Somitovasculin, a Novel Endothelial-Specific Transcript Involved in the Vasculature Development

Devi Mariappan, Rabea Niemann, Martin Gajewski, Johannes Winkler, Shuhua Chen, Suma Choorapoikayil, Marco Bitzer, Herbert Schulz, Jürgen Hescheler, Agapios Sachinidis

Objective—We recently isolated and characterized endothelial-like CD31\(^+\) cells derived from mouse embryonic stem (mES) cells and identified their transcriptome. The main objective of this study was to determine the functional relevance of the transcripts of unknown function (TUF) for vasculature development.

Methods and Results—We selected 2 TUFs of more than 27 to study their role for blood vessel development in zebrafish. Morpholino (MO) knockdown of the zebrafish orthologs of the first TUF (TUF1, mouse cDNA BC022623) showed disruption of the intersegmental vessels (ISV) at 2 days postfertilization as observed by live imaging of fl\(i:EGFP\)-transgenic embryos. The morphants showed abnormal blood circulation, but no effect on hematopoiesis was observed as demonstrated by gata-1 in situ hybridizations. Because knockdown of TUF1 resulted in disruption of the ISV patterning we named the TUF1 somitovasculin. TUF2 has been identified as cDNA clone BC020535. The MO knockdown of TUF2 resulted in a phenotype with an enlarged heart and the embryos lacked circulation completely.

Conclusion—We have shown the participation of a novel transcript (named somitovasculin) in circulatory vessel development. The combination of expression profiling in differentiating mES cells and the zebrafish model has the potential for rapid identification and functional characterization of TUFs. (Arterioscler Thromb Vasc Biol. 2009;29:1823-1829.)

Key Words: transcripts with unknown function ■ intersegmental vessel ■ morpholino-knockdown ■ CD31\(^+\) cells ■ vasculature development ■ zebrafish

S

ince the sequencing of multiple mammalian genomes, including the human genome, the functional characterization of transcripts with unknown function (TUFs) is in the focus of interest. Transcripts that show tissue-specific expression are of particular interest to study organ-specific functions and organ development. Embryonic stem (ES) cells offer an attractive model system to rapidly identify TUFs in somatic cells derived in vitro.\(^1\)\(^-\)\(^3\)

CD31 (also known as PECAM-1), a member of the immunoglobulin gene superfamily, is highly expressed on the surface of endothelial cells and moderately expressed on leukocytes and platelets.\(^4\) We recently isolated and characterized a ES cell–derived CD31\(^+\) population that exhibits endothelial-like characteristics such as incorporation of Dil-labeled acetylated low-density lipoprotein as well as formation of tubular structures on matrigel.\(^5\) Comparative bioinformatic analysis demonstrated enrichment of functional annotations related to angiogenesis, vasculogenesis, and blood coagulation in this cell population.\(^5\) The zebrafish Danio rerio has emerged as a powerful vertebrate model to study gene function. It has key advantages for studying vascular development via knockdown experiments: embryos survive even in the absence of blood circulation for approximately 7 days postfertilization (7 dpf), thus permitting screening for vascular mutants,\(^6\) and transgenic zebrafish expressing enhanced green fluorescent protein (EGFP) [Tg(fklk1:EGFP), Tg(fli1:EGFP)] throughout the vasculature greatly facilitate in vivo studies of vessel formation.\(^7\)\(^-\)\(^8\)

In zebrafish embryos, the first vascular endothelial precursors called angioblasts arise from the lateral plate mesoderm. During middle and late somitogenesis, they reside in the region known as the intermediate cell mass. The cells in the lateral posterior mesoderm express endothelial and hematopoietic markers, suggesting that they include bipotential precursors for both the hematopoietic and angioblastic lineages, termed hemangioblasts.\(^9\)\(^-\)\(^10\) A 2-step mechanism for vessel formation has been proposed based on time-
lapse multiphoton microscopy of living Tg(fli1:EGFP) zebrafish embryos. In the first step, pairs of endothelial sprouts emerge bilaterally from the dorsal aorta close to the intersomitic boundaries at ~20 hours postfertilization (hpf) and form an initial primary network of vascular cells. Once the formation of the primary aorta-derived vascular network is completed, in the second step a new secondary set of vascular sprouts begins to emerge exclusively from the posterior cardinal vein, beginning at ~1.5 dpf. These secondary sprouts interact dynamically with the primary network to form a functional vasculature.

In the present study, we identified all the TUFs specifically upregulated in endothelial-like CD31+ cells and functionally characterized their role in vascular development using the in vivo zebrafish model.

Materials and Methods

Animals

Zebrafish embryos were obtained from natural spawning of wild-type (wt) or Tg(fli1:EGFP) zebrafish line (a kind gift from Dr. Gerd-Jörg Rauch, Heidelberg, Germany). Embryos were raised and stained as described. Embryos were incubated either at 23.5 or 28.5°C and staged as described. To inhibit pigmentation, embryos were treated with 0.2 mmol/L 1-phenyl-2-thiourea (PTU, Sigma) after 24 hpf. The absence of blood circulation was determined under a stereomicroscope (Leica). Morpholino antisense oligonucleotide (MO)–injected embryos were compared with uninjected and mismatch MO (MIMO)-injected controls from the same clutch. Gata1:DsRed transgenic animals were a kind gift from Jean-Pierre Levraud and originally generated in the laboratory of Dr. Leonard Zon, Children’s Hospital Boston and the Howard Hughes Medical Institute, Boston, Mass.

Design and Microinjection of MO

cDNA sequences of transcripts corresponding to the probe sets of interest were obtained from NetAffx. Predicted peptide sequences were obtained from NCBI Unigene, and putative zebrafish orthologs were identified using BLASTx against the Ensembl Zebrafish peptide database. Among the list of hits, those with lowest e-values (not higher than 10^-5) were examined, the most probable match was identified and the start of the open reading frame was used for MO design. MOs (Gene Tools) were designed complementary to the region of translational initiation of the zebrafish orthologs to inhibit protein translation. The MOs were diluted to 0.5 to 1.0 mmol/L in H2O, 0.1 mol/L KCl and 0.2% phenol red. MO injections were performed on 1- to 2-cell embryos using Femtojet and correspondingly modified MOs were injected in the pCS2+ vector with either at 23.5 or 28.5°C and staged as described. To inhibit pigmentation, embryos were treated with 0.2 mmol/L 1-phenyl-2-thiourea (PTU, Sigma) after 24 hpf. The absence of blood circulation was determined under a stereomicroscope (Leica). Morpholino antisense oligonucleotide (MO)–injected embryos were compared with uninjected and mismatch MO (MIMO)-injected controls from the same clutch. Gata1:DsRed transgenic animals were a kind gift from Jean-Pierre Levraud and originally generated in the laboratory of Dr. Leonard Zon, Children’s Hospital Boston and the Howard Hughes Medical Institute, Boston, Mass.

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed as previously described. Embryos were processed using an automated In situPro system (Abimed) as described previously. Digoxigenin-labeled RNA probes were prepared using RNA labeling kit (Roche) and stained using BM purple (Roche). Whole-mount embryos were observed under a stereomicroscope (Leica) and photographed (Axiocam, Zeiss). The PCR primers (forward and reverse primers are flanked with T7 and T3 promoter sequences respectively) used for the generation of in situ probes are as follows: TUF1-Forw-T3-5' TACCTGCTTTTTCCAGGGCAGCATG TGU1-Rev-T7-5' TTTGACTTTCTTTACATCCTCCTCC.

Generating a Control Construct to Test the Specificity of the MOs

mRNA was isolated from 72-hpf-old zebrafish embryos using the trizol protocol with TRIZOL reagent (Gibco). cDNA was generated by first-strand synthesis using the SuperScript III-Kit (Invitrogen) according to the manufacturer’s manual. The PCR primers (forward and reverse primers are flanked with Clal and Ncol restriction sites, respectively) used for the generation of 5'UTR are as follows: TUF1-Forw-Clal-5' TACTGACTTTCTTTACATCCTCCTCC, TUF1-Rev-Ncol-5' TTTGACTTTCTTTACATCCTCCTCC. Control constructs (5'UTR including target sequence of the MO in front of GFP) were generated in the pCS2+ vector with Clal and Ncol (NEB). All constructs were validated by sequencing. Plasmids were linearized with NotI to drive transcription from the SP6 promoter and mRNA was synthesized using the mMessage mMachine kit (Ambion). mRNA was injected in the range of 250 ng/μL in a solution containing 0.1 mol/L KCl and 0.2% Phenol red into 1-cell stage embryos. After injection the embryos were transferred into a petridish with system water and incubated to the desired stage. Injections were performed using Femtojet and a micromanipulator (Eppendorf).

Results

Identification of Transcripts of Unknown Function From CD31+ Cells Isolated From Mouse Embryonic Stem Cells

As we described previously, we identified 259 transcripts that were highly expressed in CD31+ cells (t test probability value <0.01 and fold change >2) compared to undifferentiated mES cells and 8-day-old EBs. Among these, 229 transcripts have known roles in endothelial differentiation or vascular development and 27 are previously uncharacterized transcripts of unknown function (TUFs; supplemental Table I, available online at http://atvb.ahajournals.org). We extended our analysis focusing on 2 TUFs (1426734_at and 1435600_s_at, named TUF1 and TUF2, respectively) that were significantly elevated in the CD31+ cells (Figure 1). We have chosen TUF1 and TUF2, because in contrast to the other TUFs their function was completely unknown and open reading frames (ORFs) of TUF1 and TUF2 can be clearly located. A clear location is essential for designing of zebrafish morpholinos which block translation.

Identification of TUF1 and TUF2 Zebrafish Orthologs

Mouse cDNA sequence BC022623 (TUF1) corresponds to the protein FAM43A, an uncharacterized protein containing 424 amino acids with a molecular weight of 46.2 kDa. It has a conserved phosphotyrosine-binding (PTB) domain (supplemental Figure IA). PTB domains, also known as phosphotyrosine-interacting domains (PIDs), have a pleckstrin homology (PH)-like fold. PH domains are found in a large variety
signaling proteins\textsuperscript{18} and are involved in cell signaling and cytoskeletal rearrangement.\textsuperscript{19}

Using the mouse cDNA sequence as a query, we identified a putative zebrafish ortholog (similarity 50\%) for TUF1, which corresponds to an unknown protein containing 305 amino acids and a PTB domain (supplemental Figure IB and IC). As determined by in situ hybridization, this gene was expressed in vascular cells such as posterior cardinal vein (CV), dorsal aorta, and dorsal longitudinal anastomotic vessel (DLAV), and also found in nonvascular cells including the somite boundaries (Figure 2A, i, ii upper panel) and the forebrain (Figure 2A, ii, iii). Sense ISH did not show staining at all (Figure 2A, i, ii lower panel), indicating that TUF1 is also ubiquitously expressed at a low level according to the slight overall staining in the antisense ISH.

Mouse BC020535 cDNA sequence (TUF2), an uncharacterized protein C20orf160 (Chromosome 20 open reading frame 160) homolog, has high similarity to a region of cerebral cavernous malformation 2 (human CCM2), which plays a role in vasculogenesis and is associated with cerebral cavernous malformations on gene mutation.\textsuperscript{20} The mouse protein contains 564 amino acids with a molecular weight of 62 kDa.

**MO Knockdown of TUF2**

The MO knockdown of TUF2 in 17 of 20 morphants resulted in a phenotype with an enlarged heart (Figure 2Bi) compared to wt (Figure 2Bii) as detected by cmlc2 (cardiac myosin light chain 2) expression (heart indicated by arrow). Embryos lacked circulation completely (Table). During our studies, Mably et al\textsuperscript{21} published their findings on the role of the TUF2 transcript which they named valentine in their study confirming our findings and demonstrating the validity of our approach. Rather than duplicating this work, we focused on the functional characterization of TUF1.

**MO Targeting Sequence-EGFP mRNA Expression in the Presence of TUF1 MO**

To test the specificity of the MO-TUF1, mRNA was transcribed in vitro using a construct that consists of the MO targeting sequence of TUF1 in the front of the EGFP reporter sequence. Injection of this mRNA leads to a bright fluorescence in 81 embryos of 86 as well as in 73 embryos of 79 after coinjection of this mRNA with the MMO. In contrast, coinjection of the mRNA together with the corresponding MO resulted in a suppression of EGFP expression in 78 embryos of 87 because of the specific binding of MO to the 5'-UTR region (Figure 3).

**Knockdown of TUF1 Affects Blood Circulation but Did Not Affect Hematopoiesis**

To investigate the potential role of TUF1 for blood vessel or hematopoietic development, we performed a functional knockdown by injection of MO into 1 to 2 cell stage embryos obtained from the Tg(fli1:EGFP) zebrafish embryos.\textsuperscript{7} Fli1, a member of the Ets family of transcription factor genes, is expressed in hematopoietic and endothelial cells.\textsuperscript{22} Morphants were difficult to distinguish from the uninjected embryos until 24 hpf. However, they were distinguishable from the wild-type after the onset of blood circulation and displayed more prominent defects by 48 hpf (Table).

After 3 dpf, circulation defects were observed in 46\% and 37\% of the TUF1-MO1 and TUF1-MO2 morphants, respectively, whereas none of the MMO-injected embryos showed this defect. Scoring of the phenotypes demonstrated that 42%/32\% of the TUF1-MO1/TUF1-MO2 morphants lacked blood circulation completely, whereas 4%/5\% of the respective morphants showed abnormal circulation in which blood cells were circulating in an irregular manner with reduced blood flow velocity (supplemental Video files II) compared to the control animals (supplemental Video files 1). In 42\% of the morphants an almost complete blood stasis was observed, apparently because of the clogging/clumping of blood as a result of the reduced velocity of the blood flow. Diaminofluorene staining further confirmed that the blood cells in the
morphants were accumulated at various positions including brain, trunk, and above the yolk sac at 48 hpf (Figure 4A, I through IV). The hemorrhagic effect of the TUF1 knockdown has been confirmed by additional experiments performed on transgenic gata1:DsRed zebrafish animals. As demonstrated in Figure 4A (vi), knockdown of TUF1 resulted in accumulation of blood cells in the caudal vasculature, in the ventral part of the CCV as compared to the untreated embryos (Figure 4A, v). To analyze whether the knockdown affected the hematopoietic development, we performed whole-mount in situ hybridization with a gata-1 riboprobe in wild-type animals and morphants at 18 to 20 somite stage. In zebrafish, gata-1 is initially detected at the 2-somite stage in 2 stripes of cells that flank the paraxial mesoderm of the posterior embryo.23 We observed similar expression of gata-1 in both the wild-type and the morphants (Figure 4B), suggesting that TUF1 knockdown did not affect hematopoiesis and the circulatory phenotype could be attributable to a vascular defect.

Knockdown of TUF1 Causes Blood Vessel Defects and Affects the Expression of Endothelial Markers

Because the above observations suggested an important role of TUF1 for the development of blood vessels, we further investigated vessel morphology and the expression of vascular markers. Knockdown of TUF1 by MO injection resulted in the disruption of ISVs as shown by EGFP expression (Figure 5B) compared to control animals (Figure 5A). Morphants were able to form rudimentary major vessels. Interestingly, although the angiogenic sprouting of ISVs was not affected, the assembly and patterning of ISVs was disturbed. As shown in Figure 5B, DLAV formation was impaired. These alterations might be attributable to the inability of the ISV sprouts to migrate properly. To ascertain the requirement of TUF1 for correct vessel formation, we repeated these experiments using a second TUF1-MO. As shown in Figure 5C, these TUF1-MO2 morphants showed similar defects as those observed with TUF1-MO1.

To examine the vascular defects in TUF1 morphants, we analyzed the expression of vascular markers by whole-mount in situ hybridization. mRNA of the 3 endothelial markers Flk1, Fli1, and Cdh5 was detected in the 2 major vessels, dorsal aorta and posterior cardinal vein in wild-type embryos, whereas expression of these markers in the ISVs of morphants reflected the missing endothelial cells (Figure 5D and 5E) which is consistent with the aberrant EGFP expression (Figure 5B).

Discussion

The main obstacles to study the function of endothelial- or cardiac-specific TUFs in vivo are the identification of cells of the developing cardiovascular system in the early embryo and the practical difficulties in obtaining purified cells in sufficient quantities. To accelerate the identification and functional characterization of TUFs relevant for the development of the vasculature we pursued a combined strategy using ES cells and the zebrafish model along with high throughput genomics. Although gene expression profiling of endothelial cells isolated either from human or mouse ES cells has been reported previously,24–26 there are few functional validation studies of TUFs using morpholino-mediated knockdown in zebrafish27 or analyzing TUF expression patterns by in situ hybridization.3,28

Table. Summary of the Knockdown Experiments of TUF1 and TUF2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. (mmol/L)</th>
<th>No. of Experiments</th>
<th>Total No. of Embryos</th>
<th>Live Embryos (Normal) (%)</th>
<th>Circulation Defects Abnormal None (%)</th>
<th>Dead (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMO</td>
<td>0.6</td>
<td>2</td>
<td>84</td>
<td>61 (73)</td>
<td>0</td>
<td>23 (27)</td>
</tr>
<tr>
<td>TUF1-MO1</td>
<td>0.6</td>
<td>6</td>
<td>404</td>
<td>156 (39)</td>
<td>16 (4)</td>
<td>71 (42)</td>
</tr>
<tr>
<td>TUF1-MO2</td>
<td>0.6</td>
<td>2</td>
<td>112</td>
<td>54 (48)</td>
<td>6 (5)</td>
<td>36 (32)</td>
</tr>
<tr>
<td>TUF2-MO</td>
<td>0.75</td>
<td>1</td>
<td>63</td>
<td>0</td>
<td>0</td>
<td>58 (92)</td>
</tr>
</tbody>
</table>

Injected embryos were scored for circulation defects after 3 dpf. "Abnormal circulation" refers to the embryos with reduced blood flow attributable to hemorrhage at various locations including brain, trunk, and above the yolk sac. "None" refers to the embryos with absence of circulation. MMO refers to the mismatch morpholino.

Figure 3. Effect of MO-TUF1 on the expression of the MO-TUF1 targeting sequence-EGFP mRNA. The MO-TUF1 targeting sequence-EGFP mRNA is injected solitary (i) or in combination with the MO that suppressed the expression of EGFP. Fluorescent stereomicroscopic images of 20 hpf embryos are shown.
Whole-mount in situ hybridization with a \textit{gata-1} riboprobe in wild-type and morphant embryos at the 18 to 20 somite stage showed that the knockdown of TUF1 did not affect hematopoiesis. In zebrafish, \textit{gata-1} is first detected at the 2-somite stage in 2 stripes of cells that flank the paraxial mesoderm of the posterior embryo.\textsuperscript{23} Diaminofluoresce staining revealed that the gene knockdown also leads to hemorrhage. Our results suggest that TUF1 is not involved in hematopoiesis but rather is specifically involved in ISV patterning.

TUF2 has been identified as cDNA clone BC020535 and has high similarity to human CCM2. CCM2 plays a role in vasculogenesis, and CCM2 mutations are associated with cerebral cavernous malformations.\textsuperscript{20} The CCM2 locus has
been identified as the MGC4607 or malcaverin gene, encoding a protein with a putative phosphotyrosine binding domain. This protein is involved in the regulation of integrin signaling pathway, and when perturbed it causes abnormal vascular morphogenesis in the brain leading to cerebral cavernous malformations. Cerebral cavernous malformations (CCMs) are vascular anomalies characterized by abnormally enlarged capillary cavities without intervening brain parenchyma. They cause seizures and focal neurological symptoms. The CCM1 protein is expressed by endothelial cells, more specifically by the arterial and microvascular endothelium.

The above study also reports CCM2 protein expression in mouse brain, which is detected in pyramidal cells and astrocytes. Valentine (vtn) was identified as the zebrafish homolog of human CCM2 by positional cloning. The Valentine protein is highly conserved across vertebrates. Whole-mount in situ hybridization of 28 hpf embryos with a vtn probe has demonstrated vtn expression in the brain ventricular zone, with weaker expression in the vein. Our MO knockdown resulted in a phenotype with an enlarged heart (Figure 2E), and the embryos had no circulation (Table). Our results are in accordance with the above study. Notably, we identified the functional role of this gene applying an entirely different ES cell/zebrafish approach than Mably et al, illustrating that our strategy for identifying TUFs of interest is a valid approach for studying vascular development. In summary, our strategy proved optimal to discover developmentally regulated TUFs under physiological conditions and to validate their function for vascular development.

The development of vasculature includes (1) differentiation of endothelial cells which results in an assembly of vascular tubules, (2) sprout formation, and (3) the development of large vessels from smaller blood vessels. Intersomitic (ie, intersegmental) vessels are a useful model for sprouting angiogenesis and vessel pathfinding. They are the first vessels in the embryo to form by sprouting angiogenesis and their navigation between somites is guided by the same cues that guide axon growth cones (for review see ).

We found that knockdown of TUF1 induces a partial angiogenic arrest of the intersomitic vasculature (ISV). Therefore we may conclude that TUF1 is required for the angiogenic process. This phenomenon has been described also for well-characterized genes such as EphrinB2. Knockdown of EphrinB2 in mice resulted in angiogenic arrest of ISV. Also severe defects in sprouting of intersomitic vessels from the dorsal aorta have been demonstrated for C-terminal Src kinase (Csk) knockout mouse embryos. As mentioned, TUF1 has a conserved phosphotyrosine-binding (PTB) domain. PTB domains have a pleckstrin homology (PH)-like fold. Guanine nucleotide exchange factors (GEFs) include a PH domain and frequently also a DBH homology (DH) domain that catalyze the GDP-GTP exchange reaction of Rho proteins, thereby transmitting signals regulating actin-containing fibers and gene expression. We may therefore hypothesize that TUF1 encodes for a new GEF that is involved in intersomitic vascular development. Interestingly, it has been shown that Sema3E and Plexin-D1 acting through GEFs are both required for intersomitic vascular patterning.

The adaptor protein APPL1 also contains a PH domain, a PTB domain, and a leucine zipper motif 1. The specific function of APPL1 is not known; however, it is speculated that it may recruit kinases AKT2 and PI3K to the cell membrane. AKT2 and PI3K are members of the VEGF pathway which has been shown to be crucial for normal angiogenesis in the embryo. It is not so far off to conclude that TUF1 via domains similar to those found in APPL1 plays a role in regulating endothelial cell migration and vessel connectivity. In TUF1 morphants, ISVs are often truncated or branch out aberrantly, without having a specific pattern. We therefore suggest that this functional defect is likely attributable to a disruption of blood vessels, which could result from a defect during the migration of endothelial cells or during ISVs patterning. It could also be that certain guidance signals (signaling molecules) might play a role in mispatterning of the ISVs. However, defects in endothelial cell polarity (meandering endothelial cells may form such aberrant structures), in regulation of actin cytoskeletal reorganization, or in the recruitment of pericytes that are required to stabilize vessel integrity may be also responsible for ISV defects observed after knockdown of the TUF1. Further work is required to explore the mechanism(s) involved by which somitovasculin regulates the integrity of the ISVs and vessel patterning.

Sources of Funding

This work was supported by a grant from the European Commission (6th Framework Programme, Thematic Priority: Life sciences, genomics and biotechnology for health, contract No.: FunGenES LS HG-CT-2003-503494).

Disclosures

None.

References


25. Mariappan et al Unknown Transcripts and Vasculature Development 1829


Somitovasculin, a Novel Endothelial-Specific Transcript Involved in the Vasculature Development
Devi Mariappan, Rabea Niemann, Martin Gajewski, Johannes Winkler, Shuhua Chen, Suma Choorapoikayil, Marco Bitzer, Herbert Schulz, Jürgen Hescheler and Agapios Sachinidis

Arterioscler Thromb Vasc Biol. 2009;29:1823-1829; originally published online June 18, 2009; doi: 10.1161/ATVBAHA.109.190751

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/29/11/1823

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/09/16/ATVBAHA.109.190751.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Somitovasculin, a novel endothelial-specific transcript involved in the vasculature development

Running title: Unknown transcripts and vasculature development

Devi Mariappan¹*, Rabea Niemann¹²*, Martin Gajewski², Johannes Winkler¹, Shuhua Chen¹, Suma Choorapoikayil², Marco Bitzer², Herbert Schulz³, Jürgen Hescheler¹, Agapios Sachinidis¹#

¹Center of Physiology and Pathophysiology, Institute of Neurophysiology, and Center of Molecular Medicine, University of Cologne (CMMC), Robert-Koch Str. 39, 50931 Cologne, Germany

²Institute for Genetics, University of Cologne, Zülpicher Str. 47, 50674 Cologne, Germany

³Max-Delbrueck-Center for Molecular Medicine - MDC, Robert-Rössle Str. 10, 13092 Berlin, Germany

*Equally contributed

#Corresponding author

Prof. Dr. A. Sachinidis

University of Cologne

Center of Physiology and Pathophysiology

Institute of Neurophysiology

Robert Koch Str. 39

50931 Cologne, Germany

Tel: +49 221 478 73 73

Fax: +49 221 478 69 65

Email: a.sachinidis@uni-koeln.de
Results

Figure I. A, TUF1 (mouse cDNA sequence BC022623) protein shown schematically illustrates the position of the PTB domain with a pleckstrin homology-like fold (from NCBI). B, Mouse TUF1 protein sequence (NP_808300) aligned with the zebrafish ortholog (XP_00133815) using ClustalW. Accession numbers of the protein sequences are given in brackets. C, TUF1 (zebrafish ortholog) protein shown schematically illustrates the position of the pleckstrin homology-like fold (from NCBI).
Table I. Probe sets representing TUFs that are significantly upregulated in the CD31⁺ cells compared to undifferentiated ES cells and 8 days old EBs

<table>
<thead>
<tr>
<th>Probesets</th>
<th>t-test ES vs CD31⁺</th>
<th>t-test d8 EBs vs CD31⁺</th>
<th>Fold change ES vs CD31⁺</th>
<th>Fold change d8 EBs vs CD31⁺</th>
<th>Gene Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1460382_at</td>
<td>2.7E-03</td>
<td>1.7E-03</td>
<td>3.82</td>
<td>2.88</td>
<td>BC020535</td>
</tr>
<tr>
<td>1458969_at</td>
<td>5.1E-02</td>
<td>7.4E-03</td>
<td>2.71</td>
<td>4.56</td>
<td>AU019559</td>
</tr>
<tr>
<td>1458610_at</td>
<td>1.1E-02</td>
<td>4.7E-03</td>
<td>2.41</td>
<td>2.06</td>
<td>---</td>
</tr>
<tr>
<td>1458148_at</td>
<td>6.2E-03</td>
<td>6.8E-03</td>
<td>3.33</td>
<td>2.57</td>
<td>Nlrc3</td>
</tr>
<tr>
<td>1457287_at</td>
<td>7.5E-02</td>
<td>6.4E-03</td>
<td>1.62</td>
<td>2.05</td>
<td>---</td>
</tr>
<tr>
<td>1456762_at</td>
<td>2.2E-04</td>
<td>3.6E-05</td>
<td>16.66</td>
<td>14.38</td>
<td>---</td>
</tr>
<tr>
<td>1452834_at</td>
<td>3.4E-04</td>
<td>1.3E-04</td>
<td>5.36</td>
<td>2.50</td>
<td>2600010E01Rik</td>
</tr>
<tr>
<td>1451415_at</td>
<td>7.9E-04</td>
<td>2.5E-03</td>
<td>43.74</td>
<td>5.56</td>
<td>1810011O10Rik</td>
</tr>
<tr>
<td>1451321_a_at</td>
<td>3.2E-04</td>
<td>4.9E-03</td>
<td>3.76</td>
<td>2.62</td>
<td>Rbm43</td>
</tr>
<tr>
<td>1447915_x_at</td>
<td>2.3E-03</td>
<td>1.4E-03</td>
<td>7.05</td>
<td>3.45</td>
<td>Tmem204</td>
</tr>
<tr>
<td>1447621_s_at</td>
<td>4.4E-05</td>
<td>2.1E-03</td>
<td>6.08</td>
<td>3.99</td>
<td>Tmem173</td>
</tr>
<tr>
<td>1447544_at</td>
<td>1.8E-03</td>
<td>1.2E-05</td>
<td>6.64</td>
<td>3.88</td>
<td>---</td>
</tr>
<tr>
<td>1444052_at</td>
<td>1.8E-05</td>
<td>3.7E-04</td>
<td>14.30</td>
<td>4.62</td>
<td>---</td>
</tr>
<tr>
<td>1443673_x_at</td>
<td>1.4E-01</td>
<td>3.5E-03</td>
<td>1.56</td>
<td>6.37</td>
<td>---</td>
</tr>
<tr>
<td>1443088_at</td>
<td>7.9E-03</td>
<td>5.9E-03</td>
<td>1.42</td>
<td>3.31</td>
<td>9930031P18Rik</td>
</tr>
<tr>
<td>1438027_at</td>
<td>3.5E-04</td>
<td>3.4E-03</td>
<td>13.97</td>
<td>3.77</td>
<td>---</td>
</tr>
<tr>
<td>1437451_at</td>
<td>6.2E-04</td>
<td>8.4E-03</td>
<td>48.93</td>
<td>4.16</td>
<td>1110006O17Rik</td>
</tr>
<tr>
<td>1437087_at</td>
<td>2.1E-03</td>
<td>8.9E-03</td>
<td>8.48</td>
<td>3.38</td>
<td>2210408K08Rik</td>
</tr>
<tr>
<td>1435703_at</td>
<td>1.5E-04</td>
<td>3.0E-03</td>
<td>11.33</td>
<td>3.89</td>
<td>LOC677224</td>
</tr>
<tr>
<td>1435600_s_at</td>
<td>6.4E-04</td>
<td>2.9E-03</td>
<td>9.94</td>
<td>5.76</td>
<td>BC020535</td>
</tr>
<tr>
<td>1435595_at</td>
<td>1.3E-05</td>
<td>1.4E-03</td>
<td>29.35</td>
<td>5.86</td>
<td>1810011O10Rik</td>
</tr>
<tr>
<td>1434621_at</td>
<td>6.0E-05</td>
<td>6.4E-03</td>
<td>9.65</td>
<td>2.75</td>
<td>Tmem204</td>
</tr>
<tr>
<td>1433837_at</td>
<td>2.1E-05</td>
<td>7.0E-04</td>
<td>6.10</td>
<td>2.50</td>
<td>8430408G22Rik</td>
</tr>
<tr>
<td>1428452_at</td>
<td>1.1E-02</td>
<td>7.1E-03</td>
<td>1.87</td>
<td>2.51</td>
<td>2810025M15Rik</td>
</tr>
<tr>
<td>1428420_a_at</td>
<td>3.7E-03</td>
<td>8.7E-03</td>
<td>12.52</td>
<td>4.18</td>
<td>1200009J06Rik</td>
</tr>
<tr>
<td>1426734_at</td>
<td>1.6E-02</td>
<td>1.4E-04</td>
<td>3.02</td>
<td>3.58</td>
<td>Fam43a</td>
</tr>
<tr>
<td>1420277_at</td>
<td>3.3E-02</td>
<td>2.6E-03</td>
<td>2.67</td>
<td>4.49</td>
<td>---</td>
</tr>
</tbody>
</table>
Detailed figure 2, 4, and 5 legends

**Figure 2.** A, Expression of TUF1 mRNA in dorsal aorta (arrow head), posterior cardinal vein (red arrow) and dorsal longitudinal anastomotic vessel (black arrow) at 2 dpf (i) and (ii, upper panel) which represents a higher magnification view of (i) as compared to the sense ISH (ii, lower panel). Expression was also detectable in the forebrain (iii). Sense ISH showed no specific staining, indicating that TUF1 is ubiquitously expressed at a low level according to the slight overall staining in the antisense ISH. B, MO knockdown of TUF2 (ii) showing an enlarged heart evidenced by *cmlc2* (cardiac myosin light chain 2) in 17 out of 20 morphants compared to wt (i) (heart indicated by arrow). Lateral views, anterior to the left, of 2 dpf; bright field stereomicroscopic images are shown.

**Figure 4.** A, Diaminofluorene staining of wild-type embryos (i) and TUF1 morphants (ii-iv) at 3 dpf. Blood cells in the morphants accumulated at various positions including trunk (ii and iv), brain (iii), and above the yolk sac (ii). Hemorrhages are indicated by arrow. Fluorescent images of Tg(*gata1*:DsRed) wild-type embryos (v) and TUF1 morphants (vi) at 3 dpf. Detection of the blood in the vasculature of *gata1*:DsRed transgenic zebrafish animals. Gata1 is a specific marker for blood. Accumulation of blood cells is indicated by arrow heads. CCV: Common cardinal vein, CA=Caudal artery; CV=Caudal vein. B, Expression of *GATA-1* at the 18 somite stage was indistinguishable in control embryos (left) and TUF1 morphants (right). The intermediate cell mass is marked with black arrow. Lateral views, anterior to the right. Pictures were taken under identical exposure times.
**Figure 5.** Phenotypes associated with the knockdown of TUF1 and 3 dpf embryos hybridized with the endothelial-specific markers. **A-C,** Fluorescent images of Tg(*fli1:EGFP*) embryos at 3 dpf (**A**), injected with TUF1-MO1 (**B**) and TUF1-MO2 (**C**). The boxed region in B and C clearly shows the impairment of DLAV formation and irregular patterning of the ISV. Lateral views, anterior to the left, of 3 dpf embryos. **D-F,** 3 dpf embryos after injection with TUF1-MO were analysed for the expression of vascular markers by whole-mount *in situ* hybridization. mRNA of the three endothelial markers *Cdhn* (**D**), *Fli-1* (**E**) and *flk1* (**F**) was detected in the two major vessels, dorsal aorta and posterior cardinal vein in wild-type embryos, whereas expression of these markers in the ISVs of morphants reflected the missing endothelial cells. The arrow in **D-F** indicates that the morphants display disruption in the ISVs.

**References**