Early Endocardial Morphogenesis Requires Scl/Tal1

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The primitive heart tube is composed of an outer myocardial and an inner endocardial layer that will give rise to the cardiac valves and septa. Specification and differentiation of these two cell layers are among the earliest events in heart development, but the embryonic origins and genetic regulation of early endocardial development remain largely undefined. We have analyzed early endocardial development in the zebrafish using time-lapse confocal microscopy and show that the endocardium seems to originate from a region in the lateral plate mesoderm that will give rise to hematopoietic cells of the primitive myeloid lineage. Endocardial precursors appear to rapidly migrate to the site of heart tube formation, where they arrive prior to the bilateral myocardial primordia. Analysis of a newly discovered zebrafish Scl/Tal1 mutant showed an additional and previously undescribed role of this transcription factor during the development of the endocardium. In Scl/Tal1 mutant embryos, endocardial precursors are specified, but migration is severely defective and endocardial cells aggregate at the ventricular pole of the heart. We further show that the initial fusion of the bilateral myocardial precursor populations occurs independently of the endocardium and tal1 function. Our results suggest early separation of the two components of the primitive heart tube and imply Scl/Tal1 as an indispensable component of the molecular hierarchy that controls endocardium morphogenesis.

Introduction

The primitive heart tube is the first functional organ in the vertebrate embryo and is composed of a myocardial tube lined by an inner endothelial layer called the endocardium. Significant progress has been made towards elucidating the morphogenetic events and transcriptional control underlying patterning of the myocardium [1]. However, the morphogenetic events and the transcription factors involved in early development of the endocardium remain largely undefined. In fact, the specific embryonic origin of the future endocardial cells and their relationship with the future myocardial cells is still unclear [2].

Results obtained using in vitro differentiation of embryonic stem cells [3] and analysis of different mesodermal or cardiac cell lines [4,5] suggest the development of both cardiac lineages from bipotential progenitors during heart field formation and, indeed, bipotential cells have been identified in the early mouse embryo at a single-cell level [3]. However, lineage tracing experiments in the avian embryo have shown restricted myocardial or endocardial potential of precardiac cells, with both types of precursors intermingled during their migration towards the site of the primitive heart tube [6]. A similar sequestration of endocardial and myocardial cells is observed in the zebrafish where cells at late blastula stages give rise to both endocardial and myocardial cells [7]. These cells also give rise to the head vasculature and an anterior population of hematopoietic cells of the primitive myeloid lineage and become restricted in their potential to form either endocardium or myocardium during early gastrulation stages [8].

After endocardial and myocardial precursors have been specified, complex morphogenetic movements occur that shape the primitive heart tube. Again, most studies have focused on the morphogenesis of the myocardium. One of the early markers of myocardial differentiation is the transcription factor nKx2.5, expression of which is detected in the zebrafish in bilateral populations starting at the ten-somite stage [9]. Subsequently, these bilateral myocardial fields merge in the midline at the 18-somite stage, with the first contacts occurring in a relative posterior position [10]. The myocardial precursors posterior to the first junction as well as the most anterior portions then come in contact with each other, creating a ring with an anterior central portion devoid of myocardial cells that has been suggested to contain the endocardial precursors [10,11]. Around these stages, expression of cardiac contractile genes, such as such as cardiac myosin light chain (myl7), starts [12]. Finally, myocardial cells move to the left, and during a complex and poorly understood process, convert the myocardial disc into the primitive heart tube [10].

The basic helix-loop-helix (bHLH) transcription factor Scl/Tal1, hereafter referred to as Tal1, an oncogene originally identified in childhood leukemias, is expressed in endothelial, endocardial and hematopoietic, but not myocardial cells during early murine development [13]. Tal1 forms a transcriptional complex containing Lmo2 to regulate expression of target genes. Gain of function of Tal1 in combination with Lmo2 leads to an expansion of endothelial and hematopoietic...
**Author Summary**

In its earliest functional form, the embryonic heart of all vertebrates is a simple linear tube consisting of two cell types. An outer muscular cell layer called the myocardium surrounds an inner vascular cell layer called the endocardium that connects the heart to the vascular system. The integration of both cell types is an important step during heart development, but the formation of the endocardial component of the heart tube is poorly understood. Here, we analyze the formation of the endocardium in the zebrafish embryo and show using time-lapse imaging that it is a highly dynamic structure. In addition, we have identified a zebrafish mutant with a specific defect during endocardial development. This defect is caused by a mutation in T cell acute leukemia 1, a gene that—when misexpressed—causes many cases of childhood leukemias. Here, we show an additional role for this gene during heart development. In mutant embryos, both endocardial and myocardial precursors are specified, but integration of both cell types does not occur properly due to a defective migration of the endocardial precursors. Given the many interactions that occur between the endocardium and the myocardium, our results will provide a more comprehensive understanding of heart development.

**Meiotic Mapping and Sequencing**

The t21384 mutation was mapped to Chromosome 22 using standard simple sequence length polymorphism mapping. For sequencing of the tal1 gene, genomic DNA was extracted from 12 wild-type (wt) and 12 mutant t21384 embryos. The three coding exons of the zebrafish tal1 gene were PCR amplified and sequenced on both strands. A mutation in the third coding exon was confirmed in a panel of 580 single mutant and 53 single sibling embryos using PCR with primers tal1-ex3-fw (5'-CAACATTAATGCACATCTTGG-3') and tal1-ex3-rev (5'-TCTACCTGGTGCTTCTGCTC-3') and sequenced using primer tal1-ex3-seq (5'-TGGGCGAACAATTCAATTAG-3').

**Full-Length Sequence of kdr, flt1, and flt4**

We used a unidirectionally cloned, oligo dT–primed SMART cDNA library constructed from 2- and 3-d-old zebrafish larva using Advantage2 DNA Polymerase Mix (Clontech, http://www.clontech.com/). Primers used for identification of kdr, flt1, and flt4 3' and 5' ends are available upon request.

**Phylogenetic and Synteny Analysis**

The MEGA3 package was used for phylogenetic analysis [24]. Amino acid sequences were aligned with ClustalW (the resulting alignment is available upon request) and a phylogenetic tree was constructed using a neighbor-joining algorithm. The resulting tree was tested using 1,000 bootstrap resamplings. Pairwise distances were calculated with the PAM substitution matrix. Identification of vertebrate VEGF receptors and synteny analysis was performed using the Ensembl database (http://www.ensembl.org), release 44, April 2007.

**Whole-Mount In Situ Hybridization and Immunohistochemistry**

Single and double in situ hybridizations were performed essentially as described [25], except that labeled probes were purified using NucleoSpin RNA clean-up columns (Machery-Nagel, https://www.macherey-nagel.com/) and embryos were transferred to 15-mm Costar Netwells (Corning, http://www.corning.com/) after hybridization. The substrate used in the second color-reaction was INT ([4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride) (Roche Applied Science, http://www.roche-applied-science.com/). Previously described probes used were tal1, gata1, spi1, runx1, hey2, flk1/kdr, ephrin B2a, dab2, mnx2.5, amhc, and cmlc2 [9,12,26–34]. Probes for flt4, kdr, and flt1 were transcribed from the 5' part of the respective cDNAs that code for the extracellular domain of the proteins. Immunohistochemistry using anti-GFP (Torrey Pines Biolabs, http://www.chemokine.com/) and anti-tropomyosin (CH1; Sigma, http://www.sigmaaldrich.com/) antibodies was performed as described [35]. Secondary antibodies were Alexa555-anti-rabbit and Alexa488-anti-mouse (Invitrogen, http://www.invitrogen.com/).

**mRNA Injections**

Full length tal1 mRNA was transcribed from linearized plasmids using the Message-Machine kit (Ambion, http://www.ambion.com/) as described [18]. mRNA injections were done in a volume of 1 nl at the one-cell stage in Milli-Q (http://www.millipore.com/) water at a concentration of 20 pg/embryo.

**Materials and Methods**

**Zebrafish Lines**

The tal1t21384 allele was isolated in a genetic screen for ENU-induced mutations affecting vascular development [21]. The transgenic line TG(fli1agfp)3 [22] was obtained from Brant Weinstein (Bethesda, Maryland). The transgenic line TG(vegfr4: gfp)anst [23] was obtained from Didier Stainier (San Francisco, California).

**Immunohistochemistry**

The second color-reaction was INT ([4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride) (Roche Applied Science, http://www.roche-applied-science.com/). Previously described probes used were tal1, gata1, spi1, runx1, hey2, flk1/kdr, ephrin B2a, dab2, mnx2.5, amhc, and cmlc2 [9,12,26–34]. Probes for flt4, kdr, and flt1 were transcribed from the 5’ part of the respective cDNAs that code for the extracellular domain of the proteins. Immunohistochemistry using anti-GFP (Torrey Pines Biolabs, http://www.chemokine.com/) and anti-tropomyosin (CH1; Sigma, http://www.sigmaaldrich.com/) antibodies was performed as described [35]. Secondary antibodies were Alexa555-anti-rabbit and Alexa488-anti-mouse (Invitrogen, http://www.invitrogen.com/).

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Time-Lapse Analysis

Embryos were mounted in 0.25% agarose in a six-well culture plate with a cover slip on the bottom of the well and imaged with a Leica TCS SP confocal microscope (Leica Microsystems, http://www.leica-microsystems.com/) using a 40× dry objective (vegfr4:gfp) or 10× dry objective with 2× digital zoom (fli1a:gfp). Maximal z-projections of 40–50 slices at 4 μm per slice were compiled using ImageJ software (http://rsb.info.nih.gov/ij/). Time points were recorded every 5 min (fli1a:gfp) or 7.5 min (vegfr4:gfp) for 6–8 h. A heated stage was employed to keep the embryos at approximately 28.5 °C.

Results

A Truncating Mutation in Zebrafish tal1

In a large-scale forward genetic screen [21], we identified a mutant (t21384) that showed a severe reduction in endothelial alkaline phosphatase activity at 4 d post fertilization, particularly in the region of the dorsal aorta. Until 26 h post fertilization (hpf), the general morphology of mutant embryos was indistinguishable from that of their wt siblings. At this time however, the pericardium became edematous (Figure 1A), even though heartbeat was initiated normally. No erythrocytes were observed and embryos consequently lacked circulation. To identify the molecular lesion responsible for the t21384 phenotype, we used simple sequence length polymorphism mapping to position the mutation on Chromosome 22. Single embryo mapping using a limited number of mutant embryos (n = 96) positioned the mutation at 28.0 cM (±0.5 cM) between simple sequence length polymorphism markers z21515 and z938, closely linked to the tal1 gene (27.89 cM)). The combination of endothelial and hematopoietic defects seen in t21384 mutants had also been observed in mouse Tal1 knockouts [15], as well as in a morpholino knockdown of tal1 in zebrafish [18–20]. Therefore, tal1 was a very likely candidate gene. Sequencing of the
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injection of low amounts of wt mRNA (20 pg per embryo), whereas most of the remaining (8/54, 15%) display an intermediate phenotype. Class 3: short endocardium with no lumen. The endocardial phenotype can be rescued to class 1 through injection of low amounts of wt mRNA into embryos from a cross between heterozygous individuals carrying the fli1agfp transgene to visualize endothelial cells. In uninjected mutant embryos, vascular patterning was severely affected, leading to a loss of intersomitic vessels. The defect in vascular patterning was rescued by injection of wt tal1 mRNA (Figure 1D), as was the hematopoietic and the endocardial defect (see below).

Although the t21384 mutation resided in the final coding exon of the tal1 gene, and therefore the mRNA was unlikely to be subjected to nonsense-mediated decay [36], we observed reduced expression of tal1 mRNA at 28 hpf, as revealed by in situ hybridization (Figure 1E). Expression of tal1 was retained in the ventral mesenchyme of the tail, a region that has been hypothesized to contain hematopoietic progenitors [26]. In addition, we consistently observed a bilateral population of tal1 expressing cells above the yolk extension. These cells resided in the mesenchyme ventral to the pronephric tubule and lateral to the developing gut tube (Figure 1E, inset).

To confirm the loss of hematopoietic lineages, we performed in situ hybridization for genes that are required for the formation of the two major primitive hematopoietic lineages in the early embryo as well as those that are required for the formation of definitive hematopoietic stem cells. Consistent with data obtained by morpholino injection [18–20], we showed that the development of the primitive erythroid lineage (gata1) as well as the formation of definitive hematopoietic stem cells (runx1) was lost. Similarly, development of the primitive myeloid lineage was severely affected (Figure 1F), although some spil1 expressing cells were observed in the head at 28 h (unpublished data).

Based on the tight linkage, the stop codon in the tal1 gene, and the rescue of all phenotypic aspects in mutant embryos through injection of wt mRNA, we conclude that t21384 encodes tal1 and hereafter refer to the mutant allele as tal1t21384.

Tal1 Mutants Display Defects in Early Endocardial Development

In addition to the defects observed in the hematopoietic and endothelial lineages (Figure 1D and see below), we observed in tal1t21384 mutants a strong defect in the morphogenesis of the heart that has not been previously described in the zebrafish or mouse. Although myocardial differentiation does not appear to be affected and heartbeat is initiated normally, the endocardial cells in the tal1t21384 mutants do not form a single cell layer lining the myocardium, and do not form atrial endocardium. Instead, endocardial cells aggregate at the arterial pole of the heart, leading to complete ventricular stenosis (Figure 2A and 2B). Later, concentric growth of the myocardium is defective and no heart valves

Scl/Tal1 Function during Endocardial Development

![Figure 2. Defects in Endocardial Development in tal1t21384 Mutants](image)

Figure 2. Defects in Endocardial Development in tal1t21384 Mutants

(A) Confocal images of the head region from embryos transgenic for fli1agfp at 32 hpf. Arrows indicate the location of the endocardium. In the wt endocardium, a lumen is visible, which is absent in tal1t21384 mutants.

(B) Transversal sections of wt and tal1t21384 mutant heart ventricles at 56 hpf stained with hematoxylin/eosin. In wt embryos, the endocardium forms an epithelium attached to the myocardium, and blood cells are visible within the heart tube. In the tal1t21384 mutant heart, the endocardium fails to form an epithelium and is only present in the ventricular myocardium.

(C) Rescue of endocardial defects. Maximum z-projection of a stack of confocal images of embryos carrying the vegf4-gfp transgene. Embryos were divided into three classes based on their endocardial phenotype. Class 1: wt length of the endocardium with normal tube formation (wt phenotype). Class 2: wt length of the endocardium but no tube formation (Intermediate phenotype). Class 3: short endocardium with no tube forming (mutant phenotype). All embryos homozygous or heterozygous for the wt allele are in class 1, whereas all embryos homozygous for the tal1t21384 allele are in class 3, showing the high penetrance of this phenotype. In 43 out of 54 embryos homozygous for the tal1t21384 allele (83%), the endocardial phenotype can be rescued to class 1 through injection of low amounts of wt tal1 mRNA (20 pg per embryo), whereas most of the remaining (8/54, 15%) display an intermediate phenotype (class 2).

three coding exons of the zebrafish tal1 gene in mutant and sibling embryos revealed an A→T transversion in the third and final coding exon (Figure 1B). The mutation resulted in a K→ochre nonsense mutation at position 183 of the protein. The resulting putative protein had a deletion of the highly conserved bHLH domain, including the DNA binding basic region (Figure 1C).
lost the Zebrafish vegfr4 orthologue, coinciding with the emergence of the X-chromosome inactivation center XIST in the same genomic locus. (C) Expression of zebrafish VEGF receptors in the heart region at 26 hpf, dorsal view, anterior to the top. Three VEGF receptors are expressed in the endocardium: vegfr4, kdr, and flt1 (arrowheads). Note the absence of flt4 expression in the endocardium (asterisk). At this stage, both vegfr4 and kdr are expressed at high levels in all endothelial cells, whereas flt1 and flt4 have a mutually exclusive expression pattern: flt1 is expressed in all arteries (AA1, first aortic arch; LDA, lateral dorsal artery; and PICA, primitive internal carotid artery) whereas flt4 is expressed in all veins (MCeV, middle cerebral vein [which is largely out of the focal plane of this picture]). PHBC, primordial hindbrain channel; PMBC, primordial midbrain channel.

doi:10.1371/journal.pgen.0030140.g003

Figure 3. Zebrafish vegfr4(flk1) Represents a New Class of Nonmammalian VEGF Receptors

(A) Rooted neighbor-joining tree of vertebrate VEGF receptors. Different colors represent different classes of VEGF receptors. Note the clear separation of zebrafish and other teleost fish vegfr4, and the novel frog, chick, opossum, and platypus VEGF receptor genes from the three other classes of vertebrate VEGF receptors. Bootstrap value of VEGF4 node is indicated (1,000 times out of 1,000 iterations).

(B) Synteny analysis of vertebrate VEGF receptors using human, mouse, chick, and zebrafish genome assemblies. Colors used are similar to those in (A). Question marks represent missing orthologous genes, potentially due to gaps in the chick genome assembly. Clear syntenic relationships of all vertebrate VEGF receptors were observed, indicating duplication from a primordial gene cluster in a primitive chordate. Mammals have are formed, consistent with an important role for the endocardium in these processes [37,38]. This phenotype was always found in combination with the loss of primitive hematopoiesis, and both aspects of the phenotype could be efficiently rescued by injecting wt tal1 mRNA, showing the specificity of both phenotypes to the loss of tal1 function (Figure 2C).

Early Endocardial Precursors Express a VEGF Receptor Gene That Has Been Lost during Mammalian Evolution

To be able to examine the early endocardial defects observed in tal1−/− mutants, we aimed to develop several markers for endocardial morphogenesis. A previous study used the expression of a zebrafish vascular endothelial growth factor (VEGF) receptor homologue to delineate early endocardial development in the zebrafish [31], which was proposed to be the zebrafish Flk1/KDR orthologue. However, the murine Flk1 gene is expressed in multiple nonendothelial cells [39,40], and Flk1+ progenitors give rise to beating cardiomyocytes [3]. The observed endothelial-specific expression of zebrafish flk1/kdra at early developmental stages is therefore surprising. This prompted us to reassess the phylogenetic relationship of the zebrafish VEGF receptors.

We obtained the full length sequence for all zebrafish VEGF receptors: flk1/hdra, flt1, flt4 and kdrb [31,41–43], of which flk1/kdra and kdrb were proposed to be the result of a whole-genome duplication event in teleost fish [43]. However, we identified likely orthologues for all four zebrafish genes, including both flk1/hdra and kdrb in the genomes of Xenopus tropicalis, chick, platypus, and opossum. Phylogenetic and synteny analysis of the three human VEGF receptors FLT1, KDR, and FLT4 and the four receptors of zebrafish, chick, and opossum both supported the hypothesis that zebrafish flk1/hdra and the novel chick and opossum VEGF receptor genes in fact represent a separate VEGF receptor class that was lost during mammalian evolution (Figure 3A and 3B). In addition, this showed that the gene previously published as kdrb is in fact the KDR orthologue. Given these results, we propose the use of VEGF receptor 4 to designate the novel class of vertebrate VEGF receptors and will use vegfr4 instead of flk1/hdra and kdr instead of kdrb for the remainder of this manuscript.

Expression analyses revealed that kdr was the first VEGF receptor expressed during development (see Figure S1). vegfr4, flt1, and kdr but not flt4 are expressed in the endocardium of the heart at 26 hpf (Figure 3C). Importantly, and consistent with earlier results, the expression of vegfr4 was restricted to endothelial precursors and blood vessels
Figure 4. Migration of Endocardial Precursors in wt Embryos

(A) Embryos transgenic for vegfr4:gfp were subjected to time-lapse confocal microscopy, revealing rapid endocardial migration prior to heart tube formation. A movie demonstrating this process can be viewed in the Video S1. Twelve individual frames from this movie at indicated stages and time-
points are shown seen in (A), with white dashed lines indicating the position of (pre-) endocardial cells. Frames 1–5 show the fusion of the bilateral endocardial precursors between the 14- and 16-somite stages. Frames 5–8 indicate the posterior migration of endocardial cells to cover the lateral and posterior regions of the cardiac disc; note the posterior migration of the paired lateral dorsal aortas between the 18- and 22-somite stages (white arrows). The apex of the endocardial disc (pink dashed line) appears to be constricted below the aortic arches between the 18- and 22-somite stages (frames 7–10). A leftward movement of the endocardium is visible between the 20- and 26-somite stages (frames 9–12), and is coinciding with the appearance of single vegfr4:gfp-positive cells lateral to the remaining endocardium (red dashed line). Also note the migration of the venous posterior hindbrain channels (red arrows) between the 22- and 26-somite stage (frames 10–12).

(B) Relative locations of endocardial and myocardial precursors during fusion of endocardial precursor populations, revealed by two-color in situ hybridization showing cdh5 (blue, endocardium) and nkx2.5 (red, myocardium) expression. The bilateral populations of endocardial precursors (arrows) are located anterior to the myocardial precursors until the 14-somite stage, then migrate medially and posteriorly to assume a position in between the myocardial precursors at the 18-somite stage.

(C) Embryos transgenic for fli1a:gfp were subjected to time-lapse confocal microscopy, revealing slow medial movement of gfp-positive cells between the six- and 12-somite stage (frames 1–4) and rapid migration starting at the 14-somite stage. A movie demonstrating this process can be viewed in the Video S2.

doi:10.1371/journal.pgen.0030140.g004

during all stages examined and could be used as a marker for endocardial development.

Early Endocardial Development in the Zebrafish

To understand the endocardial defects in tal1<sup>21384</sup> mutants, we first characterized normal endocardial development in the zebrafish. We used time-lapse confocal microscopy in the vegfr4:gfp transgenic background [23] to analyze the morphogenetic movements during early endocardial development. Consistent with vegfr4 mRNA expression, this transgene was expressed in all the endothelial cells including the endocardium, but not the myocardium, of the primitive heart tube and therefore was used to follow endocardial precursors during their migration, although it is important to note here that higher resolution tracking of single cells or fate mapping will be required to definitely address the origin and migratory path of endocardial precursors.

The earliest time point at which fluorescence was detected was between the 10- and 12-somite stage (14–15 hpf). At this stage, the transgene was expressed in the anterior and posterior lateral plate mesoderm, representing the endothelial and hematopoietic precursors. Between the 12- and 14-somite stage (15–16 hpf), the anterior lateral plate mesoderm moved medially with more gfp-positive cells in the posterior region of the anterior lateral plate mesoderm. This region later formed part of the head vasculature, the primitive myeloid cells, the anterior dorsal aortas and, importantly, the endocardium and aortic arches (see Video S1 and Figure 4A). The coexpression of vegfr4:gfp in both the endocardium as well as the head vasculature suggests a respective origin of these cell populations within the anterior lateral plate mesoderm. Both our marker analysis and the time-lapse imaging are consistent with that notion; however, in the absence of single-cell tracking, it is not completely conclusive. Both of these lineages seem to arise as bilateral populations at the 14-somite stage (dashed lines in Figure 4A). The presumed endocardial precursors then rapidly migrated posterior and fused between the 15- and 18-somite stage (16.5–18 hpf). Fusion of these endocardial precursor populations initiated at the anterior side, progressed in a posterior direction, and was finished by 18 hpf. At the same time, further posterior migration of endocardial cells occurred. Finally, a complex leftward movement of the endocardial primordium occurred to position the endocardial component of the primary heart tube at the left side of the embryo between the 22- and 26-somite stage. Although most endocardial cells moved slowly and as an epithelial sheet at these stages, additional single vegfr4:gfp-positive cells that are separate from the endocardium were rapidly moving lateral and anterior (Figure 4A, red dashed line).

To assess the relative positions of endocardial and myocardial precursors during fusion of the endocardial precursor populations, we performed two-color in situ hybridization using precisely staged embryos and small time intervals. The endocardial precursors as well as precursors of the head vasculature were marked by expression of cadherin 5 (cdh5) and the myocardium by expression of nkx2.5 (Figure 4B). In this way, we showed that VE-cadherin expressing cells, which include the endocardial precursors, do not express nkx2.5 and are found immediately anterior to the myocardial precursors in the lateral plate mesoderm at the 14-somite stage, when migration begins. In addition, these data confirmed that endocardial and not myocardial cells are the first to arrive in the midline.

Finally, to confirm the results obtained in the vegfr4:gfp transgenic line and to analyze the movements of endocardial precursors before the 14-somite stage, we used the fli1a:gfp transgenic line that also expresses a transgene specifically in endothelial cells, including the endocardium (Figure 4C and Video S2). GFP fluorescence in this line can be detected from before the six-somite stage and thus can be used to follow tissue movements prior to endocardial migration. In addition to the endocardium, this transgene is expressed in the developing primitive myeloid population, as well as the pharyngeal mesoderm. Time-lapse imaging of this line confirmed the results obtained with the vegfr4:gfp transgenic line between the 14- and 20-somite stage. Before this stage, the anterior lateral plate mesoderm gradually moved medially. This analysis also suggests a close association of endocardial and primitive myeloid cell populations, as macrophages (identified based on their motility and being fli1a:gfp positive) start migrating from the 12-somite stage from a region that also includes the endocardial precursors.

Early Endocardial Development in the tal1<sup>21384</sup> Mutant

To assess the timing of endocardial defects in tal1<sup>21384</sup> mutants, we analyzed mutant embryos carrying the vegfr4:gfp transgene and performed time-lapse confocal microscopy, similar to wt embryos (Figure 5A and Video S3). In mutant embryos, fluorescence was first detected between the ten- and 12-somite stage, indistinguishable from wt embryos. The bilateral presumptive endocardial precursors initiated migration at the 14-somite stage normally, but there was a severe defect in the continued posterior migration of endocardial precursors. Whereas wt endocardial precursors migrated rapidly and formed a single cell layer in the

PLoS Genetics | www.plosgenetics.org August 2007 | Volume 3 | Issue 8 | e140
embryonic midline, endocardial precursors in \( \text{tal1}^{\text{t21384}} \) mutants aggregated in an anterior position (Figure 5A and Video S3).

**Tal1 Expression in Zebrafish Endocardial Precursors**

Previous reports have indicated Tal1 expression in the endocardium of the mouse [13]. However, a previous study did not identify \( \text{tal1} \) expression in endocardial cells in the zebrafish [26]. We analyzed \( \text{tal1} \) expression during early endocardial development using two-color in situ hybridization and showed \( \text{tal1} \) expression during all stages of endocardial cell migration (10–20-somite stage) (Figure 5B), consistent with a cell-autonomous role for \( \text{tal1} \) in this process. In the trunk, \( \text{tal1} \) expression was detected in angioblasts and primitive erythrocytes. Expression is downregulated during endothelial differentiation and only maintained in erythrocytes. Similarly, expression of \( \text{tal1} \) in the endocardium was downregulated during early migration and maintained in the primitive myeloid lineage.

**Endocardial Differentiation in \( \text{tal1}^{\text{t21384}} \) Mutants**

Results obtained using morpholino knockdown have shown an important role for Tal1 in the differentiation of arterial and venous endothelial cells [18–20]. This suggested that failure of endocardial differentiation could be the primary defect in \( \text{tal1}^{\text{t21384}} \) mutant hearts. Using the genetic mutant, we reassessed the role of \( \text{tal1} \) during arteriovenous differentiation and showed that indeed most arterial gene expression was lost and venous gene expression expanded (Figures 5C and S2). In addition, we resolved a difference between previous data [18,19] and showed migration of angioblasts to the region of the dorsal aorta not to be affected. Endothelial cells were present in their correct location ventral to the notochord (Figure S3).

Many arterial markers, such as \( \text{flt1} \), \( \text{hey2} \), and \( \text{dll4} \) but not venous markers such as \( \text{flt4} \) and \( \text{dab2} \) are also expressed in the endocardium at 24–28 hpf, suggesting common regulation of gene expression. Importantly, we observed that \( \text{tal1} \) differentially regulates arterial and endocardial gene differentiation as expression of \( \text{flt1} \) and \( \text{hey2} \) is severely reduced in the head and trunk arteries, but expression is maintained in the endocardium (Figure 5C). In the absence of more specific endocardial marker genes, these results suggest that defects during migration rather than during endocardial differentiation are the cause of the heart defects observed in \( \text{tal1}^{\text{t21384}} \) mutant embryos.

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**Figure 5. Migration of Endocardial Precursors and Heart Tube Formation in \( \text{wt tal1}^{\text{t21384}} \) Mutant Embryos**

(A) \( \text{tal1}^{\text{t21384}} \) mutant embryos transgenic for \( \text{vegf4:gfpl} \) were subjected to time-lapse confocal microscopy, revealing defects during early endocardial precursor migration. A movie demonstrating this process can be viewed in the Video S3. Six individual frames from this movie are shown in (A). Whereas the initial formation of bilateral endocardial precursors is not affected, posterior migration is disturbed, and endocardial precursors remain attached in a relative anterior position. Note that migration of the paired lateral dorsal aortae (arrows) proceeds normally.

(B) \( \text{tal1} \) expression in endocardial precursors. Two-color in situ hybridization revealing \( \text{tal1} \) (blue) and \( \text{nklx2.5} \) (red) expression in wt embryos at the 14- and 18-somite stage. \( \text{tal1} \) expression is observed in endocardial but not myocardial precursors during their posterior migration (arrowheads).

(C) Expression of the arterial markers \( \text{flt1} \) and \( \text{hey2} \) is retained in \( \text{tal1}^{\text{t21384}} \) mutant endocardium, but severely reduced in endothelium, as revealed by in situ hybridization. Dorsal view of \( \text{flt1} \) and \( \text{hey2} \) expression in the head, anterior to the top, and lateral view of \( \text{flt1} \) and \( \text{hey2} \) in the tail (28 hpf). In wt embryos, \( \text{flt1} \) expression is observed in all head arteries, the aortic arches and the endocardium (arrow). In \( \text{tal1}^{\text{t21384}} \) mutant embryos, expression of \( \text{flt1} \) is observed in a few remaining head arteries and the aortic arches. High levels are seen in the endocardium (arrow). In wt embryos, \( \text{flt1} \) expression is observed in the dorsal aorta and the developing intersegmental vessels. In the tail of \( \text{tal1}^{\text{t21384}} \) mutant embryos, expression of \( \text{flt1} \) is abolished, except for a few remaining cells that express \( \text{flt1} \) at low levels (arrowheads). In wt embryos, \( \text{hey2} \) expression is observed in the endocardium and the aortic arches and in some parts of the brain and spinal chord. In \( \text{tal1}^{\text{t21384}} \) mutant embryos, expression in the endocardium (arrow) is increased. In wt embryos, \( \text{hey2} \) expression is observed in the dorsal aorta and the developing intersegmental vessels, spinal chord neurons, and in ventral and dorsal cells of the somites. In \( \text{tal1}^{\text{t21384}} \) mutant embryos, expression in the dorsal aorta and intersegmental vessels is severely reduced, although some anterior intersegmental vessels and aortic cells retain low levels of \( \text{hey2} \) expression (arrowheads).

doi:10.1371/journal.pgen.0030140.g005
Heartbeat initiated normally in tal1t21384 mutant embryos, indicating normal myocardial differentiation in the absence of an endocardial lining. Indeed, differentiation of atrial and ventricular myocardium was observed at 28 hpf (Figure 6A). However, at this stage, the atrial side of the heart appeared abnormally enlarged (bracket in Figure 6A), suggesting early defects during heart tube morphogenesis. Therefore, we performed two-color immunohistochemistry labeling the myocardium (tropomyosin) and endocardium (vegfr4:gfp) in single embryos. Using this method, we confirmed the observation that fusion of the myocardial primordia is initiated in a relative posterior position to form a butterfly-like shape that changes to a horseshoe-shaped myocardium through fusion of the most posterior part of the myocardial
primordium between the 18- and 20-somite stages (Figure 6B) [10]. At the onset of myocardial fusion, most of the endocardium is positioned medially to the bilateral myocardial precursors. Connection between the endocardium and the lateral dorsal aortae is persistent throughout development and occurs through the developing first aortic arches. These cells represent the endocardial cells located dorsal to the myocardium as described by Trinh et al. [44] (brackets in Figure 6B). Fusion of the myocardium occurs dorsal to the endocardium and is initiated just anterior to the most posterior endocardial cells (arrow in Figure 6B). Indeed, around this stage, movement of endocardial cells occurs ventrally relative to the myocardial cells, and by the 20-somite stage, most endocardial cells are located ventral to the myocardium—especially in the lateral and posterior regions of the heart (Figure 6C). However, the region of the endocardium that connects to the aortic arches is still positioned medially to the myocardium.

In tal1<sup>21384</sup> mutant embryos, fusion of the bilateral myocardial precursors is initiated normally, and thus appears independent of the endocardium or tal1 function. Endocardial cells at the 22-somite stage remain located anterior to the myocardium, leading to defects in the anterior fusion of the myocardium, which in wt embryos has occurred at this stage (Figure 6D and 6E). Anterior fusion of the myocardium is delayed until the 26-somite stage (Figure 6E). The medial region of the myocardium at the 22-somite stage gives rise to the ventricle of the heart, whereas the lateral and posterior myocardium gives rise to the atrium [12]. Therefore, absence of atrial endocardial cells can be explained by an early migration defect of endocardial precursors. A summary of heart tube assembly from endocardial and myocardial precursors in wt and tal1<sup>22784</sup> mutant embryos is provided in Figure 6F.

**Discussion**

**A Second Genetic Vertebrate Model for Loss of tal1 Function**

We have identified a truncating mutation in the zebrafish tal1 gene, making this the second species for which a genetic mutation for this important hematopoietic transcription factor is available. The tal1<sup>21384</sup> allele contains a truncating mutation that deletes the conserved bHLH domain of the protein. Functional interaction of Tal1 and Lmo2 is required for the early stages of vascular and hematopoietic lineages in the zebrafish [45], and this interaction occurs at the second helix, a region that is deleted by the tal1<sup>22784</sup> mutation. Moreover, expression of only the helix-loop-helix domain was sufficient to rescue hematopoietic and endothelial development in tal1 morphants [45], indicating that the N-terminal part of the Tal1 protein is dispensable for early hematopoietic and endothelial development. Therefore, we conclude that the tal1<sup>22184</sup> mutation leads to a complete loss of tal1 function during early cardiovascular and hematopoietic development.

**Loss of a Fourth VEGF Receptor Gene during Mammalian Evolution**

We used a transgenic line under the control of the zebrafish vegfr4 promoter to follow endocardial development. Surprisingly, vegfr4 represents a fourth VEGF receptor class with orthologues in all vertebrates—except placental mammals—for which sufficient genome information is available. This fourth VEGF receptor class most likely arose as a consequence of the two proposed whole-genome duplication events that occurred before vertebrate divergence [46]. This is evidenced by the phylogenetic tree of the vertebrate VEGF receptors, which places the emergence of this fourth class prior to the divergence of teleost fish and terrestrial vertebrates. Linkage of class III receptor tyrosine kinases (which includes the VEGF receptors) to a caudal-type homeobox gene is conserved in the basal chordate amphioxus [47], suggesting that this configuration represents the ancestral state.

Our data strongly suggest the loss of a fourth VEGF receptor within the mammalian lineage, as we identified an orthologue of vegfr4 in the genome of the opossum Monodelphis domestica, tightly linked to the Cdx4 gene. This implies that the loss of a mammalian vegfr4 orthologue occurred relatively recently—after the divergence of the placential (eutherian) and marsupial mammals ~180 million years ago, but before the mammalian radiation. Interestingly, the human and mouse Cdx4 genes are found adjacent to the XIST noncoding RNA that regulates X-chromosome inactivation. XIST originated through pseudogenization of the LNX3 gene [48], of which an orthologue is also found in the zebrafish vegfr4-cdx4 locus. The differences between marsupial and placental mammals in the mechanism of X-chromosome inactivation [49], together with the observation that Cdx4 has a role during placental development [50], and the absence of a fourth VEGF receptor in placental mammals present in other vertebrate species (this study) suggest that the cdx4–vegfr4 locus has been an important hotspot during mammalian evolution.

The observation that noneutherian vertebrates have a fourth VEGF receptor has consequences for understanding of the relationship of tal1 and the VEGF receptors. The murine KDR orthologue, Flk1, was found to function upstream of Tal1, and Tal1 expression was not detected in Flk1<sup>−/−</sup> embryos [51]. However, the putative zebrafish flk1 orthologue was found to be downstream of tal1 during zebrafish hematopoietic development [26], leading to controversy in the understanding of their interactions. Here, we redefine the orthology among the different VEGF receptor classes, and importantly, we identify the true Flk1/KDR orthologue in the zebrafish (kdr). We find that early nonendothelial kdr expression, which starts 3–4 h before the onset of tal1 expression, is not affected in tal1<sup>21384</sup> mutants. Therefore, we conclude that tal1 does not function as an essential factor for the initiation of kdr expression. Rather, maintenance of high-level endothelial expression of this gene is dependent on tal1.

**Morphogenesis and Embryonic Origins of the Endocardium**

Lough and Sugi [2] reviewed data on endocardial morphogenesis in higher vertebrates and proposed that endocardial and myocardial precursors are both derived from the same anatomic location: the cardiogenic mesoderm. From there, endocardial precursors migrate as single cells in between the myocardium and the underlying endoderm. Subsequently, endocardial precursors assemble in two bilateral vascular chords that later on fuse to form the inner lining of the primitive heart tube. Our data show that endocardial morphogenesis in the zebrafish embryo differs in at least
two important aspects from that found in higher vertebrates. First, endocardial precursors appear to be derived from a distinct anterior region of the cardiogenic mesoderm, and require posterior migration to the site of heart tube formation. This difference is potentially due to the lack of dynamic observations of endocardial precursor migration in higher vertebrates. Indeed, some data indicate that in higher vertebrates, the endocardium arises from a specific region of the cardiogenic mesoderm [52]. Our results give a first look at the origin of endocardial cells during early development. However, to obtain conclusive evidence regarding the cell movements and origins of the endocardium, higher-resolution fate mapping and cell tracing will be required. Second, zebrafish endocardial precursors do not assemble into bilateral vascular chords, but form a sheet medial to the myocardial precursors. The bilayered disc that is formed through fusion of the myocardial primordia over the endocardium is then converted into the primitive heart tube.

This last difference is potentially due to differences in developmental timing between fusion of bilateral primordia and heart tube morphogenesis in zebrafish and higher vertebrates.

Using the vegfr4:gfp and fi1agfp lines and two-color in situ hybridization, we suggest that the endocardium of the primitive heart tube forms from two bilateral precursor populations that are located immediately anterior to the bilateral myocardial precursor populations that express nkx2.5. The finding that the endocardium might arise from a region anterior to the myocardial primordia is surprising, given the lineage-tracing experiments performed by Serbedzija and coworkers [53]. In their study, cells were labeled anterior to the nkx2.5-expressing myocardial primordia at the ten- to 12-somite stage. At 33 hpf, these cells were found to contribute to the “head mesenchyme” and no cells were found in the heart. One explanation for this finding is that only cells were labeled immediately anterior to the nkx2.5-expressing region. Our time-lapse imaging indicates that these cells give rise to the aortic arches and the head vasculature, whereas endocardial precursors originate from a position within the part of the anterior lateral plate mesoderm that is slightly more anterior and ventral. Therefore, Serbedzija and coworkers most likely have not labeled the cells that we show here constitute the endocardial precursors.

Recently, Kattman et al. [3] used the differentiation of murine embryonic stem cells to show the existence of a cell population expressing Flk1, with the potential to form both myocardial and endocardial cells in vitro. Our data indicate that in the zebrafish embryo, there are anatomically separate populations of endocardial and myocardial precursor cells during early developmental stages. We show that during normal development both lineages undergo different morphogenetic movements that are suggestive of early lineage separation of endocardium and myocardium. However, this does not imply that these precursors are restricted to one particular differentiation fate at this stage, and given alternative (ex vivo) cues they can still contribute to both lineages. Our data also indicate that anatomically, the endocardial precursors are found in close association with a particular subset of the hematopoietic lineage in the zebrafish: the anterior population of primitive myeloid cells. Lineage tracing experiments will identify whether there exists a lineage relationship with a common endocardial–myeloid progenitor, or whether endocardial and myeloid precursors are simply intermingled during one stage of their development. While resolving this specific question is beyond the scope of this paper, it is worth noting that we observed migrating cells with macrophage morphology that have low levels of vegfr4:gfp expression originating from the same bilateral populations of cells as those giving rise to the endocardium and the head vasculature (Figure 4A). This transgene is not expressed in differentiated primitive myeloid cells, and so the signal potentially represents residual gfp protein from a previous stage of their development.

Conclusion

How the endocardial lining of the primitive heart tube becomes established is one of the least-understood aspects of cardiac morphogenesis [2]. In recent years, studies on zebrafish embryos have provided significant insight into the genetic regulation of myocardial development. We show here that a similar approach can be taken to study endocardial development. Given the many interactions between the myocardium and endocardium, both during development as well as in adult physiology and disease [54], our findings will provide a more comprehensive understanding of the morphogenesis and genetic regulation of the heart.

Supporting Information

Figure S1. kdr Expression during Gastrulation
At the tailbud (TB) stage, kdr but not vegfr4 is expressed. Expression of kdr is diffuse, but highest around the tailbud and near the embryonic axis. Found at doi:10.1371/journal.pgen.0030140.sg001 (4.3 MB EPS).

Figure S2. Endothelial Gene Expression in the Trunk
Trunk expression of different markers in wt and tal121384 mutant embryos at 24–30 hpf for general endothelial (cdh5, vegfr4), venous (flt4, dah2), and arterial (notch3, ephrinB2) differentiation. In mutant embryos, expression of arterial marker genes is reduced or absent, whereas the expression domain of venous marker genes is expanded to the dorsal aorta. Found at doi:10.1371/journal.pgen.0030140.sg002 (8.1 MB EPS).

Figure S3. Presence of Endothelial Cells in the Region of the Dorsal Aorta
Immunohistochemistry using anti-GFP antibody in the fi1agfp background; brown labeling indicates gfp-positive endothelial cells. Although no luminized vessels in the trunk are formed in tal121384 mutants, endothelial cells are present in the location of the dorsal aorta (arrows), and some intersegmental vessels form (arrowhead). Found at doi:10.1371/journal.pgen.0030140.sv003 (7.2 MB EPS).

Video S1. Time-Lapse Movie of Normal Endocardial Development in the vegfr4:gfp Transgenic Background
Found at doi:10.1371/journal.pgen.0030140.sv001 (4.9 MB MOV).

Video S2. Time-Lapse Movie of Normal Endocardial Development in the fi1agfp Transgenic Background
Found at doi:10.1371/journal.pgen.0030140.sv002 (2.8 MB MOV).

Video S3. Time-Lapse Movie of Endocardial Development in tal121384 Mutants in the vegfr4:gfp Transgenic Background
Found at doi:10.1371/journal.pgen.0030140.sv003 (2.2 MB MOV).

Accession Numbers

The National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide) accession numbers for the nucleotide sequences of the zebrafish VEGF receptors are kdr, AY529999; flt1, AY534000; and flt4, AY5324001. The NCBI GenBank and Ensembl (http://www.ensembl.org) acces-
sion numbers for the genes and gene products (other than the zebrafish VEGF receptors noted above) used for phylogeny reconstruction are Flk1 (mouse/Mus musculus), NP_034742; Flt1 (chick/Gallus gallus), NP_059853; Flt1 (human/Homo sapiens), NP_020210; Flk1 (mouse), NP_054358; Flt1 (chick), XP_414600; Flt1 (human), NP_002011; Flt1 (mouse), NP_052055; KDR (frog/Xenopus tropicalis), ENSXETG00000020161; KDR (human), NP_002444; kdr (medaka/Oryzias latipes), GENSCAN000000045289; KDR (opossum/ Monodelphis domestica), encoded by ENSMODOG00000020673; KDR (platypus/Ornithorhynchus anatinus), ENSOANG0000000053802; kdr (stickleback/Gasterosteus aculeatus), ENSGACG000000014277; kdr (tetraodon/Tetraodon nigroviridis), GSTENG00032761001; KDR (chick), NP_000104568; VEGFR4 (chick), XP_420292; VEGFR4 (frog), GENSCAN0000000035479; vegfr4 (fugu/Takifugu rubripes), SINFRG00000013563; vegfr4 (medaka), ENSLRG000000002936; VEGFR4 (platypus), ENSMODG00000020842; VEGFR4 (human), ENSHUMG000000002222; vegfr4 (stickleback), ENSSCORL00000011717; vegfr4 (zebrafish), GSTENG000312295001; and vegfr4 (zebrafish), NP_571547.

References

1. Olson EN (2006) Gene regulatory networks in the evolution and development of the heart. Science 313: 1922–1927.
2. Lough J, Sugi Y (2000) Endoderm and heart development. Dev Dyn 217: 327–342.
3. Kato Y, Huber TL, Keller GM (2006) Multipotent flk-1(+) cardiovascular progenitor cells give rise to the cardiovascular, endothelial, and vascular smooth muscle lineages. Dev Cell 11: 725–732.
4. Schultz-Ehms TM, Burch JB, Lassar AB (1997) A role for bone morphogenetic protein inhibitors in the formation of cardiac myocytes. Dev Genes Dev 11: 565–578.
5. Nemer G, Nemer M (2002) Cooperative interaction between GATA5 and NF-ATc regulates endothelial-endocardial differentiation of cardiogenic cells. Development 129: 4045–4055.
6. Colman-Gould LI, Mikawa T (1996) The fate diversity of mesodermal cells within the heart field during chick early embryogenesis. Dev Biol 177: 265–273.
7. Lee RK, Stainier DY, Weinstein BM, Fishman MC (1994) Cardiovascular development in the zebrafish. II. Endodermal progenitors are sequestered within the heart field. Development 120: 3363–3366.
8. Keegan BR, Meyer D, Yelon D (2004) Organization of cardiac chamber progenitors in the zebrafish blastula. Development 131: 3081–3091.
9. Chen JN, Fishman MC (1996) Zebrafish tinman homolog demarcates the heart field and initiates myocardial differentiation. Development 122: 3896–3918.
10. Glickman NS, Yelon D (2002) Cardiac development in zebrafish: Coordination of form and function. Semin Cell Dev Biol 13: 507–515.
11. Stainier DY, Lee RK, Fishman MC (1993) Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation. Development 119: 31–40.
12. Yelon D, Horne SA, Stainier DY (1999) Restricted expression of cardiac muscle genes reveals regulated aspects of heart tube assembly in zebrafish. Dev Biol 214: 23–37.
13. Drake CJ, Fleming PA (2000) Vascularization in the day 6.5 to 9.5 mouse embryo. Blood 95: 1671–1679.
14. Gotoh Y, Yamada K, Rabbits TH, Patient RK (2003) Lmo2 and Scl/Tal1 convert non-axial mesoderm into haemangioblasts which differentiate into endothelial cells in the absence of Gata1. Development 130: 6187–6199.
15. Shvidrasani RA, Mayer EL, Orkin SH (1995) Absence of blood formation in mice lacking the T-cell leukaemia oncprotein tal1-scl. Nature 373: 432–434.
16. Robb L, Lyons IL, Ri L, Hartley L, Kontgen F, et al. (1995) Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. Proc Natl Acad Sci U S A 92: 7075–7079.
17. Visvaenser JE, Fujiiwara Y, Orkin SH (1998) Unsuspected role for the T-cell leukemia protein SCL/Tal1 in vascular development. Genes Dev 12: 473–483.
18. Pettersson J, Gering M, Patient R (2005) Scl is required for dorsal aorta as well as blood formation in zebrafish embryos. Blood 105: 3502–3511.
19. Dooley KA, Davidson AJ, Zon LI (2005) Zebrafish scl functions independently in hematopoietic and endothelial development. Dev Biol 277: 322–336.
20. Jareau MA, Su F, Chun S, Kiel MJ, Lyons SE (2005) Distinct roles for SCL in erythroid specification and maturation in zebrafish. J Biol Chem 280: 41636–41644.
21. Holzbach R, Olethra J, Waldenreich B, Mainschein H, Schulte-Merker S (2002) Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. Curr Biol 12: 1405–1412.
22. Cassinoni LD, Villefranche JA, Kacergis MC, Weinstein BM, Lawson ND (2006) Distinct genetic interactions between multiple Vegf receptors are required

Acknowledgments

We thank Christine Mummery and Ben Hogan for editorial review, Youri Adolfs and Jeroen Korting for immunohistochemistry and tissue processing, Josi Peterson-Maduro and Hinrich Habeck for cloning the zebrafish VEGF receptors, and the Tübingen 2000 screen consortium for identifying the icf23/tal1 allele. The allele was identified by S. Schulte-Merker while working at Exelixis Deutschland GmbH. We also thank members of the Schulte-Merker laboratory for comments and scientific discussions.

Author contributions. J. Bussman conceived and performed experiments and wrote the paper. J. Bakers conceived the experiments in Figure 3. S. Schulte-Merker conceived experiments and wrote the paper.

Funding. J. Bussman is supported by a Boehringer Ingelheim Fonds PhD Scholarship.

Competing interests. The authors have declared that no competing interests exist.
for development of different blood vessel types in zebrafish. Proc Natl Acad Sci U S A 103: 6554–6559.

44. Trinh LA, Stainier DY (2004) Fibronectin regulates epithelial organization during myocardial migration in zebrafish. Dev Cell 6: 371–382.

45. Patterson LJ, Gering M, Eckfeldt CE, Green AR, Verfaillie CM, et al. (2006) The transcription factors, Scl and Lmo2, act together during development of the haemangioblast in zebrafish. Blood 109: 2389–2398.

46. Dehal P, Boore JL (2005) Two rounds of whole genome duplication in the ancestral vertebrate. PLoS Biol 3: e314. doi:10.1371/journal.pbio.0030314

47. Ferrier DE, Dewar K, Cook A, Chang JL, Hill-Force A, et al. (2005) The chordate ParaHox cluster. Curr Biol 15: R820–R822.

48. Duret L, Chureau C, Samain S, Weissenbach J, Avner P (2006) The Xist RNA gene evolved in eutherians by pseudogenization of a protein-coding gene. Science 312: 1653–1655.

49. Cooper DW (1993) X-inactivation in marsupials and monotremes. Seminars in Developmental Biology 4: 117–128.

50. van Nes J, de Graaff W, Lebrin F, Gerhard M, Beck F, et al. (2006) The Cdx4 mutation affects axial development and reveals an essential role of Cdx genes in the ontogenesis of the placental labyrinth in mice. Development 133: 419–428.

51. Ema M, Faloon P, Zhang WJ, Hirashima M, Reid T, et al. (2005) Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse. Genes Dev 17: 380–393.

52. Sugi Y, Markwald RR (1996) Formation and early morphogenesis of endocardial endothelial precursor cells and the role of endoderm. Dev Biol 175: 66–83.

53. Serbedzija GN, Chen JN, Fishman MC (1998) Regulation in the heart field of zebrafish. Development 125: 1095–1101.

54. Hsieh PC, Davis ME, Lisowski LK, Lee RT (2006) Endothelial-cardiomyocyte interactions in cardiac development and repair. Annu Rev Physiol 68: 51–66.