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Assessing the effects of threonyl-tRNA synthetase on angiogenesis-related responses

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Abstract

Several recent reports have found a connection between specific aminoacyl-tRNA synthetases and the regulation of angiogenesis. As this new area of research is explored, it is important to have reliable assays to assess the specific angiogenesis functions of these enzymes. This review provides information about specific *in vitro* and *in vivo* methods that were used to assess the angiogenic functions of threonyl-tRNA synthetase including endothelial cell migration and tube assays as well as chorioallantoic membrane and tumor vascularization assays. The theory and discussion include best methods of analysis and quantification along with the advantages and limitations of each type of assay.

Keywords

aminoacyl-tRNA synthetase; angiogenesis methods; endothelial cells; CAM assay; ImageJ; MetaMorph

1. Introduction

Angiogenesis is defined as the formation of new blood vessels from a pre-existing vasculature that occurs during both development and in tissues responding to injury, hypoxia and/or nutrient stress. The regulation of angiogenesis is tightly controlled by substances secreted by the cells experiencing stress, the responding endothelial and mural cells, as well as the surrounding blood cells including immune cells and platelets [1]. Our laboratory has recently discovered that threonyl-tRNA synthetase (TARS)¹ is among a subgroup of aminoacyl-tRNA synthetases that is secreted by hypoxic cells and stimulates an angiogenic response [2, 3]. The methods described below outline both *in vitro* and *in vivo* methods used to identify the angiogenic properties of TARS including troubleshooting tips and advantages and limitations of each individual assay. Because we found that TARS secretion underlies

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¹**Abbreviations:** TARS, threonyl-tRNA synthetase; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor; CAM, chorioallantoic membrane; PECAM-1, platelet endothelial cell adhesion molecule; MVA, microvascular area

the angiogenic response, most of these assays use purified recombinant TARS applied to the extracellular space which will be considered within the discussion along with future applications of angiogenic assays in the study of non-canonical functions of aminoacyl-tRNA synthetases.

2. Materials and Methods

2.1 Expression and Purification of human threonyl-tRNA synthetase

The assays described below use recombinant amino-terminal His₆-tagged human TARS (also known as ThrRS) expressed and purified from *E. coli*. The quality of the purified protein is important for consistent results in the angiogenesis assays and the following protocol produces TARS that has confirmed activity in both aminoacylation and angiogenesis assays [2].

1. *E. coli* Rossetta™ 2(DE3)pLysS competent cells (EMD) are transformed with the TARS plasmid (pET28a hcfThrRS) as in Bovee et al., 2003 [4]. Transformant cultures are grown in terrific broth including 100 mg/ml kanamycin and 100 mg/ml chloramphenicol at 37°C to a cell density of A₆₀₀ = 0.6. Expression of TARS is induced with 1 mM isopropyl 1-thio-β-D-galactoside overnight at 15°C. *Note:* after induction, cell pellets can be frozen, which improves the cell lysis and protein recovery.
2. The resulting bacterial pellets are 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 17050 × g for 30 minutes. Nucleic acids are precipitated by the addition of protamine sulfate to a final concentration of 0.3% and removed by centrifugation.
3. The TARS-rich supernatant is loaded onto a HisTrap™ FF column (GE Healthcare) equilibrated with buffer A and eluted by an imidazole gradient of 35–250 mM in buffer A over 20 column volumes in 5 ml fractions. The fractions containing TARS are identified by separating 10 μl of the fractions by SDS-PAGE followed by GelCode™ Blue staining (Thermo Scientific). TARS-containing fractions are then pooled, and dialyzed into buffer B (100 mM potassium phosphate buffer pH 6.8 and 5 mM β-mercaptoethanol).
4. The partially purified TARS is then 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). TARS-containing fractions are determined by SDS-PAGE as above, pooled, and dialyzed into buffer D (10 mM HEPES pH 8.0, 100 mM KCl, 2.5 mM β-mercaptoethanol, and 40% glycerol). Protein concentration is determined by A₂₈₀ and aliquots are then stored at –20°C. *Note:* Prior to dialysis, save 0.5 ml of buffer D (storage buffer) to use as a protein blank and as a control in angiogenesis assays. This second purification step is critical for generating consistently pure TARS for experiments. Stock concentrations of TARS are made at 100 μM to generate final experimental concentrations in the nM range.

2.2 *In vitro* Angiogenesis Assays

Endothelial responses are key to the initiation and progression of angiogenesis [5]. The *in vitro* angiogenesis assays assess the impact of compounds on the capacity of endothelial cells to migrate and form tubes which indicates the propensity to break down extracellular matrix and assemble in a directed fashion. These assays are simple to conduct and relatively easy to quantify, thus are often the first assays used to screen for angiogenic potential. In our assessment of TARS effects on angiogenesis, we use a model of low passage (passage 2–6) cultured human umbilical vein endothelial cells (HUVECs) that are grown on plates coated with 0.2% gelatin and maintained in endothelial growth media (EGMTM-2, Clonetics).

2.2.1 Transwell Migration Assay—The transwell migration assay uses a modified Boyden chamber that consists of a porous membrane between 2 chambers. Cells are applied to the upper chamber and cells that cross the membrane are counted to determine the migration effects [6]. Vascular endothelial growth factor (VEGF) is used as a positive control and migration should be initially examined at several time points between 2 and 24 hr. The challenge is getting the appropriate control media conditions that allow survival but do not induce migration; below is the protocol that produced consistent results in measuring the impact of TARS on endothelial cell migration.

1. HUVECs are serum-depleted overnight in low-serum EGMTM-2 (0.2% FBS). Cells trypsinized, counted and 5×10^4 cells are plated in the upper chamber of 0.2% gelatin-coated 24-well 8 μ m TranswellTM inserts (Corning) containing control media (90% endothelial basal media (EBMTM-2), 10% EGMTM-2). The lower chamber contains control media plus the test compounds (i.e. 50 ng/ml VEGF, 100 nM TARS protein).
2. Culture chambers are incubated for 4 hr and then fixed in 10% formalin. Cells present on the top of the membrane are removed with a cotton swab. Cells that migrate to the bottom of the membrane are stained with 10 μ g/ml DAPI solution (Roche) and imaged using a 4 $\times$ objective on the Olympus IX70 inverted microscope (Olympus). DAPI-stained nuclei are quantified using ImageJ software as described below.

2.2.2 Endothelial Tube Formation Assay—Another important *in vitro* method that indicates angiogenic activity is the endothelial cell tube formation assay. This assay uses HUVECs that are plated in the presence of extracellular matrix components (MatrigelTM), and fetal bovine serum is used as a positive control to stimulate tube formation [7, 8]. The advantage of this assay is that it measures formation of endothelial cell tubes in an environment which more closely mimics conditions during new blood vessel formation. For experiments with TARS, a relatively short incubation period is used to reduce background tube formation effects [2].

1. The 48-well plates are first coated with MatrigelTM (Basement Membrane Matrix Growth Factor Reduced; BD Biosciences). *Important Note:* The MatrigelTM solution is solid at -20°C , liquid at 0°C , and solidifies into a gel at 25°C . The solution cannot be thawed quickly; it must be thawed overnight on ice at 4°C and approximately 100 μ l aliquoted into wells using pipet tips that have been stored

at -20°C . Work quickly and change tips if the solution becomes thick. Avoid bubbles by setting the pipet at 110 μl and not ejecting the entire amount. Place the plate in the culture incubator for 30 min while preparing the cells.

2. To prepare the cells, start with low-passage HUVECs grown on a gelatin-coated 10 cm plate. Cells are trypsinized, removed from the plate in a solution of trypsin neutralizing solution and EGMTM-2, and then collected by centrifugation. The HUVEC pellet is gently, but fully, resuspended in 300 μl serum-free EBMTM-2 media (Lonza) and cells are counted. Sterile microfuge tubes are prepared that contain the desired components (EBM media only (negative control), EGMTM-2 media + 2% FBS (positive control) and EBMTM-2 media + 200 nM TARS). Cells are added such that there are 5.5×10^4 cells/well in a total of 200 μl solution/well. Samples should be prepared in duplicate or triplicate.
3. Plates are incubated for 4 to 6 hr in a 37°C incubator, stopping the reaction when tubes are visible in the positive control wells. The tubes are then fixed with 10% formalin, stained with Oregon Green 488 phalloidin (1:150; Molecular Probes), and imaged using an inverted light microscope. *Note:* when aspirating cells after the incubation period, take care when suctioning to prevent aspiration of the gel matrix. Imaging is optimal in the 48-well format as the small diameter of the 96-wells results in distortion of tubes for imaging. The number of tube branches (in pixels) are quantified using the Angiogenesis Analyzer plug-in on ImageJ software as described below [9].

2.3 *In vivo* Angiogenesis Assays

In vivo assays have the obvious advantage of the physiological environment under which new vessels are formed, but the disadvantage of difficulties in application of test compounds and variability in quantification. For measurements of TARS effects on angiogenesis, we utilized the chicken chorioallantoic membrane (CAM) assay and a syngenic mouse ovarian tumor model.

2.3.1 Ex Ova Chicken Chorioallantoic membrane assays—CAM assays provide a relatively simple and inexpensive method for the assessment of angiogenesis *in vivo*. The procedure investigates changes in blood vessel growth within the CAM of developing chicken embryos in response to various test conditions. The CAM is a highly vascular, extra-embryonic membrane that forms from the fusion of chorion and allantois on developmental day 3 and rapidly expands until developmental day 10 [10]. Blood vessels are highly visible within the clear membrane and can be easily monitored using a standard stereo microscope. As the immune system of the embryo is incomplete during these developmental stages, the likelihood of artifacts resulting from compound immunogenicity is low [11]. Unlike the other *in vivo* methodologies, such as mouse or zebrafish models, CAM assays can be performed using the same facilities as standard cell culture. Furthermore, IACUC standards do not apply to embryos prior to their typical hatching date, which is typically recognized as developmental day 15 of chickens. Therefore, the CAM assay can provide rapid *in vivo* data without the need for specialized equipment or an established animal protocol.

In order to maximize the number of test compounds, embryos are often cultured *ex ova* in sterile, 10 cm diameter tissue culture dishes and test compounds soaked into 1 mm³ medical sponges distributed over the surface. Fertilized chicken eggs are purchased directly from farms that are familiar with research needs and shipped overnight. For best results the eggs should be collected together soon after laying since synchronized embryos produce the most consistent results. Furthermore, because the process of *ex ova* culture can be harsh on the embryos, extra eggs should always be purchased to offset the number that die (sometimes 50% depending on the weather).

1. Upon arrival, eggs are cleaned using dry cotton gauze to remove feathers and excrement in order to minimize contaminants. The eggs are then placed in an incubator with the long axis laying horizontal and incubated at 37 °C at normal oxygen with a humidity pan for 72 hours.

The choice of 72 hours has been empirically determined as the optimal age for cracking. Earlier developmental stages typically die before the assay can begin while older embryos become too fragile to survive the cracking procedure. For incubation, poultry egg incubators are particularly suited for this function, however, a standard cell culture incubator at 37°C with CO₂ turned off and lots of water in the pan is sufficient.

2. After 72 hours eggs are removed from the incubator and immediately marked on top with a pencil for reference. This mark denotes the location of the embryo within the egg as it will naturally rotate to the top during incubation.
3. To avoid contamination eggs are coated in Betadyne using cotton gauze and allowed to dry. Eggs should be kept mark up as much as possible to avoid changing the location of embryo, however, the embryo's position is fairly resistant to movement over short periods and is unlikely to change from minor manipulations during the coating process.

The cracking process can be difficult at first and extra eggs should be purchased until the researcher is comfortable with the technique. In addition, even successfully cracked embryos are very sensitive and may die for unknown reasons. Therefore, even individuals well acquainted with this protocol should prepare more embryos to improve the chances of statistically relevant data. A helpful video presentation can be found in [12].

4. Hold eggs with the mark facing upwards, away from the cracking surface, in two hands, keeping the thumbs and middle fingers around the equator of the egg meeting at the middle and the index fingers meeting at the mark on the top of the egg. The orientation of the mark is very important as it reduces the likelihood that the embryo or yolk will be damaged in the cracking process (Figure 1).
5. Hold arms extended away from the body and crack the egg with one to two hits of the egg onto a solid sharp edge (we used the edge of a stainless steel pipette container). For greater control, the motion should include the entire arm and not extension from the elbow. The amount of force used should be enough to put a

small opening in the bottom of the egg just enough that the contents begin to seep out but not enough to rupture the yolk sac.

6. Move the egg immediately over an awaiting culture dish and lower it so that the egg contents are just touching the dish's surface. The egg should then be raised very slowly while gently pulling with your thumbs and middle fingers and pushing with your index fingers so that crack grows along the meridian of the egg up to the top mark. This process should create a vacuum that helps pull the embryo out of the egg and into the dish. If successful, the yolk should be intact and the embryo clearly visible on the surface. Sometimes minor ruptures to the yolk can be tolerated but major ones will likely lead to the death of the embryo. Nonviable embryos should be immediately discarded to prevent contamination of the other embryos.
7. Return eggs to the incubator and incubate for another 10 days. Movement of the dishes and external commotion should be minimized during this period as the embryos are very sensitive to vibrations and dead embryos (identifiable by their lighter color) should be removed. It is not uncommon for yolks to rupture during this process, but these dishes can be kept so long as the embryos survive.
8. For compound application, 1mm³ gelatin surgical sponges (Surgifoam™) are placed on the outer third of the membrane near larger vessels and 10 µl of the desired compounds are slowly soaked into the sponges. Typically enough space is available for up to six compounds. Positive controls include human basic fibroblast growth factor (400 ng) and human VEGF (20 ng); retinoic acid (1 µg) (Sigma-Aldrich) is used as an angiostatic control. Images are captured using a Leica MZ6 stereomicroscope at initial application (time=0) and then compounds are reapplied and images captured every 24 hr for 72 hr. Following the final images, embryos should be sacrificed by an appropriate method: typically decapitation or exsanguination by severing a major artery.
9. CAM images are analyzed using the Intensity Scoring method modified from [11] and described below.

2.3.2 Tumor angiogenesis—Measuring TARS effects on microvascular area within tumors—The most relevant means of evaluating the angiogenic effects of TARS is in a complex angiogenic environment such as that produced during tumor growth. Because ovarian cancer is highly angiogenic and affected by the immune response, the model used in this study is a syngenic mouse model of ovarian cancer [13, 14]. Tumor cells are injected intraperitoneally to simulate the peritoneal spread seen in ovarian cancer. Although compounds cannot be easily applied locally, TARS can be manipulated in tumor cells prior to injection and compounds that block TARS activity can be administered. The advantages of the complexity of this model are offset by its inherent variability. The following protocol has been used to assess the role of TARS activity in the progression of ovarian tumor angiogenesis by measuring the endothelial cell marker PECAM-1 (also known as CD31) as a means of determining microvascular area.

1. The ID8 cell line used to generate ovarian tumors was originally generated by self-transformation of normal ovarian epithelial cells [15]. Following intraperitoneal injection, these cells form aggressive tumors that follow a progression of invasion that parallels that of stage IV human ovarian cancer [16]. To generate tumors, cells are grown in culture, collected by trypsinization and centrifugation, and then resuspended in PBS. Between 1 and 3×10^6 cells are injected into normal mice (C57BL/6) either intraperitoneally or subcutaneously into the flank.
2. For experiments that involve overexpression or siRNA knockdown of TARS, cells are stably transfected with the relevant TARS expression or shTARS plasmids prior to injection.
3. For experiments that include application of the TARS inhibitor BC194, starting at 3 weeks post-tumor cell injection, mice are administered vehicle (DMSO) or the TARS inhibitor BC194 (2 mg/kg intraperitoneally 3 times per week for 3 weeks).
4. After injections, the mice are monitored for the development of swelling from ascites and are euthanized 6–8 weeks after the initial injection. Ascites and blood are collected and tumors in the abdominal cavity are photographed for staging and tumors excised, especially along the colon, diaphragm and any observed distant sites. For flank injections, tumors are measured with calipers as tumors develop and then tumors with extended margins are excised. For immunostaining, samples are fixed by immersion in 10% formalin and processed for paraffin embedding.
5. For immunofluorescence to analyze inflammation and microvascular area, serial sections (5 μ m) from paraffin-embedded specimen are cut, transferred to slides, and incubated in a Coplin jar with Dako S1699 target retrieval solution for 20 min at 95–98°C. After cooling, slides are blocked with 3% BSA/0.2% TX-100/PBS for one hour at room temperature.
6. The following primary antibodies are used: GTX116359 Rabbit anti-TARS (1:400) and SC-1506 Goat anti PECAM-1 (M-20,1:100) in 3% BSA/0.1% TX-100/PBS. Note: to save on the amount of antibody used, 100 μ l of antibody solution is applied directly to the section and carefully covered with a pre-cut piece of parafilm. Slides are incubated in a humidified box at 4°C for 16–24 hr and then washed several times in a Coplin jar with PBS.
7. Secondary antibodies used are Invitrogen's Alexa 488 donkey anti-goat IgG (1:500) and Alexa 594 donkey anti-rabbit IgG (1:500) in 3% BSA/0.1% TX-100/PBS. DAPI (10 μ g/ml) is included as a nuclear marker. Solutions are applied similarly as with the primary antibody, but for 1 hr at 37°C. After staining with secondary antibodies, the slides are washed with PBS and mounted with coverslips using AquaPolyMount. Images are collected using a fluorescence microscope using either a 10 $\times$ or 20 $\times$ objective. Analysis of microvascular area using MetaMorph™ (Molecular Devices) software is described below.

3. Theory/Calculation

3.1 Quantification of Migration

A major advantage of the transwell migration assay is the ease of quantification. Experiments can be set up in triplicate and quantification of nuclei that have migrated across the porous membrane is performed by collecting DAPI images and then counting nuclei using ImageJ (<https://imagej.nih.gov/ij/>) [17]. For multiple conditions, analysis of results is through one way ANOVA with a post-hoc test such as the Tukey honestly significant difference test [18]. The ease of performing and analyzing the data is offset by the limitation that migration is stimulated by compounds dispersed evenly within the lower chamber. There are also no means to measure concentration-dependent effects or migration that is based on complex intracellular interactions, thus this assay must be paired with another more angiogenesis-specific assay.

3.2 Quantification of endothelial tubes using ImageJ with angiogenesis analyzer

The endothelial tube assay is an effective means to screen compounds for angiogenic activity as it is relatively fast and several conditions can be compared simultaneously. The quantification is more complicated than the transwell assay because the resulting tube matrix can include unconnected tubes, tubes with branches and the development of a tubular mesh containing master junctions. To compensate for this difficulty, the Image J algorithm has an Angiogenesis Analyzer tool that extracts characteristics of the tube network and provides output including color coding and number of tubes, branches, and segments (Figure 2, Table 1). This type of quantification also provides clues as to the mechanisms of effect since the formation of individual tubes is regulated differently than the formation of branch points [5]. For practical purposes, all images are taken at the same fluorescence settings with intensity settings slightly higher than that used for representative images to avoid false negatives. During the ImageJ analysis, equally increasing the contrast of the images has been found to improve the consistency of the output data. Once numerical data is obtained, statistical analysis includes one-way ANOVA with a post-hoc Tukey Test.

3.3 Quantification of CAM angiogenesis

CAM images are usually analyzed using the Intensity Scoring method described in [11]. To avoid bias, the analysis should be performed in a blinded manner. Images are ranked on a 0–5 scale based on the change in the extent of vessel convergence in proximity to the sponge from day 0 to day 3. Specifically, the scoring method focuses on new, smaller vessels growing directly towards the sponge, but dilation of the surrounding larger vessels contributes to the score (Figure 3). A score of 0 indicates that no new vessels are seen growing towards the sponge; scores of 1 to 3 imply vasodilation and increasing numbers of unbranched vessels; and scores of 4 or 5 imply increasing numbers of branched vessels. In addition, scores of 4 and 5 are often associated with a slight haze of blood around the sponge due to the leakiness of the nascent vasculature. The total score is averaged from individual experimental condition scores from at least 10 replicates.

3.4 Quantification of Microvascular area using MetaMorph™

There are differences of opinion regarding the best method to quantify angiogenic vessels in tissue sections. Microvascular density has been used to measure areas with “hotspots” of neovasculature, especially in tumor biology. In this measurement the number of vessels in each vascular “hot spot” are manually counted at both low and high magnification to generate a numerical value of angiogenesis. The use of microvascular density has been an accurate measure of tumor aggressiveness, however the subjective nature of determining which vessels to count leads to bias [19]. Recent advances in software capabilities have allowed for a more objective criteria for analyzing large sections of tissue that measure vessels as a ratio of total tissue area. This measurement is termed microvascular area (MVA) and is considered to be a more accurate parameter than microvascular density [20]. In our experiments, we measured MVA to determine the effects of TARS inhibitors on angiogenesis in developing ovarian tumors.

To determine the MVA, immunofluorescence images are obtained from tumor sections that are stained with TARS to mark the tumor compartment and PECAM-1 (CD31) to mark microvessels. It is recommended that both low (10×) and high (20×) images are captured to improve the flexibility in the analysis. The TARS area is selected and the MetaMorph™ software is then used to generate masks of the PECAM-1 compartment. The MVA is then defined as PECAM-1 positive pixel area relative to the tumor tissue area. An example is shown in Figure 4.

Others have reported a possible disadvantage of PECAM-1 to define microvessels because there is the potential for cross-reactivity with infiltrating immune cells. In our experience, this is not the case; we do not see co-localization of PECAM-1 with the immune cell-specific antigens C3 or F4/80 (KML and PW, unpublished observation). We are thus confident that our masked images were generated due to microvessels and not due to infiltrating immune cells.

4. Results and Discussion

The methods described here will assist researchers in choosing the appropriate means for assessing angiogenic activity of aminoacyl-tRNA synthetases based on our experiences with the study of TARS [2, 21]. Each of the assays has its own advantages and limitations. The *in vitro* assays are simple to quantify and can be used to test multiple conditions in a relatively short time, yet they do not include the complex factors that contribute to physiological angiogenesis. The *in vivo* assays include these complexities but at the cost of increased experimental variability and subjective analysis. It is clear that choosing multiple assays from each category is the most reliable way to both generate convincing evidence as well as to formulate hypotheses towards the mechanisms underlying the angiogenic impact.

There are several methods that were not described here that have been used by our lab and others to study angiogenesis. Additional *in vitro* assays include the scratch wound assay and matrix degradation assay [22]. The scratch wound assay measures endothelial cell migration after creating a wound to the monolayer using a pipette tip. Analysis is by imaging the wound over time to determine effects of substances on the speed and extent of wound

closure. The matrix degradation assay examines the breakdown of extracellular matrix by measuring activity of matrix metalloproteases. For these assays, plates are coated with fluorescence-linked matrix components such as collagen or fibrinogen and loss of fluorescence indicates activity. The aortic ring organ culture is another assay that has been used; however, in our experience this method is prone to high variability due to the detachment of the aortic rings in culture thus is not recommended. Additional *in vivo* assays include the retinal angiogenesis assay and the zebrafish vascular development assay. Our laboratory has used the latter to demonstrate vascular malformations when zebrafish embryos are exposed to a TARS inhibitor or when TARS expression is reduced using a morpholino knockdown [21]. The power of the zebrafish model is the ability to genetically manipulate and that are readily measured using genetically encoded endothelial GFP-expression to visualize vessel development. This aspect allows analysis of genetically encoded TARS mutations and does not use purified TARS protein, which is an unavoidable weakness in many of the other angiogenesis assays.

Based on the factors discussed above, the following overall conclusions can be drawn. First, the study of angiogenesis includes an analysis of specific endothelial cell responses as well as interactions with the surrounding tissue, which cannot be measured accurately using any single assay. Second, while the CAM assay has a long list of troubleshooting tips, it has the most advantages with respect to applying multiple compounds and assessing *in vivo* effects. Third, because many of the assays used to study angiogenesis require image analysis, it is essential that high-resolution images are captured and that image analysis software is used to reduce bias whenever possible. As microscopy and imaging software advances, the ability to accurately measure angiogenesis in more complex models including humans will continue to improve. Deep tissue angiograms using computed tomography (CT) scanning are currently performed with the possibility that this technology can be used as an aid in diagnosing vascular pathologies associated with dysfunctional angiogenesis such as that seen in ischemic diseases and cancer. Thus, discoveries made through the angiogenesis models described here can be translated into clinical applications.

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References

1. Welti J, Loges S, Dimmeler S, Carmeliet P. Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. *The Journal of Clinical Investigation*. 2013; 123:3190–3200. [PubMed: 23908119]
2. Williams TF, Mirando AC, Wilkinson B, Francklyn CS, Lounsbury KM. Secreted Threonyl-tRNA synthetase stimulates endothelial cell migration and angiogenesis. *Scientific reports*. 2013; 3:1317. [PubMed: 23425968]

3. Mirando AC, Francklyn CS, Lounsbury KM. Regulation of Angiogenesis by Aminoacyl-tRNA Synthetases. *International journal of molecular sciences*. 2014; 15:23725–23748. [PubMed: 25535072]
4. Bovee ML, Pierce MA, Francklyn CS. Induced fit and kinetic mechanism of adenylation catalyzed by *Escherichia coli* threonyl-tRNA synthetase. *Biochemistry*. 2003; 42:15102–15113. [PubMed: 14690420]
5. Mettouchi A. The role of extracellular matrix in vascular branching morphogenesis. *Cell adhesion & migration*. 2012; 6:528–534. [PubMed: 23257831]
6. Svensson KJ, Kucharzewska P, Christianson HC, Skold S, Lofstedt T, Johansson MC, et al. Hypoxia triggers a proangiogenic pathway involving cancer cell microvesicles and PAR-2-mediated heparin-binding EGF signaling in endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:13147–13152. [PubMed: 21788507]
7. Arnaoutova I, Kleinman HK. In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract. *Nat Protoc*. 2010; 5:628–635. [PubMed: 20224563]
8. Cassavaugh JM, Hale SA, Wellman TL, Howe AK, Wong C, Lounsbury KM. Negative regulation of HIF-1 α by an FBW7-mediated degradation pathway during hypoxia. *Journal of Cellular Biochemistry*. 2011; 112:3882–3890. [PubMed: 21964756]
9. Carpentier G. Contribution: angiogenesis analyzer. *ImageJ News*. 2012; 5
10. Nowak-Sliwinska P, Segura T, Iruela-Arispe ML. The chicken chorioallantoic membrane model in biology, medicine and bioengineering. *Angiogenesis*. 2014; 17:779–804. [PubMed: 25138280]
11. Ribatti D, Nico B, Vacca A, Presta M. The gelatin sponge-chorioallantoic membrane assay. *Nat Protocols*. 2006; 1:85–91. [PubMed: 17406216]
12. Dohle DS, Pasa SD, Gustmann S, Laub M, Wissler JH, Jennissen HP, et al. Chick ex ovo Culture and ex ovo CAM Assay: How it Really Works. *Journal of Visualized Experiments : JoVE*. 2009:1620. [PubMed: 19949373]
13. Bamberger ES, Perrett CW. Angiogenesis in epithelial ovarian cancer. *Mol Pathol*. 2002; 55:348–359. [PubMed: 12456770]
14. Kulbe H, Chakravarty P, Leinster DA, Charles KA, Kwong J, Thompson RG, et al. A dynamic inflammatory cytokine network in the human ovarian cancer microenvironment. *Cancer Res*. 2012; 72:66–75. [PubMed: 22065722]
15. Roby KF, Taylor CC, Sweetwood JP, Cheng Y, Pace JL, Tawfik O, et al. Development of a syngeneic mouse model for events related to ovarian cancer. *Carcinogenesis*. 2000; 21:585–591. [PubMed: 10753190]
16. Erickson BK, Conner MG, Landen CN Jr. The role of the fallopian tube in the origin of ovarian cancer. *American journal of obstetrics and gynecology*. 2013; 209:409–414. [PubMed: 23583217]
17. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature methods*. 2012; 9:671–675. [PubMed: 22930834]
18. Kao LS, Green CE. Analysis of variance: is there a difference in means and what does it mean? *The Journal of surgical research*. 2008; 144:158–170. [PubMed: 17936790]
19. Nico B, Benagiano V, Mangieri D, Maruotti N, Vacca A, Ribatti D. Evaluation of microvascular density in tumors: pro and contra. *Histology and histopathology*. 2008; 23:601–607. [PubMed: 18283645]
20. Chantrain CF, DeClerck YA, Groshen S, McNamara G. Computerized quantification of tissue vascularization using high-resolution slide scanning of whole tumor sections. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*. 2003; 51:151–158. [PubMed: 12533523]
21. Mirando AC, Fang P, Williams TF, Baldor LC, Howe AK, Ebert AM, et al. Aminoacyl-tRNA synthetase dependent angiogenesis revealed by a bioengineered macrolide inhibitor. *Scientific reports*. 2015; 5:13160. [PubMed: 26271225]
22. Goodwin AM. In vitro assays of angiogenesis for assessment of angiogenic and antiangiogenic agents. *Microvascular research*. 2007; 74:172–183. [PubMed: 17631914]

Highlights

- Methods are provided to study aminoacyl-tRNA synthetase functions in angiogenesis.
- Troubleshooting tips for obtaining data and using analysis software are detailed.
- The discussion stresses a need for multiple assays to produce convincing evidence.

Figure 1.
Drawing illustrating the orientation of the fertilized egg for best results in the CAM assay.

Figure 2.

Using ImageJ Angiogenesis Analyzer to quantify endothelial tube formation. Image represents HUVECs incubated in endothelial growth medium containing Oregon green Phalloidin 488. Images were taken using inverted fluorescence microscope and analyzed using angiogenesis analyzer. Yellow lines represents the master segments, tubes that connect together to different junctions; green lines represents branches; light blue surrounding the master segment is the mesh; dark blue represents isolated tubes; Red circles represents the master junction points. Legend: Image (a) represents HUVECs in low serum (EBM only), Image (b) is HUVECs in Full serum (EGM + 2% FBS). This plug-in provides numerical information including number of tubes (#pieces) and branches (see Table 1).

Figure 3.

Image showing progression of microvessels after treatment with TARS. At embryonic day 10, agents are applied to gelfoam sponges on the CAM. Shown are photographs taken immediately after placement of the gelatin surgical sponge and at 24 and 72 hr after applying 10 pmol of purified recombinant TARS. Note the vasodilation at 24 hr and the microvessel haze at 72 hr, achieving a score of 4 on a scale of 0–5.

Figure 4.

Using MetaMorph™ to quantify microvascular area within tumors. Shown is an image of an intraperitoneal tumor from the ID8 mouse model of ovarian cancer that is stained with antibodies recognizing PECAM-1. The vessels are identified by a mask algorithm in MetaMorph™. The outline shows the area of the tumor that was identified by TARS staining in the section.

Table 1

Data Table from Angiogenesis analyzer (from Figure 1)

Treatments	# pieces	# branches
Low Serum	79	15
Full Serum	192	47