Noncanonical Activity of Seryl-tRNA Synthetase Is Involved in Vascular Development

Hajime Fukui, Ryuki Hanaoka, Atsuo Kawahara

Abstract—Vascular endothelial growth factor (Vegf) plays central roles in the establishment of stereotypic vascular patterning in vertebrates. However, it is not fully understood how the network of blood vessels is established and maintained during vascular development. A zebrafish ko095 mutant presented the disorganized vessels with abnormal branching of the established intersegmental vessels (ISVs) after 60 hours postfertilization. The gene responsible for ko095 encodes seryl-tRNA synthetase (Sars) with a nonsense mutation. The abnormal branching of ISVs in ko095 mutant was suppressed by the introduction of either wild-type Sars or a mutant Sars (T429A) lacking the enzymatic activity that catalyzes aminoacylation of transfer RNA for serine (canonical activity), suggesting that the abnormal branching is attributable to the loss of function of Sars besides its canonical activity. We further found the increased expression of vegfa in ko095 mutant at 72 hours postfertilization, which was also reversed by the introduction of Sars (T429A). Furthermore, the abnormal branching of ISVs in the mutant was suppressed by knockdown of vegfa or vegfr2 (kdra and kdrb). Knockdown of vegfc or vegfr3 rescued the abnormal ISV branching in ko095 mutant. These results suggest that the abnormal ISV branching in ko095 mutant is caused by the activated Vegfa-Vegfr2 signal and requires the Vegfc-Vegfr3 signal, because the latter is needed for general angiogenesis. Hence, we conclude that noncanonical activity of Sars is involved in vascular development presumably by modulating the expression of vegfa. (Circ Res. 2009;104:00-00.)

Key Words: angiogenesis ■ intersegmental vessel ■ seryl-tRNA synthetase ■ Vegf

Vascular development is coordinated by multiple signaling pathways.1–3 Transparent zebrafish embryo expressing enhanced green fluorescent protein (EGFP) under the control of fli1a promoter (fli1a:EGFP) allowed us to visualize and analyze the vascular development.4,5 The network of trunk vessels along somite boundaries is well conserved among vertebrates. Primary angiogenic sprouts emanate bilaterally from the dorsal aorta and forms the intersegmental vessels (ISVs) along the somites from ventral to dorsal and the dorsal longitudinal anastomotic vessels along the head to tail axis at the most dorsal side. The secondary sprouts from the posterior cardinal vein connecting to the primary ISVs becomes the intersegmental veins. The remaining secondary sprouts branch to form the parachordal vessels near the horizontal myoseptum that develops toward the head or tail.5,6

Angiogenic sprouting is regulated by tip cells and the cells following the tip cells, the stalk cells.2,3,7 In these 2 types of cells, the importance of vascular endothelial growth factor (Vegf)-mediated regulation has been unraveled. Vegfa induces the migration of the tip cells and the expression of a Notch ligand Dll4 that is exclusively expressed in the endothelial cells, via Vegfr2 in the tip cells. Subsequently, Dll4 represses the expression of Vegfr2 and Vegfr3 via Notch expressed in the stalk cells to inhibit the responses to Vegfs.2,3,8 Primary ISV sprouts express both Vegfr2 and Vegfr3, suggesting that differential expression of Vegfrs affects the vascular development via modulating the responsiveness of endothelial cells to Vegfs.7,9 Consistently, deletion of a single Dll4 allele in mice and knockdown of dll4 in zebrafish results in the defects of vascular formation.10–13 Although the Vegfr-mediated signal is proven to be important for the vascular development, it is not fully understood how Vegf-Vegfr signaling regulates vascular remodeling.

For embryonic development, general protein synthesis is required. Aminoacyl-tRNA synthetases (ARSs) catalyze aminoacylations of their cognate tRNAs that are indispensable for protein synthesis. In addition to their aminoacylation activities (canonical activity), several ARSs are known to possess diverse biological functions (noncanonical activity), such as inflammation, angiogenesis, transcription regulation, and apoptosis.14,15 Human tyrosyl-tRNA synthetase (YARS) released on apoptosis is processed into 2 fragments: the N-terminal fragment of YARS acts as an angiogenic factor,
whereas the C-terminal fragment functions as a cytokine that regulates inflammatory responses. Proteolytic form of tryptophanyl-tRNA synthetase (WRS) is produced on interferon-γ stimulation and acts as an angiotensin factor. Glutamyl-prolyl-tRNA synthetase (EPARS) suppresses angiogenesis through the translational silencing of VEGF-A. Human glutamyl-tRNA synthetase (QARS) in cytoplasm inhibits apoptosis through an association with ASK1 (apoptosis signal-regulating kinase). Therefore, noncanonical activity as well as canonical activity of ARSs is involved in various types of cellular responses. In this study, by analyzing a novel zebrafish ko095 mutant defective in vascular patterning, we demonstrate that the noncanonical activity of seryl-tRNA synthetase (Sars) is involved in the regulation of the trunk vessel patterning.

**Materials and Methods**

**Isolation of ko095**

The ko095 is an embryonic lethal mutant with abnormal branching in the head and trunk blood vessels. The ko095 was crossed to TL strain to create hybrid lines used in meiotic mapping. The ko095 locus was defined by genotyping of ko095 mutant embryos using simple sequence length polymorphic (SSLP) markers on the linkage group 23. We first identified a nonsense mutation in the zebrafish sars gene at 1204 bp (substitution from C to T), resulting in the premature stop at amino acid 402 of Sars protein. Genomic DNA from ko095 mutant was sequenced to confirm the mutation. The sequence of zebrafish sars was deposited at the DNA Data Bank of Japan (accession no. AB453157). To perform genotyping of the ko095 mutation, the ko095 locus was amplified from the isolated genomic DNA by PCR using the following primers: ko095 sense, 5'-ACATCGTGTCAGGTTTGTTCAC-3'; ko095 antisense, 5'-ATCCAGAAGCTTGCTCACA-3'. The ko095 mutation was determined by direct sequencing of the amplified fragments.

**Microinjection of Morpholinos or Synthetic mRNAs**

We designed sars morpholino (MO) (5'-AGGAGAAATGT-GAAAACCTGCAC-3') and 5'- antisense (5'-AGGACACTCT-GAAGAAGCTCAC-3'). As previously described, we designed dial edema in the trunk (Figure 1B, arrowheads). In the neural tube in the trunk (Figure 1B, arrowheads). In the neural tube in the mutant started to exhibit ectopic ISV branching adjacent to the neural tube in the mutant (Figure 1A and 1B). At 60 hpf, the mutant allele contained a C-to-T transition at base pair 1204 in the sars gene, which converted the gene responsible for sars to a SSLP marker (z141) on the linkage group 23 (Figure 2A). We identified sars as the gene responsible for ko095 mutant. The ko095 mutant allele contained a C-to-T transition at base pair 1204 in the sars gene, which converted a glutamine to a premature stop codon at amino acid 402 (Figure 2B). Zebrafish Sars is highly homologous to mammalian Sars (Online Figure II). The ko095 mutant form

**Results**

ko095 Mutant Exhibits Vascular Patterning Defects in the Trunk and Head Vessels

To identify a novel regulator involved in vascular development, we carried out N-ethyl N-nitosourea mutagenesis screening for mutants defective in vascular patterning. Using the Tg(fli1a:EGFP)y1 transgenic fish in which the expression of EGFP is controlled under the fli1a (an endothelial cell specific gene) promoter, we isolated an embryonic lethal mutant ko095 that had defects in vascular patterning. By 48 hpf, we observed no clear difference between wild-type (WT) and ko095 mutant (Figure 1A and 1B). At 60 hpf, the ko095 mutant started to exhibit ectopic ISV branching adjacent to the neural tube in the mutant (Figure 1B, arrowheads). In the following few days, the abnormal ISV branching became more severe exclusively in the dorsal side of the horizontal myoseptum. In addition, we observed an increased number of abnormal branching in cranial vessels of ko095 mutant (Figure 1E and 1F, asterisks). Although we observed pericardial edema in the ko095 mutant, somites, notochord, and neural tube in the ko095 mutant were normally formed by 24 hpf and maintained at least by 72 hpf (Figure I in the online data supplement, available at http://circres.ahajournals.org). These results indicate that ko095 is defective in vascular patterning.

**The Gene Responsible for ko095 Encodes Zebrafish Sars**

To identify the gene affecting in ko095 mutant, we performed the genome mapping of ko095. The ko095 locus was closely linked to a SSLP marker z141 on the linkage group 23 (Figure 2A). We identified sars as the gene responsible for ko095 mutant. The ko095 mutant allele contained a C-to-T transition at base pair 1204 in the sars gene, which converted a glutamine to a premature stop codon at amino acid 402 (Figure 2B). Zebrafish Sars is highly homologous to mammalian Sars (Online Figure II). The ko095 mutant form...
The expression of sars at 32 hpf was significantly suppressed in sars<sup>ko095/ko095</sup> mutants, suggesting that the nonsense mutation in sars induces the decreased stability of zygotic sars Mut mRNA, which is presumably caused by nonsense-mediated mRNA decay. In agreement with the suppression of sars mRNA in sars<sup>ko095/ko095</sup> mutants, Western blotting using anti-human Sars antibody detecting both zebrafish Sars WT and Sars Mut forms revealed that the expression of Sars was reduced in the sars<sup>ko095/ko095</sup> mutants at 72 hpf (Online Figure III). These data indicate that the expression of Sars is attenuated by 72 hpf in sars<sup>ko095/ko095</sup> mutants.

To confirm that sars is the gene responsible for ko095, we examined the effect of depletion of Sars in WT embryo. We designed sars antisense morpholino (sars MO) targeting for the exon 8–intron 8 splicing site of sars. sars morphant but not the embryo injected with control 5-mis MO (5-base mismatched morpholino for sars MO) exhibited abnormal ISV-branching defects that resembled to the sars<sup>ko095/ko095</sup> mutant phenotype (Online Figure IV). We also tested whether the injection of sars WT mRNA into the mutants rescues the vascular-patterning defects of sars<sup>ko095/ko095</sup> mutants. Severity of the abnormal branching in sars<sup>ko095/ko095</sup> mutants varied from mild phenotype (6%, n=412) to severe phenotype (19%, n=412) (Online Figure V). The abnormal ISV branching was observed in 25% of the embryos obtained by the intercross of heterogeneous Sars<sup>WT/ko095</sup>, as expected according to Mendelian inheritance. Injection of zebrafish and human sars WT mRNA, but not sars Mut, effectively rescued the abnormal ISV branching in the mutant, as indicated by the decreased number of the embryos with abnormal branching (the Table and Figure 3C), presenting the conserved role of Sars between zebrafish and mammals. Given the decrease in Sars of sars<sup>ko095/ko095</sup> mutants at 72 hpf, the phenocopy by knockdown of sars in WT embryos and the rescue of the vascular-patterning defects of sars<sup>ko095/ko095</sup> mutants by sars WT mRNA injection, the abnormal ISV branching in the mutant appeared to be ascribed to the loss of function of zygotic Sars.

**Involvement of Noncanonical Activity of Sars in Trunk Vascular Patterning**

We assumed that impairment of general protein synthesis attributable to the loss of zygotic Sars canonical activity in

---

**Table.**

<table>
<thead>
<tr>
<th>Dose (pg)</th>
<th>Normal Branching</th>
<th>Abnormal Branching</th>
<th>Phenotype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Noninjected</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mut</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th>Noninjected</th>
<th>Injected mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>412 311 (75)</td>
<td>23 (6) 78 (19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05, ‡P<0.01.
the mutant did not cause the abnormal branching, because we observed the increase instead of the decrease in the number of abnormal vessels and the extension of abnormal branch until 96 hpf (Figure 1). To test whether canonical activity of Sars is needed for abnormal branching, we examined the effect of the expression of a mutant Sars (T429A) unable to bind to Ser on the abnormal ISV branching in sarsko095/ko095 mutants. We first confirmed that Sars (T429A) as well as Sars Mut completely lacked the canonical activity in contrast to Sars WT (Figure 3A and 3B). The aberrant ISV branching in the mutant was substantially suppressed by mRNA injection of the enzymatically inactive mutant T429A (100 pg) to the same extent as sars WT (the Table, Figure 3C, and Online Table I). These data suggest that the noncanonical activity of zygotic Sars is involved in the regulation of vascular patterning.

We further excluded the possibility that the inhibition of general protein synthesis is the cause of abnormal ISV branching by examining the effect of a protein synthesis inhibitor cycloheximide (CHX) on angiogenesis in zebrafish. Treatment of zebrafish embryos with CHX (10 μg/mL) significantly suppressed protein synthesis, because heat-induced EGFP expression in embryos containing the reporter gene (HG9B), in which the heat shock protein 70 promoter was connected to EGFP, was strongly inhibited by CHX treatment compared to that of control embryo (Online Figure VI). On the same condition, treatment of WT embryos with CHX (10 μg/mL) between 60 and 72 hpf did not induce abnormal branching of ISVs (Online Figure VI). These data suggest that loss of function of canonical activity of Sars is not the cause of abnormal branching in sarsko095/ko095 mutants.

Sars Regulates Vascular Development in a Cell Nonautonomous Manner

We addressed the question of how the noncanonical activity of Sars regulates the vascular development. sars is strongly expressed in somites during early embryogenesis. This raises the possibility that the depletion of Sars in somites may influence the trunk vascular patterning. We examined whether zygotic Sars cell autonomously or cell-nonautonomously affects the trunk vascular patterning by cell transplantation analysis. Transplanted WT cells incorporated into the myotome of WT embryos did not affect
vascular patterning at 72 to 96 hpf (n=16) (Figure 4, top images). The cells from sars\(^{ko095\times ko095}\) mutants that were transplanted and incorporated into the myotomes of WT embryos induced the abnormal ISV branching around the transplanted area (n=4) (Figure 4B, arrows in middle images). Conversely, WT cells transplanted and incorporated into the myotomes inhibited the extension of abnormal branching in sars\(^{ko095\times ko095}\) mutants (n=11) (Figure 4, arrow in bottom images, and Online Figure VII).

Most of the branched vessels sprouting from one ISV in sars\(^{ko095\times ko095}\) mutants almost reached to the neighboring ISVs. Abnormal branching was observed bilaterally in right ISV and left ISV in the sars\(^{ko095\times ko095}\) mutant. The disruption of the growing branches by the implantation of WT cells in sars\(^{ko095\times ko095}\) mutants may indicate the direct effect of the implanted cells and exclude the possibility of the inherent variation. Because it was very rare that the transplanted cells were incorporated into the endothelial cells of ISVs, we could not examine how zygotic Sars-deficient endothelial cells in WT recipients behave in vascular development. These data, therefore, indicate that Sars at least functions in a cell-nonautonomous manner in the trunk vascular patterning, although we cannot exclude the possibility that Sars cell autonomously functions in endothelial cell of sars\(^{ko095\times ko095}\) mutants.

**Vegfa-Vegfr2 Signal and Vegfc-Vegfr3 Signal Are Required for Abnormal Branching of ISVs**

Vegfa-Vegfr2 (Kdr) and Vegfc-Vegfr3 (Flt4) signaling pathways are essential for vascular development in zebrafish and mammals. In the vascular sprouting, tip cells responding to Vegfa express Dll4, thereby suppressing Vegfr2 and Vegfr3 through Notch signaling in the stalk cells. In sars\(^{ko095\times ko095}\) mutants, the abnormal branching started after the establishment of ISVs. We hypothesized that the inhibitory signaling mediated by Dll4-Notch on Vegfr3 is disturbed in sars\(^{ko095\times ko095}\) mutants. In addition, we noticed that the abnormal ISV branching of sars\(^{ko095\times ko095}\) mutant resembled that of dll4 morphant.\(^{10,12}\)

We examined the involvement of Vegfr3 in the abnormal branching of sars\(^{ko095\times ko095}\) mutants by testing whether depletion of Vegfr3 affects the phenotype. As reported previously,\(^{10,12}\) ectopic branching was observed in the trunk and head vessels of dll4 morphants at 72 hpf, whereas vascular patterning was normal at 48 hpf (Online Figure VIII). In addition to the similarity of the onset and consequence of the abnormal branching between dll4 morphants and sars\(^{ko095\times ko095}\) mutants, we found the increase in the cell number in ISVs at 50 hpf, when the abnormal branching was not yet observed, in these 2 embryos (Online Figure VIII). Furthermore, we examined the effect of depletion of Vegfr3/Flt4 in sars\(^{ko095\times ko095}\) mutants on the cell number and found that depletion of Vegfr3/Flt4 inhibited the increased cell number of the ISVs in sars\(^{ko095\times ko095}\) mutants (Online Figure VIII). We also observed that low dose of vegfa MO (2 ng) or vegfc/flt4 MO (1 ng) rescued the abnormal ISV branching in sars\(^{ko095\times ko095}\) mutants at 72 hpf (Figure 5). We evaluated the abnormal ISV branching using the embryos derived from the intercross of heterogeneous sars\(^{WT\times ko095}\) carriers and found the suppression of abnormal branching in vegfc MO–injected embryos (abnormal ISV branching; 2%, n=92) and in vegfr3/flt4 injected embryos (abnormal ISV branching; 4%, n=97).

We further examined the involvement of Vegfa-Vegfr2 signaling in the abnormal branching, because Vegfr2 is known to be suppressed by Notch in the stalk cells. In zebrafish, there are 2 vegfr2/kdr genes (kdra and kdrb).\(^{9}\) As shown in Figure 5, the abnormal branching in sars\(^{ko095\times ko095}\) mutants was effectively suppressed not only by coinjection of kdra MO and kdrb MO (1 ng each, abnormal ISV branching; 6%, n=109) but also by injection of low dose of vegfa (0.5 ng, abnormal ISV branching; 5%, n=162).

By using Vegfr inhibitors, we confirmed the requirement of Vegfa-Vegfr2 and Vegfc-Vegfr3 signaling for the abnormal branching in sars\(^{ko095\times ko095}\) mutants. The embryos derived from heterogeneous sars\(^{WT\times ko095}\) carriers were treated either with SU5614\(^{25}\) (Vegfr2 and Vegfr3 inhibitor; 2 \(\mu\)mol/L) or with MAZ51\(^{26}\) (Vegfr3-specific inhibitor; 2 \(\mu\)mol/L) between 54 and 72 hpf. The abnormal ISV branching was significantly suppressed by MAZ51 and SU5614 but not AG1478\(^{32}\) (EGFR-specific inhibitor; 1 \(\mu\)mol/L) (Online Table II). These results indicate that both Vegfa-Vegfr2 and Vegfc-Vegfr3 signaling pathways are required for the vascular patterning defects in sars\(^{ko095\times ko095}\) mutant.

![Figure 5. Vegfa-Vegfr2 signal and Vegfc-Vegfr3 signal are required for the induction of abnormal ISV branching in sars\(^{ko095\times ko095}\) mutants. Effect of knockdown of Vegfs-Vegfrs on the vascular patterning defects in sars\(^{ko095\times ko095}\) mutant. The abnormal ISV branching in sars\(^{ko095\times ko095}\) mutant was suppressed by injection of vegfr3/flt4 MO (1 ng, top left), vegfc MO (2 ng, bottom left), kdra + kdrb MO (1 ng each, top right), or vegfa (0.5 ng, bottom right).](http://circres.ahajournals.org/Content/early/2017/07/19/hsa8681/Figure5.jpg)
Noncanonical Activity of Sars Affects the vegfa Expression

To clarify the relevance of Vegfs and Vegfrs to the noncanonical activity of Sars, we examined the expression of Vegf- and Notch-signaling molecules (vegfa, vegfc, vegfr1/flt1, kdra/flk1, vegfr2/flt4, dll4 and notch1b). Whole-mount in situ hybridization analysis revealed no difference in the expression of these genes between WT and sarsko095/ko095 mutant at 32 hpf (Online Figure IX). We analyzed the subsequent expression of Vegf-signaling molecules by quantitative real-time PCR at 72 hpf, when the abnormal ISV branching in sarsko095/ko095 mutant was observed. The expression of vegfa mRNA but not vegfc, kdra/flk1, or vegfr3/flt4 was significantly increased in the sarsko095/ko095 mutants (Figure 6). The increased vegfa expression was effectively inhibited by the introduction of the enzymatically inactive sars (T429A), whereas the expression of sars (T429A) did not affect the expression of other molecules we tested (Figure 6). These results suggest that the noncanonical activity of Sars may negatively regulate the vegfa expression.

Discussion

We demonstrate for the first time that the noncanonical activity of Sars is involved in the vascular patterning of trunk blood vessels. The previously unidentified noncanonical activity of Sars was confirmed by the finding that the vascular patterning defects in sarsko095/ko095 mutants were restored by the injection of enzymatically inactive sars (T429A). We suppose that the noncanonical activity of Sars negatively regulates the expression of vegfa, thereby being involved in the suppression of ectopic branching from the established ISVs. EPARS has been reported to suppress VEGF-A expression on interferon-γ-stimulated monocytes. Interferon-γ induces the formation and activation of GAIT (interferon-γ-activated inhibitor of translation) consisting of heterotetrameric RNA-binding complexes including EPARS. EPARS negatively regulates the translation of VEGF-A mRNA. ARSs including Sars are localized not only in the cytoplasm but also in the nucleus. Sars has a potential to bind to mRNA. Thus, it is possible that Sars in the nucleus may directly regulate the transcription and translation of vegfa in zebrafish. Further analysis is required to clarify how Sars regulates the vegfa expression.

It is unclear why the abnormal ISV branching did not start until around 60 hpf. Functional zygotic Sars is not produced in the embryos of sarsko095/ko095 mutants. Why is the protein synthesis preserved during angiogenesis? One possibility is that the amount of maternally supplied tRNASer and Sars detected by the whole-mount in situ hybridization at 1-cell stage, and the Western blotting with anti-Sars (Online Figure III) may be enough for protein synthesis but not for the noncanonical activity of Sars that may negatively regulate the vegfa expression. It is noteworthy that the maternal supply of Sars, Qars, Kars, Mars, and Sars for early embryogenesis in zebrafish is also reported by other group. Transplantation analysis showed that Sars in myotome cells influences at least the trunk vascular patterning in a cell-nonautonomous manner. When the cells from sarsko095/ko095 mutants were incorporated into the myotomes, the aberrant ISV branching is induced around the transplanted cells in WT embryo. Consistent with the requirement of Sars function in myotomes, sars is detected in somites at 24 hpf, and the sars expression is decreased in somites of sarsko095/ko095 mutants (Online Figure III). Vegfa released from the somites regulates the vascular development. Therefore, the noncanonical activity of Sars in the somites appears to be important for the maintenance of trunk vascular patterning in zebrafish. Because we could not analyze how loss of function of zygotic Sars in endothelial cells behaves in trunk vessel formation using the transplantation analysis, we cannot rule out the possibility that Sars cell-autonomously regulates the vascular patterning in endothelial cells.

Both Vegfa-Vegfr2 and Vegfc-Vegfr3 signaling pathways are required for angiogenesis. We think that the abnormal ISV branching in sarsko095/ko095 mutant is attributable to the increased expression of vegfa and requires the Vegfc-Vegfr3 signaling because it is generally required for angiogenesis. We found no alteration of vegfc and vegfr3 expression between WT and sarsko095/ko095 mutant. We further need to investigate the phosphorylation of Vegfr3 and Vegfr3-mediated downstream events in sarsko095/ko095 mutants to clarify the relevance of noncanonical activity of Sars to Vegfc-Vegfr3 signal.
Acknowledgments
We thank M. Sone and M. Minamimoto for technical assistance; N. Mochizuki, M. Matsuda, S. Fukuhara, M. Kamei, and S. Isogai for critical reading and valuable comments; Y. Kaziro for suggestions and encouragement; M. Masuda and K. Wakasugi for suggestions about experimental procedures; W. Herzog and D. Y. R Stainier for discussion and sharing unpublished data; N. Lawson, M. Hibi, and H. Okamoto for fish and reagents; K. Kawakami and the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology of Japan for the HG9B line.

Sources of Funding
This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and by the Japan Society for the Promotion of Science.

Disclosures
None.

References
Noncanonical Activity of Seryl-tRNA Synthetase Is Involved in Vascular Development
Hajime Fukui, Ryuki Hanaoka and Atsuo Kawahara

Circ Res. published online May 7, 2009;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2009/05/07/CIRCRESAHA.108.191189.citation

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/05/07/CIRCRESAHA.108.191189.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research 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 Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Online Materials and Methods

Whole-Mount In Situ Hybridization. Anti-sense RNA probes labeled with digoxigenin (DIG) were prepared by using the RNA labeling kit (Roche). Fixed embryos were hybridized with antisense RNA probes at 65 ºC overnight in hybridization buffer (5 X SSC, 50% formamide, 5mM EDTA, 0.1% Tween 20, 50 μg/ml heparin, 1 mg/ml RNA torula). After hybridization, embryos were washed twice at 65 ºC for 30 min in washing buffer I (50% formamide, 2 X SSC, 0.1% Tween 20), twice at 65 ºC for 15 min in washing buffer II (2 X SSC, 0.1% Tween 20), twice at 65 ºC for 30 min in washing buffer III (0.2 X SSC, 0.1% Tween 20). Embryos were incubated with maleic acid solution (0.1 M maleic acid [pH 7.5]) at room temperature (r.t.) for 15 min. After preincubation with blocking buffer (0.1 M maleic acid [pH 7.5], 5% sheep serum, 2% blocking reagent [Roche]) at r.t. for 2 hr, embryos were incubated with anti-DIG antibody conjugated with alkaline phosphatase (Roche) in blocking buffer at r.t. for 4 hr. Embryos were washed six times in phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBST). Colorimetric reaction was carried out using BM purple as substrate and was terminated by washing the embryos with PBST.

Western Blotting. WT and ko095 embryos were discriminated by morphology of blood vessel patterning at 72 hpf. Each twenty embryos were incubated with lysis buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 3 mM EDTA, 1% Nonidet P-40, 2 mM sodium orthovanadate, and protease inhibitor mixture Complete [Roche]). Total cell lysates were centrifuged at 15 000g for 15 min to remove insoluble materials and proteins from each lysates were separated by SDS-PAGE (9% gel), and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane filters (Millipore). The filters were incubated with mouse anti-Sars antibody (clone 1H4, Abnova corporation, 1:2000 dilution) or rabbit anti-Erk1/2 antibody (Cell Signaling Technology, 1:2000 dilution). Proteins reacting with primary antibodies were visualized by an enhanced chemiluminescence system with peroxidase-conjugated and species-matched secondary antibodies and analyzed with an LAS-1000 system (Fuji Film).
**Blocking of protein synthesis.** A transgenic line HG9B under heat inducible hsp70 promoter was used in protein synthesis inhibition assay. The embryos were exposed to heat shock (37 °C) for 20 min (between 59.7 hpf and 60 hpf), and the embryos were subsequently treated with protein synthesis inhibitor cycloheximide (CHX; 10 μg/ml) between 60 hpf and 72 hpf. The embryos derived from heterogeneous \( sars^{ko095/WT} \) carriers were treated with protein synthesis inhibitor cycloheximide (CHX; 10 μg/ml) between 60 hpf and 72 hpf.

**Real-time reverse transcription-PCR**

Total RNA was prepared by using Trizol (Invitrogen) from 72 hpf embryos. The concentration and quality of total RNA were determined by spectrophotometer and agarose electrophoresis, respectively. Quantitative real-time reverse transcription (RT)-PCR was carried out using QuantiFast SYBR Green RT-PCR kit (Qiagen). Total RNA (20 ng) was reverse transcribed for 10 min at 50 °C, followed by a denaturing step at 95 °C for 5 min and 40 cycles of 10 sec at 95 °C and 30 sec at 60 °C. Fluorescence data were collected and analyzed using Mastercycler ep realplex (Eppendorf). The primers used for amplification were as follows: \( sars \) primers, 5’-TGCTTTGAACCACGCAGCTAG-3’ and 5’-ATGACACGAGTGGTCGCACAC-3’; \( vegfa \) primers, 5’-TCCTGTGTGTTCTCATGC-3’ and 5’-TGCATTACACTTGGTGTTGTC-3’; \( vegfc \) primers, 5’-GACCACACCATTACCTAGACAAG-3’ and 5’-ATTTGTTGCAGGTAGGTGTG-3’; \( kdra/flk1 \) primers, 5’-TACCTGAGAAGTAAGAGGTG-3’ and 5’-GTACAAATCCTCTTGCTCTTCC-3’; \( flt4 \) primers, 5’-CTCCACTCACAATAGAGGATC-3’ and 5’-AAGATGCTCTCTTGAGCCATC-3’.
eflα primers, 5’-CTGGAGGCCAGCTCAAACAT-3’ and 5’-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3’. For normalization, expression of eflα was determined in parallel as an endogenous control.
Legends for Online Figures

**Online Figure I. Morphology of heart, somites, notochord and neural tube in ko095 mutants.** A through E, Anterior is left (lateral view) in all pictures. A and B, Wild-type (WT). C and D, ko095 mutant. ko095 mutant embryo at 72 hpf had severe pericardial edema (C and D, arrows) compared to that of WT (A and B). D, Overall morphology of somites (SO), floor plate (FP; arrow), notochord (NC) and neural tube (NT) was comparable between WT and ko095 at 30 hpf, 54 hpf and 72 hpf stages. After examining morphology, genotypes of embryos were determined by direct sequence of the ko095 locus.

**Online Figure II. Predicted amino acid sequence of Sars proteins.** Identical amino acids among zebrafish (Dr), human (Hs), mouse (Mm) and chicken (Gg) Sars are boxed. Dash indicates gaps to optimize the sequence alignment.

**Online Figure III. Expression pattern of sars during early embryogenesis.** A and C through J, Whole-mount in situ hybridization with antisense sars probe. sars was maternally expressed (A), because any signal in 1-cell stage was not detected by sense sars probe. Ubiquitous expression of sars (C through H) was observed throughout embryogenesis (from bud to 48 hpf). E through G, The expression of sars was strongly expressed in lens, midbrain and somites at 24 hpf. I and J, The sars expression in ko095 mutant (J) was significantly suppressed compared to that of WT (I). K, Western blotting with Sars antibody. Sars antibody recognized purified proteins for Sars (10 ng) and Sars Mut (10 ng). Protein expression of Sars Mut was strongly decreased in the lysates from ko095 (72 hpf), while Sars was expressed in the lysates from WT (72 hpf). Erk expression was comparable in both WT and ko095 lysates.

**Online Figure IV. Morphology of abnormal ISV branching in sars\textsuperscript{ko095/ko095} mutant.** We observed morphology of ten ISVs over the yolk tube at 72 hpf. By the severity of the
branching (arrowheads), the vascular phenotypes was categorized to the three groups: normal, mild (one or two in ten ISVs) and severe (from three to ten in ten ISVs).

**Online Figure V. Knockdown of sars leads to the abnormal branching in trunk blood vessels.** *sars* MO is an antisense morpholino for the exon8-intron8 splicing site of *sars*. 5-mis MO is 5 base mis-matched morpholino for *sars* MO. RT-PCR analysis (right panel) revealed that injection of *sars* MO led to the predominant production of aberrant forms (asterisks) of *sars* mRNA, resulting in the creation of premature stop codon. Ectopic abnormal branching of ISVs was observed in *sars* MO (5 ng)-injected embryo, but not in uninjected- and 5-mis MO (5 ng)-injected embryos (left panel).

**Online Figure VI. Blocking of protein synthesis results in thin blood vessels in wild-type embryo.** Embryos were treated either by protein synthesis inhibitor cycloheximide (CHX; 10 μg/ml) or by DMSO (control) between 60 hpf and 72 hpf. A Wild-type embryo treated with CHX exhibited thin blood vessels compared to that of control embryos. B, Transgenic line HG9B possesses the reporter gene (hsp70 promoter+EGFP). Exposure of heat shock (37 °C) for 20 min (between 59.7 hpf and 60 hpf) induced strong EGFP expression in whole body of HG9B embryo at 72 hpf. Heat-induced EGFP expression in HG9B embryo was suppressed by CHX treatment.

**Online Figure VII. Sars can cell non-autonomously regulate vascular patterning.** The aberrant ISV branching in *sars* ko095/ko095 mutant was suppressed around WT-derived donor cells in myotomes at 72 hpf in transplanted left side (lower panel; arrowhead), whereas the patterning defects was not affected in un-transplanted right side (upper panel).

**Online Figure VIII. Phenotypes of dll4 morphant resemble the phenotypes of sars ko095/ko095 mutant.** A, *dll4* MO (5 ng)-injected embryos at 72 hpf. The pericardial edema (arrow) and abnormal branching in head (asterisk) and trunk vessels (arrowheads) in *dll4*
morphants are exactly similar to those found in the \textit{sars}^{ko095/ko095} mutant as shown in Figure 1. B and C, Cell number in the ISVs of \textit{sars}^{ko095/ko095} mutants as well as of \textit{dll4} morphant embryos was counted by the number of nuclear accumulating EGFP (arrows) using a confocal microscopy (B) and quantified as shown in (C) (\textit{sars}^{WT/WT}; n=42, \textit{sars}^{ko095/ko095}; n=49, \textit{dll4} MO-injected \textit{sars}^{WT/WT}; n=51, \textit{flt4} MO-injected \textit{sars}^{WT/WT}; n=55, \textit{flt4} MO-injected \textit{sars}^{ko095/ko095}; n=49). The effect of depletion of \textit{vegfr3/fht4} MO (1 ng) on the cell number is analyzed by the distribution of cell number per ISV and of percentage of cells in ISV (C).

**Online Figure IX. Expression of marker genes involved in Vegf- and Notch-mediated signaling in \textit{sars}^{ko095/ko095} mutants.** We examined the expression of marker genes in \textit{sars}^{ko095/ko095} mutants at 32 hpf by whole-mount \textit{in situ} hybridization. Probes are indicated at left of each panel. The expression of \textit{sars} was strongly suppressed in \textit{sars}^{ko095/ko095} mutant, whereas the expression of indicated genes involved in Vegf- and Notch-mediated signaling was comparable between WT and \textit{sars}^{ko095/ko095} mutant. After examining the expression of marker genes, genotypes of embryos were determined by direct sequence of the \textit{ko095} locus.
Online Figures

Online Figure I

Supplement Material.
Online Figure I (H. Fukui et al.)

E

30hpf 54hpf 72hpf

WT

ko095

30hpf 54hpf 72hpf
Online Figure III

Supplement Material.

Online Figure III (H. Fukui et al.)

A. 1-cell
B. sense-probe 1-cell
C. bud
D. 15s
E. 24hpf
F. 24hpf
G. 24hpf
H. 48hpf
I. WT
J. ko095 32hpf
K. Sars protein
   embronic lysates
   WT Mut
   WT ko095
   WT Sars
   Mut Erk
Online Figure IV

Supplement Material.
Online Figure IV (H. Fukui et al.)

Online Figure V
Supplement Material.
Online Figure V (H. Fukui et al.)
Online Figure VI

Supplement Material.
Online Figure VI (H. Fukui et al.)

A

DMSO

CHX (10 μg/ml)

B

HG9B

HG9B + heat shock

HG9B - heat shock

HG9B + heat shock

Online Figure VII

Supplement Material.
Online Figure VII (H. Fukui et al.)

fli1α:EGFP

right ISV

rhodamine dextran

merge

left ISV
Online Figure IX

Supplement Material.
Online Figure IX (H. Fukui et al.)

Supplement Material.
Online Table I (H. Fukui et al.)

<table>
<thead>
<tr>
<th>injected mRNA</th>
<th>number</th>
<th>normal</th>
<th>abnormal branching</th>
<th>mild</th>
<th>severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>uninjected</td>
<td>152</td>
<td>118 (77%)</td>
<td>7 (5%)</td>
<td>27 (18%)</td>
<td></td>
</tr>
<tr>
<td>sars T429A</td>
<td>104</td>
<td>92 (88%)&quot;</td>
<td>7 (7%)</td>
<td>5 (5%)</td>
<td></td>
</tr>
</tbody>
</table>
Supplement Material.
Online Table II (H. Fukui et al.)

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>number</th>
<th>normal branching</th>
<th>mild</th>
<th>severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>125</td>
<td>94 (75%)</td>
<td>5 (4%)</td>
<td>26 (21%)</td>
</tr>
<tr>
<td>MAZ51 (2 μM)</td>
<td>187</td>
<td>170 (91%)**</td>
<td>7 (4%)</td>
<td>10 (5%)</td>
</tr>
<tr>
<td>SU5614 (2 μM)</td>
<td>175</td>
<td>153 (88%)**</td>
<td>9 (5%)</td>
<td>13 (7%)</td>
</tr>
<tr>
<td>AG1478 (1 μM)</td>
<td>128</td>
<td>97 (76%)</td>
<td>6 (5%)</td>
<td>25 (18%)</td>
</tr>
</tbody>
</table>