exclude potential off target effects of tnt2 knock-down. We are therefore required to subject differentially expressed genes to the same evaluation process as CXCR4a to determine the site of expression and the effect of gene knock-down on collateral vessel development. The duplication of many genes (including CXCR4) in zebrafish means that drawing exact parallels between the function of orthologs is not possible. However, there is significant functional conservation between mammalian and teleosts, and key mediators of vascular development in mammals (such as VEGF) play similar roles in zebrafish (11). The zebrafish therefore represents an informative model with which to study the vascular response to alteration in hemodynamic force and to arterial occlusion.
EFFECT OF HEMODYNAMIC FORCE ON ZEBRAFISH TRANSCRIPTOME

GRANTS

This work was supported by a GlaxoSmithKline Clinician Scientist Fellowship and British Heart Foundation Project Grant 06/052 awarded to T. J. A. Chico, a National Institute of Health Research Biomedical Research Unit grant to D. C. Crossman, and a Medical Research Council Centre Development Grant (G0400100) awarded to P. W. Ingham. The light microscopy facility was funded by the Wellcome Trust (GR077544/1A).

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