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TITLE:

Whole-Mount In Situ Hybridization in Zebrafish Embryos and Tube Formation Assay in iPSC-ECs to Study the Role of Endoglin In Vascular Development

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KEYWORDS:

whole-mount in situ hybridization, iPSC-ECs, tube formation, vascular development, HHT, cardiovascular disease

SUMMARY:

Presented here is a protocol for whole-mount in situ RNA hybridization analysis in zebrafish and tube formation assay in patient-derived induced pluripotent stem cell-derived endothelial cells to study the role of endoglin in vascular formation.

ABSTRACT:

Vascular development is determined by the sequential expression of specific genes, which can be studied by performing in situ hybridization assays in zebrafish during different developmental stages. To investigate the role of *endoglin(eng)* in vessel formation during the development of HHT, morpholino-mediated targeted knockdown of *eng* in zebrafish are used to study its temporal expression and associated functions. Here, whole-mount in situ RNA hybridization (WISH) is employed for the analysis of *eng* and its downstream genes in zebrafish embryos. Also, tube formation assays are performed in HHT patient-derived induced

pluripotent stem cell-derived endothelial cells (iPSC-ECs; with *eng* mutations) together with autologously corrected ECs. A specific signal amplifying system using the WISH method provides higher resolution and lower background signal than traditional methods. To obtain a better signal, the post-fixation time is adjusted to 30 min after probe hybridization. Because fluorescence staining is not sensitive in zebrafish embryos, it is replaced with diaminobezidine (DAB) staining here. In this protocol, hereditary hemorrhagic telangiectasia (HHT) patient-derived iPSC lines containing an *eng* mutation are differentiated into endothelial cells. After coating a plate with basement membrane matrix for 30 min at 37 °C, iPSC-ECs are seeded as a monolayer into wells and kept at 37 °C for 3 h. Then, the tube length and number of branches are calculated using microscopic images. Thus, with this improved WISH protocol, it is shown that reduced *eng* expression affects endothelial progenitor formation in zebrafish embryos. This is further confirmed by tube formation assays using iPSC-ECs derived from a patient with HHT. These assays confirm the role for *eng* in early vascular development.

INTRODUCTION:

A single mutation on *eng* (CD105) has been reported in patients with HHT. The mutation leads to increased EC proliferation and reduced flow-mediated EC elongation[1,2]. It has also been previously reported that ENG deficiency reduces endothelial markers expression (i.e., *kdrl*, *cdh5*, *hey2)* in zebrafish[3]. *Endoglin,* mainly expressed in endothelial cells, is a transmembrane glycoprotein and functions as a co-receptor for transforming growth factor β (TGF-β) family members. It directs BMP9 binding on the cell surface to regulate downstream gene, including *Id1* expression, to induce stem cell differentiation toward ECs[4]. Thus, the *eng* gene plays important roles in vasculogenesis and human vascular disease[5,6]. We have previously examined the effects of *endoglin* knockdown on vessel formation in zebrafish embryos, followed by analysis of iPSCs-derived ECs acquired from an HHT patient bearing an *eng* mutation[7]. This protocol demonstrates the effects of ENG deficiency on endothelial progenitor marker expression and tube formation, which is a quantifiable method for measuring in vitro angiogenesis.

To study *eng* spatial and temporal expression, WISH is employed to detect gene expression in vivo[8]. In situ hybridization (ISH) is a method of using labeled probes with complement sequences of target nucleic acids (DNA or mRNA) to detect and visualize target nucleic acid hybrids in a fixed specimen. The process amplifies gene expression signals in vivo and is used to detect the expression of genes by microscopy. WISH has been widely used in various model animals, especially in zebrafish[9]. It is also used to acquire the following data: 1) gene spatial/temporal expression patterns, which provide information about gene function and classification; and 2) specifically expressed gene markers that are used in high-throughput drug or mutant screening[10].

Chromogenic probes are easily degradable with traditional chromosome in situ hybridization (CISH), which results in high background noise and nonspecific signals[11,12]. The WISH method uses two independent double Z probes, which are designed to hybridize to target RNA sequences. Each probe contains an 18–25 sequence complementary to the target RNA and a 14

base tail sequence (conceptualized as Z). The target probes are used in a pair (double Z). The two tail sequences together form a 28 base hybridization site for the preamplifier, which contains 20 binding sites for the amplifier. The amplifier, in turn, contains 20 binding sites for the label probe and can theoretically yield up to 8,000 labels for each target RNA sequence.

This advanced technology facilitates simultaneous signal amplification and background suppression to achieve single-molecule visualization while preserving tissue morphology[13]. Further modification of the WISH methods is based on previous research[14], including extra fixation and DAB staining. Provided here is an improved WISH protocol that can work even if the target RNA is partially decreased or degraded. Advantages include that it can be completed in 24 h without RNase-free conditions. Signals can also be simultaneously detected through multiple channels from multiple targets, and the results are consistent and compatible with results from different high-throughput automation platforms.

Results from animal models do not necessary reflect the same phenomenon that occurs in humans. ENG contains two pairs of conversed cysteines, C30-C207 and C53-C182, which form disulfide bridges in orphan regions. To further study the role of *eng* in HHT patients, tube formation assays with iPSCs derived from HHT patients have been carried out in cells without/with *eng* mutations (Cys30Arg, C30R)[15]. After Kubota et al. first reported the tube formation experiment[16], the assay has been developed in several ways. It has been used to identify angiogenic or antiangiogenic factors, define the signaling pathways in angiogenesis, and identify genes regulating angiogenesis[17].

Prior to the availability of patient-derived iPSC-ECs, researchers used primarily cultured ECs to study angiogensis[16]. However, for endothelial cells, it is a technical challenge to transduce exogenous genes with a virus, because of the limited passage number that ECs can undergo. This is because there is hardly enough cellular material to be collected from human vessels either from surgery or matched approved donors. With the invention of the iPSC generation technique by Shinya Yamanaka, human ECs derived from iPSCs can be used reliably in in vitro experiments, as reported previously[18].

Using virally transduced ECs with limited numbers and passages may be sufficient for signaling studies, but for functional studies, it is better to generate mutant pluripotent stem cell lines, (either iPSCs or CRISPER/Cas9-targeted hESCs), then differentiate them into ECs for angiogenesis studies that use tube formation assays[19]. Tube formation can be used to evaluate the function of endothelial cells bearing mutations. This protocol also describes tube formation on an μ-slide angiogenesis plate, which is an easy, cost-effective, and reproducible method.

The protocol below provides a reliable and systemic method for studying the role of specific genes in vascular formation, along with details for in vivo expression pattern and in vitro functional quantification for modeling human disease.

PROTOCOL:

All animal experiments described were approved by the Research Ethics Committee of Zhejiang University school of medicine.

1. Whole-mount in situ hybridization

1.1 Zebrafish line husbandry and reproduction

1.1.1 Feed and raise all adult zebrafish at 26–28 °C in a recirculating aquaculture system with 14 h light/10 h dark cycle for each day. Use AB (wild-type) zebrafish lines for the following procedures.

1.1.2 Put a layer of pebbles in the bottom of the breeding box as a shelter for eggs. Group parental fish in the ratio of 2:1 (i.e., two females and one male) into each box. Let the female fish spawn for 1 day. At the end of spawning, remove the parent fish immediately to prevent them from eating the eggs.

1.1.3. Inject the 2 ng of morpholinos and 500 pg of mRNA into one-cell stage embryos using the Femto Jet injection system under a microscope (endoglin-MOs sequence: 5'-GATGAACTCAACAC TCGTGTCTGAT-3'; 5-mispair control MOs sequence: 5'-AAACAGACCACATCCTCTTCATCTC-3').

1.1.4. Incubate the zygotes in acidic sea water at 27 ˚C for approximately 48 h.

1.2 Collection and fixation of zebrafish embryos

1.2.1 Use a plastic transfer pipette to collect 20–40 zebrafish embryos from the circulating system water (pH = 5.0) in a 1.5 mL tube when they are dechorionated and reach a specific period of development. Add 1 mL of freshly prepared 4% PFA solution at room temperature (RT).

NOTE: The period at which the embryo chosen is based on the purpose of the experiment**.** **Table 1** shows the fixation time required for different embryonic periods.

1.2.2 Remove the fixation solution, wash embryos with phosphate-buffered solution with Tween-20 (PBST, dilute 1 mL of Tween into 1000 mL of PBS solution) 3x for 5 min each at RT.

1.2.3 Dehydrate the embryos through incubation in a series of 25%, 50% and 75% methanol (diluted in PBST) for 5 min each. Transfer embryos to 100% methanol for 5 min and replace with fresh methanol. Store the embryos at -20 °C for overnight or longer.

1.3 Hydration and digestion

1.3.1 Hydrate embryos that were preserved in 100% methanol using a sieve with nylon mesh at the bottom to sequentially wash embryos in the wells of 12 well plates with a series of 75%, 50%, and 25% methanol (diluted in PBST) for 5 min each. Wash the embryos with PBST 3x for 5

min each at RT.

1.3.2 Add 50 µL of proteinase K (10 mg/mL) into the tube with embryos and follow the digestion time in the **Table 2** at RT.

1.3.3 Remove the proteinase K and wash embryos with PBST 3x for 5 min each at RT.

1.4 Probe hybridization and post-fixation

1.4.1 Put the probes designed according to each gene's mRNA sequences in a 40 °C hybridizatin system until the precipitate is dissolved, which should take ~10 min. Mix the target probes of *eg* (XM_007116.7) and an additional two important genes involve in the early mesoderm endothei al progenitor formation (here, *aplnr* [NM_001075105.1*]* and *nrp1a* [NM_001040326.1]) in a 1.5 mL tube at a 50:1:1 ratio.

1.4.2 Add 50–100 µL of mixed target probes into each tube with embryos, then incubate overnight at 40 °C.

NOTE: Pre-mixed probes must be pre-warmed to 40 °C and cooled to RT before use.

1.4.3. Prepare a 0.2x saline sodium citrate solution with Tween-20 (SSCT): dissolve 175.3 g of NaCl and 88 g of sodium citrate into 1 L of dH_2O to obtain a 20x SSC solution. Then, dilute the solution 1:100 in dH_2O to obtain a 0.2 x SSCT solution. Dilute Tween-20 1:1000 in the 0.2x SSCT solution.

1.4.3 Transfer the recycled probes to a new tube. Wash the embryos in 0.2x SSCT solution 3x for 15 min each at RT. The recycled probes can be reused 5x–10x.

1.4.4 Use 4% paraformaldehyde (PFA) to fix the embryos for 30 min at RT. Wash the embryos in 0.2x SSCT solution 3x for 15 min each at RT.

NOTE: The work of Gross-Thebing et al. suggests a post-fixation period of 10 min[14], but the actual amount must be adjusted accordingly. Here, we tested 10 min post-fixation 3x, and the sensitivity of DAB dying is significantly influenced. After investigating, ensure that 0.5–1.0 h of fixation is the optimal condition (a shorter fixation period cannot produce a clear signal from the target RNA, and the appropriate extension of fixation time helps strengthen the signal and provide less background noise).

1.5 Sequential amplifier and label probe hybridization

1.5.1 Remove the SSCT solution and replace with 50 µL of Amp1. Allow the embryos to settle in Amp1 at 40 °C for 30 min. Then, wash the embryos in 0.2x SSCT solution 3x for 15 min each at RT.

1.5.2 Remove the SSCT solution and add 50 µL of Amp2. Allow the embryos to settle at 40 °C for 15 min. Then, wash the embryos in 0.2x SSCT solution 3x for 15 min each at RT.

1.5.3 Remove the SSCT solution, add 50 µL of Amp 3 and tap the tube mildly. Then, incubate the embryos at 40 °C for 30 min. Wash the embryos in 0.2x SSCT solution 3x for 15 min each at RT.

1.5.4 Remove the SSCT solution, add 50 µL of Amp 4 dropwise, and carefully tap the tube. Then, incubate the embryos at 40 °C for 15 min. Wash the embryos in 0.2x SSCT 3x for 15 min each at RT.

1.6 Counterstaining and microscopy

1.6.1 Remove the SSCT solution and add 50 µL of DAPI to each tube. Incubate the embryos overnight at 4 °C.

1.6.2 Wash the embryos with PBST and prepare them for imaging using a 1% low melting point agarose (LMP) solution (1 g of LMP dissolved in 100 mL of dH_2O) in a Petri dish filled with PBST.

1.6.3. Use a DAB peroxidase substrate kit to stain the specimen. Add color reagents A, B, and C (50 µL each) to 1 mL of distilled water and mix well to obtain a complete DAB working fluid. Add the fluid to the specimen and cover for 10 min.

1.6.4 Wash the specimen thoroughly with PBST after staining.

1.6.5 Use an optical microscope with a photographic function for imaging the samples.

NOTE: If taking images later, tubes should be wrapped in aluminum foil and stored at 4 °C.

2. Tube formation assay

NOTE: The *eng* mutant and wildtype ECs (control) were differentiated from iPSCs derived from an HHT patient (here, a 62 year-old female patient with recurrent epistaxis since 22 years old, gastrointestinal bleeding, pulmonary arteriovenous malformations, carrying a missense *eng* mutation in position c.88T>C of exon 2) and a healthy donor (without *eng* mutation), which were provided by Peking Union Medical College Hospital with approval from the college research ethics committee.

2.1 Control and HHT iPSC cell cultures

2.1.1 Add a certain volume of basement membrane matrix (e.g., Matrigel) to a 6 well cell culture plate to cover the bottom of the well and incubate the plates for 1 h at 37 °C.

NOTE: When first using the basement membrane matrix stored at -20 °C, incubate on ice or in a

frost-free 4 °C refrigerator until thawed. Then, dilute at 1:100 in DMEM/F12, then distribute the diluent into 200 µL tubes containing 100 µL each. Store the tubes at -20 °C and avoid repeated freezing and thawing. When adding the diluted basement membrane matrix, do not create bubbles. After adding the diluted basement membrane matrix, shake the 6 well plate gently by hand so that it evenly covers the bottom.

2.1.2 After coating the plate, remove the used basement membrane matrix solution from the wells. Then, add 2 mL of mTeSR1 medium into each well, and plate the iPSCs harvested from the last passage at approximately 1 x 10^6 per well.

2.2 Generation and expansion of ECs from iPSCs

2.2.1 After approximately 4 days of iPSC culture, exchange the culture medium with BEL medium supplemented with activin A (25 ng/mL), BMP4 (30 ng/mL), VEGF165 (50 ng/mL), and CHIR99021 (1.5 µM). Grow the cells for 3 days to generate mesoderm cells.

2.2.2 Replace the medium described in step 2.2.1 with BEL medium supplemented with VEGF (50 ng/mL) and SB431542 (10 µM) for 4 days to expand vascular ECs. Treat the cells with the same medium and culture for another 3–4 days.

2.2.4 Use CD31-dynabeads to purify the mature vascular ECs: refer to the instructions in the CD31-dynabeads kit, which links human CD31 antibody with magnetic beads to combine CD31 molecules expressed on ECs, then collect the CD31-positive cells by elution buffer. Maintain and expand the purified ECs in EC-SFM medium containing VEGF165 (30 ng/mL), bFGF (30 ng/mL), and FBS (1%).

2.2 Plating endothelial cells on Matrigel-coated plates and microscopy

2.3.1 Add 10 µL of basement membrane matrix per well to coat the angiogenesis plates (see **Table of Materials**) and incubate at 30 min at 37 °C.

2.3.2 Harvest endothelial cells after checking endothelial markers CD31 and VE-cadherin with immunofluorescence staining and resuspend them in endothelial growth medium 2 by pipetting repeatedly. Add 50 µL of cell suspension (2 x 10^5cells/mL) per well onto the solidified matrix and incubate the cells for 3–5 h at 37 °C.

NOTE: Before the tube formation experiment, endothelial markers should be checked to make sure the proper phenotype of functional endothelial cells. 1 x 10^4 cell per well is an ideal number for tube formation. Too many or too few cells are not conducive to tube formation. Add cells from the side of the dish and do not touch the basement membrane matrix. Use light microscope in high magnification field to check the cells and take photos.

2.4. Quantitative results of tube formation

2.4.1. Observe the results with a microscope with high resolution and take pictures of at least 10 areas for each group to obtain reliable data statistics.

2.4.2. Examine endothelial tube formation: estimate the extent of tube formation by inspecting the overall tube length, tube number, and branch points. Assess and count the number and length of branches using ImageJ software.

REPRESENTATIVE RESULTS:

Whole mount in situ hybridization is based on a principle similar to fluorescence resonance energy transfer. It is designed to improve both sensitivity and specificity in zebrafish ISH as well as amplify target-specific signals without affecting the background signal.

In 24 hpf zebrafish embryos, *endoglin* is highly expressed in the posterior cardinal vein (PCV), intersegmental vessels (ISVs), and blood islands[3]. It is hard to control the staining time in traditional chromogenic in situ hybridization (CISH), with non-positive signals occurring in some regions, such as yolk sac (**Figure 1A**). To investigate the role of *eng* in early vascular development, a hemogenic endothelial marker was used (*aplnra*) as well as another endothelial progenitor marker (*nrp1a)* to examine which type of endothelium was affected by *eng* silencing[20]. The expression of *aplnra* and *nrp1a* was weak in 24 hpf embryos. Compared to CISH, the weak signal from the two genes was clearly demonstrated in the tail region after *eng* knockdown in WISH (**Figure 1B**). The knockdown of *eng* RNA caused reduced expression of two types of endothelial cells marker genes expression in an HHT animal model.

Before conducting experiments on differentiated iPSCs, the cells were identified and confirmed as ECs. Morphologically, they appeared as cobblestone shape. Expression of endothelial cell surface molecular markers CD31, CD146, VE-cadherin, and vWF was confirmed with IF staining[21]. After magnetic-based isolation using CD31, the functional assay was conducted as described below.

Here, the tube formation assay was performed using the *eng* mutant and control iPSC-derived endothelial cells. Statistical analysis was performed based on the number, branches, and lengths of tubes. A novel parameter was also introduced based on previous work[3], points of angiogenesis, in order to reflect tube formation of endothelial cells. Eventually, it was found that *eng* mutant endothelial cells formed fewer branches than control endothelial cells, and that branches in *eng* mutant endothelial cells significantly increased after stimulation with vascular endothelial growth factor (VEGF) (**Figure 2A,B**). The mutation in *eng* resulted in defective vessel tube formation.

FIGURE AND TABLE LEGENDS:

Figure 1: Endoglin knockdown decreased the expression of endothelial markers. (A) CISH and WISH were performed to determine the expression of endoglin in 24 hpf embryos (n > 30). The red box represents the enlarged region. Scale bars = 200 µm. (**B)** *aplnra and nrp1a* expression

by WISH in 24 hpf zebrafish embryos of the control-MO and *eng*-MO group. The red arrow indicatesregions where the expression of these genes significantly decreased.

Figure 2: Tube formation of *eng* mutant and control iPSC-ECs. (A) The tube formation by the four groups, including the control group, the control + VEGF (control endothelial cells + 30 ng/mL VEGF), the *eng* mutant (*eng* mutant endothelial cells) group, and the *eng* mutant + VEGF (*eng* mutant endothelial cells + 30 ng/mL VEGF) group. Tube formation was assessed and photographed after 3 h. Scale bars = 100 μm. (**B)** Quantitative results of tube formation are shown. At the covered area the number of branches and their length of tubes were calculated by Image J. Error bars represent the SD of the mean values from three independent experiments. A value of P was considered statistically significant when $*p < 0.05$.

Table 1: Fixation time required for different embryonic stages.

Table 2: Digestion time required for different embryonic stages.

DISCUSSION:

This protocol applied an improved whole-mount in situ RNA analysis platform for zebrafish and tube formation assays on iPSC-ECs derived from an HHT patient. The traditional ISH method requires a longer experimental cycle with extra experimental steps. The protocol has some important improvements, the use of independent double Z probes and iPSCs derived from a patient with HHT that were applied in WISH assays and tube formation, respectively. These refinements are crucial for enhancing the detection sensitivity compare with what is observed with traditional ISH. The uniquely designed probes and target signal amplification allow the detection of rarely expressed transcripts (*aplnra* is a poorly expressed gene in 24 hpf embryos). One modification is the extra fixation after probe hybridization. The extra fixation can enhance the combination of probes and transcripts. DAB staining is also applied rather than fluorescence staining, because it was found that it was difficult to perform fluorescence staining in certain low-abundance genes. Then, iPSCs were used for angiogenesis assays, which increases the significance and clinic relevance of this work. Generation of iPSCs requires strict culture conditions and represents a limitation of the protocol.

Some critical steps must be highlighted. In WISH analysis, the digestion time of zebrafish embryos should be followed in strict accordance with the text. A shorter digestion time cannot guarantee that the probe combines with transcripts successfully. In contrast, extended digestion time will destroy the integrity of embryos. In the tube formation experiments, the imaging timing should be well-controlled. Generally, the endothelial cells will become tubes within 12h but the tubular structure tends to disintegrate after 24 h. The cell number is also important for tube formation, as a cell density that is too high or too low can lead to failed tube formation. If it is difficult to induce endothelial cells to form tubes, it is helpful to include adding growth factors such as VEGF to the culture medium as a positive control to test the ability of ECs to form tubular structures.

In summary, the improved WISH method has been considered an advantageous technique for laboratory workflows. Recently, our research group has started to test this assay in clinical samples. Considering higher sensitivity and more efficient detection than observed in traditional methods, this improved WISH method holds significant promise for developing and implementing RNA-based molecular diagnostics[22].

Performance of tube formation assay using patient iPSC-ECs allows confirmation of results from animal studies as well as identification of disease-causing mutations from single nucleotide polymorphism in the *eng* gene. The combination of these methods clarifies the role of *eng* in vascular formation and hold potential in gene correction of patient iPSC-ECs for cardiovascular cell therapy[23].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

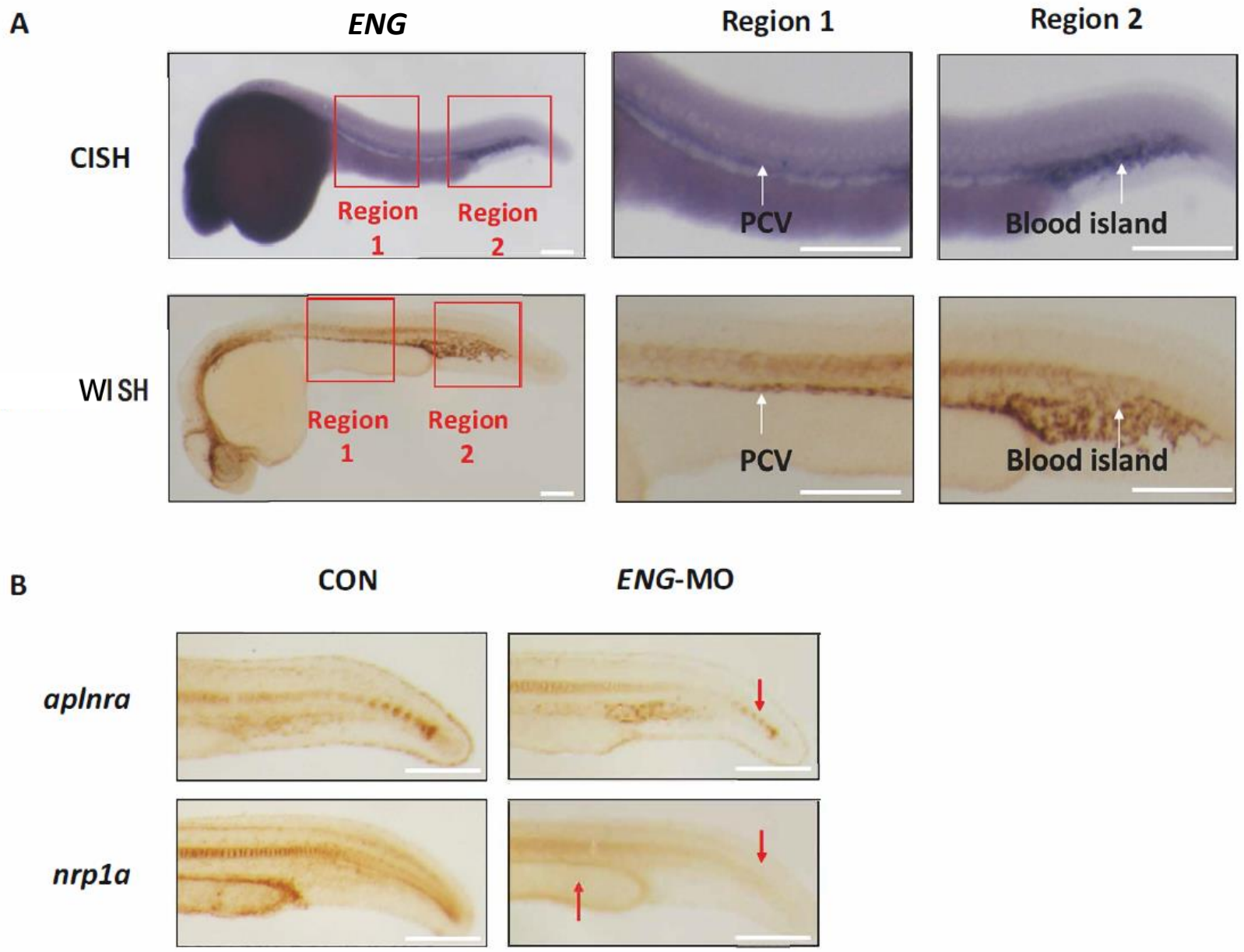

Figure 2

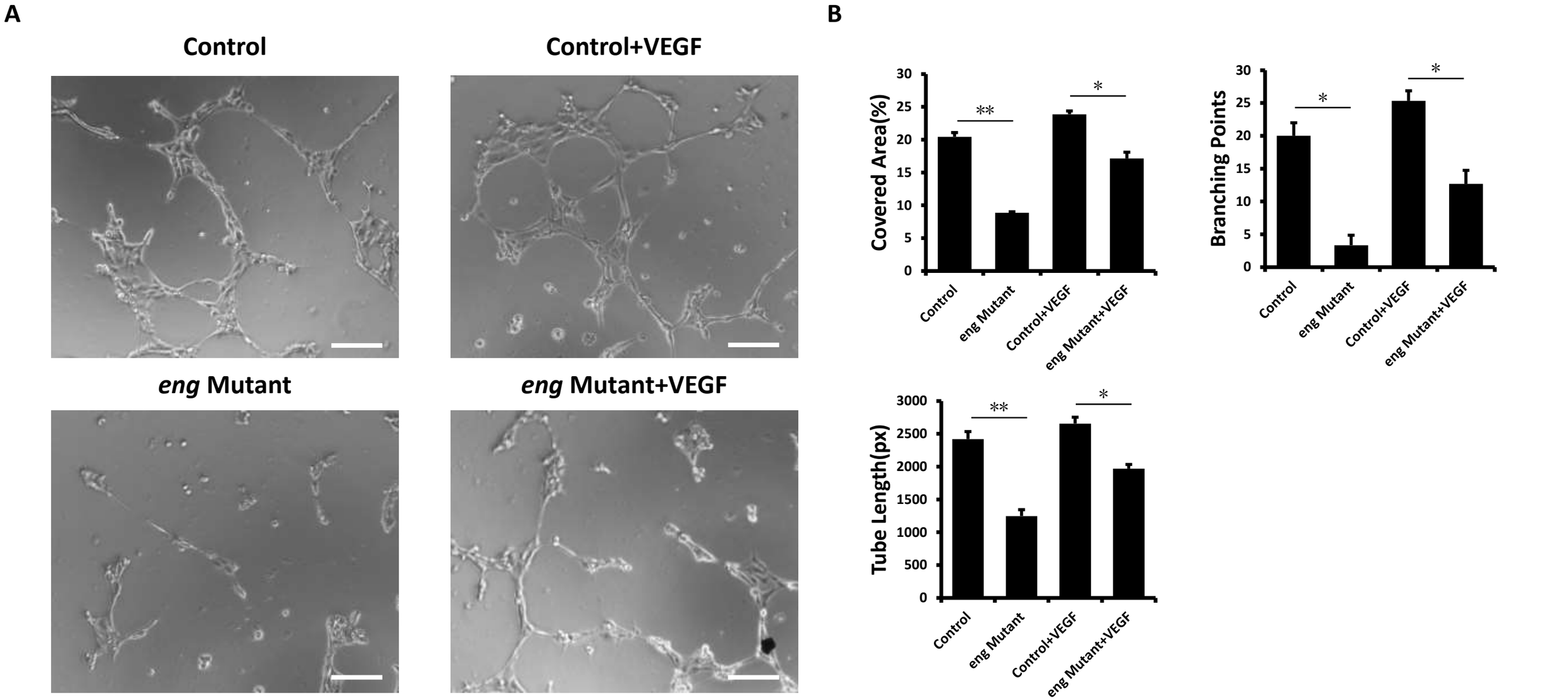

Table 1

Embryonic period	Fixation time
4-cells to 8 hpf	4 hours
8 hpf to 24 hpf	1 hours
24 hpf to 4 dpf	30 minutes

Table 2

Embryonic period	Digestion time
4-cells to 8 hpf	2 minutes
8 hpf to 24 hpf	5 minutes
24 hpf to 4 dpf	10 minutes

Name of Material/Equipment	Company	Catalog Number
µ-Slide Angiogenesis	ibidi	81506
Amp 1-FL	ACD	SDS 320852
Amp 2-FL	ACD	SDS 320853
Amp 3-FL	ACD	SDS 320854
Amp 4 Alt B-FL	ACD	SDS 320856
Corning Matrigel Matrix	Corning	354234
DEPC	Sigma	D5758
Human Endothelial-SFM	Thermofisher	11111044
Paraformaldehyde	Sigma	30525-89-4
Paraformaldehyde	Sigma	30525-89-4
Protease K	ACD	SDS 322337
Sodium Citrate	sigma	6132-04-3
VEGF-165	STEMCELL Technologies	78073

Comments/Description	
Cell culture	
Signal Amplification	**WISH**
Signal Amplification	
Signal Amplification	
Signal Amplification	
Growth factor-reduced Matrigel	
RNAase-free Water	
Cell culture	**Tube formation**
Fixed embryos	
Fixed Cells	
Digest tissue	
SSCT solution：Wash Buffer	
Growth factor	

Dear Editor,

Thank you for formatting the manuscript and the arrangement of reviewing of our manuscript entitled " Apply a new developed in Situ RNA analysis platform on Zebrafish and iPSC-EC tube formation assay to study Cardiovascular disease " (JoVE60498R1). Editor and Reviewers raised some concerns that required further revision. Following the instruction, we revised carefully the whole manuscript point by point. We fully addressed the concern and corrected all minor errors metioned by reviewers and highlighted the filming part in the protocol. We believe that the process has significantly enhanced the message and impact of our manuscript. We are very keen to resubmit our work to JoVE.

We address all questions the reviewers raised:

Response to Reviewer 1 comment:

Reviewer #1:

Manuscript Summary:

The authors have revised the manuscript to state that they have 'improved' a model for in situ hybridization in zebrafish as opposed to generating a novel model.

Major Concerns:

The manuscript is still difficult to read and lacks novelty and originality. Any findings are incremental at best.

Response to the reviewer: We followed editor's annotation and revised one by one to fully address all the questions. We simplified the abstract and remove the redundant parts in the text to make it easy to read. To add in the value of tube formation we not only carried experiment with patient-derived iPSC-ECs for tube formation assay, also metioned *eng* mutation autologous corrected line in this revision. Although we haven't added new data in figures, but we would carry out tube formation assay on Crispr/CAS9 single mutation corrected iPSC-ECs when we could be filmed. This result hasn't been reported yet. Also we will perform the assay on µ-Slide angiogenesis plate, which reduced the volumn of Matrigel used, and allows for live cell microscopy with all cells in focus on one 2D cell layer. We believe these increase the novelty and originality of our manuscript.

Reviewer #2 and Reviewer #3 accepted revision 1.

Response to Reviewer 4 comment:

Reviewer #4:

Manuscript Summary:

The manuscript describes two methods to study the role of angiogenic factors in vessel formation. The authors greatly improved the manuscript after revision. There are, however, several unresolved issues.

Major Concerns:

The protocol of the whole-embryo in situ hybridization is a refinement of the RNAscope based protocol involving a post-fixation step and DAB staining. The authors claim in the introduction (line 86), the whole protocol can be finished in one day as a major advantage, but in their step-by-step description they write the probe is hybridized overnight (line 172). Have they tried shorter hybridization times? They claim the post-fixation being a major refinement, but as they cite, this has been already published by Gross-Thebing et al. This reference is completely missing in the References section.

Response to the reviewer: We thank your constructive advices. Regards 'the authors claim in the introduction (line 86), the whole protocol can be finished in one day as a major advantage, but in their step-by-step description they write the probe is hybridized overnight (line 172)'. In fact, with the longest step-hybridization takes about 12hours, other steps including fixation, digestion, amplification and staining plus wash won't take more than one hour each, all procedure can be finished in 24 hours (one day). The reference cited as Ref. 10.

Other discrepancies:

line 86: "…previous researcher" - Reference is missing

line 135: The yolk does not disappear after 2 days. The yolk sac is present until the embryo reaches free-swimming free-feeding stage at 120hpf.

line 225: Why the fluorescent confocal miscroscope is used to take images if the DAB staining is the immunohistochemical staining?

page 12: Add the name of the probe used in Fig1A.

page 16: The information about the morpholinos used is missing. What concentration was used?

Response to the reviewer: We have corrected the discrepancies listed:

Line 86 (updated to line 92): We added in ref. 10.

Line 135: We remove the unnecessary sentence.

Line 225 (updated to line 238): We corrected it to 'Use an optical microscope with a photographic function for imaging the samples.'

Page 12: we added the name of the probe used in Fig1A –'ENG'.

Page 16: We added the imformation about the morpholinos used as below:

1.1.3. Inject the 2ng morpholinos and 500pg mRNA into one cell stage embryos using the Femto Jet injection system under a microscope. (Endoglin-MOs sequence: 5'-GATGAACTCAACACTCGTGTCTGAT-3'; 5-Mispair control MOs sequence: 5'-AAACAGACCACATCCTCTTCATCTC-3'). Incubate the zygotes in acidic sea water at 27 ◦C for approximately 48 hours.

Regarding the method of the tube formation, it is not clear what kind of refinement the authors add to the current protocols.

In the introduction (lines 59-60), they claim they corrected the eng mutation in iPSC derived from HHT patients. This is not shown or elaborated on anywhere in the manuscript.

line 232: "con" should be spelled out.

Response to the reviewer: We improve the tube formation through using μ-slide angiogenesis plate that can reduce the volume of matrigel and allow for live cell microscopy with all cells in focus on one 2D cell layer.
The eng mutation correction was a result of our previous work that has been published by another group member in Pulmonary Circulation online 2019, DOI: 10.1177/2045894019885357. (We applied Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) /Cas9 technique: the gRNA (5'-ACCACTAGCCAGGTCTCGAA-3'; score 92) was designed using online "CRISPR Design" software (http://crispr.mit.edu/), and was inserted into the BbsI sites of CRISPR/Cas vector px458 (Addgene). For donor plasmids, 1500bp DNA fragments including exon2 were obtained using normal human genomic DNA as template, and cloned into piggyBac plasmid (pPB –puDtk) to generate donor plasmids.
'con' has been spelled out as 'control' with clear definition-wild type ECs in line244.

In summary we believe that we have made every effort to address the concerns of the editor and the reviewers, which have substantially increased the quality of our manuscript. We look forward to hearing from you.

Yours sincerely,

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Title of Article:	In situ RNA analysis of zebrafish embryos and tube formation assays with iPSC-ECs for studying hereditary hemorrhagic telangiectasia
Author(s):	Yong Wang, Ding Zhang, Fang Zhou, Meijun Zhou, Jingyu Chen, Jun Yang

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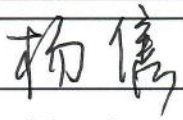
Signature:	
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