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Vap (Vascular Associated Protein): A novel factor involved in erythropoiesis and angiogenesis

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ABSTRACT

Both endothelial and erythroid cells are generated in the intermediate cell mass (ICM) during zebrafish embryogenesis, but the nature of the genes that contribute to the processes of erythrocyte maturation and blood vessel network formation is not fully understood. From our *in situ*-based screening, we have identified a novel factor, Vap (Vascular Associated Protein) that is predominantly expressed in the ICM, and subsequently enriched in endothelial cells. Vap expression in the ICM was drastically suppressed in the *cloche* mutant that has defects in both vasculogenesis and hematopoiesis, whereas Vap expression was not affected in the *vlad tepes/gata1* mutant. Knockdown of Vap using anti-sense morpholinos (VAP-MO) not only resulted in decreased numbers of erythrocytes but also in the strong suppression of hemoglobin production. Further, we found that Vap knockdown caused the disorganization of the intersegmental vessels (ISVs), which show irregular branching. We propose that Vap plays an important role in the maturation of endothelial and erythroid cells in zebrafish.

1. Introduction

The processes of vascular and hematopoietic development are well conserved among vertebrates. It is thought that both angioblasts (endothelial precursors) and hematopoietic stem cells are generated during early embryogenesis from a common precursor cell, the hemangioblast [1]. In fact, there is *in vivo* evidence for the existence of the hemangioblast that contributes solely to hematopoietic and endothelial cells [2]. Thus, vascular and hematopoietic development is established in close functional interaction as well as in close spatial and temporal association.

Vascular development is mediated by two distinct processes, vasculogenesis and angiogenesis. In vasculogenesis, angioblasts derived from hemangioblasts differentiate and become lumenized to form the arteries and veins of the primary vasculature [3]. In angiogenesis, new blood vessels such as intersegmental vessels (ISVs) sprout from pre-existing vessels and are remodeled to establish a complex network. Although vasculagenesis and angiogenesis occur in distinct contexts, they are regulated by similar molecular processes. The vascular endothelial growth factor (Vegf) signaling pathway is essential for both vasculogenesis and angiogenesis [4]. Interestingly, knockdown of several factors including *neuropilin-1* [5], *angiopoietin-like proteins* [6], *semaphorin 3a1* [7,8] and *netrin1a* [9,10], selectively caused the ISV defects in the processes of angiogenesis, whereas vasculogenesis appeared to be unaffected. On the other hand, hematopiesis involves two well characterized processes, primitive and definitive hematopoiesis, which are evolutionarily conserved programs in vertebrates. Progenitor cells originating from hemangioblasts predominantly produce mature embryonic erythrocytes and myeloid cells during primitive hematopoiesis [11,12]. Subsequently, the definitive hematopoietic stem cells supply all lineages of adult blood cells consisting of definitive erythrocytes, lymphocytes and myeloid cell [13].

Zebrafish is a powerful vertebrate model system to study vascular and hematopoietic development. Both erythroid and endothelial cells are produced in close proximity in the ICM during early zebrafish embryogenesis. Although knockdowns of a few genes affect both erythroid cell and blood vessel development [14], the molecular components that regulate erythropoiesis and angiogenesis are still incompletely understood. To identify molecules involved in the formation of blood cells and vessels, we have screened candidate genes that are selectively expressed in the ICM by whole-mount *in situ* hybridization. In this paper, we report

Abbreviations: ICM, intermediate cell mass; ISV, intersegmental vessel; EGFP, enhanced green fluorescent protein; Vegf, vascular endothelial growth factor; DA, dorsal aorta; PCV, posterior cardinal vein.

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the isolation and characterization of a novel factor, Vap that is required for both erythropoiesis and angiogenesis.

2. Materials and methods

2.1. Isolation and construction of Vap

Total RNA was isolated from blood cells that were collected after tail amputation at 48 h post-fertilization (hpf). A uni-directional cDNA library was prepared by using the SMART cDNA library construction kit (Clontech) in the pCS2P-SfiI expression vector [15]. Clone b819 is predominantly expressed in the ICM during embryogenesis. Sequence analysis revealed that b819 encodes a novel factor (Vap: Vascular Associated Protein) with no obvious homology to known genes. Full-length Vap mRNA was isolated by PCR amplification using Vap-S, 5′-CGGGATCCTAGCTTAAATAT CGTCTACG-3′ and Vap-AS, 5′-GCTCTAGAGTTGGGCACAACAATCA TG-3′. For the construction of pCS2P-Vap, the amplified fragments were digested with BamHI and XbaI, the resulting fragments were inserted into BamHI/XbaI-cleaved pCS2P expression vector. The Vap GenBank accession number is AB265140. For the construction of pCS2-Vap-EGFP, full-length Vap (1-1196 a.a.) was fused to enhanced green fluorescent protein (EGFP). For the construction of pCS2-Vap-N-EGFP, the 5′-UTR and Vap N-terminal domain (1-70 a.a.), which contains the sequence complementary to VAP-MO and VAP-MO2, was fused to EGFP [16].

2.2. RNA probes and whole-mount in situ hybridization

Anti-sense RNA labeled with digoxigenin (DIG) was prepared using the RNA labeling kit (Roche). Whole-mount *in situ* hybridization was performed as previously described [15].

2.3. Zebrafish mutants

Mutant alleles of *vlad tepes* (*vlt*)^{m651} and *cloche* (*clo*)^{m39} were used [17,18]. Genotyping of *vlt* was done by using PCR after whole-mount *in situ* hybridization as previously described [17]. To monitor the endothelial cell development, we used a transgenic line, *Tg(fli1a:EGFP)*^{y1}[19].

2.4. Microinjection of morpholinos or synthetic RNAs

VAP-MO, VAP-MO2 and 4mMO (4 base mis-matched control morpholino) morpholinos were obtained from Gene Tools, LLC, as follows; VAP-MO, 5′-AATGACGCATTTTCGGAGCCAAAAG-3′; VAP-MO2, 5′-ATTCCAGGAAGAAATCCGTTGCCAC-3′; 4mMO, 5′-AATGAGGCATATTCGCAGCCTAAAC-3′. The nucleotides complementary to the VAP mRNA initiation sites are underlined. Morpholinos were injected into the yolk of 1–2 cell stage embryos. Capped RNAs were prepared using the mMESSAGE mMACHINE (Ambion) according to the manufacture's instruction. Synthetic RNAs were injected into one blastomere of 8–16 cell stage zebrafish embryos.

2.5. Visualization of hemoglobin production

Dechorionated unfixed embryos were incubated in staining buffer (0.6 mg/ml *o*-dianisidine, 10 mM sodium acetate [pH 5.2], 0.65% hydrogen peroxide, and 40% Ethanol) for 15 min in the dark to detect hemoglobin [13].

2.6. Transfection of plasmid DNA and microinjection of FluoSpheres

Human 293T cells were transfected with plasmid DNAs (pCS2P-EGFP or pCS2P-Vap-EGFP) by using the Lipofectamine Reagent (Invitrogen) according to the manufacture's instruction [20]. Dechorionated embryos are anesthetized with tricaine in embryo media. FluoSpheres (carboxylate-modified microspheres, 0.04 μm, red–orange fluorescent; Molecular Probes) were injected into the sinus venosus on the yolk by a glass microneedle.

3. Results

3.1. Isolation and molecular nature of the novel factor Vap

We hypothesize that there are still important novel molecules expressed in the ICM where both endothelial and blood cells are produced. To identify candidate genes selectively expressed in the ICM, we have performed *in situ*-based screening using a primitive hematopoietic cDNA library (see Section 2). Anti-sense RNA probes were prepared from the library and ICM-specific genes were identified by whole-mount *in situ* hybridization using embryos from several different stages. From this screening, we have identified a novel gene, clone b819, that is predominantly expressed in the ICM and is subsequently detected in endothelial cells (see below, Fig. 2). Therefore, we named this molecule Vap (Vascular Associated Protein). The primary structure of Vap (Fig. 1A) exhibits no obvious similarity to known genes in GenBank, and presently it is not clear whether mammals have a counterpart for Vap. A putative signal sequence occurs at the N-terminal region of Vap (Fig. 1A; red characters), and a putative transmembrane domain is indicated near the C-terminus (Fig. 1A; blue characters), suggesting that Vap is a type-I membrane protein. To examine this possibility, we made a construct (Vap-EGFP) by fusing the entire Vap sequence to EGFP. Vap-EGFP chimeric molecules were predominantly localized in the membrane in human 293T cells (Fig. 1B), suggesting that Vap functions as a membrane protein. Further, domain search analysis of Vap suggests that the Vap extracellular domain contains weak similarity to the Herpes virus major outer envelope glycoprotein BLLF1 (also called gp350/220) (green underline: 229–477 a.a.), and the EGF/laminin domain (black underline: 955–1000 a.a)(Fig. 1A). Thus, Vap is a novel putative membrane protein, showing only weak similarity to domains of known proteins.

3.2. Vap expression during early zebrafish embryogenesis

The developmental expression pattern of Vap during embryogenesis was examined by whole-mount *in situ* hybridization. Vap expression is weak or undetectable through the 5-somite stage (Fig. 2A). VAP expression starts in both the anterior LPM (ALPM, blue arrowhead) and the posterior LPM (PLPM, red arrowhead) around the 10-somite stage. Cells expressing Vap in the LPM migrate to the midline under the notochord during somitogenesis stages (Fig. 2C and D). Subsequently, Vap was gradually extended in the ICM (orange arrowheads) (Fig. 2E and F) where both erythroid and endothelial cells are generated. In zebrafish vasculogenesis, differentiation of primary vessels such as the DA and PCV occurs in the ICM [21]. Subsequently, sprouting of ISVs (green arrowhead in Fig. 2H and I) at 25 hpf seems to be the initial step of angiogenesis in zebrafish (Fig. 2H and I). The expression of Vap was also detected in the ISVs at 30 hpf. In addition, Vap was expressed at the edge of the tail tip in a domain that probably does not have a relationship to vascular development (Fig. 2E, F and H). The expression of Vap in endothelial cells was maintained at 48 hpf. A transverse section through the trunk region shows that Vap is expressed in both artery (DA; red arrowhead) and vein (PCV; blue arrowhead) (Fig. 2L). These results suggest that Vap has a role in vascular and hematopoietic development.

A

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MRHFVYLFLF ILPPGNTLLN SSQDCGGKIL GEHSGSIRYS LHPSRLLFDR   50
ITNDSKEKQL DITCTWIIDA HENQTVWIDA ISVGKGARIG ITFGNGDTVI  100
YEKDEQEIFS GTGRTVVEWS LKRTDKTFAT LHLRWNTSEE NQTSPLIPYT  150
HSEADPVSPS PSSSNGVTYT PDIPVNSNET PKTHSIDGKG IRQGGGVSDL  200
ADKHRPLYPQ ETTNNGQETA GVWISDLAFT GTHSYSDTHT SSGLDAHMDT  250
LAVLSDEPPL AQSSTKDQSP LLSAQTTPSS TAPNAAKTST LLDTNDVAFT  300
ELHRGSSQPT HTHAFTQIQS KTAKRGDLSS IFPTSSYMTV SPPNIEPFNQ  350
TSTEADFISD SATKDKPTTR AGFNRDVFTM SEKIVTNPFH GTFWSSNIIT  400
DDSQSIASES DRTDRSPRST NPNKTINEDL VMTTSTLATV SRFPNQTNPL  450
TFPDSETTEY SGYSASPPTN VTTDIEPTTS SSTLATSEAL TTQNLHTVQS  500
HHTSAQETSS ASAPVFDLTS HNNNKISAFT TFSTTDVTPI ESTSTPVVDE  550
GFSTSGRDCL GLGCMSYLPT TMQREEETPI TSPPSSSASP QISPNMTFDD  600
RTTVGVENRT NVEQSDTSSS QVQNTNTKSS IQTDLYTSTP QYLHPSSKTT  650
QSHVGFSDVT DINEMTSGQM ESTSQLSTST TSSKTTLGLS RFNTESSENT  700
NGFTSSYHFT TPSVFQVSTT DHTIQTRTTD SITTPHWETS QTSVVHTSSV  750
TIPQVNPQTT TTETTSARVH KQSQPGTFRP LQHTTTSSFG FYTKARKIPT  800
QTVSHSTASS FDKKWPHGRH YFIVEDQPVI FKEKTFQVLL QIVLEGDYVP  850
SVRLLEVETF LRKVAGFQNQ HVTWHSGPVL QTVIQFQTVQ ALSWLGRVES  900
LLREAGLNPL PKKGLFVGGV RVKNITEGGL QTDACEWLLE CPSGFQCVSS  950
KGNATCTSVC HSEYCKHQGI CVHRLGQQPI CQCPVGEDYW FMGQRCDLRM 1000
TRPRLVGVCF GVLVAVAAVM ALLSYLAVRR FKSMLMQAKV EQTRSSYRRF 1050
NHFDELSARF WGRSWPGSED SLDNPGFTRS DELLHLRALD RTCCYHDDTL 1100
SVVSTYHGSG AHLNTVYPHG SQYGWDLSNC SLADGVVDSG KASDLSVCSW 1150
PIEPIQWTPF PLLQQLSRNT TTVKASRPRS YCEGMELVDL EKSWTA     1196
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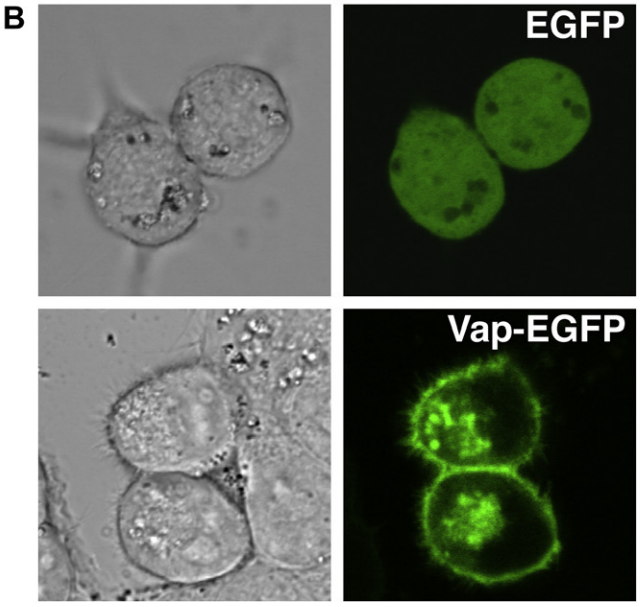

Fig. 1. Primary structure and cellular distribution of zebrafish Vap. (A) Predicted amino acid sequence of Vap. Red, putative signal sequence. Blue, putative transmembrane domain. Domain search analysis shows that the Vap extracellular domain has weak similarity to the Herpes virus envelope glycoprotein (green underline: 229–477 a.a.) and EGF/Laminin (black underline: 955–1000 a.a). (B) Cellular distribution of Vap-EGFP in human 293T cells. EGFP or Vap-EGFP was transfected into human 293T cells. Vap-EGFP was predominantly localized at the membrane, whereas EGFP was widely distributed in cytoplasm.

We examined how Vap expression is regulated by analyzing mutant zebrafish with altered vascular and hematopoietic development. The zebrafish *cloche* mutant has defects in both hematopoietic and vascular development [18], suggesting that the *cloche* gene plays an important role in the formation or maintenance of hemangioblasts. In the *cloche* mutant, Vap expression in the ICM, but not at the edge of the tail tip, was completely suppressed (Fig. 2M and P). On the other hand, the *vlad tepes* (*vlt*)/*gata1* mutant has a defect in the maturation of erythroid cells, but blood vessels develop normally [17]. Consequently, the expression of *βe3-globin* was partly inhibited in the *vlt/gata1* mutant (Fig. 2O and R), but Vap expression was not affected (Fig. 2N, and Q). These results indicate that Vap expression in the ICM is dependent on *cloche* function, but not on the function of *vlt/gata1*.

3.3. Knockdown analysis of Vap

Injection of antisense morpholino for a target gene in zebrafish embryos effectively prevents the translation of the gene. To examine the developmental function of Vap, we interfered with the translation of Vap by using VAP antisense morpholinos (VAP-MO and VAP-MO2). VAP-MO and VAP-MO2 were designed to interact

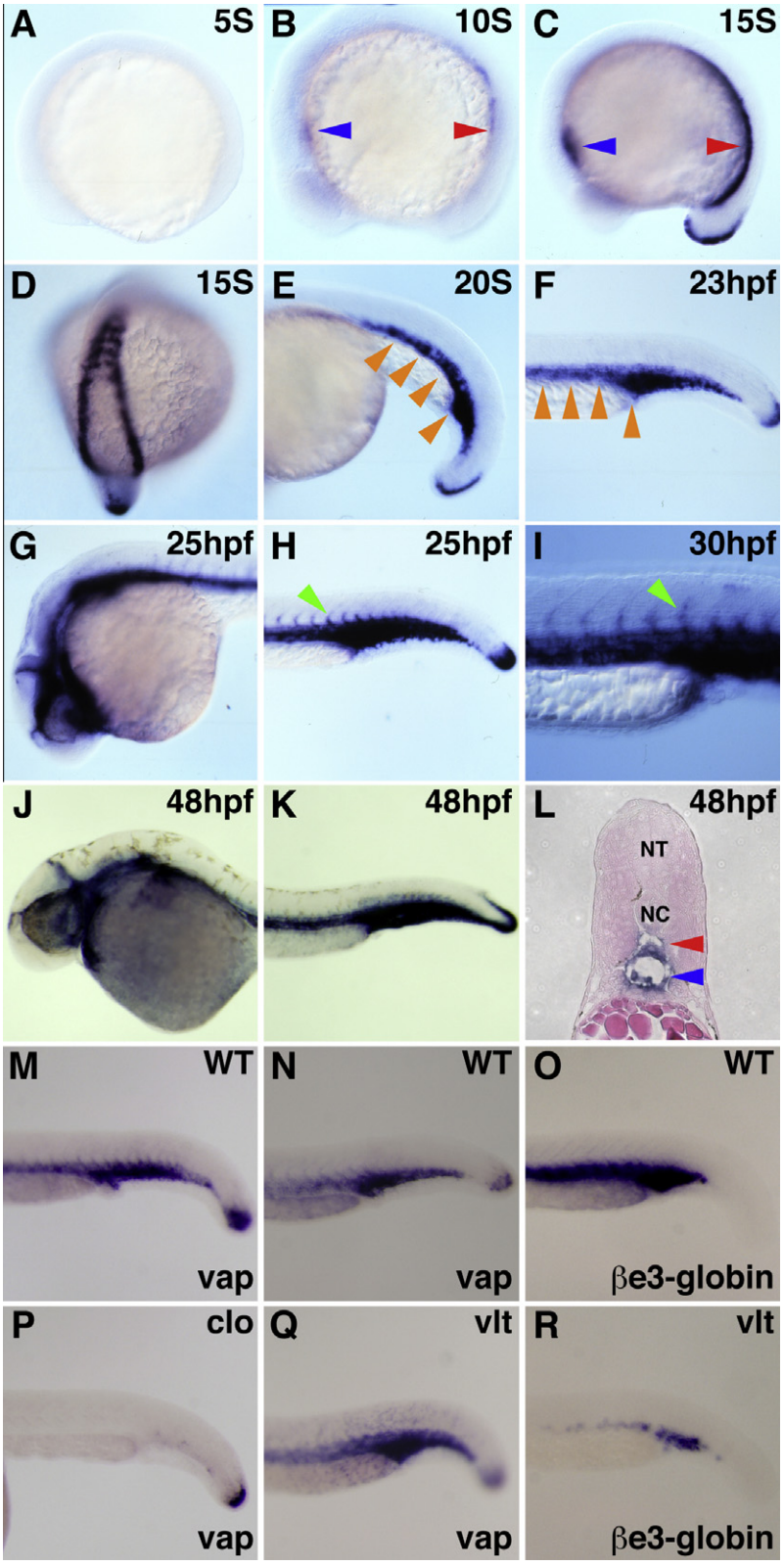

Fig. 2. Expression pattern of **Vap** during zebrafish early embryogenesis. (A–L) Whole-mount *in situ* hybridization with anti-sense **Vap** probe. Stages are indicated at the upper right of each photograph. S, somite; hpf, hours post-fertilization. **Vap** was not expressed at the 5-somite stage (A). **Vap** expression started in the anterior LPM (blue arrowhead) and the posterior LPM (red arrowhead) at the 10-somite stage (B). Expression of **Vap** in the LPM is gradually increased (C, D) and cells expressing **Vap** migrated to the midline under the notochord (E, F). **Vap** expression was newly detected at the edge of tail tip (C, E, F). **Vap** was expressed in blood vessels including the dorsal aorta (DA), posterior cardinal vein (PCV) and intersegmental vessels (ISVs) (G-I). **Vap** expression of blood vessels in both brain and trunk was still maintained at 48 hpf (J, K). Eosin stained cross section at the trunk level demonstrated that Vap is expressed in both DA (red arrowhead) and PCV (blue arrowhead) just below the notochord. (M–R) Vap expression in vascular and hematopoietic mutants. Vap expression in the ICM was completely suppressed (P) in the *cloche* mutant that has defects in both vascular and hematopoietic development. In contrast, Vap expression at the edge of tail tip was maintained in all embryos (M, P). Vap expression was not affected in the *vlad tepes/gata1* mutant embryos (N, Q), while *βe3-globin* expression was partly suppressed (O, R).

with different parts of the 5′ UTR and the region surrounding the initiation codon of Vap, whereas 4mMO is a 4-base mismatched control morpholino for the VAP-MO. The expression of VAP-N-EGFP containing the VAP-MO target sequences was strongly inhibited by co-injection of VAP-MO or VAP-MO2, but not 4mMO (Supplemental Fig. 1). We did not observe any obvious defects on epiboly movements during gastrulation stages in VAP-MO-injected embryos (data not shown). The overall morphology in somitogenesis was normal expect that slightly bent and short tails were observed in the VAP-MO-injected embryos, but not the 4mMO-injected control embryos (Fig. 3G and J). We believe that Vap expression at the tail tip affects the growth and extension of the tail in later stages. The number of circulating blood cells was greatly decreased in the VAP-MO-injected embryos at 52 hpf compared to those of uninjected and 4mMO-injected embryos. Consistently, the heart in VAP-MO-injected embryos was transparent and colorless at 52 hpf, while red blood cells were abundant in the heart of uninjected and 4mMO-injected embryos (Fig. 3D–F). Further, injection of VAP-MO caused strong suppression of hemoglobin production (suppressed: 87%, $n = 30$) as monitored by *o*-dianisidin chemical staining (Fig. 3A–C). These defects are specific for Vap knockdown, because 4mMO-injected embryos were completely normal, and injection of the non-overlapping VAP-MO2 caused the same defect in hemoglobin production (suppressed: 86%, $n = 21$). These results suggest that Vap is required for the erythrocyte maturation.

We noticed that small numbers of blood cells were moving in the DA and PCV of VAP-MO-injected embryos, but were hardly detectable in the ISVs of these embryos. Therefore, we examined the function of Vap on the formation of the blood vessel network. Since we have used embryos from the transgenic fish *Tg(fli1a:EGFP)*, endothelial cell development could be monitored by EGFP expression [19]. At 52 hpf stage, FluoSphere beads conjugated with red fluorescent dye were injected near the heart of the embryos to monitor blood flow. In both uninjected and 4mMO-injected embryos, GFP expression was well correlated with the signal of red FluoSphere beads expect in the parachordal vessels (Fig. 3H, I, M and N), suggesting that the parachordal vessels were not completely lumenized at 52 hpf. We found that the formation of ISVs was disorganized, showing thin vessels and irregular branching, in the VAP-MO-injected embryo (Fig. 3K, L, O and P). Interestingly, a signal derived from red FluoSpheres was detected in both DA and PCV, but hardly detected in the ISVs. We assume that vascular lumen formation was inhibited in the ISVs or that the ISVs were not properly linked to the primary vessels in the affected embryos. Notably, the differentiation of the DA and PCV was largely

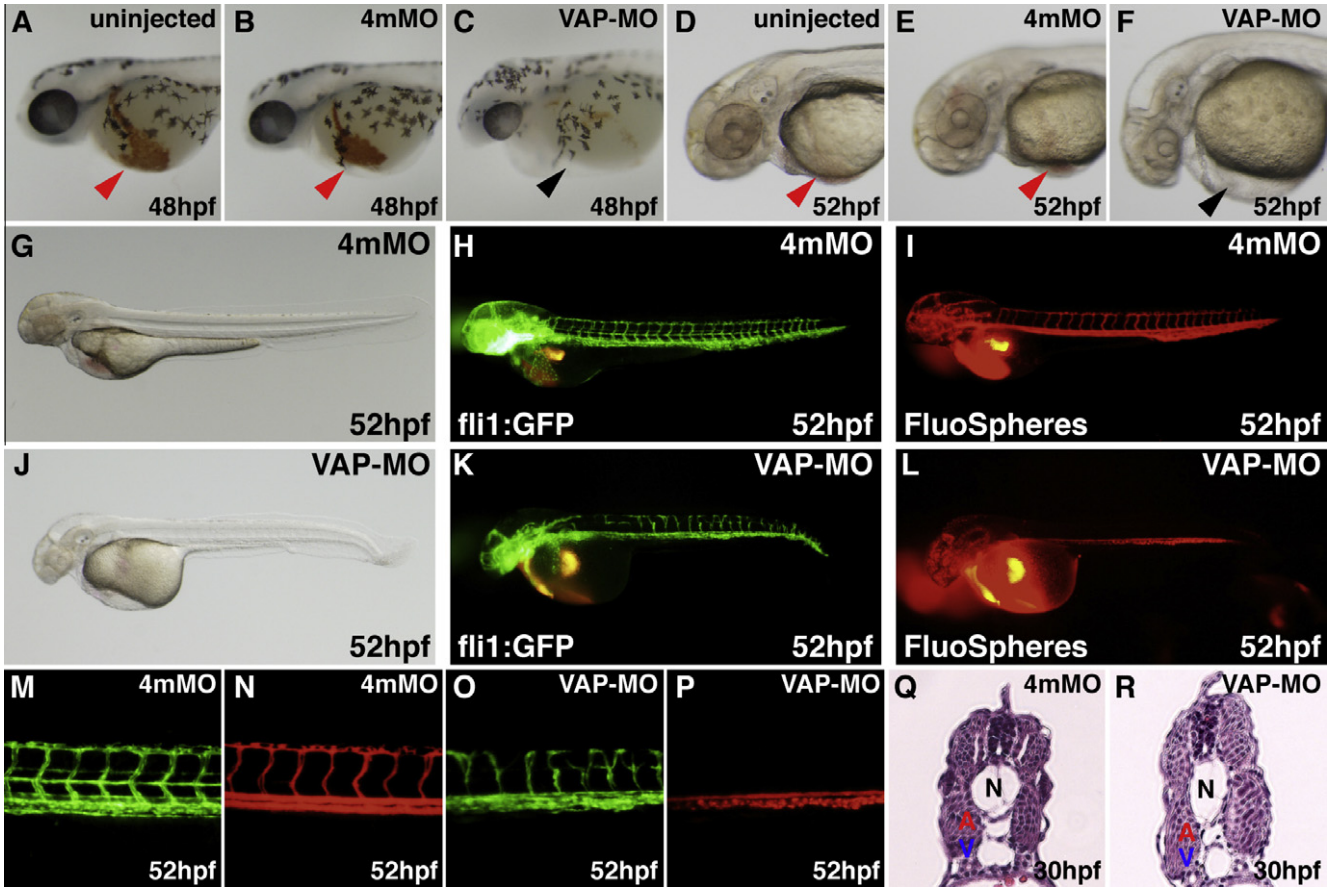

Fig. 3. Vap knockdown phenotypes. Morpholinos (VAP-MO, 10 ng; or 4mMO [4 base mis-matched control morpholino for VAP-MO], 10 ng) were injected into 1–2 cell stage embryos from wild-type or *Tg(fli1a:EGFP)* transgenic fish. At 52 hpf stage, red fluorescent-labeled microspheres (FluoSpheres) were injected near the heart to visualize circulation in lumenized blood vessels. Lateral view, anterior is left (A–P). Transverse section, dorsal is up (Q, R). (A–F) Vap knockdown phenotype on erythropoiesis. (A–C) Chemical staining (*o*-dianisidine staining) to detect hemoglobin. Injection of VAP-MO, but not 4mMO caused strong suppression of hemoglobin production. (D–F) Live embryos at 52 hpf stage. The color around the heart was red due to erythroid cells in uninjected and 4mMO-injected embryos, whereas VAP-MO-injected embryos were transparent around the heart with few erythroid cells. (G–P) Vap knockdown phenotype in angiogenesis. In 4mMO-injected embryos, vessels marked by EGFP-positive endothelial cells showed blood flow (red) except in the parachordal vessel (G–I, M, N). In contrast, the ISV formation was disorganized in VAP-MO-injected embryos, whereas both the DA and PCV were normal. Blood flow visualized by red microspheres was seen in the DA, PCV and brain vessels, while the ISVs in the trunk were not labeled. (Q, R) Trunk transverse section stained with Hematoxylin-Eosin showed tube formation in DA and PCV was detected in both 4mMO- and VAP-MO-injected embryos.

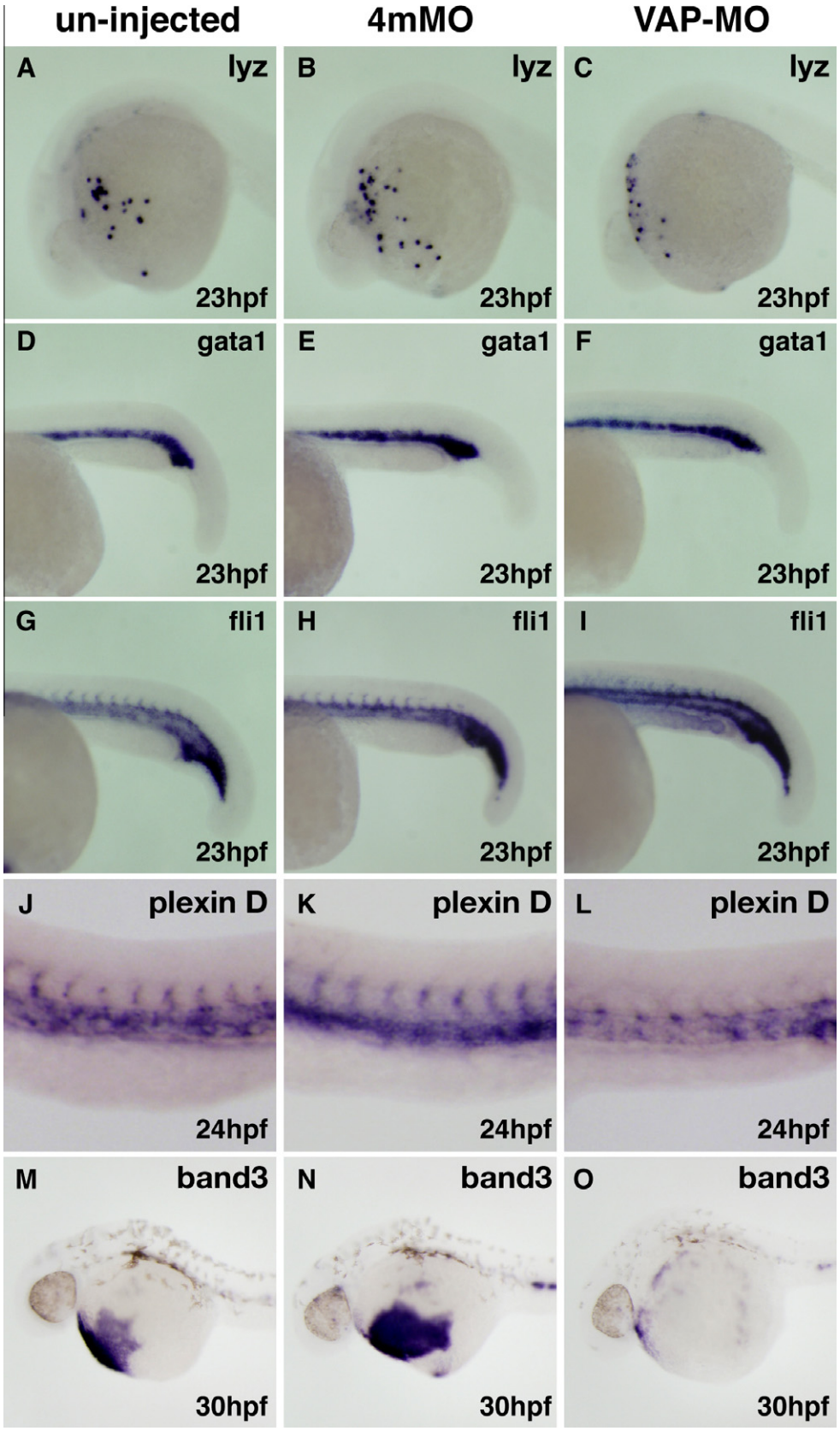

Fig. 4. Effect of VAP-MO on marker gene expression. Morpholinos (10 ng) were injected into 1–2 cell stage embryos, and the expression of marker genes was examined by whole-mount *in situ* hybridization. Stages are indicated at the lower right, and markers are indicated at the upper right of each panel. The expression of *lyz*, *gata1* and *fli1* was comparable in control and VAP-MO-injected embryos (A–I). The expression of *plexin D* was inhibited in the ISVs, but not in the DA or PCV (J–L). *band3* expression was strongly suppressed in VAP-MO-injected embryos (M–O).

unaffected in the VAP-MO-injected embryos, because the FluoSphere signal marked both the DA and PCV in the trunk, and transverse sections at the trunk level showed that tube formation had taken place at the normal position below the notochord (Fig. 3Q and R).

To characterize the Vap knockdown phenotype in both erythropoiesis and angiogenesis, we investigated the effect of VAP-MO on the expression of hematopoietic and vascular marker genes. In zebrafish, myeloid cells including macrophages and granulocytes develop predominantly from the ALPM located in the head region. The expression of a myeloid maker, *lysozyme C* (*lyz*), was not affected in the VAP-MO-injected embryo (Fig. 4A–C). The expression pattern of the erythroid markers *gata1* and *biklf* and the endothelial markers *fli1*and *flk1* in the ICM was comparable among control and VAP-MO-injected embryos at 23 hpf stage (Fig. 4D–I and data not shown), suggesting that Vap is not essential for the initial differentiation of endothelial and erythroid cells. We found that expression of *band3*, encoding an anion exchanger, was suppressed at 30 hpf in the VAP-MO-injected embryo (Fig. 3M–O). *band3* is the gene responsible for the *retsina* mutant that shows anemia with mitotic defects in erythropoiesis [22]. Further, expression of *plexin D* and *flk1* in the ISV was effectively inhibited in the VAP-MO-injected embryos, while *plexin D* and *flk1* expression could be detected in the DA and PCV (Fig. 3J–L and data not shown). Thus, *band3* expression during erythroid cell maturation and *plexin D* and *flk1* expression in the ISVs during angiogenesis are severely inhibited in the VAP-MO-injected embryos.

4. Discussion

In this study, we report the isolation and characterization of a novel factor Vap. Vap is initially expressed in the LPM around the 10-somite stage, and Vap-positive cells in the LPM appear to migrate to the midline below the notochord during late somitogenesis stages (Fig. 2). Thus, Vap is predominantly expressed in the ICM where both erythroid and endothelial cells are generated. Subsequently, Vap is enriched in the blood vessels including the DA, PCV and ISVs. Vap expression at the ICM is selectively suppressed in the *cloche* mutant, which is defective in both vasculogenesis and hematopoiesis, but not the *vlad tepes/gata1* mutant, which is defective in erythropoiesis only (Fig. 2). The primary structure of Vap has no apparent similarity to known genes, but possesses a signal sequence and one transmembrane domain (Fig. 1A), suggesting that Vap is a type-I transmembrane protein. This idea is supported by the fact that Vap-EGFP chimeric molecules are specifically localized at the membrane in human 293T cells (Fig. 1B). Thus, Vap is a novel ICM-enriched molecule that is a candidate regulatory factor in the maturation of erythroid and certain types of endothelial cells.

Knockdown analysis of Vap presented two distinct defects in erythropoiesis and angiogenesis. In erythropoiesis, Vap knockdown resulted in a decreased number of erythrocytes and the strong suppression of hemoglobin production (Fig. 3A–F). Since the expression of erythrocytes regulators, *gata1* and *biklf*, was comparable in 4mMO- and VAP-MO-injected embryos at 23 hpf (Fig. 4), Vap is not essential for the initial differentiation of erythrocytes. Knockdown of Vap might affect the process of erythropoiesis directly in the ICM, or Vap-negative primary vasculature might indirectly affect the environment in which erythrocytes mature. We found that a membrane protein, *band3*, was effectively suppressed at 30 hpf in the VAP-MO-injected embryos (Fig. 4M–O). It has been reported that the *retsina/band3* mutant shows anemia with mitotic defects in erythropoiesis [22], and therefore the inhibition of *band3* expression might be a cause for the decreased number of erythrocytes in the VAP-MO-injected embryos. Thus we propose that the novel membrane protein Vap is required for the maturation of erythrocytes in zebrafish development.

Although Vap is predominantly expressed in the DA and PCV, knockdown of Vap seems not to severely affect the formation of the primary vasculature, as lumenization of the DA and PCV was observed in transverse sections of VAP-MO-injected embryos, these vessels carried blood flow with small numbers of blood cells (Fig. 3P and R), and the expression of endothelial cell regulators *flk1* and *fli1* at 23 hpf was normal (Fig. 4D–I, data not shown). In contrast, the ISVs sprouting from the primary vessels were deformed, showing gaps and irregular branching, in VAP-MO-injected embryos. Further, while injection of FluoroSpheres visualized blood circulation in the DA and PCV, this could not be seen in the ISVs; thus ISVs were not lumenized or their connection to the DA was defective in these embryos. We found that the expression of *plexin D* and *flk1* in the ISVs was effectively suppressed in the VAP-MO-injected embryos, while *plexin D* and *flk1* expression in the DA and PCV was present although apparently reduced (Fig. 4J–L). Recently, it has been reported that the zebrafish *out of bounds (obd)/plexin D1* mutant shows drastic mispatterning of ISVs [8]. Further, zebrafish mutants disrupted in the Vegf receptors exhibit defects in the angiogenic sprouting of ISVs [23]. It is possible that the suppression of *obd*/*plexin D1* and *flk1* expression of VAP-MO-injected embryos is connected to the defects we observed. In summary, we present a novel membrane protein, Vap that involved in both erythropoiesis and angiogenesis in zebrafish.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.019.

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