Introduction

The cardiovascular system of vertebrates provides a means to transport nutrients and to waste away from cells throughout the organism. As such, this system is critical for the survival of the organism. The cardiovascular system is made up of the vasculature and the blood that flows through it. It has been proposed that a common cell type, known as hemangioblasts, contribute to the development of vascular and blood cells [1,2,3,4,5]. This idea is supported by experimental evidence from several different organisms, notably the derivation of both blood and vascular endothelial cells from cultured mouse embryonic stem cells [6,7,8], and through in vivo lineage tracing studies in zebrafish and chick/quail chimeras [9,10]. The zebrafish mutant line cloche provides genetic evidence since homozygous mutants lack both hematopoietic and vascular endothelial cells but organogenesis is otherwise normal [11]. As bi-potential precursor cells, hemangioblasts produce cells with more restricted potentials, angioblasts and hematopoietic progenitors [3]. Angioblasts subsequently produce the endothelial cells that line the vasculature during both vasculogenesis and angiogenesis, while hematopoietic progenitors differentiate into distinct blood lineages through hematopoiesis. Although some of the molecular factors that orchestrate these processes are known, many remain to be identified and studied.

Microarray studies were previously used to identify multiple novel hematopoietic and vascular genes misexpressed in the cloche mutant [12,13,14]. A particularly important gene identified in these studies was the transcription factor, Ets1-related protein (Etsrp), which is both necessary and sufficient to direct the development of the vascular and primitive myeloid lineages [13,15,16]. In zebrafish, hematopoietic/vascular development is separated into two distinct anatomical locations, the anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM). The ALM gives rise to both primitive myeloid cells as well as the endothelial cells of the head vasculature. The PLM gives rise to primitive erythroid cells and the endothelial cells of the trunk and tail. Etsrp is expressed in both the ALM and PLM. Loss of etsrp function by morpholino antisense or genetic mutation, termed y11, results in loss of primitive myeloid cells and disrupted vasculogenesis and angiogenesis in the head and trunk. The defective vasculature in etsrp knockdown or mutant fish appears to be due to altered gene expression and cell behavior and not simply a loss of cells as the number of flk1/gfp transgene positive cells is similar to control animals [15]. In contrast, the complete loss of pu.1 staining in ects knockdown and mutants suggests that primitive myeloid cells are never specified [16,17]. Interestingly, the primitive erythroid population in the PLM appears relatively normal when ects function is blocked. Thus, ects is critical for primitive myeloid and endothelial development from the ALM and endothelial development from the PLM but not the primitive erythroid cell population of the PLM.

A mammalian homolog of ects, ER71/Etv2, has recently been identified. ER71/Etv2 knockout mice exhibit loss of vasculature and primitive erythrocytes suggesting it functions in the developing hemangioblast [18]. Additionally, overexpression of ER71/Etv2 in zebrafish embryos causes the ectopic induction of the hemangioblast marker scl/tal1 and the flk1/gfp transgene, identical to the results of ects overexpression. Therefore, both ects and ER71/Etv2 play an evolutionarily conserved role in hematovascular development.
As members of the Ets transcription factor gene family, Etsrp and ER71/Etv2 have a conserved DNA binding domain and presumably act as transcriptional activators. Limited in situ hybridization analysis of etsrp morpholino gene knockdown or y11 mutant embryos demonstrate that etsrp is necessary for the expression of flk1, scl, fli1, pu.1 and a few other known genes specific to vascular and hematopoietic cells [13,19]. Additionally, overexpression of etsrp in zebrafish embryos can ectopically induce vascular and myocardial gene expression [16]. We therefore decided to search for novel blood and vascular related genes downstream of etsrp by analyzing expression profiles of zebrafish embryos overexpressing etsrp. Here we report the identification of 14 genes with little or no prior investigations that are expressed in blood and/or vascular endothelial cells and demonstrate the function of two of these genes, hapln1b and sh3gl3, in vascular development using morpholino antisense in transgenic zebrafish embryos.

Results

In order to identify novel blood or vascular related genes downstream of etsrp, the expression profiles of embryos ectopically expressing etsrp at late gastrulation stages, 80% epiboly to tail bud were compared to control embryos by microarray analysis. The flk1:gfp transgenic line was used to identify embryos successfully expressing etsrp since ectopic etsrp induces flk1:gfp expression. Neither endogenous or transgenic flk1:gfp is normally expressed at 80% to tail-bud stage [20,21], while early ectopic expression of flk1:gfp is evident in embryos injected with 75 pg of synthetic etsrp mRNA (Figure 1A). To ensure that the ectopic expression of flk1:gfp is induced by etsrp specifically, we injected up to 500 pg of synthetically transcribed mRNA encoding RFP tagged histone H2B, H2B-RFP1, as a control. Similar to un.injected controls, flk1:gfp expression is not induced in H2B-RFP1 injected embryos (Figure 1C and 1D).

Prior to microarray analysis total RNA was extracted from pools of 70 embryos per treatment group and the change in expression of genes known to be induced by etsrp overexpression (OE) were assessed by quantitative RT-PCR. Both flk1a (11-fold) and scl (7.1×) were significantly induced by etsrp OE, while overexpression of H2B-RFP1 resulted in no difference from un injected controls (Figure S1). Gene expression analysis was then performed using a microarray chip containing 34,647 oligos representing approximately 20,000 genes, 686 genes were identified as being induced greater than two-fold by ectopic etsrp expression. Although some of the genes affected are not restricted to the circulatory system (Figure S2), a great number of previously identified hematopoietic and vascular genes were induced, validating the microarray strategy used in this study. Table 1 lists the 59 genes with the greatest fold induction as measured on the microarray.

From the genes affected by etsrp OE, we set up four criteria to select candidates for further studies: 1. They have not been well studied in hematopoiesis and/or vasculogenesis; 2. They are induced by ectopic etsrp in embryos as validated by whole mount in situ hybridization (WISH); 3. They are expressed in hematopoietic and/or vascular tissues; and 4. Expression is reduced by etsrp deficiency.

To identify genes with few or no prior studies for further analysis, the list of genes induced by etsrp OE was compared to the data sets for cloche mutants [13,14]. We reasoned that genes identified in both etsrp OE and cloche mutant microarrays were likely to be involved in blood and/or vascular development. From this group of overlapping genes we screened for genes that had not been well studied using the PubMed database at the NCBI website. This resulted in the selection of 31 genes (Table 2), which were cloned and analyzed further.

To validate the results obtained with the etsrp OE microarray, WISH was performed on embryos at the same developmental stage as was done for the microarray, 80% epiboly to tailbud. To ensure that the OE samples examined were indeed ectopically expressing etsrp, embryos were injected with plasmid DNA encoding an Etsrp-mcherry fusion protein. This fusion protein was functionally active since it efficiently induced ectopic expression of flk1:gfp when injected into zebrafish embryos (data not shown). Embryos expressing Etsrp-mcherry and flk1:gfp were selected to test for ectopic induction for 23 of the 31 genes identified in Table 2. 21 of 23 genes were clearly induced ectopically by etsrp OE. Figure 2 shows the ectopic induction of the 14 genes identified below to be relevant to blood and/or vascular expression. There was ubiquitous endogenous expression in 2/23 genes, arhgef9 and znf383, prohibiting any qualitative discernment of their changes in expression by etsrp OE (data not shown). Overall, this demonstrates that the microarray data is low in false positives and reliable.

To screen the 31 selected genes for blood and/or vascular expression, their expression patterns were examined in wild type embryos by WISH at 24–30 hours post fertilization (hpf). Of the genes examined, two are expressed in blood lineages (Figure 3), and twelve in vasculature (Figure 4), while sixteen other genes demonstrate tissue specificity but are irrelevant to blood or vessels (Figure S2).

The dependence of the new blood and vascular specific genes on etsrp was then examined by comparing their expression in etsrp morphants to uninjected control embryos (Figure 5). In etsrp morphants, the expression of kmrl2 (Figure 5A) and trp33

![Figure 1. Early ectopic expression of transgenic flk1:gfp is induced by etsrp overexpression.](image-url)
Table 1. Top 59 genes induced by overexpression of etsrp.

| GB accession | Unigene ID | Location | Gene name | Fold induction |
|--------------|------------|----------|-----------|----------------|
| BI980779     | Dr.6315    | chr14    | Cdc42 guanine nucleotide exchange factor (GEF) 9 | 55.92          |
| AI965222     | Dr.45597   | chr14    | Cadherin 5 (cdh5) | 54.96          |
| DQ021472.1   | Dr.47591   | chr16    | Ets1b/etsrp `Y` | 47.01          |
| AW233044     | Dr.24158   |          | Serglycin | 39.62          |
| NM, 131626   | Dr.81423   |          | HAND2     | 34.52          |
| AF045432     | Dr.75812   | chr22    | Tal1/SCL  | 34.35          |
| NM, 152964   | Dr.17548   | chr6     | Gastrulation brain homeo box 2 (Gbx2) | 27.73          |
| NM, 131768   | Dr.3499    | chr3     | Claudin I | 26.36          |
| AW420632     | Dr.12726   | chr21    | Yes-related kinase | 25.02  |
| NM, 131530   | Dr.5729    | chr11    | Homeo box C6b | 24.88          |
| Al601685     | Dr.5365    | chr22    | Dual specificity phosphatase 5 (dusp5) | 24.52          |
| BI673802     | Dr.113768  | chr11    | Fgd5      | 23.26          |
| AI477500     | Dr.4847    | chr11    | Wnt7A     | 20.14          |
| BII80801     | Dr.83871   | chr17    | Rasgrp3   | 20.10          |
| AW115759     | Dr.23967   | chr17    | Complement receptor C1qR-like | 19.35          |
| AI477956     | Dr.76040   | chr8     | Nexillin f actin binding | 19.32          |
| AW595297     | Dr. 105109 | chr8     | UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3 | 18.21          |
| YI4538       | Dr.5730    | chr19    | Homeo box A9a | 17.82          |
| BII82764     | Dr.14186   | chr17    | Schc211-214p16.1 | 17.50          |
| AI793554     | Dr.104443  | chr16    | Cellular retinoic acid binding protein 2a | 15.98          |
| BII82214     | Dr.13039   | chr15    | Solute carrier family 43, member 2 | 14.62          |
| BM181739     | Dr.8453    | chr2     | PL2A-like otoconin | 14.23          |
| AI959344     | Dr.28288   | chr7     | Matrix metalloproteinase precursor | 13.34          |
| BM036580     | Dr.84866   | chr16    | Similar to hemicentin | 12.93          |
| AB055670     | Dr.30464   | chr23    | Wnt1      | 12.77          |
| YI4532       | Dr.75789   | chr12    | Homeo box B6b | 12.71          |
| AI626374     | Dr.2644    |          | Dehydrogenase/reductase (SDR family) member 3 | 12.60          |
| AI477237     | Dr.77024   | chr13    | Similar to Olig3 protein | 12.31          |
| AW279740     | Dr.22713   | chr23    | Zinc finger protein 385 | 12.18          |
| BI982208     | Dr.11052   | chr2     | Ornithine decarboxylase antizyme 2, like | 11.42          |
| BM103177     | Dr.24950   |          | Creatine kinase CKM3 | 11.27          |
| AW076731     | Dr.23392   | chr25    | integrin alpha, VLA protein type | 10.63          |
| AW233713     | Dr.81043   |          | Dusp2     | 10.56          |
| BG892475     | Dr.11064   | chr2     | amyloid precursor-like protein 2 (aplp2) | 10.31          |
| AA605884     | Dr.38472   |          | cDNA clone IMAGE:7137566 | 10.22          |
| YI4527       | Dr.5721    | chr17    | Homeo box A10b | 10.15          |
| NM, 131359   | Dr.75781   |          | Bone morphogenetic protein 2a | 9.90           |
| NM, 131419   | Dr.272     |          | NK2 transcription factor related 7 | 9.81           |
| AI626636     | Dr.45596   |          | Adrenomedullin receptor (admr) | 9.76           |
| NM, 131070   | Dr.20373   |          | Midkine-related growth factor | 9.68           |
| AW233616     | Dr.121119  |          | Calpain 8 | 9.52           |
| AI522349     | Dr.44232   |          | Rhoub     | 9.46           |
| NM, 131000   | Dr.20912   |          | Activated leukocyte cell adhesion molecule | 9.29           |
| BG985561     | Dr.8617    | chr2     | Hyaluronan and proteoglycan link protein 1b | 9.21           |
| NM, 131767   | Dr.26572   |          | Claudin h (cldh) | 9.09           |
| NM, 131101   | Dr.5572    |          | Homeo box B5a | 8.81           |
| AF071249     | Dr.28647   | chr17    | Homeo box A9b | 8.47           |
| BG727645     | Dr.11977   | chr16    | Leucine rich repeat 33 | 8.00           |
| BG985673     | Dr.24989   | chr10    | Myosin light chain 2 like | 7.80           |
| Y13948       | Dr.21032   | chr9     | Homeo box D3a | 7.71           |
Table 1. cont.

| GB accession | Unigene ID | Location | Gene name                        | Fold induction |
|---------------|-----------|----------|----------------------------------|----------------|
| AI601355      | Dr.77902  |          | Similar to Fraser syndrome 1 isof 1 | 7.61           |
| NM_131419_1   | Dr.272    |          | NK2 transcription factor related 7 | 7.60           |
| BM025541      | Dr.3373   | chr9     | Collagen type XVIII, alpha 1      | 7.56           |
| AF071568      | Dr.20962  | chr19    | Homeo box B2a                     | 7.43           |
| AY028584      | Dr.18294  |          | Prostaglandin-endoperoxide synthase 1 | 7.38       |
| AI601770      | Dr.21376  | chr23    | Plexin precursor                  | 7.27           |
| AY721522      | Dr.8674   |          | Septin 9b                         | 6.78           |
| BG028550      | Dr.81575  | chr15    | Cytochrome b5 reductase 4         | 6.58           |
| AI964237      | Dr.76050  |          | ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1 | 5.68 |

Note: Genebank accession number and unigene ID correspond to the probes on the array. The fold change column is the average ratio of eight measurements. ¥ The large induction ratio of etsrp reflects array probe hybridization to both the exogenously injected RNA as well as the induced expression of endogenous etsrp. (See Supplementary Figure 1).

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(Figure 5B) was abolished in myeloid cells, while the remaining 12 vascular genes were also significantly reduced in expression, most noticeably in the trunk region containing the dorsal aorta, posterior cardinal vein, and intersomitic vessels (Figure 5, C-N). Note that gene expression in the cranial vasculature was less affected in etsp morphants but still reduced. Gene expression in tissues outside of blood such as somites for krml2 (Figure 5A), neurons for rasgrp3 (Figure 5D) and ldb2 (Figure 5L), and tailbud for tem8 (Figure 5E) were not affected by etsp knockdown. This demonstrates that etsrp is not only sufficient but also required for normal expression in primitive myeloid and/or vascular cells in the 14 genes identified here.

Genes downstream of etsrp with endogenous expression in blood

**Krml2.** Krml2 is a member of the Maf family of basic leucine zipper transcription factors that have high evolutionary conservation with orthologs existing in chicken, *Xenopus*, mice, and humans [22,23]. The expression of krml2 in zebrafish was previously found in somites, reticulospinal oculomotor neurons, and lens [24]. In this study we have also detected its expression in primitive myeloid cells and the vasculature (Figure 3A). The mouse and avian orthologs of krml2 have also been detected in macrophages [25], and it has been demonstrated to regulate hematopoiesis by repressing erythropoiesis in favor of the myeloid fate through an interaction with Ets1 [26].

**LRRP33.** Leucine rich repeat containing protein 33 is a member of the leucine-rich repeat protein family. Lrrp33 is a single pass transmembrane protein that contains 21 leucine-rich repeats, and has orthologs in mice, rats, and humans, which remain to be characterized. The leucine-rich repeat family of proteins consists of over 60,000 members with diverse biological activities, but a common feature shared by proteins with LRR motifs is their propensity to associate with other proteins through LRRs [27]. Prominent members of this family with characterized roles in inflammatory and innate immunity are the toll-like receptors, which are comprised of ten members in humans, with homologs for all encoded in the zebrafish genome [28,29]. Lrrp33 is excluded from this subfamily because it lacks the cytosolic toll-interleukin (TIR) receptor domain through which TLRs transduce intracellular signals [30,31]. In this study we have detected lrrp33 expression in primitive myeloid cells and the caudal hematopoietic tail region (Figure 3B).

**Ras guanyl releasing protein 3 (RASgrp3).** RASgrp3 is a member of the RAS family of GTPases which link cell surface receptor activation and RAS activation by switching its state of activity between inactive GDP and active GTP bound states [32]. With orthologs in mice and humans, zebrafish RASgrp3 encodes a protein with 708 residues containing an N-terminal and catalytic RhoGEP domains, followed by a pair of calcium binding EF-hand domains and a zinc dependent DAG/phorbol-ester binding domain. RASgrp3 is expressed at low levels in both axial and cranial vasculature, and the caudal vascular plexus region, as well as the neural tube and hindbrain (Figure 4A). A role for RASgrp3 in endothelial cells was initially identified in an embryonic stem cell gene trap assay [33]. In endothelial cells RASgrp3 is up-regulated by VEGF signaling, and functions as a phorbol ester receptor in both B-cells and endothelial cells, where it has been ascribed with forming a disorganized angiogenic vasculature in disease states [33,34]. There are currently four RASgrp genes encoded in mammals, RASgrp1, -2, -3, and -4, while zebrafish only have three, RASgrp1, -2, and -3. The mouse ortholog is expressed in the vasculature during developmental stages, but the knockout is viable [33].

**Similar to tumor endothelial marker 8 (tem8)/Anthrax Receptor 1 (antxr1).** Tem8 was initially identified as a gene up-regulated in tumors, and is one of two anthrax toxin receptors, the other being capillary morphogenesis gene 2 (CMG2)/ANTXR2 which together recognize and internalize anthrax toxins [35,36,37]. Zebrafish tem8 encodes a 552 amino acid transmembrane protein with a von Willebrand factor type A domain, an extracellular Ig capillary morphogenesis gene 2 (CMG2)/ANTXR2 which together recognize and internalize anthrax toxins [35,36,37]. Zebrafish tem8 encodes a 552 amino acid transmembrane protein with a von Willebrand factor type A domain, an extracellular Ig anchrtox toxin receptor domain and the cytoplasmic c-terminal anthrax receptor domain. It is expressed at low levels in neurons with higher expression levels in the tailbud as well as the axial and cranial vascular endothelial cells (Figure 4B). Two cmg2 orthologs have been identified in zebrafish, cmg2a and cmg2b, but their expression remains to be examined [38]. Tem8 regulates cell adhesion and migration by coupling extracellular stimuli to changes in the actin cytoskeleton [39,40]. Regulation of cell
adhesion and migration by tem8 plays a role in angiogenesis and is relevant to tumor angiogenesis, anthrax toxin responses, and potentially circulatory system development.

RhoGTPase-activating protein 29 (arhgap29). Arhgap29 was identified in a screen for proteins that interact with the protein-tyrosine phosphatase PTPL1 (which is expressed in many tissues), and has the alternative name PTPL1-Associated RHOGAP1 (PARG1) [41]. Zebrafish arhgap29 is a guanine activating protein of RhoGTPases that is 1365 amino acids long with a protein kinase C domain and a RhoGAP domain which are conserved in orthologs in multiple species including humans. The expression of ARHGAP29 has been detected in the hearts of mouse embryos among other tissues [42], and in zebrafish it is expressed in the cranial vasculature, dorsal aorta, cardinal vein, intersomitic vessels, caudal vascular plexus, and hatching gland at 24 hpf (Figure 4C).

**Table 2. Genes screened for blood and/or vascular expression.**

| GB Accession | Unigene ID | Gene location | Gene name                                                                 | Fold Induction | WISH for etsrp OE | down in cloche |
|--------------|------------|---------------|---------------------------------------------------------------------------|----------------|------------------|----------------|
| BI980779     | Dr.6315    | chr14         | Cdc42 guanine nucleotide exchange factor GEF 9 (arhgef9)                   | 55.92          | X                | X              |
| AW171479     | Dr.2433    | chr12         | Carboxylesterase 2-like(cbe2)                                             | 3.32           | X                | X              |
| AF109780     | Dr.81287   |               | Kreisler mar-related leucine zipper homolog 2 (krmll2)                     | 4.82           | X                | X              |
| AF071255     | Dr.117289  | chr12         | Hoxb8b                                                                    | 3.57           | X                | X              |
| BI880801     | Dr.83871   | chr17         | RAS guanyl releasing protein 3 (rasgrp3)                                   | 20.51          |                  |                |
| BG727645     | Dr.11977   | chr16         | Leucine rich repeat containing 33 (lrp33)                                  | 8.00           | X                | X              |
| AW279790     | Dr.22273   | chr23         | Zinc finger protein 385 (znf385)                                           | 12.18          |                  |                |
| BI673727     | Dr.14541   | chr6          | Similar to leucine rich repeat containing 51 (lrp51)*                      | 6.08           |                  |                |
| AI544475     | Dr.33183   | chr13         | SPARC related modular calcium binding 2 (smoc2)                            | 6.68           |                  |                |
| BI867572     | Dr.9564    | chr21         | Similar to testis-expressed sequence 2(tex2)                               | 4.91           |                  |                |
| AW421939     | Dr.5562    | chr20         | Apolipoprotein B (apolipo b)                                               | 3.07           |                  |                |
| BI430050     | Dr.418     |               | Protocadherin2 gamma20 (pcdhg)                                             | 3.50           |                  |                |
| BI672391     | Dr.14449   |               | weakly similar to XP_422395.1 (novel 2)                                    | 5.61           |                  |                |
| BC093143     | Dr.20125   | chr1          | hypothetical LOC550501                                                     | 6.80           |                  |                |
| BI864031     | Dr.13409   | chr12         | Anthrax toxin receptor 1/similar to tumor endothelial marker 8 (antxr1/tem8) | 7.29           |                  | X              |
| BM095233     | Dr.17582   | chr23         | C1orf192 homolog                                                           | 4.47           |                  |                |
| BI671403     | Dr.120608  | chr24         | PTPL1-associated RhoGAP 1 (arhgap29)                                       | 5.65           |                  | X              |
| BI673802     | Dr.113768  | chr11         | Fyve rhogef and ph domain containing zinc finger fye domain containing protein 23 (Fgd5) | 23.26           | X                | X              |
| AY028584     | Dr.18294   |               | Prostaglandin-endoperoxide synthase 1 (ptgs1)                              | 7.38           |                  |                |
| BG985561     | Dr.8617    | chr2          | Hyaluronan and proteoglycan link protein 1 precursor (haplin1B)            | 9.21           |                  |                |
| BI842967     | Dr.76442   | chr8          | hypothetical LOC55335                                                      | 3.68           |                  |                |
| BI880784     | Dr.12941   |               | Similar to testican 3                                                      | 3.81           |                  |                |
| AW420632     | Dr.12726   | chr21         | Yes-relayed kinase (yrk)                                                   | 25.02          | X                | X              |
| BM036580     | Dr.84866   | chr16         | Similar to hemicentin                                                      | 12.93          |                  | X              |
| BI983776     | Dr.12218   | chr25         | SH3-domain GRB2-like 3 (sh3g3l3)                                           | 2.76           |                  | X              |
| AI884043     | Dr.24       | chr24         | Similar to immune costimulatory protein *                                 | 3.34           |                  | X              |
| X600095      | Dr.510     | chr23         | Hoxc3a                                                                    | 3.37           |                  |                |
| BI428793     | Dr.1218    |               | Lim domain binding 2 (ldb2)                                                 | 4.21           |                  | X              |
| AI721944     | Dr.21605   | chr3          | Est- AI721944                                                             | 2.92           |                  | X              |
| AW019729     | Dr.2015    | chr8          | Est- AW019729                                                             | 2.68           |                  | X              |
| AA542593     | Dr.7552    | chr23         | C20orf112                                                                 | 5.09           |                  |                |

Note: Genebank accession number and unigene ID correspond to the probes on the array. The fold change column is the average ratio of eight measurements. (*) ectopic induction was observed for lrp51 but no expression was detected between 24-30 hpf. Genes in bold indicate those that are expressed in blood or vascular endothelial cells at 24–30 hpf.

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**FYVE, rhoGEF and PH domain containing protein 5 (fgd5).** Fgd5 is a member of the Dbl homology (DH) protein family that functions as guanine nucleotide exchange factors for the RhoGTPases Rho, Rac and Cdc42 [43]. There are six paralogs of this gene in mammals. The family’s name is derived from the founding member, fgd1, which causes faciogenital dysplasia in humans when mutated [44]. Fgd5 has been widely conserved through evolution and, in zebrafish, Fgd5 is 1603 amino acids long with the common domains of all DH family members, which includes a DH domain followed by two plextrin homology PH domains that flank a FYVE containing zinc finger domain. Fgd5 is expressed in the endocardium of mice during development.
kinase domains. Unlike its ortholog in chicken, the zebrafish yrk is expressed exclusively in vascular endothelial cells where its function remains to be investigated (Figure 4E).

**Similar to hemicentin.** Hemicentin is an extracellular matrix ECM protein of the immunoglobulin superfamily identified originally in *c.elegans* and found to facilitate tissue organization and cell migration [47]. The human ortholog has been associated with age related macular degeneration [48] and in mice its expression has been observed at the pericellular ECM of various epithelial cells including embryonic trophoderm, skin, tongue, in addition to the ECM of some blood vessels [49]. Distinct from hemicentin, zebrafish similar to hemicentin differs in size, structure and possibly function. While hemicentin contains a von willberand A domain, 48 tandem Ig domains, multiple tandem epidermal growth factor repeats (EGFs), and a single fibulin-like C-terminal domain, zebrafish similar to hemicentin is 264 amino acids long, has a single Ig domain and a transmembrane domain. Its specific expression in the vascular system suggests a potential role for this gene in endothelial cells (Figure 4F).

**SH3 domain Growth factor Receptor Bound protein 2-like 3 (sh3gl3).** Sh3gl3 encodes for a protein that is 396 amino acids long which contains a Bin-Amphiphisin-Rvs (BAR) domain implicated in synaptic vesicle internalization, actin regulation, differentiation, and cell survival, and a src homology 3 (SH3) domain [50]. The mammalian SH3gl3, also referred to as endophilin A3 and EEN-B2, is a member of a small family of SH3 containing proteins known as the SH3GL family that includes three members, EEN, EEN-B1 and EEN-B2 [51]. In adult mice, EEN-B2 is expressed in the brain, testis and spinal cord, while its embryonic expression is ubiquitous and there appears to be a marked transient expression in the vascular system [52]. In zebrafish, sh3gl3 expression is vascular specific at the stage examined here (Figure 4G). Besides sh3gl3, paralogs in zebrafish include sh3gl1a, sh3gl1b, sh3gl2, sh3gl2b, and zgc:158742.

**Similar to immune costimulatory protein.** Similar to immune costimulatory protein is a novel gene that encodes a 372 amino acid long transmembrane protein with two tandem Ig-like domains at the N-terminus followed by a transmembrane domain. It is expressed in the cranial vasculature, the caudal vascular plexus, and is restricted to the artery in the axial vessels. It is also expressed in a spotted pattern throughout the ectoderm (Figure 4H).

**LIM domain binding protein 2 (ldb2).** LIM domain binding protein 2 (ldb2) is a LIM homeodomain co-activator that is one of four ldb genes found in zebrafish. Ldb2 has been reported to be induced in human vascular endothelial cells following stimulation with VEGF [53]. The expression of ldb2 in zebrafish has been described in the central nervous system and vasculature [54], which we confirmed in Figure 4I. We have also found that ldb2 is silenced in the axial vasculature of *etsrp* morphants (Figure 5L).

**Novel Expressed Sequence Tags (ESTs).** The overexpression of *etsrp* resulted in the identification of two ESTs that could not be linked to any annotated genes but which are clearly induced ectopically (Figure 2L and 2N), demonstrate vascular specific expression, including cranial, axial, caudal and intersomitic vessels (Figure 4J and 4K), and whose expression is absent in the axial and intersomitic vessels in *etsrp* morphants (Figure 5M and 5N). Their accession numbers are: AI721944 and AW019729.

**Hapln1b.** Hyaluronan and proteoglycan link protein 1b is a member of the vertebrate hyaluronan and proteoglycan link protein family which consists of four members in mice and
Figure 3. Expression of genes identified in primitive myeloid blood cells. Gene expression was examined at 24–28 hours post fertilization by whole mount in situ hybridization in wildtype embryos. (A) krml2 is expressed in primitive myeloid cells (dispersed cells labeled throughout yolk and head) in the posterior cardinal vein, caudal hematopoietic tail region, and somites. (B) Lrp33 is expressed in primitive myeloid cells and in the caudal hematopoietic tissue. Abbreviations: pm, primitive myeloid; pcv, posterior cardinal vein; cht, caudal hematopoietic tail region; ss, somites. Scale bar: 250 μm. doi:10.1371/journal.pone.0004994.g003

Figure 4. Expression of genes identified in vascular endothelial cells. Gene expression was examined at 24–28 hours post fertilization by whole mount in situ hybridization in wildtype embryos. (A) rasgrp3 is expressed weakly in the forebrain, hindbrain, spinal cord, intersomitic vessels, dorsal aorta, and caudal hematopoietic tail region. (B) tem8 is expressed highly in the cranial vasculature, the dorsal aorta and posterior cardinal vein, tailbud and weakly in the intersomitic vessels and spinal cord. (C) arhgap29 is expressed in the cranial vessels, dorsal aorta, posterior cardinal vein, intersomitic vessels, caudal hematopoietic tail region, and hatching gland. (D) fgd5 is expressed in the cranial vasculature, dorsal aorta, posterior cardinal vein, intersomitic vessels, and caudal hematopoietic tail region. (E, F, and G) yrk, similar to hemicentin, and sh3gl3 are all expressed in the cranial vasculature, dorsal aorta, posterior cardinal vein, intersomitic vessels, and caudal vascular hematopoietic tail region. (H) similar to costimulatory protein is expressed in the cranial vasculature, dorsal aorta, intersomitic vessels, and caudal hematopoietic tail region; additional expression is present in the ectoderm layer throughout the embryo. (I) ldb2 is expressed in the forebrain, midbrain-hindbrain boundary, dorsal aorta, intersomitic vessels, and caudal hematopoietic tail region. (J) est:A172944 and est:AW019729 are expressed in the cranial vasculature, dorsal aorta, posterior cardinal vein, intersomitic vessels, and caudal hematopoietic tail region. (L) hapln1b is expressed in the cranial vasculature, posterior cardinal vein, and hypochord. Abbreviations: f, forebrain; h, hindbrain; sc, spinal cord; cv, cranial vasculature; da, dorsal aorta; pcv, posterior cardinal vein; isv, intersomitic vessels; cht, caudal hematopoietic tail region; tb, tail bud; h, hatching gland; and hc, hypochord. Scale bar: 250 μm. doi:10.1371/journal.pone.0004994.g004
humans, HAPLN-1-2,-3,-4 [55]. Hapln1, also known as cartilage link protein, is a secreted extracellular matrix protein that stabilizes aggrecan-hyaluronan complexes in cartilage and other tissues, with the knockout in mice resulting in dwarfism and craniofacial abnormalities [56]. Zebrafish also contain four hapln genes, hapln-1a, -1b, -2, and -3. During early stages of zebrafish development, hapln1b is expressed in the hypochord, cranial vasculature, posterior cardinal vein, and caudal hematopoietic tail region [Figure 4L and [57]].

Functional Analysis of hapln1b and sh3gl3

To determine if the genes highlighted here might play a functional role in vascular development we used antisense morpholino (MO) oligos to knockdown hapln1b and sh3gl3 in flk::gfp transgenic fish. Hapln1b knockdown using 2 ng of morpholino results in the arrested angiogenic sprouting of many intersomitic vessels (asterisks in Figure 6B, 6D and 6F), preventing them from forming stereotypical dorsolateral anastamosing vessels (dlavs) by 48 hpf (arrow in Figure 6D). Furthermore, the vascular
remodeling that occurs at the caudal vascular plexus around 28–30 hpf is severely defective in hapln1b morphants (compare bracketed region in Figure 6E and 6F). These defects were observed in 87 out of 92 (92.6%) embryos injected with hapln1b-MO while standard control MO (5 ng) injected embryos never exhibited this phenotype (0 out of 70 embryos).

Sh3gl3 morphants (3 ng) had a less severe phenotype than hapln1b morphants. Sh3gl3-MO treated embryos had relatively normal intersomitic vessel development, but displayed reduced circulation as visualized by red blood cell movement in the axial vasculature when compared to controls (data not shown). A closer examination of the axial vessels at 72 hpf revealed a significant reduction in the diameter of the dorsal aorta with a concomitant increase in the diameter of the posterior cardinal vein in morphants (arrow in Figure 6H compared to 6G). This constriction of the dorsal aorta was likely the cause of the observed decrease in circulation. 61 of 83 (73.5%) embryos injected with sh3gl3-MO displayed the shrunken aorta/reduced circulation phenotype while none of the standard control MO injected (5 ng, 0 out of 70 embryos) or uninjected control embryos did. The demonstration that hapln1b or sh3gl3 knockdown results in distinct vascular phenotypes suggest that other genes identified in this gene expression screen will be functionally important and should be studied in further detail.

**Discussion**

The transcription factor Etsrp is a critical mediator of vasculogenesis, angiogenesis, and primitive myelopoiesis in zebrafish. In this study we have used gene expression profiling to identify a group of genes that are induced by etsrp OE during early
have found that hox repressive mechanisms and thereby transactivate non-specific overexpressed at abnormally high levels it might also overcome normally regulated by other Ets family members. When (Figure S2). It is likely that fli1a, induced by antisense. Fourteen genes were found to be both ectopically induced by etsp OE and down regulated in etsp knockdown embryos. Of these genes two, kmr2 and lrp33, are found in myeloid cells and the remaining twelve are vascular. Any expression of these genes outside of hematopoietic or vascular tissue was unaffected by etsp knockdown demonstrating the specificity of etsp’s function. Two newly identified vascular genes, hapln1b and sh3gl3, were then independently knocked down by morpholino antisense. Each knockdown resulted in distinct vascular phenotypes demonstrating the utility of the screening method used and the importance of the genes identified. Together, these studies have identified genes downstream of etsp that are likely to mediate its observed role in primitive myeloid and early vascular development in zebrafish.

The Er71/Etv2 mammalian homolog of etsp has been shown to mediate vascular development in mice. Given that etsp has a similar function in zebrafish, it is likely that many of the genes identified in this microarray screen will have mammalian homologs functionally important for angiogenesis and/or vasculogenesis. Of the fourteen genes we focused on, ten have confirmed mammalian homologs. The remaining four cannot yet be determined due to uncertainty in the assembly of the zebrafish genomic sequence. However, it is expected that mammalian homologs for these genes will be found in the future.

Interestingly, Er71/Etv2 is critical for primitive erythropoiesis in mice, while etsp knockdown or mutation in zebrafish does not affect primitive erythropoiesis. The data presented here supports the previous finding that etsp regulates primitive myelopoiesis and vasculogenesis but not erythropoiesis since genes related to the former group are highly induced by etsp OE while no erythroid genes are induced. So it appears that Er71/Etv2 and etsp have evolutionarily divergent functions in the context of primitive erythropoiesis. One possible explanation for this difference is that zebrafish primitive erythropoiesis may not progress through an endothelial cell intermediate while mammalian primitive erythropoiesis in the yolk sac blood islands does. Alternatively, the evolutionary function of etsp in primitive erythrocytes may have been co-opted by another ets transcription factor in zebrafish, possibly due to a historical genome duplication event [58]. Another possibility is that the observed difference is due to differential timing of primitive erythrocyte cell death in etsp/Er71/Etv2 null fish and mammals. In etsp mutant fish, primitive erythroid cells marked by gate1 expression are initially specified but then later lost through apoptosis [15]. In Er71 null mice, no primitive erythrocytes are observed but an increase in apoptosis was noted [18]. This suggests the possibility that primitive erythrocytes are initially specified in Er71 null mice, but quickly die due to compromised vasculature as happens in fish.

An issue arising from these studies is that etsp OE results in the induction of many nonhematopoietic and nonvascular genes (Figure S2). It is likely that etsp OE directly mis-targets genes normally regulated by other Ets family members. When overexpressed at abnormally high levels it might also overcome repressive mechanisms and thereby transactivate non-specific genes. Included in the list of genes ectopically induced are many hox genes that may represent a secondary response to etsp OE. We have found that kmr2 is expressed in myeloid cells and axial vasculature. It was reported that the expression of hoxb3 was induced by kmr2 overexpression [59]. In this study, not only is kmr2 induced, but hoxb3 is also induced about five-fold. Ectopic induction of nonvascular/nonmyeloid target genes may also be the result of secondary effects of etsp overexpression. A possible mechanism is that etsp induces premature differentiation of vascular and myeloid cells and these cells signal to adjacent cells to differentiate into additional nonvascular/nonmyeloid cell types.

The strong ectopic induction of genes in the early embryo emphasizes that Etsp is a very potent, early acting transactivator whose roles in gene regulation must be tightly controlled.

It should also be noted that the overexpression of etsp alone is sufficient to induce many other transcription factors such as scl and fli1a. The overexpression of scl has also been demonstrated to induce other blood and vascular specific genes alone [60]. Recently, it was proposed that fli1a appears to be at the top of the transcriptional network driving blood and vascular development. Constitutively active fli1a, in which the artificial trans-activation domain of the viral VP16 protein was fused to full-length fli1a coding sequences, can ectopically induce blood and vascular genes [61]. However, overexpression of fli1a alone is not sufficient to induce blood or vascular markers as demonstrated here for etsp. This argues that etsp is more likely the top regulator of hemangioblast development.

The two etsp target genes, hapln1b and sh3gl3, examined by morpholino gene knockdown each displayed interesting vascular phenotypes. Hapl1n1b is a member of a protein family that functions as extracellular linkers for hyaluronans and proteoglycans. Hapl1n1b MO treated embryos display truncated intersomitic vessels and disorganized caudal vascular plexus. This phenotype is potentially due to decreased angioblast cell migration or adhesion due to extracellular matrix problems. Future studies will be necessary to establish the exact cellular defect responsible for the vascular phenotype observed in hapln1b morphants. Sh3gl3 is an intracellular protein whose homologs have been suggested to regulate endocytosis and intracellular membrane trafficking [62]. The phenotype observed in the sh3gl3 morphant, reduced dorsal aorta and increased posterior cardinal vein diameter, is suggestive of disrupted notch signaling as notch signaling mediates artery/vein specification and relative size [63]. Indeed endocytic mechanisms are critical for proper notch signaling [64]. We hypothesize that sh3gl3 may regulate artery/vein size and notch signaling through receptor endocytosis.

In summary, we have identified a set of genes induced by etsp OE in zebrafish embryos. Within this set we have identified multiple genes that have restricted expression in the vascular or myeloid lineages and are reduced by morpholino knockdown of etsp. These genes are likely to play important roles in the development of their respective tissues. Additionally, this data can be used in the future to clarify gene hierarchies necessary for hemangioblast differentiation by comparing with mammalian stem cell or zebrafish mutant, morphant, or overexpression gene array data, as we have already done with cloche. The results presented here provide a basis for future studies of the hemangioblast.

Materials and Methods

Microarray

fli1a gfp transgenic embryos were injected with 75 pg of synthetic etsp mRNA, transcribed with Ambion’s mMessage mMachine T3 polymerase kit from Xbal linearized etsp-T3TS as previously described [19], and harvested at ~80% epiboly in pools of 70 embryos per group. The transcription profile of uninjected transgenic embryos was used as controls. Total RNA was isolated
with Trizol reagent (Invitrogen), according to manufacturer’s instructions. The detailed protocol for microarray hybridization and normalization procedures were described by Horak et al [65] and are available upon request. First-strand cDNA probes were generated by amionoallyl dUTP incorporation and then coupled to either Cy3 or Cy5. The resulting cDNA probes were purified, concentrated, and then hybridized to arrays. Each chip contains 34,647 printed oligo elements (Compugen, Operon, and MWG) designed from zebrafish EST assemblies and representing approximately 20,000 genes, approximately 60% of the total predicted genes according to the public Ensembl database. After hybridization, the slides were washed, dried, and scanned using an Agilent DNA microarray scanner (Agilent Technologies) at 635 nm (Cy5) and then at 532 nm (Cy3). Fluorescence intensities were quantified using Agilent feature extraction software (Agilent Technologies), and changes in expression levels were determined by comparing the signal intensities between etsrp OE and uninjected control groups. Hybridizations were performed four times, switching the fluorescent labeling to eliminate biases caused by the labeling process. Samples were normalized using the Lowess calculations and cutoffs for significance were set at a two-fold change in either direction. Oligo sequences were mapped to multiple databases, including RetSeq, UniGene, Ensembl, TIGR, and genomic coordinates to maximally determine gene identity and function. Data were deposited into searchable FilemakerPro and Excel databases for analysis.

Quantitative RT-PCR

Total RNA was purified with Trizol reagent (Invitrogen) from equal numbers of embryos between experimental groups, and single strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed using the iCycler iQ Real-Time PCR Detection System (BioRad, Hercules, CA) with iQ SYBR Green Supermix (BioRad). Gene expression levels were measured by the ΔΔCt method, comparing etsrp OE or H2B-mRFP injected groups relative to uninjected controls, with β-actin used as the reference gene. Pre-microarray validation was performed from the same samples used for the microarray experiment, and biological triplicates were examined for the injection control experiment reported in Figure S1. Quantitative RT-PCR primers are listed in Table S1.

Gene cloning and RNA whole mount in situ hybridization (WISH)

Genes were cloned into the pCRII Topo vector using the TOPO TA cloning kit (dual promoter) according to manufacturer’s instructions (Invitrogen), after amplifying genes of interest from a 24 hpf wild type embryo cDNA library. The primers used are noted in Table S2. Positive clones were analyzed by restriction digest and confirmed by sequencing. WISH was performed as described in [66]. DIG labeled RNA probes were generated by linearizing TOPO-cloned genes with restriction endonucleases (New England Biolabs), and transcribing with SP6 or T7 RNA polymerase (Promega).

Embryo injections

Injections were performed with borosilicate glass micropipettes (Harvard apparatus) that were pulled with a flaming/brown micropipette puller, model P-97 (Sutter Instrument Co) on a PL-90 microinjector (Harvard apparatus). For microarray validation by WISH, an etsrp-mcherry construct was generated by sub-cloning mcherry from mcherry-tol2 (a gift from Hong Zhang) into pSPORT6 between XbaI and XhoI, and etsrp was subsequently subcloned into this vector at the 5’ end of mcherry between BamHI and XbaI sites. To test the requirement of etsrp for the expression of the blood/vascular related genes a mixture of etsrp morpholinos, MO1 and MO2 (4 ng each) described in [19] was injected into embryos at 1–2 cell stage, and harvested for WISH at 24–28 hpf. Translation blocking morpholinos targeting the first codon of hapln1b and sh3gl3 were synthesized by Gene-Tools, LLC. Hapln1b MO sequence: 5’-GAGCGAGAGGTCTATGGATTAT3’; sh3gl3 MO sequence: 5’-TTAAACCGCCACTGAGATGCTTC3’; Standard control MO sequence: 5’-CCTCTTACCTGATACAATTATA-3’.

Image acquisition and processing

In situ stained embryos were further processed by a serial dehydration in ethanol, followed by rehydration into 1×PBS. Embryos were imaged either in 1×PBS on agarose coated dishes, or in 2% methylcellulose in depression microscope slides. Images were captured with a color digital CCD camera (Axioacam, Zeiss) mounted on a dissecting microscope (Stemi SV11, Zeiss) with Openlab 4.0.2 software (Improvision, Lexington, MA). Fluorescence images were obtained with the same equipment but on an Axioskop2 plus compound microscope with 10× and 20× objectives. In several cases separate images were captured per embryo and areas in focus were compiled with Adobe Photoshop CS version 8.

Supporting Information

Figure S1 Quantitative RT-PCR of etsrp overexpression and H2B-mRFP injection controls for selected genes. Relative gene expression levels were calculated for etsrp or H2B-mRFP1 expressing as compared to uninjected control embryos at 80 percent epiboly to gastrulation stages for the genes indicated on the x-axis. The induction of endogenous etsrp (3’UTR amplicon from injections encoding etsrp lacking the 3’UTR), fli1a, scl, yrk, and atx1/tem8 are statistically significant (p<0.05, Student’s t-test). Although slightly increased hapln1b and sh3gl3 are not significantly different from controls.

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Figure S2 Genes induced by etsrp overexpression but not expressed in blood or vessels. (A) arhgef9; (B) pchdg; (C) novel2; (D) LOC555375 (expression is in ventral somites not vasculature); (E) ceb2; (F) smoc2; (G) LOC555051; (H) similar to testican; (I) hoxb8; (J) tex2; (K) c1orf192; (L) e2orf112; (M) znf385; (N) apolipoB; (O) ptmps1; and (P) hoxa3a. Scale bar: 250 um.

Found at: doi:10.1371/journal.pone.0004994.s002 (6.74 MB TIF)

Table S1 Quantitative RT-PCR primers

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Table S2 Primers used to clone in situ hybridization probes

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Author Contributions

Conceived and designed the experiments: SL. Performed the experiments: GAG MBV YZ SB. Analyzed the data: SL GAG MBV. Wrote the paper: SL GAG MBV.
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