SUPPLEMENTAL INFORMATION

Secreted Threonyl-tRNA Synthetase Stimulates Endothelial Cell Migration and Angiogenesis

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EXPANDED MATERIALS AND METHODS

Cell Culture and Reagents- Human umbilical vein endothelial cells (HUVEC) (supplied by C. Holmes, University of Vermont) were grown in Clonetics® EGM®-2 complete media (Lonza) and used between passages 4 and 6. BC194 was generated by Biotica (Cambridge, UK) according to published procedures[1]. Purified basic-fibroblast growth factor (bFGF) was a gift from J. Spees, Univ. of Vermont. Retinoic acid and cycloheximide were purchased from Sigma-Aldrich, and VEGF and TNF-α were purchased from Cell Signaling and Calbiochem, respectively.

ELISA and Lactate Dehydrogenase Assays- Confluent HUVEC cultures were incubated at 37°C in Clonetics® EGM®-2 with low serum (0.2% FBS). Human VEGF or TNF-α (50 ng/ml) were added for 6 h as indicated. Levels of secreted TARS protein were measured in culture media supernatants using the threonyl-tRNA synthetase ELISA Kit (USCN Life Science) according to manufacturer's instructions. Cell membrane integrity was confirmed using the lactate dehydrogenase assay CytoTox-ONE™ (Promega) according to manufacturer's instructions and reported as percent cytotoxicity relative to a lysis control. Levels of secreted VEGF were measured after 24 h using the Human VEGF ELISA kit (Thermo Scientific) per manufacturer's instructions.

In vitro Tube Formation Assay- Tube formation assays were performed as described[2,3]. Briefly, HUVECs were seeded in 48-well plates (1.5×10^4 cells/well) coated with 100 µl of Matrigel™ Basement Membrane Matrix Growth Factor Reduced (BD Biosciences) and

incubated in Clonetics® EGM®-2 complete media (Lonza) or EGM®-2 with reduced serum (0.2% fetal bovine serum (FBS)). Cells were incubated at 37°C for 6 h then fixed in 10% formalin. Fixed samples were stained with Oregon Green 488 Phalloidin (Molecular Probes) then imaged with fluorescence microscopy. Number of tube branches (in pixels) were quantified using the Simple Neurite Tracer plug-in on ImageJ software (NIH)[4]. Statistical analysis of one-way ANOVA was performed with GraphPad Prism 5 Software. Multiple comparisons were performed using the Tukey Test.

Western blot- After treatments, cells were harvested into sample buffer containing: 0.2 M Tris-HCL, 4% SDS, 4% β-mercaptoethanol, 40% glycerol, 4 μM pyronin Y. Extracts were sheared through a 24-gauge syringe. For cell culture media samples, cells were incubated for 16 h in 0% serum EGM-2 media. Media were centrifuged 5 min at 200 *x g* to remove any cells and the supernatant was concentrated 10-fold using Amicon Centricon Ultracel YM-10 centrifugal filter devices (Millipore). Samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane and probed with specific antibody as described[5]. Primary antibodies are as follows: Rabbit polyclonal anti-ThrRS (1:1000; Santa Cruz), rabbit monoclonal anti-P-eIF2α (1:1000; Cell Signaling), rabbit monoclonal anti-Cleaved Caspase-3 (1:1000; Cell Signaling). Loading control antibodies were rabbit monoclonal anti-β-actin and anti-β-tubulin (1:1000; Cell Signaling). Secondary antibody was HRP-goat-anti-rabbit IgG (1:5,000; Jackson Laboratories).

Endothelial Cell Proliferation Assay- The MTT-based alamarBlue® (Invitrogen) reagent was used to assess cell proliferation[6]. HUVECs were seeded in a 96-well dish (1x103 cells/well) and grown for 48 h in EGM®-2 media. Cells were incubated in 0.2% FBS EGM®-2 media or EGM®-2 complete media as indicated; VEGF (50 ng/ml) and media alone served as controls. After 48 h, 72 h, or 96 h in culture, 10 μl/well premixed alamarBlue® (Invitrogen) was added and after 3 h at 37ºC the amount of reduced alamarBlue® was quantified by fluorescence (excitation at 530 nm, emission at 590 nm) on a microplate reader (Synergy™ HT, BioTek).

Nascent Protein Synthesis Assay- Nascent protein synthesis was measured using Invitrogen Click-iT® metabolic labeling reagents according to the manufacturer's instructions[7]. Briefly, HUVEC cultures were pre-incubated in methionine-free Dulbecco's Modified Eagle Medium (D-MEM) high glucose (Invitrogen) supplemented with 10% dialyzed FBS (Invitrogen) containing the control or test compounds. Cycloheximide (50 μM) was used as a synthesis inhibitor control. After 45 minutes, 25 μM Click-iT® AHA (L-azidohomoalanine) was added and cultures were incubated for 3 h. Cell lysates were labeled with biotin alkyne, separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Labeled bands were visualized using streptavidin-HRP reagent (Pierce Thermo Scientific) followed by chemiluminescence (ECL, Pierce).

Chick Chorioallantoic Membrane Assay- Fertilized chicken eggs (Sunrise Farms, Catskill, NY) were incubated at 37°C for 3 days, and then eggs were cracked and plated in a 10 cm^2 tissue culture dish and incubated for another 7 days. On developmental day 10, 1 mm^3 sterile gelatin sponge pieces (Surgifoam™) were placed within the outer one-third of the membrane between large vessels. 400 ng human bFGF and 20 ng human VEGF were used as pro-angiogenic control compounds; 1 μg retinoic acid (Sigma-Aldrich) was used as an angiostatic control. All compounds were applied in 10 μl to the CAM every 24 h for 72 h. Images were taken using a Leica MZ6 stereomicroscope every 24 h. Compounds were scored according to a modified version of Intensity Scoring as previously described[8]. Briefly, each CAM was given a blinded score from 0-5 based on the change in the extent of vessel convergence in proximity to the sponge from day 0 to day 3. Total score is averaged individual experimental condition scores from at least 8 replicates.

Note that because the chicken embryos were sacrificed in the CAM assay prior to 15 days after fertilization, they were not considered vertebrate animals by IACUC standards. Despite this qualification, care was taken to use the least number of eggs possible to obtain the data, and 6 sponges were applied to each CAM for analysis to reduce the number of embryos needed

in experiments. At the completion of experiments, a major CAM vessel was cut to ensure the embryos were euthanized prior to disposal.

Transwell Migration Assay- HUVECs were serum-depleted overnight in Clonetics EGM®-2 with low serum (0.2% FBS) then $5x10^4$ cells were plated in 90% EBM®-2, 10% EGM®-2 in the upper chamber of 0.2% gelatin-coated 24-well 8 μm Transwell™ inserts (Corning) with 90% EBM®-2, 10% EGM®-2 media plus 50 ng/ml VEGF, 100 nM TARS protein, or 10 nM BC194 in the lower chamber[9]. Cultures were incubated for 4 h, fixed in 10% formalin, and stained with 10 μg/ml DAPI solution (Roche) after removal of cells from the top layer of the chamber with a cotton swab. Migrated cells were imaged using a 4x objective on the Olympus IX70 inverted microscope (Olympus). DAPI-stained nuclei were counted using ImageJ software.

Quantitative RT-PCR-Total RNA was extracted from cells using the RNeasy column protocol and cDNA was generated using an Omniscript reverse transcriptase assay according to the manufacturer's instructions (Qiagen). Primers and probes for TARS and β2-microglobulin were Assays-on-Demand (Applied Biosystems). RT-qPCR was performed using an ABI prism 7700 Sequence Detection System (Applied Biosystems). The relative quantity of mRNA level was determined using the comparative CT (ΔΔCT) method using β2-microglobulin to normalize mRNA level[3].

SUPPLEMENTAL DATA

Expression and Purification of human aminoacyl-tRNA synthetases- N-terminal His_6-tagged human TARS (also known as ThrRS) was expressed and purified from *E. coli* Rossetta™ 2(DE3)pLysS competent cells (EMD) transformed with derivatives of plasmid pET28a hctThrRS as in Bovee et al., 2003 (obtained from Dieter Soll, Yale Univ.)[10]. Transformant cultures were grown in terrific broth supplemented with 100 mg/ml kanamycin and 100 mg/ml chloramphenicol at 37°C to a cell density of A_{600} = 0.6. Expression of TARS was induced with 1 mM isopropyl 1-thio-β-D-galactoside overnight at 15°C. The bacterial pellet was

lysed by sonication in buffer A (20 mM potassium phosphate buffer pH 8.0, 100 mM KCl, 35 mM imidazole, and 5 mM β-mercaptoethanol) and cleared by centrifugation at 17,050 *x g* for 30 minutes. Nucleic acids were precipitated by the addition of protamine sulfate to a final concentration of 0.3% followed by centrifugation. The supernatant was loaded onto a HisTrap™ FF column (GE Healthcare) in buffer A and eluted by an imidazole gradient of 35-250 mM in buffer A over 20 column volumes.

TARS-containing fractions were identified by SDS-PAGE and GelCode™ Blue (Thermo Scientific), pooled, and dialyzed into buffer B (100 mM potassium phosphate buffer pH 6.8 and 5 mM β-mercaptoethanol). The sample was loaded onto a CHT-Tricorn Hydroxyapatite column and eluted over 20 column volumes by using a gradient of buffer B to buffer C (500 mM potassium phosphate pH 8.0 and 5 mM β-mercaptoethanol). Fractions containing TARS were pooled, dialyzed into buffer D (10 mM HEPES pH 8.0, 100 mM KCl, 2.5 mM β-mercaptoethanol, and 40% glycerol), and then stored at -20°C. Protein concentration was determined by Abs_{280}. The protein purity and stability were evaluated by Coomassie stain following SDS-PAGE (**Fig. S1a**).

The L567V mutant is the human counterpart to a L489W borrelidin-resistant *E. coli* TARS[11]. The wildtype TARS sequence was mutated using Quikchange II Site-Directed Mutagenesis (Stratagene) with forward primer 5' CAC CAG TGT GCA ACC ATC CAG CTG GAT TTC CAG GTG CCC ATC AGA TTT AAT C 3' and its reverse compliment and the resulting construct was transformed into XL1-Blue cells. Colonies positive for the mutation were isolated, grown in LB media, and the plasmid purified via Qiagen miniprep kit. The plasmid was then transformed into Rosetta II cells for use in protein expression using the same protocol as for wildtype TARS (**Fig. S1a**).

The aminoacylation activities of the TARS constructs were determined using modifications to established procedures[12]. Briefly, reaction mixtures consisted of 20 mM HEPES pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, 2.5 U pyrophosphatase (Roche), 80 μM

threonine, 20 μM [^{14}C]-threonine, and 5 μM of human tRNA^{Thr} (prepared as in Francklyn et al.[12]). Reactions were initiated with the addition of 50-100 nM TARS or L567V TARS and run at 37°C. Aliquots were taken every 30 sec and spotted onto Whatmann 3MM paper filters pre-soaked in 5% trichloroacetic acid (TCA). Upon completion, the filters were washed 3 times in excess TCA, once in 95% ethanol, and dried under a heating lamp. The formation of Thr-tRNA^{Thr} was detected by scintillation counter and the activity determined by linear regression of Thr-tRNA^{Thr} formed per active site per unit time. The apparent Ki values for BC194 were 4.09 nM for wildtype TARS and 32.24 nM for L567V TARS.

N-terminal His_6-tagged human leucyl-tRNA synthetase (LARS) was expressed using the plasmid pPROEX hTb-LARS (obtained from Susan Martinis, Univ. of Illinois, Urbana-Champaign) and purified using a similar purification scheme to the TARS purification with minor modifications described in[13]. The protein purity and stability were confirmed using SDS-PAGE and Coomassie stain (**Fig. S3a**).

LARS steady state ATPase activity was determined using the same procedure as for TARS aminoacylation with the following modifications. The reaction mixture did not include labeled threonine or tRNA^{Thr} and 1 nM [α-^{32}P] ATP (PerkinElmer) was added. Reactions were incubated for 3 minutes at 37°C and initiated with the addition of 1 μM human LARS. At various time points 5 μl aliquots were quenched in 45 μl of 500 mM sodium acetate and 0.1% sodium dodecyl sulfate. For each sample, 1 μl was spotted onto a CCM cellulose PEI F plates (EMD) and resolved via thin-layer chromatography in 0.75 M potassium phosphate buffer mobile phase. Radioactive signals were detected via phosphorimaging and AMP production overtime was quantified using Quantity One v 4.6.6 software (Bio-Rad) (**Fig. S3b**).

SUPPLEMENTAL DATA REFERENCES

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SUPPLEMENTAL DATA FIGURE LEGENDS

FIGURE. S1. Human TARS or L567V TARS were expressed and purified from *E. coli* Rosetta™ cells as described. (**a**) Coomassie stain of TARS and L567V TARS proteins separated by SDS-PAGE indicating purified intact proteins. (**b**) Purified TARS exhibits aminoacyl synthetase activity and activity is not compromised in the borrelidin-resistant mutant L567V. Numbers represent Thr-tRNA^{Thr} formed per active site (pmol/pmol); mean ± s.e.m., n=3. TARS activity was comparable to *E. coli* TARS and commercially available human TARS expressed in CHO cells[12].

FIGURE. S2. Representative images for **Fig. 4a**: BC194 effects on *in vivo* angiogenesis. Fertilized chicken embryos were cultured *ex-ova* and agents were applied daily to gelfoam sponges on the CAM. Shown are images recorded at 24 h and 72 h for Control (PBS), bFGF (400 ng), VEGF (20 ng), and BC194-resistant mutant TARS (L567V, 10 pmol). Where indicated, BC194 was added (0.1 pmol). Arrows indicate spoke-wheel response; Scale bar=1.0 mm.

FIGURE. S3. Purification, activity, and CAM effects of leucyl-tRNA synthetase (LARS). Human LARS was purified from *E. coli* as described. (**a**) Coomassie stain of 2 µg LARS separated by SDS-PAGE. (**b**) LARS exhibits enzyme activity as measured by conversion of ^{32}P-ATP to AMP.

Numbers represent labeled AMP determined by thin layer chromatography followed by phosphorimaging for triplicate samples (mean ± s.e.m.). (**c,d**) LARS has no effect on angiogenesis measured in the CAM assay. Purified LARS (10 pmol) was added to CAMs as in **Fig. S2**. (**c**) Representative images showing no affect of LARS on CAM vascularity; Scale bar=1.0 mm. (**d**) Graph representing the average CAM vascularity score over 72 h as compared to Control (PBS) or TARS; mean ± s.e.m., n=18, **P*<0.05 (one-way ANOVA, Tukey test).

FIGURE S1

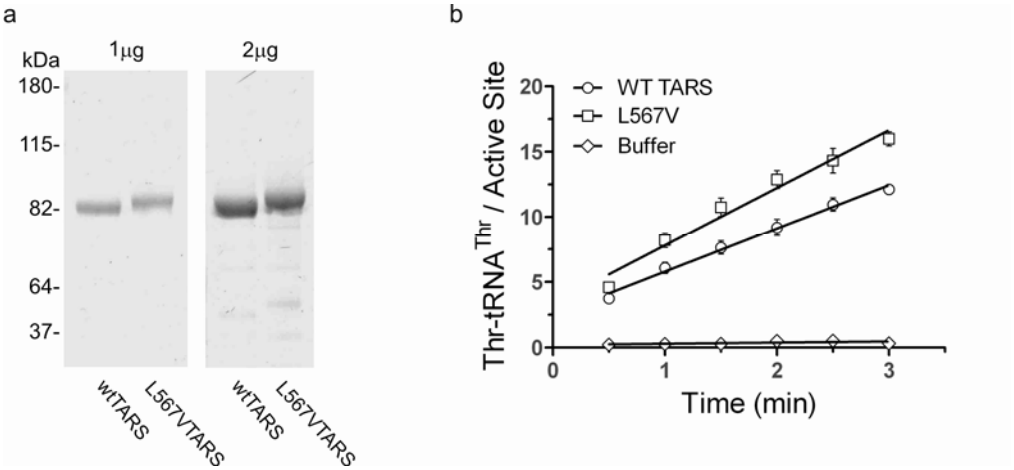
a
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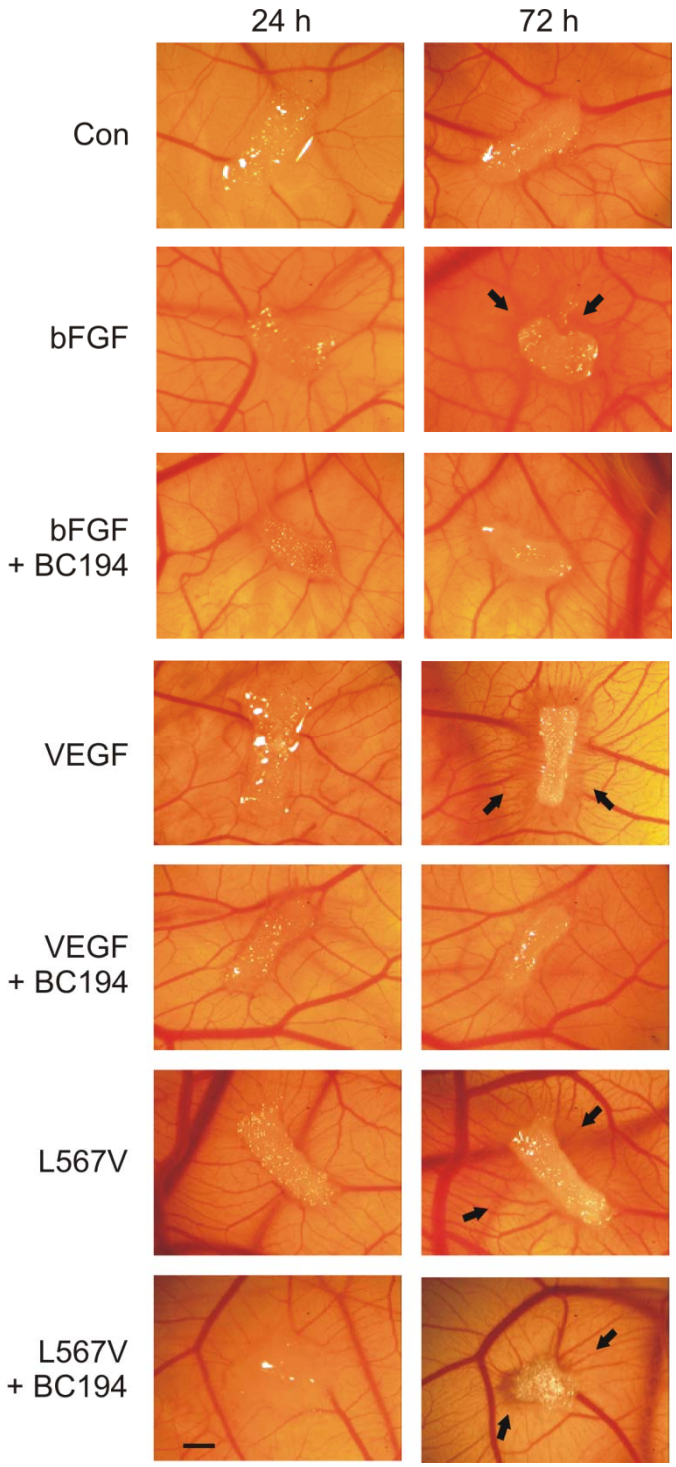
FIGURE S2


24 h
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Con
bFGF
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VEGF
+ BC194
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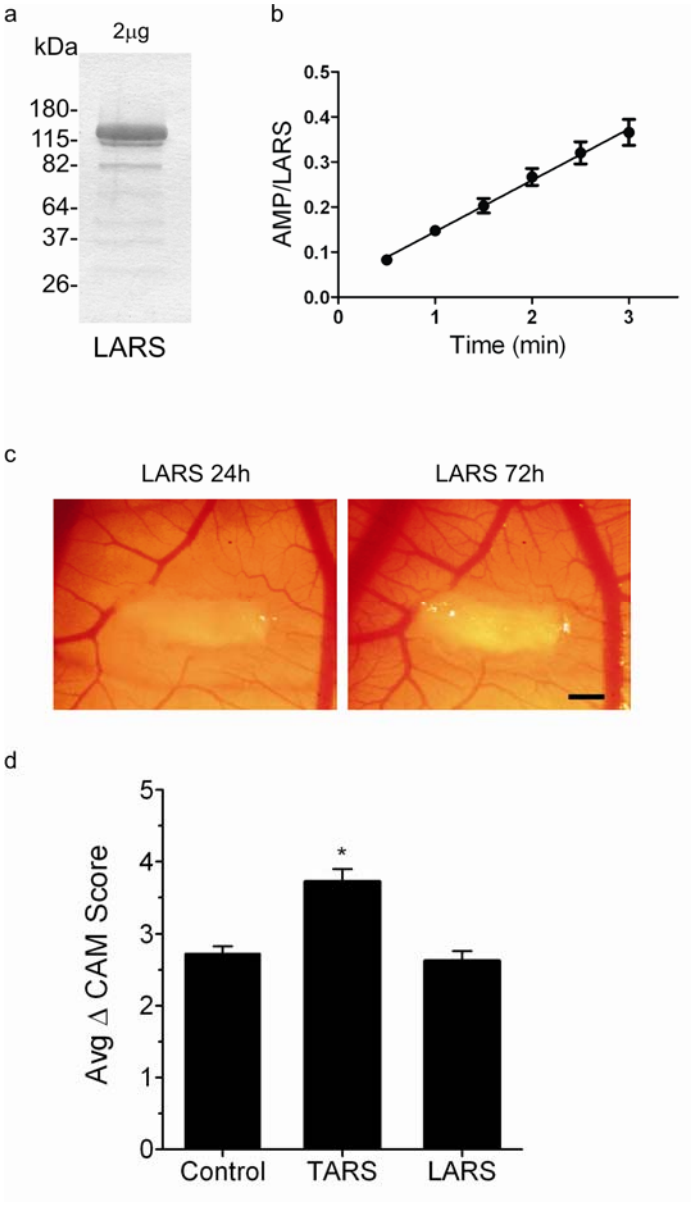
L567V
+ BC194

FIGURE S3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2
3
Time (min)
c
LARS 24h
LARS 72h
d
Avg Δ CAM Score
5
4
3
2
1
0
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Control
TARS
LARS