Genetic Evidence for a Noncanonical Function of Seryl-tRNA Synthetase in Vascular Development

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Abstract—In a recent genetic screen, we identified mutations in genes important for vascular development and maintenance in zebrafish (Jin et al. Dev Biol. 2007;307:29–42). Thirty-two mutations at the adrasteia (adr) locus cause a pronounced dilatation of the aortic arch vessels as well as aberrant patterning of the hindbrain capillaries and, to a lesser extent, intersomitic vessels. This dilatation of the aortic arch vessels does not appear to be caused by increased cell proliferation but is dependent on vascular endothelial growth factor (Vegf) signaling. By positional cloning, we isolated seryl-tRNA synthetase (sars) as the gene affected by the adr mutations. Small interfering RNA knockdown experiments in human umbilical vein endothelial cell cultures indicate that SARS also regulates endothelial sprouting. These analyses of zebrafish and human endothelial cells reveal a new noncanonical function of Sars in endothelial development. (Circ Res. 2009;104:1260–1266.)

Key Words: seryl-tRNA synthetase ■ SerRS ■ zebrafish ■ angiogenesis ■ vascular dilatation

The formation and maintenance of the cardiovascular system is essential for supplying nutrients and oxygen throughout the organism. To ensure survival, the vasculature needs to be able to respond quickly to hypoxic situations, eg, during wound repair. On the other hand, an imbalance in the vasculature and its surrounding tissues, as well as in the dependence of one for the survival of the other. Whereas mammals are dependent on the vascularization of the placenta for embryonic development, zebrafish embryos have the unique advantage that they can survive without a functional vasculature, or heart beat, for up to 7 days postfertilization, which is well into free swimming larval stages.6–8 Additionally, the optical clarity of zebrafish embryos allowing for in vivo analysis of fluorescent transgene expression9 and their amenability to genetic screens10–12 have rendered them ideal to study vascular development and identify and analyze new genes regulating that process.

In the present report, we analyze the effect of mutations in the zebrafish gene adrasteia (adr), and show that adr corresponds to the seryl-tRNA synthetase (sars) gene. Aminoacyl-tRNA synthetases catalyze the ligation of specific amino acids to their cognate tRNAs and thereby assemble the building blocks for RNA translation and protein synthesis. Whereas this essential function is based on the catalytic activity of these enzymes, there is increasing evidence that these proteins are versatile and have acquired functions in processes not directly related to protein synthesis. These so-called noncanonical functions range from interactions of tRNA-synthetases with themselves and other proteins in a multisynthetase complex (reviewed elsewhere13) to specific functions in silencing of gene translation14 or as angiogenic or angiostatic cytokines.15,16 Other acquired functions of mammalian tRNA-synthetases include antiapoptosis and translational control (reviewed elsewhere17).

Although their catalytic enzymatic core is highly conserved from bacteria to mammals, there is evidence that in some aminoacyl-tRNA synthetases, new functions were acquired by appending extra domains to the core domain.18–20 For example, human tyrosyl-tRNA synthetase has gained an extra C-terminal domain of ~170 aa21 that has cytokine-like effects on leukocytes and monocytes.22 In contrast, human tryptophanyl-tRNA synthetase acquired 60 aa at its N terminus, the presence or absence of which is regulated by alternative splicing. Both of the isoforms possess aminoacylation activity, but only the shorter form also exhibits angiostatic activity, blocked by the N-terminal appendage in the longer form.15,23 Although Sars has not been described to exhibit noncanonical activities, the vertebrate enzyme also contains a C-terminal appendage of ~40 aa, which appears to be a vertebrate invention (data not shown).
Here, we show for the first time that Sars has indeed acquired a noncanonical function in vertebrates, one that plays a key role in vascular development.

Materials and Methods

Zebrafish Husbandry and Positional Cloning

Zebrafish (Danio rerio) embryos were obtained from the mixed wild-type strain in the laboratory and raised at 28°C as previously described. Zebrafish maintenance and procedures were approved by the University of California at San Francisco institutional animal care and use committee.

Genomic mapping and positional cloning was performed using simple sequence length polymorphism (SSLP) markers as previously described. Sequences of published SSLP markers can be found at http://zebrafish.mgh.harvard.edu.

Pharmacological Manipulations and Vessel Diameter Analysis

Embryos were treated with either 1 μmol/L SU5416 (Calbiochem) or with the indicated amounts of cycloheximide (Sigma) or the seryl analog 5'-O-[N-(l-seryl)sulfamoyl]adenosine (IDT) from 60 to 72 or 74 hours postfertilization (hpf). As a control, embryos from the same batch were treated with DMSO at the same concentration (0.01% to 0.05%).

Embryos were imaged with selective plane illumination microscopy or Zeiss Lumar stereo microscopy. The vessel diameter was determined with ImageJ.

The mean values are depicted, error bars denote SD. Statistical significance was determined using unpaired (2 sample unequal variance) t test.

5-Bromodeoxyuridine Incorporation and Staining

Embryos were injected with a 5-bromodeoxyuridine (BrdUrd) solution (10 mmol/L BrdUrd, 0.2 mol/L KCl, 1% phenol red) into the pericardial sac at 58 hpf, fixed in 4% paraformaldehyde at 74 hpf, treated with 2 mmol/L HCl for 1 hour, embedded in 4% agarose, and sectioned using a Leica Vibratome. Sections were stained for anti-mouse Alexa546 (Invitrogen) as secondary label. Processed samples were mounted in Fluoromount-G (Southern Biotech), and the images were acquired using a Leica confocal microscope.

Evans’s Blue Injection

Larvae were injected with a 1% Evans blue (Sigma) solution into the circulation before the heart inflow (fusion of the common cardinal veins) at 74 hpf, fixed in 4% paraformaldehyde, embedded in 4% agarose, and sectioned using a Leica Vibratome. Sections were stained for incorporated BrdUrd with anti-BrdUrd antibody (Roche), using anti-mouse Alexa546 (Invitrogen) as secondary label. Processed samples were mounted in Vectashield (Vector Laboratories), and the images were acquired using a Leica confocal microscope.

Small Interfering RNA Transfection of Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) (Invitrogen) were grown in Medium 200 with Low Serum Growth Supplements (Invitrogen) according to the instructions of the manufacturer. They were transfected using Oligofectamine (Invitrogen) only (mock transfection) or either 70 or 150 nmol/L of On-Target plus SMARTpool small interfering (si)RNA (Thermo Scientific, L-013477-01) against seryl-tRNA-synthetase (GenBank accession no. NM_006513) in OptiMEM medium (GIBCO). Sixteen hours after transfection, cells were subjected to an in vitro angiogenesis assay: Tube Formation (Trevigen) according to the instructions of the manufacturers. Briefly, endothelial cells were evenly seeded onto a basement membrane extract and stimulated with 10 ng/mL vascular endothelial cell (VEGF) in Medium 200 with Low Serum Growth Supplements (Invitrogen). After 6 hours, they were labeled with 2 mmol/L calcine and visualized using a Leica stereomicroscope using Volocity. Each experiment was carried out in triplicates. After image acquisition, the number of branch points was counted in equal size areas and statistical significance was determined using unpaired (2-sample unequal variance) t test. The probability value was considered statistically significant when P<0.05.

Results

The adr mutations were identified in a recent genetic screen for vascular regulators. adr mutants appear morphologically unaffected for the first 3 days of development (Figure 1). By observing Tg(fli1:EGFP) expression, we noticed a pronounced dilatation of the aortic arch vasculature (Figure 1F) on day 3 (72 hpf) and subsequent aberrant branching of the hindbrain capillary network and, to a lesser extent, the intersomitic vessels (Figure 1J). Although initially unaffected, circulation gradually diminishes until at day 5 of development, it is confined to a minimal circulatory loop (data not shown). It is important to note that at 60 hpf, adr mutants are still indistinguishable from their wild-type siblings (data not shown), so that the manifestation of the dilatation phenotype happens within 12 hours. We quantified the extent of dilatation by measuring the vessel diameter in wild-type and adr mutant embryos. Figure 1H shows that in 80-hpf adr mutants, the diameter of the aortic arch vessels was increased by up to 2-fold.

To investigate the increase in vessel diameter, we analyzed cell division, as well as the physical environment of the aortic arch vasculature. We labeled dividing cells by injecting BrdUrd into the circulation of 58-hpf embryos, allowed for incorporation until 74 hpf at which time we fixed the animals. We then cut transverse sections containing the second or third visible aortic arch vessels (AA3 and AA4) and examined them for incorporated BrdUrd. We observed very few (0–1) cell divisions in the endothelial cells of the second or third aortic arch vessels of wild-type embryos during that time, although the surrounding tissue was very actively dividing (Figure 2A). Likewise, no cell division was observed in the aortic arch vessels of adr mutants (Figure 2B), indicating that the increase in vessel diameter is not caused by an increase in cell number. Additionally, we observed no obvious difference in the extracellular matrix as assessed by fibronectin and laminin expression (data not shown). Therefore, it is likely that adr mutants represent a model for vascular dilatation rather than vascular enlargement, the latter resulting from an increase in endothelial cell number.

To further analyze the physical effects of the mutation, we crossed adr heterozygous carriers to a transgenic line expressing dsRed under the gata1 promoter, so that we could examine the distribution of red blood cells in detail. No Tg(gata1:dsRed) adr-positive cells were found outside the vasculature in adr mutants (data not shown). We further analyzed the permeability of the vessels by injecting Evans blue into the circulation at 74 hpf. After 30 minutes, we observed no leakage of Evans blue in the tissue surrounding the vessel in single plane confocal sections (Figure I in the online data supplement, available at http://circres.ahajournals.org). We did not observe any obvious defect in vascular integrity in adr mutant larvae.
To begin to understand the process leading to this vascular dilatation, we treated embryos from 60 to 76 hpf with the Vegf receptor inhibitor SU5416. Inhibition of Vegf signaling in adr mutants abolished dilatation of the aortic arch vessels, although the inhibitor did not appear to have any effect on the wild-type arch vasculature (Figure 2C and 2D). Therefore, functional Vegf signaling seems to be required for the process of vascular dilatation downstream of the adr mutation.

To isolate the gene affected by the adr mutations, we used SSLPs for mapping and subsequent positional cloning. We located adr on linkage group 23 (LG23), between z20725 and Z5141, 34 to 36.6 cM from the top of the linkage group (MGH map). Consecutive mapping with individually selected SSLP markers placed adr on the fingerprint contig 2305 within close proximity of the sars gene. By sequencing cDNAs from wild-type and mutant adr embryos, we found significant mutations in the sars gene in each of the 2 adr alleles identified. The sars277 allele shows a T 1194-to-G transition, which replaces the highly conserved phenylalanine (F383) with a valine. The phenylalanine at this position is highly conserved from bacteria to human. The sars228 allele shows a G-to-T transition, resulting in a premature stop codon after amino acid 420 (Figure 3A). Additionally, another zebrafish mutation, hst3827, identified in an insertional mutagenesis screen was found to affect the sars gene and described with the same breakdown of circulation at 120 hpf. The tight genetic linkage and existence of 3 different sars mutant alleles, all displaying the same vascular phenotype, indicate that mutations in sars are causing the phenotype.

A well-known function of Sars is to catalyze the ligation of serine to its cognate tRNAs, resulting in seryl-tRNA complexes necessary for translation of RNA into protein. To analyze whether the vascular phenotype observed in adr mutants was caused by defective protein synthesis, we chemically inhibited protein synthesis between 60 and 72 hpf by addition of cycloheximide. Treatment with cycloheximide led to very thin, stretched aortic arch vessels and a reduction in hindbrain capillary formation (Figure 3C and 3D). We also examined the effects of a reduction of protein synthesis by using either reduced concentrations of cycloheximide or a seryl analog, which inhibits Sars activity. We did not observe any dilatation of the aortic arch vessels or aberrant capillary patterning with either treatment (Online
Thus, reduction or lack of protein synthesis did not appear to cause vascular dilatation or aberrant capillary formation, similar to the phenotypes observed in *adr* mutants. Additionally, zebrafish mutants in methionine (*mars*), lysine (*kars*), glutamine (*qars*), and valine (*vars*) tRNA-synthetase genes have been described, none of which exhibits the breakdown in circulation observed in 5 day old *sars* mutants.

To begin to analyze whether the effect of the *adr* mutations on the vasculature is endothelial cell–specific, we reduced SARS expression in HUVECs by transfection with siRNA against human SARS. At a concentration of 75 and 150 nmol/L siRNA per 1×10⁴ cells, after 48 hours, we observed significant cell death in SARS-siRNA transfected but not in mock transfected HUVEC cultures. This effect was concentration-dependent and could be reduced by either decreasing the amount of siRNA or by increasing the number of cells (data not shown). We assume that the increased cell death is related to the general function of SARS in protein synthesis or other functions. Future experiments will aim to test this hypothesis.

**Figure 2.** A and B, Confocal cross-sections of 74-hpf wild-type (A) and *adr*²²⁷⁷ mutant (B) larvae visualized for Tg(*flk1:EGFP*)<sup>s843</sup> expression (green) and BrdUrd (red). BrdUrd labeling indicates that no cell proliferation takes place between 59 and 74 hpf within the endothelial cells (green fluorescent protein [GFP]-positive) of the second visible aortic arch vessel (AA3) of wild-type (A) or *adr*²²⁷⁷ mutant (B) animals. Also note the clear dilatation of the aortic arch vessel (small white bars corresponding to 25 μm in A and B). Scale bars, 50 μm. C and D, Treatment of wild-type and *adr* mutant animals with the VegfR inhibitor SU5416 from 60 to 76 hpf. C, Epifluorescence micrographs of 76-hpf untreated and SU5416-treated wild-type and *adr*²²⁷⁷ mutant larvae (red bars indicate the aortic arch diameter of untreated *adr* mutant larvae). D, Diagram showing the diameter of the aortic arch vessel (AA1) in untreated and SU5416 treated wild-type and *adr*²²⁷⁷ mutant larvae. The aortic arch vessel diameter was measured at the positions indicated in Figure 1G (4 wild-types, 3 mutants, 4 to 5 SU5416-treated wild types and 3 to 6 SU5416-treated mutants were examined; for these latter 2 groups, not all positions were easily visible in all embryos).

**Figure 3.** A, Schematic representation of the Sars protein and the location of the lesions in the 2 mutant alleles Sars<sup>²²⁷⁷</sup> and Sars<sup>²²²⁸</sup>. The blue bar outlines a highly homologous area containing the serine-recognition site, the red bar the conserved enzymatic core, and the green bar the vertebrate-specific C-terminal appendage. B through D, Cycloheximide (cyc) treatment of zebrafish embryos between 60 and 72 hpf; epifluorescence micrographs of DMSO control (B), 100 μmol/L cycloheximide-treated (C), and 200 μmol/L cycloheximide-treated (D) larvae visualized for Tg(*flk1:EGFP*)<sup>s843</sup> expression.
synthesis and would be observed in other cell types as well.

To analyze whether SARS-siRNA had a specific effect on endothelial cells, 16 hours after transfection, we subjected HUVECs to a network formation assay. For this assay, endothelial cells were evenly seeded onto a gel-like extracellular matrix substitute (basement membrane extract) and after Vegf stimulation, underwent a rapid reorganization, during which they migrated on the matrix, formed cell–cell contacts and formed a network within ~6 hours. Other assays based on different matrices and less stimulating factors can result in complete tube formation but take several days. We elected to use the rapid network formation assay to minimize the effects on cell viability from knocking down SARS. We found a strong change in network formation and branching behavior of SARS-siRNA transfected HUVECs. Reduction of SARS expression resulted in an increase in the number of tube-like structures formed (Figure 4F) and in a much less organized pattern of these network connections (Figure 4D and 4E). Interestingly, the aberrant network formation was very similar to the phenotype observed when examining the patterning of the hindbrain capillaries (Figure 4B) and intersomitic vessels in adr zebrafish mutants. The hindbrain capillary network is among the few sites in the developing animal where active angiogenesis and vascular patterning occur between 60 and 90 hpf, possibly explaining why a phenotypic effect can be observed in these capillaries but not in vessels formed before 60 hpf.

**Discussion**

Although tRNA-synthetases are well known for their important role in protein synthesis, only in recent years have some of these proteins been revealed to possess additional noncanonical functions. Recently, a number of specific diseases, including neuronal, autoimmune, and metabolic conditions, as well as cancer, have been linked to heritable mutations in tRNA-synthetase genes (reviewed elsewhere). Some of these mutations do not affect the aminoacylation activity or enzyme stability but instead seem to interfere with noncanonical functions. In mammals, previously described noncanonical functions range from transcriptional and trans-
lational control, antiapoptosis, and viral assembly to extra-cellular roles as inflammatory, angiogenic, or angiostatic cytokines. In addition, fragments of Tyrosyl- and Tryptophanyl-tRNA-synthetases have been shown to elicit angiogenic or angiostatic responses in endothelial cells. 

Because deletion of tRNA-synthetase genes also interferes with their essential aminoacylation activity and therefore impairs survival, the ability to uncover these noncanonical functions has been limited. So far, no specific noncanonical function of Sars has been discovered. A significant advantage of analyzing the roles of tRNA-synthetases in zebrafish is the maternal provision of mRNA and/or protein, allowing for normal development during early stages. This maternal contribution is likely the reason that all described zebrafish tRNA-synthetase mutants are viable until 5 days postfertilization. Therefore, maternal supply of Sars might also account for the relatively late onset of the vascular specific phenotype and the confinement of the aberrant sprouting to sites of late-onset angiogenesis.

 adr mutants show 2 different vascular-specific phenotypes: a pronounced dilatation of the aortic arches (and to a lesser degree other head vessels) and the aberrant sprouting of the brain capillaries and intersomitic vessels. These different phenotypes might reflect the developmental stage of the different vessels at the time point of loss of Sars. Whereas the aortic arches are stable, previously established vessels, the brain capillaries, are undergoing active angiogenesis.

Trying to distinguish between the different roles of Sars in protein synthesis and the development of the vasculature, we blocked protein synthesis for the 12 hours during which the dilatation phenotype starts to manifest itself in adr mutants. Although inhibition of protein synthesis did not cause adr/ sars-like phenotypes, one should consider that cycloheximide is also blocking mitochondrial protein synthesis, whereas the adr mutation is not. (The zebrafish genome, like other vertebrate genomes, contains a complete set of mitochondrial-specific tRNA-synthetases, including mitochondrial sars.) On the other hand, loss of other tRNA-synthetases in zebrafish or inhibition of canonical Sars function did not cause adr/sars-like vascular phenotypes, arguing that a previously unknown direct function of Sars on endothelial cells is impaired.

However, we also analyzed the extracellular matrix because it contains serine-rich proteins likely to affect the vasculature. For example, microfibrils and associated proteins have been shown to play a role in vascular development. Therefore, we examined wild-type and adr mutants by immunohistochemistry but found no obvious difference in fibronectin or laminin deposition. Additionally, the tube-formation assay we used to analyze SARS function in HUVEC cultures is based on supplying the extra cellular matrix components in form of a basement membrane extract. If the adr phenotype was based on extracellular matrix deficiency, it probably could not have been phenocopied by SARS-deficient HUVEC cultures.

Taken together, our observations in zebrafish and human endothelial cells indicate that Sars possesses an angiogenesis-regulating capacity, with different effects representing the different developmental stages of the endothelial cells affected. Because regulation of angiogenesis is still a poorly understood subject with high medical relevance we hope that uncovering this new noncanonical function of Sars will pave the way for further insights into regulatory pathways controlling angiogenesis.

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Disclosures

None.

References


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Correction

Genetic Evidence for a Noncanonical Function of Seryl-tRNA Synthetase in Vascular Development: Correction

In the article that appears on page 1260 of the June 5, 2009, issue, “Thirty-two” was incorrectly inserted into the abstract during the editing process. The correct sentence should read as follows:

Mutations at the adrasteia (adr) locus cause a pronounced dilatation of the aortic arch vessels as well as aberrant patterning of the hindbrain capillaries and, to a lesser extent, intersomitic vessels.

On page 1261, in the last paragraph of the Materials and Methods, VEGF was expanded incorrectly. The sentence should read as follows:

Briefly, endothelial cells were evenly seeded onto a basement membrane extract and stimulated with 10 ng/mL vascular endothelial growth factor (VEGF) in Medium 200 with Low Serum Growth Supplements (Invitrogen). The publisher regrets these errors.

These errors have been noted in the online version of the article, which is available at http://circres.ahajournals.org/cgi/content/full/104/11/1260.

Reference


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Online Figure I

Analysis of vascular integrity by 1% Evans Blue injection

(A, B) Sagittal confocal crossections (single plane) of 76hpf wild-type (A) and *adr*<sup>277</sup> mutant (B) larvae visualized for *Tg(flk1:EGFP)<sup>s843</sup>* expression (green) and Evans blue (red). No evidence for a compromised vascular integrity was found in *adr* mutant larvae. Sections show the 5 visible aortic arch vessels (AA1-6). Scale bars, 50µm.

Online Figure II

Reduction or block of protein synthesis results in reduced growth of the hindbrain capillary network as well as thinner vessels, clearly visible at the aortic arches.

(A-I) Epifluorescence micrographs of 75hpf untreated (A), cycloheximide treated (cyc, B-F) and Seryl Analogue treated (SerA, G-I) *Tg(flk1:EGFP)<sup>s843</sup>* larvae. Cycloheximide is a general protein synthesis inhibitor whereas the Seryl analogue inhibits Seryl-tRNA Synthetase specifically.

Note that neither inhibitor caused a dilatation of the aortic arch vessels or aberrant patterning of the hindbrain capillaries.

The concentrations of the inhibitors are indicated on the bottom right and given per ml.