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Zebrafish enhancer trap line recapitulates embryonic *aquaporin 1a* expression pattern in vascular endothelial cells

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ABSTRACT Aquaporin 1 (Aqp1) is a water channel protein, expressed widely in microvascular endothelial cells and implicated in mammalian tumor angiogenesis. However, its developmental expression has not yet been characterized in great detail. An enhancer trap screen was performed using a Tol2-derived GFP reporter in zebrafish embryos. An insertional Et(GBT-B1)tpl1 line was identified that has reporter insertion in the vicinity of the *aqp1a* gene. We further characterized the embryonic expression pattern of this GFP reporter line, as well as that of endogenous *aqp1a*. Both endogenous *aqp1a* and reporter GFP expression were restricted to the vascular endothelial cells within the dorsal aorta, cranial, intersegmental and other secondary vessels, but were absent in the axial venous vasculature. In addition, endogenous *aqp1a* expression was observed in both primitive and definitive hematopoietic erythroid progenitors, as well as in the otic vesicle, swim bladder, pneumatic duct, intestine and a subset of neurons within the retina and the midbrain-hindbrain region. We further show that *gata1* and *etsrp/etv2* function is required for hematopoietic and endothelial *aqp1a* expression, respectively. *Aqp1a* expression is restricted to endothelial and erythroid cells during early embryogenesis. The transgenic Et(GBT-B1)tpl1 line recapitulates endogenous endothelial *aqp1a* expression. Because currently very few reporter lines can differentiate between arterial and venous endothelial cells, the Et(GBT-B1)tpl1 transgenic line and characterization of the *aqp1a* expression pattern will be useful for future studies of endothelial and arterial-venous differentiation.

KEY WORDS: *aquaporin, angiogenesis, enhancer trap, vascular endothelial, erythroid, tumor*

Aquaporin 1 is a water channel protein expressed in vascular endothelial cells in multiple different vertebrates. Its strong expression has been observed in microvascular endothelium in rats and mice within adult kidney, lung, skin, secretory glands, pleura and peritoneum (Hasegawa *et al.*, 1994, Nielsen *et al.*, 1993a, Verkman, 2006). In addition, AQP1 is a major component of red blood cell and renal tubule membranes (Harris *et al.*, 1991, Nielsen *et al.*, 1993b). AQP1 is expressed strongly in proliferating microvessels in multiple different tumors in humans including mammary carcinomas and glioblastomas, brain tumors such as astrocytomas and metastatic carcinomas as well as bone marrow microvessels in patients with active multiple myeloma (Endo *et al.*, 1999, Saadoun *et al.*, 2002, Vacca *et al.*, 2001, Verkman *et al.*, 2008). Endothelial AQP1 expression has been also observed within the chick chorioallantoic membrane (CAM) (Ribatti *et al.*, 2002). Disruption of AQP1 function results in impaired tumor microvessel proliferation in mice tumor model, resulting in extensive tumor necrosis (Saadoun *et al.*, 2005). AQP1 function is required for endothelial cell motility, as demonstrated by mammalian cell culture and CAM

Abbreviations used in this paper: Aa, aortic arches; Aqp, aquaporin; CAM, chorioallantoic membrane; CrDI, internal carotid artery; DA, dorsal aorta; DLAV, dorsal longitudinal anastomotic vessel; Dpf, days-post-fertilization; GFP, green fluorescent protein; Hpf, hours-post-fertilization; ICM, intermediate cell mass; ISV, intersegmental Vessels; LDA, lateral dorsal aorta; MCeV, middle cerebral vein; MO, morpholino; PCV, posterior cardinal vein; PHBC, primordial hindbrain channel; PICA, primitive internal carotid artery; PMBC, primordial midbrain channel; RT-PCR, reverse transcriptase polymerase chain reaction.

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assays (Camerino *et al.*, 2006, Saadoun *et al.*, 2005). While these results demonstrate a critical role for AQP1 function during tumor angiogenesis, AQP1-null mice are viable (Saadoun *et al.*, 2005), therefore its role in embryonic development and angiogenesis, if any, is currently unknown.

During fetal and prenatal rat development, AQP1 expression has been observed in choroid plexus, periostium, endocardium and cornea (Bondy *et al.*, 1993). During the early development of killifish *Fundulus heteroclitus aqp1a* is expressed in the developing blood vessels including the caudal artery and intersegmental vessels (Tingaud-Sequeira *et al.*, 2009). However, it is not known if the embryonic vascular expression pattern of *aqp1* is evolutionarily conserved and comprehensive analysis of embryonic *aqp1* expression during different developmental stages is still not available.

Zebrafish has emerged as an advantageous model system to study embryonic vascular development. Similar to other vertebrates, zebrafish contains homologs for all thirteen different mammalian Aqp family members, which in some cases have undergone genomic duplication (Tingaud-Sequeira *et al.*, 2010). In zebrafish, similar to other teleost fish, two *aqp1* homologs are present in close genomic location. *Aqp1b / aqp1a.2* gene is located immediately downstream of *aqp1a / aqp1a.1* within the chromosome 2 (Tingaud-Sequeira *et al.*, 2008). DNA and protein sequences of the two zebrafish *aqp1* homologs have been previously described (Tingaud-Sequeira *et al.*, 2008). Interestingly, based on RT-PCR analysis, *aqp1a* is expressed in multiple zebrafish adult tissues while adult *aqp1b* expression is restricted to the ovary, testis and brain (Tingaud-Sequeira *et al.*, 2010, Tingaud-Sequeira *et al.*, 2008). However, embryonic expression of either zebrafish *aqp1* homolog has not been previously investigated.

Arterial-venous (A-V) differentiation is a critical step in the establishment of functional vasculature. In zebrafish, similar to other vertebrates, A-V differentiation is initiated prior to the establishment of circulation during formation of the major axial vessels, the dorsal aorta and the posterior cardinal vein (Jin *et al.*, 2005, Zhong *et al.*, 2001, Zhong *et al.*, 2000). According to the current model, notochord-derived Sonic Hedgehog induces expression of the vascular endothelial growth factor (Vegf) within the medial part of the somites. Vegf through PLCγ-dependent ERK phosphorylation activates the expression of Notch and its ligand Delta within the arterial precursors which then act to promote arterial and repress venous marker expression (Hong *et al.*, 2006, Lawson *et al.*, 2001, Lawson *et al.*, 2002). While multiple arterial and venous specific markers have been previously identified in zebrafish including an *aquaporin* homolog *aqp8* which is expressed in the arterial but not venous endothelial progenitors (Sumanas *et al.*, 2005), it is currently not known if *aqp1* exhibits differential A-V expression pattern.

Enhancer trap screens have been previously used to generate new fluorescent reporter lines and to identify novel gene expression patterns (Asakawa *et al.*, 2008, Balciunas *et al.*, 2004, Ellingsen *et al.*, 2005, Parinov *et al.*, 2004, Poon *et al.*, 2010). While there are multiple vascular endothelial specific lines generated either by traditional transgenesis or enhancer trap methods, most of them show ubiquitous endothelial expression and are not useful to specifically identify arterial or venous blood vessels.

In this study, we report generation of a new GFP reporter line Et(GBT-B1)tpl1 discovered in a Tol2-mediated enhancer trap screen. This line displays embryonic GFP expression restricted to the arterial axial vessels as well as cranial and intersegmental vessels while excluded from the axial venous vessels. We further show that the reporter insertion is located in the vicinity of *aqp1a* in the zebrafish genome. We characterize developmental expression of *aqp1a* which is primarily restricted to the arterial vasculature including the dorsal aorta and is excluded from the cardinal vein, similar to the reporter GFP expression pattern. In addition, endogenous *aqp1a* is also expressed in erythroid progenitors. These findings suggest that *aqp1a* may play a specific function during embryonic endothelial and / or hematopoietic development. Furthermore, Et(GBT-B1) tpl1 line will be a useful tool to study endothelial development and arterial-venous differentiation.

Results

Tol2 mediated transgenesis was used in an enhancer trap screen to generate reporter lines that display novel expression patterns. Transgenesis was performed using GBT-B1 vector which is designed for both enhancer trap and gene trap. To trap novel enhancers, GBT-B1 contains zebrafish β-actin minimal promoter in front of eGFP (see Experimental Procedures and Suppl. Fig. S1). Detailed construction of the vector and the screening procedure will be presented elsewhere (Balciuniene *et al.*, in preparation).

One of the generated lines, Et(GBT-B1)tpl1, displayed specific GFP fluorescence within vascular endothelial cells starting at the 26 hours-post-fertilization (hpf) stage (data not shown). At 31-48 hpf stages, GFP expression was observed within the dorsal aorta, intersegmental vessels, dorsal longitudinal anastomotic vessel, the plexus region of the cardinal vein and cranial vasculature (Fig. 1 A-E). Interestingly, axial vessel expression was restricted to the dorsal aorta and mostly absent from the posterior and anterior cardinal veins except for the caudal vein plexus region which showed GFP fluorescence (Fig. 1 C,E). Similar endothelial-specific expression pattern was also observed during later stages at least until 5 dpf (data not shown). No expression was observed within the caudal fin vasculature of adult Et(GBT-B1)tpl1 zebrafish (data not shown). Many embryos showed mosaic GFP expression pattern where only some of the endothelial cells displayed GFP fluorescence. The observed mosaicism is likely caused by expression silencing due to DNA methylation which has been previously observed in transgenic lines that contain synthetic 14x Gal4-UAS sequences (Goll *et al.*, 2009). Therefore only the embryos that showed the most robust GFP expression were propagated.

As determined by inverse PCR, the transgenic reporter was inserted within the chromosome 2, 23.7 kb upstream of *aqp1a* gene (Fig. 1F, Suppl. Fig. S2). Therefore we hypothesized that endothelial-specific enhancer of *aqp1a* was present in the vicinity of GFP insertion and thus resulted in endothelial GFP expression pattern. To confirm this, we investigated embryonic expression pattern of *aqp1a*, the sequence and protein structure of which have been previously described (Tingaud-Sequeira *et al.*, 2008). As determined by *in situ* hybridization, *aqp1a* is expressed in vascular endothelial cell progenitors as early as the 18-22- somite stages (Fig. 2A, and data not shown). At the 22-somite – 26 hpf stages, *aqp1a* expression is restricted to vascular endothelial cells within the dorsal aorta (DA), intersegmental vessels and some of the cranial vasculature including the lateral dorsal aorta (LDA), the primitive internal carotid artery (PICA), the primordial midbrain channel (PMBC), the middle cerebral vein (MCeV) and the cranial division of the internal carotid artery (CrDI) (Fig. 2 A-D). In addition,

aqp1a is expressed in the hematopoietic cells within the posterior intermediate cell mass (ICM) region which mostly include erythroid progenitors (Fig. 2 A-D). By 26 hpf *aqp1a* expression is present in only few erythroid cells in the ICM region, because most erythroid progenitors have entered circulation at this stage (Fig. 2D). Interestingly, no *aqp1a* expression was observed within the posterior cardinal vein (PCV) or the primordial hindbrain channel (PHBC) while some cranial venous vessels including the MCeV and the PMBC show *aqp1a* expression. At 30-48 hpf stages, *aqp1a* is strongly expressed in the newly forming vessels such as the dorsal longitudinal anastomotic vessel (DLAV) while its expression within the DA is less intense, particularly by 48 hpf (Fig. 2 E-G). At 48 hpf *aqp1a* expression is also apparent in intersegmental and cranial vessels and excluded from the PCV, similar to the earlier stages (Fig. 2 F,G). By 74 hpf *aqp1a* expression is mostly restricted to endothelial cells within the secondary vessels including the DLAV, the ISVs and the aortic arches (aa) (Fig. 2H). In addition, *aqp1a* is expressed in a subset of neural cells within the retina and the midbrain-hindbrain region, the dorsal part of the otic vesicle and ionophores (Fig. 2 H,I). *Aqp1a* expression is also apparent within the swim bladder and the pneumatic duct (Fig. 2H, inset). By 4 dpf *aqp1a* expression is apparent in the endothelial cells of ISVs and the definitive hematopoietic progenitors within the posterior blood island region (Fig. 2J). In addition, *aqp1a* is expressed in the swim bladder, the intestine and neural cells in the midbrain and hindbrain regions (Fig. 2 J,K).

To confirm that *aqp1a* is expressed in both endothelial and hematopoietic cells, we analyzed its expression in *gata1* and *etsrp* morpholino (MO)-injected embryos (morphants). *Gata1* knockdown results in the inhibition of erythropoiesis and loss of expression of multiple erythroid-specific genes (Galloway *et al.*, 2005, Rhodes *et al.*, 2005). Erythroid-specific *aqp1a* expression was absent in *gata1* morphants at 23 hpf while its endothelial expression was not affected (Fig. 2L). It is apparent that endothelial *aqp1a* expression in *gata1* morphants is restricted to the DA and absent from the PCV (Fig. 2L). Based on the previous studies, expression of multiple endothelial-specific genes is downregulated in the absence of *etsrp* function (Pham *et al.*, 2007, Sumanas and Lin, 2006). Endothelial specific *aqp1a* expression was absent in *etsrp* morphants at 23 hpf while erythroid expression was not affected (Fig. 2M). These data argue that endothelial and hematopoietic expression domains of *aqp1a* are controlled by different signaling pathways and require *etsrp* and *gata1* function, respectively.

Discussion

Based on our findings, vascular endothelial *aqp1a* expression is restricted to the DA and excluded from the PCV. In addition, we show that *aqp1a* is expressed in erythroid progenitors as early as 24 hpf stage. While this manuscript was in preparation, an independent study was published describing zebrafish *aqp1a* expression pattern (Chen *et al.*, 2010). In the latter study, it is reported that *aqp1a* is expressed in both DA and PCV while hematopoietic *aqp1a* expression was not observed until 48 hpf stage. Because erythroid

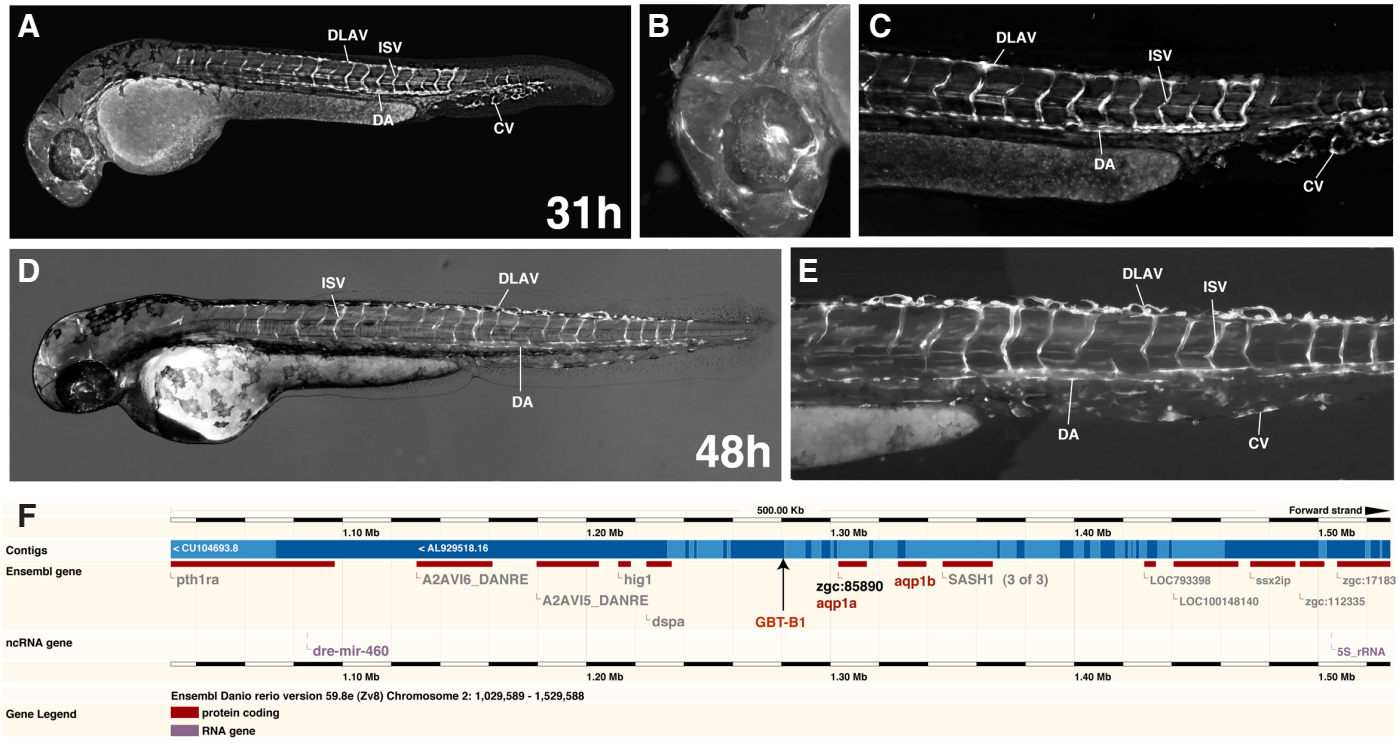

Fig. 1. Et(GBT-B1)tpl1 enhancer trap line shows endothelial-specific GFP expression pattern. (A-E) *Et(GBT-B1)tpl1 line displays GFP expression in the dorsal aorta (DA), intersegmental vessels (ISV), the dorsal longitudinal anastomotic vessel (DLAV), the plexus region of the caudal vein (CV) and the cranial vasculature at 31 h* **(A-C)** *and 48 hpf* **(D,E)** *stages. 5x* **(A,D)** *and 10x lateral views of the head* **(B)***, trunk and tail regions* **(C,E)** *are shown. Note that GFP fluorescence is absent from the posterior cardinal vein. GFP expression displays some mosaicism, particularly in the cranial vessels.* **(F)** *Map Viewer diagram shows that GBT-B1 insertion is located 23.7 kb upstream of* aqp1a *gene within the chromosome 2 based on Zv8 zebrafish genome assembly. The schematic location of* aqp1b *was added manually since it was not shown in Zv8 annotation.*

and PCV staining can overlap prior to the onset of circulation, it may be difficult to distinguish the two cell types based on solely microscopic observations. Our results obtained using *gata1* MO clearly show that *aqp1a* is expressed in erythroid progenitors but not PCV during early embryogenesis.

Interestingly, Et(GBT-B1)tpl1 expression closely recapitulates endothelial expression pattern of the endogenous *aqp1a* transcript yet it is not observed in hematopoietic cells. It is likely that endothelial and hematopoietic expression domains of *aqp1a* are controlled by different enhancers, consistent with *gata1* and *etsrp* MO knockdown results. The transgenic insertion is likely located in the vicinity of the endothelial but not hematopoietic *aqp1a* enhancer resulting in the observed endothelial GFP expression pattern.

While zebrafish endothelial *aqp1a* expression is present in the dorsal aorta at 24 hpf, it becomes restricted mostly to microvasculature including intersegmental vessels by 2 dpf. Such expression pattern is also observed in adult mice and rats where AQP1 expression is restricted to microvascular endothelia (Hasegawa *et al.*, 1994, Nielsen *et al.*, 1993a). It is intriguing that zebrafish *aqp1a* expression is also observed in both primitive and definitive hematopoietic cells. AQP1 is one of the major membrane components in human erythrocytes where it is thought to transport CO2 across the membrane (Prasad *et al.*, 1998, Preston and Agre, 1991). Erythroid-specific expression pattern of zebrafish *aqp1a* suggests that its transport function is likely to be evolutionarily conserved. *Aqp1* expression has also been reported in the adult intestinal epithelia of multiple fish species (Aoki *et al.*, 2003, Martinez *et al.*, 2005, Raldua *et al.*, 2008). Similar to the other species, zebrafish

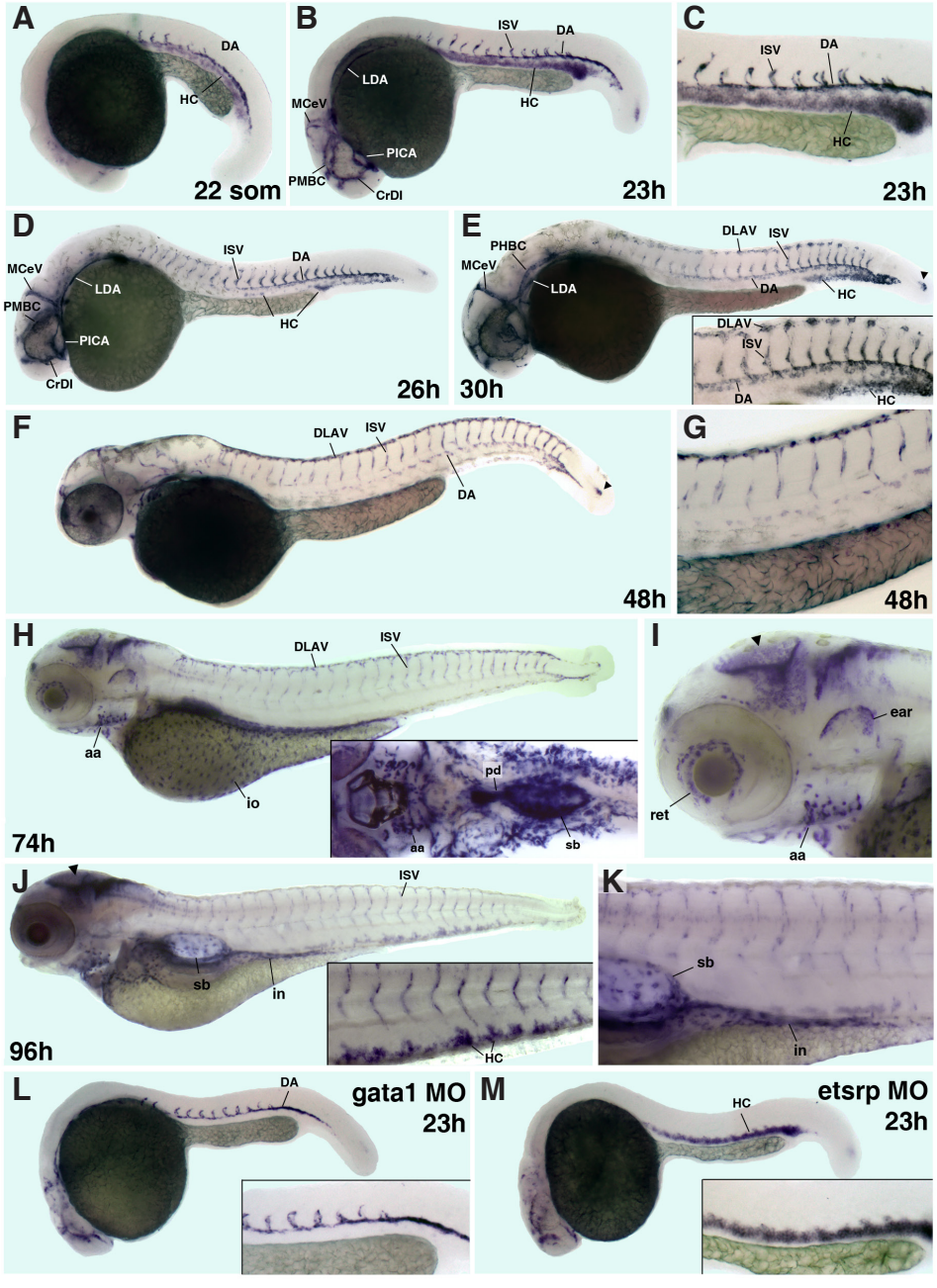

Fig. 2. *Aquaporin 1a* is expressed in vascular endothelial and erythroid progenitors at the 22-somite – 96 hpf stages. (A-G) *At the 22-somite – 48 hpf stages* aqp1a *is expressed in vascular endothelial cells that include the dorsal aorta (DA), intersegmental vessels (ISV), the dorsal longitudinal anastomotic vessel (DLAV), the lateral dorsal aorta (LDA), the primitive internal carotid artery (PICA), the primordial midbrain channel (PMBC), the primordial hindbrain channel (PHBC) and the cranial division of the internal carotid artery (CrDI). In addition,* aqp1a *expression is observed in hematopoietic cells (HC) in the posterior intermediate cell mass region that mostly include erythroid progenitors. Note that* aqp1a *expression is absent from the trunk venous vasculature including the posterior cardinal vein.* **(H,I)** *At 74 hpf* aqp1a *is expressed in the endothelial cells of the secondary vessels including the DLAV, the ISV as well as the aortic arches (aa). In addition,* aqp1a *is expressed within the swim bladder (sb), the pneumatic duct (pd), ionocytes (io), dorsal part of the otic vesicle (ear), a subset of retinal cells (ret) and a group of cells in the midbrain and the anterior hindbrain regions (arrowhead). (H) 5x lateral view; (I) 10x lateral view of the head region of the same embryo. Inset, (H), 10x ventral view of the mid-trunk region, anterior is to the left; embryo has been flat-mounted with the yolk removed.* **(J,K)** *At 96 hpf* aqp1a *is expressed in the endothelial cells of ISVs and definitive hematopoietic clusters (HC, inset, 10x view of the ventral tail region) in the posterior blood island. In addition, the expression is apparent in the swim bladder (sb), the intestine (in) and neural cells in the midbrain-hindbrain region (arrowhead). (K) Magnified lateral view of the trunk region, anterior is to the left.* **(L)** Aqp1a *erythroid expression is lost in* gata1 *morphants while endothelial expression is not affected. Note that* aqp1a *expression in the trunk region (magnified view, inset) is restricted to the DA and absent from the PCV.* **(M)** *Endothelial* aqp1a *expression is absent in* etsrp *morphant embryos at 23 hpf (inset, magnified view of the trunk region) while hematopoietic expression is not affected.*

aqp1a expression is apparent in the developing intestine by 4 dpf.

In summary, our study describes generation of a novel transgenic line which expresses reporter GFP under endothelial-specific *aqp1a* enhancer in the arterial but not venous axial vasculature. We further characterize *aqp1a* expression pattern which includes erythroid progenitors and vascular endothelial cells of the dorsal aorta as well as the secondary and cranial vasculature. Generation of Et(GBT-B1)tpl1 transgenic line and characterization of *aqp1a* expression pattern will be useful for future studies of endothelial, hematopoietic and arterial-venous differentiation. The observed *aqp1a* expression pattern raises questions about possible functional roles for *aqp1a* during early endothelial or hematopoietic development that may be answered in the future studies.

Materials and Methods

Generation of Et(GBT-B1)tpl1 line

To generate transgenic embryos, *tol2* transposase mRNA was microinjected together with GBT-B1 vector which is a dual purpose eGFP enhancer trap and Gal4-VP16 gene trap vector (Suppl. Fig. S1). The key components of the vector are a gene trap cassette based on GBT-R15 vector (Petzold *et al*., 2009) with AUG-less mRFP replaced by AUG-less Gal4-VP16 (Koster and Fraser, 2001). In addition, the vector has a 14XUAS:eGFP (Koster and Fraser, 2001) flanked by FRT sites. The gene enhancer trap cassette is flanked by miniTol2 transposon sites (Balciunas *et al*., 2006).

Inverse PCR

To identify the integration site, Et(GBT-B1)tpl1 embryos were sorted for GFP expression at 1-3 dpf. Batches of 20 GFP-positive and GFP-negative embryos were frozen at 5 dpf. Both sets of embryos were used for inverse PCR using our published protocols (Balciunas *et al*., 2006, Hermanson *et al*., 2004, Petzold *et al*., 2009) with Tol2-R4 (ATAATACTTAAGTACAGTAATCAAG) ant Tol2-F13 (GTACTTATTTTTTGGAGATCACTTC) primers for the first reaction and Tol2-R5 (TAATCAAGTAAAATTACTCAAGTAC) and Tol2-F11 (CCCTTGCTATTACCAAACCAATTGA) for the nested PCR step, and inverse PCR products were separated on agarose gel. PCR fragments were subsequently purified using GENJet Gel extraction kit (Fermentas) and sequenced with Tol2-R5 primer. Integration we confirmed by PCR using primers Tol2-R3 (ACTGGGCATCAGCGCAATTCAATTG) and 3B1-F1 (CTCGGAGTGCATCGAGTTCAGAA), and resulting PCR fragment was sequenced with Tol2-R3. The genomic sequence next to the integration site is shown in Suppl. Fig. S2.

In situ *hybridization*

cDNA clone corresponding to *aqp1a* gene was obtained from Open Biosystems (cat. No. EDR1052-7534520). The cDNA sequence contained full *aqp1a* coding sequence including 5' and 3'-UTRs and matched the *aqp1a* reference sequence NM_207059.1. To synthesize DIG-labeled probe for *in situ* hybridization, pExpress-1 vector containing *aqp1a* clone was linearized with SmaI (Fermentas) and transcribed with T7 RNA polymerase (Promega) in the presence of DIG-UTP (Roche). *In situ* hybridization was performed as previously described (Jowett, 1999).

Imaging

Live Et(GBT-B1)tpl1 embryos were dechorionated and mounted in 2% methylcellulose in the presence of 0.002% Tricaine (Sigma). Embryos were imaged under 5x and 10x objectives (Plan-Neofluar 5x/0.16 NA and 10x/0.3 NA) with an epifluorescent microscope (AxioImager, Zeiss). Monochrome camera (AxioCam MRm, Zeiss) and AxioVision 4.6 software (Zeiss) was used to capture Z-stacks of fluorescent images. Projection images were obtained using Extended Focus software module (Zeiss).

To capture images after *in situ* hybridization, embryos were mounted in 2% methylcellulose and imaged under 10x objective (Plan-Neofluar 10x/0.3, Zeiss) and an AxioImager compound microscope using color Axiocam Icc3 camera (Zeiss). Multiple focal planes were collected and the projection image was produced using Extended Focus module within AxioVision 4.6 software.

Morpholino knockdown

To knockdown *etsrp* function, 10 ng total of 1:1 *etsrp* MO1 and *etsrp* MO2 mixture (Sumanas and Lin, 2006) was injected at the 1-2 cell stages. For *gata1* knockdown, 8 ng of translation-blocking *gata1* MO (Open Biosystems, sequence TTCTCCATCTGCAAGTGTAGTATTG) was used.

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