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In Vitro Model of Coronary Angiogenesis

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Cover Letter

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Dr. Nandita Singh

Senior Science Editor, JOVE

Dear Dr. Singh,

Please find enclosed our revised manuscript entitled "*In vitro* model of coronary angiogenesis." We have carefully revised the manuscript by incorporating all the editorial and reviewers' comments, which has significantly improved the quality of the manuscript. We have also provided point-by-point response to each editorial and reviewers' comments. We are hopeful that you will find our revised manuscript ready for the publication. Should you have any further questions regarding the revision, please feel free to contact me via email at bsharma@bsu.edu and by phone at 765-285-8817.

Sincerely Yours,

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TITLE:

In Vitro Model of Coronary Angiogenesis

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KEYWORDS:

sinus venosus (SV), endocardium (Endo), coronary angiogenesis, ex vivo, in vitro, explant culture

SUMMARY:

In vitro models of coronary angiogenesis can be utilized for the discovery of the cellular and molecular mechanisms of coronary angiogenesis. In vitro explant cultures of sinus venosus and endocardium tissues show robust growth in response to VEGF-A and display a similar pattern of COUP-TFII expression as in vivo.

ABSTRACT:

Here, we describe an in vitro culture assay to study coronary angiogenesis. Coronary vessels feed the heart muscle and are of clinical importance. Defects in these vessels represent severe health risks such as in atherosclerosis, which can lead to myocardial infarctions and heart failures in patients. Consequently, coronary artery disease is one of the leading causes of death worldwide. Despite its clinical importance, relatively little progress has been made on how to regenerate damaged coronary arteries. Nevertheless, recent progress has been made in understanding the cellular origin and differentiation pathways of coronary vessel development. The advent of tools and technologies that allow researchers to fluorescently label progenitor cells, follow their fate, and visualize progenies in vivo have been instrumental in understanding coronary vessel development. In vivo studies are valuable, but have limitations in terms of speed, accessibility, and flexibility in experimental design. Alternatively, accurate in vitro models of coronary angiogenesis can circumvent these limitations and allow researchers to interrogate important biological questions with speed and flexibility. The lack of appropriate in vitro model systems may have hindered the progress in understanding the cellular and molecular mechanisms of coronary vessel growth. Here, we describe an in vitro culture system to grow coronary vessels from the sinus venosus (SV) and endocardium (Endo), the two progenitor tissues from which many of the

coronary vessels arise. We also confirmed that the cultures accurately recapitulate some of the known in vivo mechanisms. For instance, we show that the angiogenic sprouts in culture from SV downregulate COUP-TFII expression similar to what is observed in vivo. In addition, we show that VEGF-A, a well-known angiogenic factor in vivo, robustly stimulates angiogenesis from both the SV and Endo cultures. Collectively, we have devised an accurate in vitro culture model to study coronary angiogenesis.

INTRODUCTION:

Blood vessels of the heart are commonly called coronary vessels. These vessels are comprised of arteries, veins, and capillaries. During development, highly branched capillaries are established first, which then remodel into coronary arteries and veins[1-5]. These initial capillaries are built from endothelial progenitor cells found in the proepicardium, sinus venosus (SV), and endocardium (Endo) tissues[1,6-8]. SV is the inflow organ of embryonic heart and Endo is the inner lining of the heart lumen. Endothelial progenitor cells found in the SV and Endo build the majority of coronary vasculature, whereas the proepicardium contributes to a relatively small portion of it[2]. The process by which the capillary network of coronary vessels grow in the heart from its preexisting precursor cells is called coronary angiogenesis. Coronary artery disease is one of the leading causes of death worldwide and yet an effective treatment for this disease is lacking. Understanding the detailed cellular and molecular mechanisms of coronary angiogenesis can be useful in designing novel and effective therapies to repair and regenerate damaged coronary arteries.

Recently, a surge in our understanding of how coronary vessels develop has been in part achieved through the development of new tools and technologies. In particular, in vivo lineage labelling and advanced imaging technologies have been very useful in uncovering the cellular origin and differentiation pathways of coronary vessels[9-12]. Despite the advantages of these in vivo tools, there are limitations in terms of speed, flexibility, and accessibility. Therefore, robust in vitro model systems can complement in vivo systems to elucidate the cellular and molecular mechanisms of coronary angiogenesis in a high-throughput manner.

Here, we describe an in vitro model of coronary angiogenesis. We have developed an in vitro explant culture system to grow coronary vessels from two progenitor tissues, SV and Endo. With this model, we show that the in vitro tissue explant cultures grow coronary vessel sprouts when stimulated by growth medium. Additionally, the explant cultures grow rapidly compared to control when stimulated by vascular endothelial growth factor A (VEGF-A), a highly potent angiogenic protein. Furthermore, we found that the angiogenic sprouts from the SV culture undergo venous dedifferentiation (loss of COUP-TFII expression), a mechanism similar to SV angiogenesis in vivo[1]. These data suggest that the in vitro explant culture system faithfully reinstates angiogenic events that occur in vivo. Collectively, in vitro models of angiogenesis that are described here are ideal for probing cellular and molecular mechanisms of coronary angiogenesis in a high-throughput and accessible manner.

PROTOCOL:

Use of all the animals in this protocol followed Ball State University Institutional Animal Care and Use Committee (IACUC) guidelines.

1. Establishing mouse breeders and detecting vaginal plugs for timed pregnancies

1.1. Set up a mouse breeding cage with wild type male and female mice. Ensure that the age of the breeding mice is between 6–8 weeks. Set up either a pair (1 male and 1 female) or as a trio (1 male and 2 female) for breeding.

1.2. Check for a vaginal plug the following morning. Use an angled metal probe to detect a deep plug by inserting it into the vaginal opening. Designate the morning of a positive vaginal plug to be embryonic day 0.5 (e0.5).

NOTE: A vaginal plug can be either superficial (which is easily visible, see **Figure 1**) or deep (which is not easily visible). Presence of a deep plug will block full insertion of the probe whereas the absence of a plug will allow full insertion without resistance.

1.3. Maintain timed pregnancy until the embryos reach e11.5 at which they will be harvested. To confirm pregnancy before harvesting embryos, record the weight of female mice between e7.5 and e11.5.

NOTE: Daily increase in the mother's weight will indicate a successful pregnancy, whereas no change in weight will indicate a failed pregnancy.

2. Harvesting embryos from pregnant mice

NOTE: Before beginning, make sure to have the following equipment and reagents: a CO_2 euthanasia chamber, 70% ethanol, paper towels, regular forceps, fine forceps, scissors, 1x sterile phosphate-buffered saline (PBS), 10 cm sterile Petri dishes, container with ice, perforated spoon, dissection stereomicroscope.

2.1. Place an e11.5 pregnant mouse in a clean CO_2 euthanasia chamber to sacrifice it. Close the lid of the chamber to prevent the mouse from escaping.

2.2. After the mouse is secured in the euthanasia chamber, turn on CO_2. Make sure to regulate the flow rate of CO_2 per IACUC recommendations (i.e., 10–30% displacement per minute). After the mouse is completely euthanized, perform cervical dislocation to ensure death.

2.3. Spray the mouse with 70% ethanol. Lift the skin over the belly using forceps, make a small incision using a pair of scissors and extend the incision laterally. Enlarge the incision anteriorly up to the diaphragm and expose the uterine horn containing the embryos (**Figure 2**).

2.4. Pull out the string of embryos (uterine horn + embryos) by grasping the uterus and cutting it free. Place the string of embryos in ice-cold sterile 1x PBS.

2.5. Dissect out the individual embryos from the uterus horn by peeling off the uterine muscle, yolk sac, and amnion one by one (**Figure 3A**-**F**) under a stereomicroscope. Transfer the cleaned embryos using a perforated spoon to a Petri dish containing sterile 1x PBS on ice. Make sure to keep the embryos cold.

3. Isolating hearts from e11.5 embryos

NOTE: Before beginning, make sure to have the following equipment and reagents: regular forceps, fine forceps, 1x sterile PBS, 10 cm sterile Petri dish, 6 cm sterile Petri dish, container with ice, perforated spoon, dissection stereomicroscope.

3.1. Set up a new Petri dish with ice-cold sterile 1x PBS under a stereomicroscope. Transfer an embryo from step 2.5 into the Petri dish to dissect out the heart.

3.2. Remove the head of the embryo using forceps. First, squeeze the head between the forceps with one hand and then remove the head by scraping it away with the other hand using closed forceps (**Figure 4A**-**C**).

3.3. After removing the head, orient the embryo with its ventral side up by holding the embryo with forceps at its belly with one hand (**Figure 4D**).

3.4. With the other hand, open the chest wall of the embryo by first making a small incision in the chest slightly above the diaphragm using fine forceps. Then, enlarge the incision very carefully by inserting closed forceps and tearing the chest wall by opening the forceps. Make sure to not thrust too deep, which can damage the heart. With the help of the forceps, keep the chest wall wide open to expose the heart and lungs in the thoracic cavity (**Figure 4E**).

3.5. Using fine forceps, gently move the heart anteriorly (90°) and expose the dorsal aorta/vein. Pull out the heart/lungs anteriorly by capturing the dorsal aorta/vein at the base of the heart (**Figure 4F**-**H**).

NOTE: Be gentle while pulling out the heart/lungs to avoid tearing of the SV, which is located at the dorsal side of the heart.

3.6. Rinse the heart/lungs with cold 1x PBS to remove blood cells.

3.7. Repeat steps 3.1–3.6 to remove the heart/lungs from the remaining embryos. Make sure to keep the isolated heart/lungs on ice.

4. Isolating SVs and ventricles from e11.5 embryonic mouse hearts

4.1. Place the Petri dish with heart/lungs from step 3.7 under a stereomicroscope to isolate the SVs and whole ventricles. Peel off the attached lobes of the lungs one-by-one from their root

using fine forceps.

4.2. Orient the heart on its dorsal side and remove atria and the adjacent tissue that surrounds the SV anteriorly without tearing the SV. Remove the left and right atria from the heart by holding at its base and scraping it off using fine forceps (**Figure 5B**). Remove the adjacent tissue surrounding the SV using a similar technique (**Figure 5C**).

NOTE: Keep in mind that the right atrium is attached to the SV, so be careful to only remove the atrium.

4.3. To isolate the SV, first orient the heart with its dorsal side facing up (because the SV is on the dorsal side) and keep the heart still in this position by gently holding the heart at its ventricles with forceps.

NOTE: The SV is an inflow organ of an embryonic heart that lies in between the atria on the dorsal side of the heart.

4.4. Remove the SV by carefully peeling it off the heart where it is attached or by holding the SV at the base of its attachment with fine forceps and scraping it off with closed forceps (**Figure 5 D**,**E**).

4.5. Transfer the isolated SV into a new 6 cm Petri dish with ice-cold sterile 1x PBS on ice using a sterile transfer pipette and label the Petri dish as SV.

4.6. To isolate the whole ventricles, remove the outflow tract (aorta and pulmonary trunk) from the heart after the SV is removed (**Figure 5F**,**G**).

4.7. Transfer the whole ventricles into a new 6 cm Petri dish containing sterile 1x PBS on ice using a sterile transfer pipette and label the Petri dish as ventricles. Keep the isolated SV and ventricles on ice.

4.8. Repeat steps 4.1–4.7 to isolate SVs and ventricles from the remaining hearts.

5. Setting up tissue culture plates with inserts and extracellular matrix coating

NOTE: Before beginning, make sure to have the following equipment and reagents: commercial extracellular matrix solution (ECM; e.g., Matrigel), 8.0 µM polyethylene terephthalate (PET) culture inserts, 24 well plates, 37 °C, 5% CO_2 incubator.

5.1. Let the ECM solution thaw on ice. Keep the ECM solution on ice to avoid solidification.

5.2. Place the PET membrane culture inserts (pore size = 8.0 µm, filtration area = 0.3 cm^2, filter diameter = 6.5 mm) into the wells of non-tissue culture treated 24 well plates. Label the plates as SV or ventricles for the SV or the endocardial angiogenesis assays, respectively.

NOTE: Set up the inserts in separate plates for the SV and the ventricles when performing both cultures simultaneously. Make sure to set up enough wells for all the experimental samples and controls.

5.3. After the ECM solution is thawed, immediately dilute ECM 1:2 in precooled basal medium (i.e., EBM-2 basal medium, see **Table of Materials**) to a sufficient volume (100 µL/insert x number of inserts).

NOTE: For instance, if there are six inserts, then the total volume will be 100 µL x 6 = 600 µL. Add 200 µL of ECM into 400 µL of basal medium.

5.4. Coat the inserts with 100 µL of freshly diluted ECM by adding it directly on top of the membrane. Incubate the plate at 37 °C for at least 30 min to allow the ECM to solidify.

NOTE: This must be performed under a laminar flow tissue culture hood to avoid contamination.

6. SVs and whole ventricles cultures

NOTE: Before beginning, make sure to have the following equipment and reagents: 70% ethanol, transfer pipette, stereomicroscope, forceps, laminar flow tissue culture hood, microvascular endothelial cell supplement kit (**Table of Materials**), basal medium, 1x sterile PBS). **Figure 6** shows the workflow of SV and ventricle culture.

6.1. Thaw out the contents of the supplement kit on ice. Prepare the complete medium by adding all the contents of the supplement kit into 500 mL of basal medium under a certified laminar flow tissue culture hood. Mix the medium well and distribute into 50 mL aliquots.

6.2. Sterilize the base of the stereomicroscope and surrounding working area with 70% ethanol.

6.3. Obtain the tissue culture plates from step 5.4. With the aid of a transfer pipette, carefully transfer the explants from step 4.7 on top of the insert membrane. Under a stereomicroscope and with the aid of clean forceps, position the explants at the center of the inserts to ensure they are not stuck in the corner of the inserts or attached to the side walls.

6.4. After the explants are placed and centered on the inserts, carefully remove any extra PBS from the inserts and close the lids of the plates.

6.5. Under a laminar flow tissue culture hood, add 100 µL of the prewarmed complete medium on top of the inserts and 200 µL into the wells to culture the explants at the air-liquid interface such that the basal surface of the insert is in contact with the medium, but the top surface is exposed to the air.

NOTE: Make sure to adjust the volume to obtain an air-liquid interface if using different size

inserts/well plates.

6.6. Add 300 µL of PBS into the unused wells of the 24 well plates and cover with the lid. Incubate the plate in a 37 °C, 5% CO_2 incubator, and grow the cultures for 5 days.

6.7. In the following days, routinely observe the cultures under an inverted light microscope to assess the status of the explant cultures. Make sure that the explants exhibit contractile beating and that all the explants are attached to the bottom of the membrane embedded with ECM. Take note of any floating explants.

NOTE: The periodic contraction of the explants indicates that they are alive. Floating explants should be omitted from the analysis.

6.8. After assessing the culture status, put the culture plate back into the incubator and continue to grow the culture for up to 5 days.

7. Treatment of cultures with VEGF-A (positive control)

NOTE: Before beginning, make sure to have the following equipment and reagents: laminar flow tissue culture hood, 1x PBS, basal medium + 1% fetal bovine serum (FBS), basal medium + VEGF-A, pipettes, and pipette tips.

7.1. Prepare the basal medium + 1% FBS and the basal medium + VEGF-A.

7.1.1. To prepare the basal medium + 1% FBS, first determine the number of control wells needed. For instance, if there are three control wells, then 300 µL/well x 3 = 900 µL is the total volume needed. Add 9 µL of FBS into 891 µL of basal medium to make the basal medium + 1% FBS.

7.1.2. To prepare the basal medium + VEGF-A, first determine the total number of wells needing VEGF-A medium. If there are three wells, then 300 µL/well x 3 = 900 µL is the total volume and 50 ng/well x 3 = 150 ng VEGF-A. Add 150 ng of VEGF-A into 900 µL of basal medium to make the basal medium + VEGF-A.

NOTE: Assemble this solution at a larger volume than calculated to insure a sufficient number of smaller aliquots for each experiment.

7.2. On day 2, remove the media from both chambers (the inserts and the wells). Wash cultures with 300 µL of 1x PBS by adding 100 µL to the inserts and 200 µL into the wells. Firmly swirl the plates a few times and remove the PBS.

7.3. Add 300 µL of basal medium + 1% FBS (100 µL into the insert and 200 µL into the wells) to starve the cultures for 24 h.

7.4. On day 3, after starvation, add 300 µL of basal medium + 1% FBS (100 µL into the insert and 200 µL into the wells) into the control wells and basal medium + VEGF-A (50 ng/well) into the treatment wells, respectively.

7.5. After treatment, continue to grow the cultures in the incubator.

8. Fixation and immunostaining

NOTE: Before beginning, make sure to have the following equipment and reagents: 4% paraformaldehyde (PFA), 1x PBS, primary and secondary antibodies, a shaker, 0.5% nonionic surfactant in PBS (PBT).

8.1. On the sixth day of culturing, remove the medium and wash cultures with 1x PBS at room temperature (RT).

8.1.1. Fix the cultures by adding 200 µL of 4% PFA solution into the wells and 100 µL into the inserts. Fix cultures in 4 °C for 20 min while rocking.

8.1.2. After 20 min fixation, remove PFA from the cultures in a fume hood and wash the cultures with 1x PBS by adding 200 µL into the wells and 100 µL into the inserts.

8.1.3. Repeat washes 3x, 10 min each, while rocking. Then proceed to perform immunostaining.

NOTE: All the wash steps are performed on a benchtop at RT.

8.2. Dilute primary antibodies (anti-VE-Cadherin, anti-ERG 1/2/3) in blocking solution (5% donkey serum, 0.5% PBT). Add 300 µL of primary antibody solution (200 µL in the bottom wells and 100 µL into the inserts). Incubate cultures in primary antibodies overnight at 4 °C while rocking.

NOTE: Anti-VE-Cadherin is used to label the endothelial cell membrane and anti-ERG 1/2/3 is used to label the endothelial cell nucleus in order to visualize the angiogenic sprouts of endothelial cells.

8.3. The next day, wash and rock the culture plates 10x in 0.5% PBT, changing PBT every 10 min.

8.4. Dilute the secondary antibodies (donkey anti-rat Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 555) in blocking solution. Add 300 µL of the secondary antibody as in step 8.2 and incubate the cultures overnight at 4 °C while rocking. The next day, wash the secondary antibodies 10x in PBT, changing PBT every 10 min.

NOTE: Wash a minimum of 10x but more washes are better. After the washes are complete, the cultures can be stored with 1x PBS until they are mounted onto slides.

9. Mounting cultures onto slides, imaging, and analysis

NOTE: Before beginning, make sure to have the following equipment and reagents: fine forceps, slides, mounting medium with 4',6-diamidino-2-phenylindole (DAPI), coverslips, and confocal microscope. After secondary antibody staining, mount the cultures onto slides for imaging using the following steps.

9.1. Peel off the membrane carefully from the insert using fine forceps and transfer it onto the slides by putting the membrane side down and placing the explant cultures upward. Place the replicate samples into the same slides and label the slides as control or VEGF-A. Add a few drops of mounting medium with DAPI directly onto the membrane and cover the slides with cover slips.

NOTE: Make sure to avoid air bubbles while placing the coverslips.

9.2. Seal off the edges of the slides with clear nail polish and let dry.

NOTE: Slides can be stored in -20 °C for long-term storage.

9.3. Image slides using a confocal microscope.

9.4. Perform analysis to measure the length of angiogenic outgrowth. Quantify angiogenic outgrowth length by measuring the distance of the endothelial cells (Ve-Cadherin+/ERG 1/2/3+) extended from the inside boundary of the ERG 1/2/3+ cells in the ventricle cultures and from the center of the SV explants in the SV cultures.

9.4.1. To perform quantification using FIJI/ImageJ, first download FIJI software.

9.4.2. Open image files in FIJI: go to **File | Open | Folder | Filename | Open**.

9.4.2. Go to **Analyze | Set Measurements | Select Perimeter**.

9.4.3. Select the **Straight Line** tool from the main window.

9.4.4. Draw a line across the length of a sprout as suggested in step 9.4.

9.4.5. Go to **Analyze | Measure**.

NOTE: Length measurements are displayed in the new window.

9.4.6. Perform quantification in images that represent at least three randomly selected fields of view. Average the sprout length measurements and report them as mean ± standard deviation.

REPRESENTATIVE RESULTS:

One of the most striking features of SV angiogenesis in vivo is that it follows a specific pathway and involves cell dedifferentiation and redifferentiation events that occur at stereotypical times

and positions[1]. As initial SV cells grow onto the heart ventricle, they stop producing venous markers such as COUP-TFII (**Figure 7**). Subsequently, coronary sprouts take two migration paths, either over the surface of the heart or deep within the myocardium. Surface vessels eventually become veins while invading vessels become arteries and capillaries[1,6,7]. This distinction is preserved as the heart grows and the coronary vasculature expands.

To facilitate the discovery of the molecular underpinnings of coronary development, we have devised an in vitro model of SV sprouting that can be utilized for loss-of-function and gain-of-function experiments. SV tissue from embryonic mouse hearts are dissected, placed on the top of diluted ECM (which is commercially available, see **Table of Materials**), and maintained at the air-liquid interface in endothelial cell growth medium. The SV myocardium continues to beat throughout the culture period. After 2 days in culture, epicardial cells that line the tissue leave the explant and migrate out onto the matrix. After 5 days, SV endothelial cells sprout out and migrate onto the epicardial tissue (**Figure 8A**, black arrowheads). Collectively, this process recapitulates angiogenic aspects of coronary development.

Cultured SV sprouts also undergo venous dedifferentiation. As in the embryonic heart, COUP-TFII expression is reduced as the vessels migrate away from the SV (**Figure 8B**, compared to **Figure 7**). Control vessels such as umbilical or yolk sac arteries and veins can produce sprouting vessels. However, they are fewer in number and shorter in length and do not change their arterial or venous identity (not shown). Thus, venous reprogramming is specific to SV sprouting and not a general feature of angiogenesis or a response to ECM components. These data also indicate that the cell types contained within the SV itself are necessary and sufficient to induce dedifferentiation.

It is well known that vascular endothelial growth factor A (VEGF-A) is a potent inducer of angiogenesis[7,13-18]. Previous studies have shown that myocardial VEGF-A stimulates endocardial angiogenesis to grow coronary vessels[7]. In addition, coronary endothelial cells have been shown to express VEGF-A receptor, and SV-derived coronary vessels in the myocardium might grow in response to VEGF-A in vivo[2]. To show that our in vitro model of coronary angiogenesis faithfully responds to VEGF-A, we stimulated SV and Endo cultures with VEGF-A. As expected, our results showed that both SV and Endo are highly responsive to VEGF-A. Cultures stimulated with VEGF-A show increased growth of angiogenic sprouts both in density and length (**Figure 9A**,**C**). SV cultures stimulated by VEGF-A show an almost 3-fold increase in sprout length compared to the control (**Figure 9B**). These results suggest that endothelial sprouts from SV and Endo respond to similar cues that are known in vivo.

Taken together, our data suggest that these in vitro explant cultures share similar cellular and molecular events that occur during in vivo coronary development, including venous dedifferentiation and robust growth in response to VEGF-A stimulation. Therefore, our data suggest that these explant culture models are useful for studying coronary angiogenesis in vitro.

FIGURE LEGENDS:

Figure 1: Vaginal plug identification. (**A**) A vaginal plug (white spot). (**B**) Magnified view of boxed

region including the vaginal plug (indicated by arrow).

Figure 2: Accessing the embryo string in the uterus of a pregnant mouse. A euthanized pregnant mouse is positioned with its ventral surface up and sprayed with 70% ethanol to wet skin for dissection. The skin is pulled up at the pelvic region using forceps and a small incision is made. The incision is extended laterally. An additional incision is made anteriorly up to the diaphragm. In the uterus, a string of embryos is visible (indicated by arrowheads).

Figure 3: Dissection and removal of embryos from the uterus. (**A**) Image showing the uterus containing a string of embryos. The membrane on the dorsal side of the embryo (opposite of the side where the placenta is located) is peeled off. (**B**) The embryo in the yolk sac becomes visible after peeling off the uterus membrane. (**C**,**D**) Embryo attached to the placenta with the umbilical cord is visible after peeling off the yolk sac. Amnion is peeled off if still present. (**E**) Image showing an embryo attached to the placenta by the umbilical cord. (**F**) Image showing the detaching of the embryo from the placenta by pulling on the umbilical cord (Umb. Cord) using forceps.

Figure 4: Removal of the heart/lungs pluck from embryos. (**A,B**) The head is removed from the embryo by capturing at the neck and scraping it off. (**C**) Image showing the removal of the head from the body. (**D**) The correct positioning of the embryo for heart dissection. The embryo is positioned with the ventral side up to have easy access to the thoracic cavity for the removal of the heart. Once the embryo is positioned as shown in D, the chest cavity is opened with forceps. (**E**) Image showing the open chest cavity with the heart exposed on its ventral side. (**F**) To remove the heart without damaging the SV, the heart is flipped anteriorly, and the dorsal aorta becomes visible. (**G**) Image showing the position at the base of the heart/lungs where the dorsal aorta/vein is captured with forceps and the heart/lungs pluck is pulled out anteriorly. (**H**) The image shows the e11.5 heart/lungs pluck removed from the embryo. V. heart = ventral heart; d. heart = dorsal heart; d. aorta = dorsal aorta; LA = left atrium; RA = right atrium; LV = left ventricle; RV = right ventricle.

Figure 5: Isolating SV and ventricles from embryonic hearts. (**A**) Image showing the dorsal view of the isolated heart/lungs pluck from the e11.5 embryo. Location of the SV in the heart is indicated. (**B**) Image of the heart after the lung buds have been removed. (**C**) Atria and adjacent tissues surrounding the SV are removed, revealing the aorta. Location of the SV is labelled. (**D**) Image showing removal of the SV. Using forceps, the SV is gripped at its base and is scraped off from the heart. (**E**) SV removed from the heart is shown along with the remaining tissue of the heart. The aorta and pulmonary trunk (PT), collectively called the outflow tract (OFT), become visible in the heart after SV is removed. The SV will be used for in vitro culture, as described in **Figure 6** (left panel). (**F**) Position of the OFT to be dissected is marked by a dashed white line. (**G**) Aorta/PT (outflow tract) is removed from the ventricles by dissection at the position shown in panel F. The remaining ventricles without the OFT are used for ventricle cultures as shown in **Figure 6** (right panel).

Figure 6: Schematic showing the workflow of SV and ventricle culture. Left: Procedure for SV culture. First, the SV is removed from e11.5 hearts and placed onto a culture insert. Insert

contains porous membrane at the bottom (8 µm pore) and is coated with growth factor reduced ECM. Inserts are placed into the wells of a 24 well plate. Medium is added both in the bottom and top chamber without covering the explant. The culture is grown for 5 days. After 5 days, the membrane is removed from the insert and is mounted onto the slides for imaging and analysis. **Right**: Procedure for ventricle culture. Ventricles without the SV, atria, and OFT are removed from the e11.5 hearts and are cultured as described above for the SV.

Figure 7: COUP-TFII expression is lost in the SV sprouts in vivo. (**A**) Dorsal view of e11.5 hearts. Confocal image of endothelial marker (red) that labels the sinus venosus (SV), coronary sprouts (cs, dotted line), and endocardium (endo) lining the heart lumen. (**B**) The venous maker COUP-TFII (green), a vein transcription factor (trx), expressed in the SV is progressively lost as sprouts leave the SV and grow onto the ventricle. **Right**: Higher magnification of the coronary sprout shown in the boxed region of panel A. Scale bar = 100 µm.

Figure 8: In vitro models of venous identity and reprogramming. (**A**) Brightfield image of cultured SV tissue. Vascular sprouts (arrowheads) grow out from the original explant (black dotted line) over the ECM substrate. (**B**) Immunofluorescence of SV cultures labeled with antibodies that mark the endothelial cell surface (VE-Cadherin), endothelial cell nuclei (ERG 1/2/3), and vein and epicardial nuclei (COUP-TFII). Nuclei are positive for both ERG 1/2/3 and COUP-TFII at the SV, but ERG 1/2/3 only in sprouts migrating over the ECM. Scale bar = 50 µm.

Figure 9: SV and endocardium respond to VEGF-A in vitro. (**A**) Confocal image of the 5-day-old control and VEGF-A treated SV explant culture. Endothelial cells are immunostained with VE-cadherin (green), endothelial cell nuclei stained with ERG 1/2/3 (red) antibodies, and cell nuclei with DAPI (blue). (**B**) Angiogenic outgrowth of endothelial cells is quantified by measuring the length of sprout extension (the distance migrated by ERG 1/2/3+ endothelial cells from the explant, indicated by white solid lines in the bottom panels of (**A**). (**C**) Confocal image of a 5-day-old culture of a control and VEGF-A treated ventricles. Endothelial cells are immunostained with VE-cadherin (green) and endothelial cell nuclei are stained with ERG 1/2/3 (blue) antibodies. Dots are individual measurements and error bars are mean ± SD. Scale bar = 200 µm (**A**,**C**).

DISCUSSION:

Some of the most critical steps for successfully growing coronary vessels from the SV and Endo progenitor tissues are: 1) Correctly identifying and isolating the SV tissue for SV culture; 2) using ventricles from embryos between the ages of e11−11.5 for accurate Endo culture; 3) maintaining sterile conditions throughout the dissection period and keeping the tissues cold at all times; and 4) keeping the explants attached to the ECM coated membrane to avoid tissue floating in the medium.

First, isolation of SV tissue can be challenging. It is important to realize that the SV lies on the dorsal side of the heart between the left and right atria hidden within the lung lobules. Therefore, it is difficult to separate and isolate. Instead, the whole heart/lungs pluck must be extracted first. The SV is then isolated from the pluck by carefully removing the lung lobules and cleaning up the adjacent tissues with the help of fine forceps. Furthermore, one must be very careful while

cleaning up the adjacent tissues to not lose the SV.

Second, for accurate endocardial angiogenesis, it is important to culture ventricles from embryos no older than e11.5. In e12.5 and older embryos, coronaries from the SV grow into a significant proportion of the ventricles. Therefore, the coronary vessels that grow from older ventricles can be comprised of both the SV- and Endo-derived coronary vessels[1,2,10,19,20]. For this reason, to accurately assay the coronary vessel growth from the Endo, it is critical to culture ventricles (minus SV and outflow tract) from e11.5 embryos[20]. In addition, the SV is relatively larger and is easier to isolate at e11.5.

Third, because the protocol involves a substantial amount of work outside the tissue culture hood, it is critical to maintain a sterile working environment. It is important to sterilize the dissection tools (forceps, scissors, etc.) and the tissue culture plates and avoid contact with unsterile areas at all times. It is important to spray the working area and dissection scope with 70% ethanol. To avoid contamination, it is important to perform the dissection procedure quickly and minimize work outside the tissue culture hood.

Fourth, to obtain healthy explant cultures, it is important that the explants remain attached to the base of the membrane at all times. Floating explants in the medium must be avoided to successfully grow the explant cultures. To avoid floating, it is critical to maintain an air-liquid interface where the basal surface of the insert is in contact with the medium, but the top surface is exposed to the air. Such an interface is obtained when the explant is sufficiently covered by the medium but is not fully submerged. It is important to monitor the volume of the medium daily, as volume can be lost to evaporation. To prevent this, the culture plate can be humidified by adding PBS into the unused wells of the culture plates.

Similar explant cultures of SV and Endo are described elsewhere[20]. In these methods, the explants were cultured directly into the wells of tissue culture plates, which limits high-resolution confocal imaging. The images captured in this setting are relatively poor in quality compared to the method proposed here, limiting detailed microscopic analyses. To circumvent this, we utilized culture inserts from which the membranes containing the culture can be peeled off and mounted onto the glass slides for imaging. This allows for high-resolution confocal imaging where cellular details such as expression patterns and morphology can be viewed. In addition, culturing directly on plates requires a separate staining protocol for DAPI or other pan-nuclear marker staining. This protocol does not require separate staining steps because the slides can be mounted with mounting medium containing DAPI. Furthermore, mounting on slides allows for long-term storage without fluorescent fading, whereas cultures stored in wells with liquid medium will result in quick fading and are not amenable to long-term storage and imaging.

The in vitro culture models described here are ideal for interrogating cellular and molecular mechanisms of coronary angiogenesis in a high-throughput and accessible manner. This in vitro system can be used to screen for potential drugs or molecular targets to assess their effect on coronary angiogenesis. In addition, this system can also be used to study the gain-of-function and loss-of-function of various genes for their autonomous function in coronary angiogenesis. We

observed that coronary sprouts from SV followed the epicardial migration in our in vitro cultures, suggesting an important interaction between coronary endothelial cells and epicardial cells. It is well known that the epicardium is a rich source of growth factors for coronary angiogenesis from the SV. For instance, epicardial-derived growth factors such as VEGF-C and ELABELA have been previously shown to regulate SV-derived coronary angiogenesis[2,19]. We can use this SV culture system to further investigate the interaction between the epicardium and coronary endothelial cells and identify novel epicardial-derived molecular pathways of coronary angiogenesis. Additionally, our in vivo data suggest that myocardial hypoxia may be involved in endocardial angiogenesis[19]. Our in vitro culture system offers an excellent model to study the role of hypoxia in endocardial angiogenesis by incubating the cultures in hypoxic or normoxic conditions. These are difficult experiments to conduct in vivo because it requires a pregnant mouse to be placed in a controlled hypoxic chamber. From our own experience, it is very challenging to obtain consistent and reliable results from in vivo experiments. In summary, our in vitro model of coronary angiogenesis provides a reliable system to interrogate a wide range of questions relating to the cellular and molecular biology of coronary angiogenesis.

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DISCLOSURES:

The authors declare no conflict of interest.

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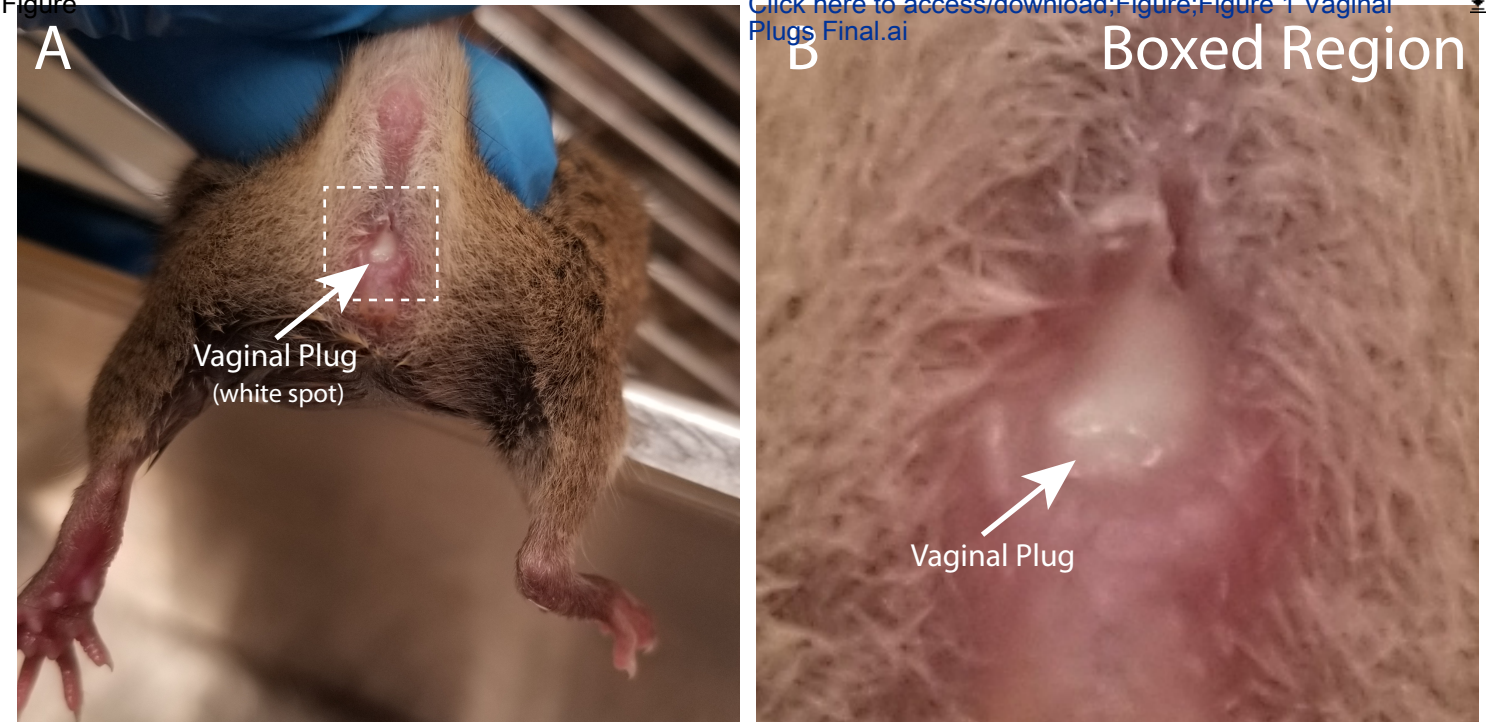
Figure



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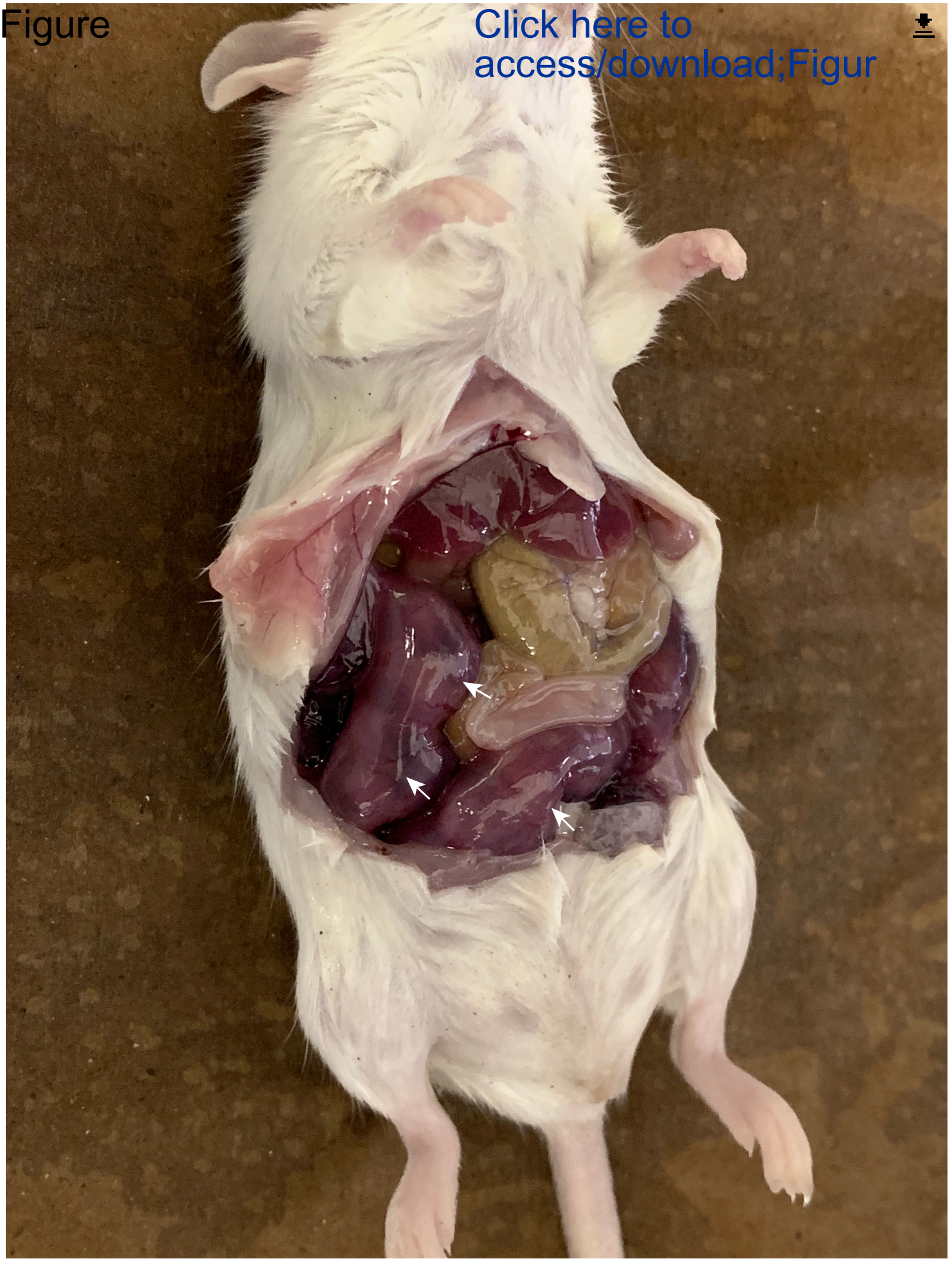

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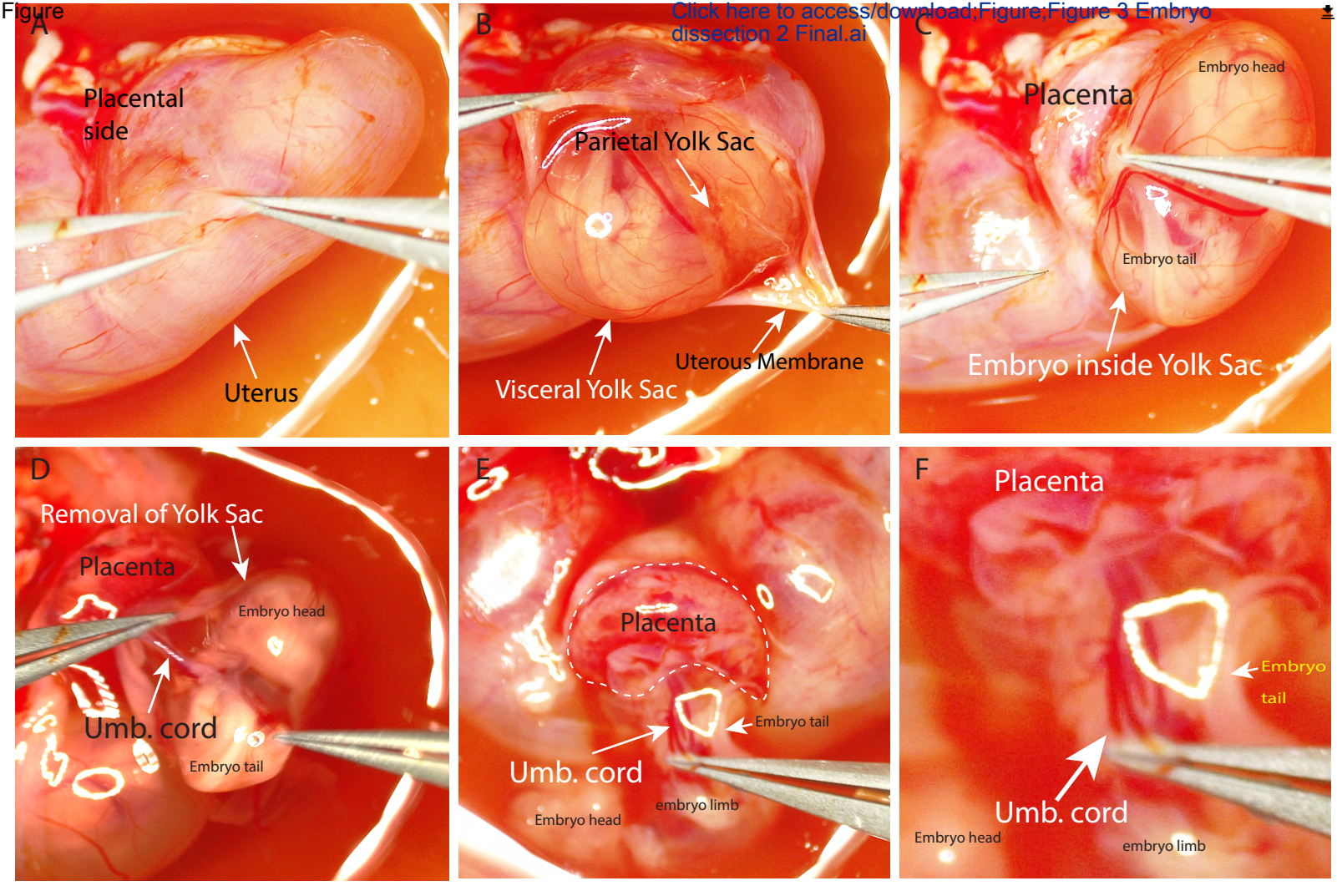

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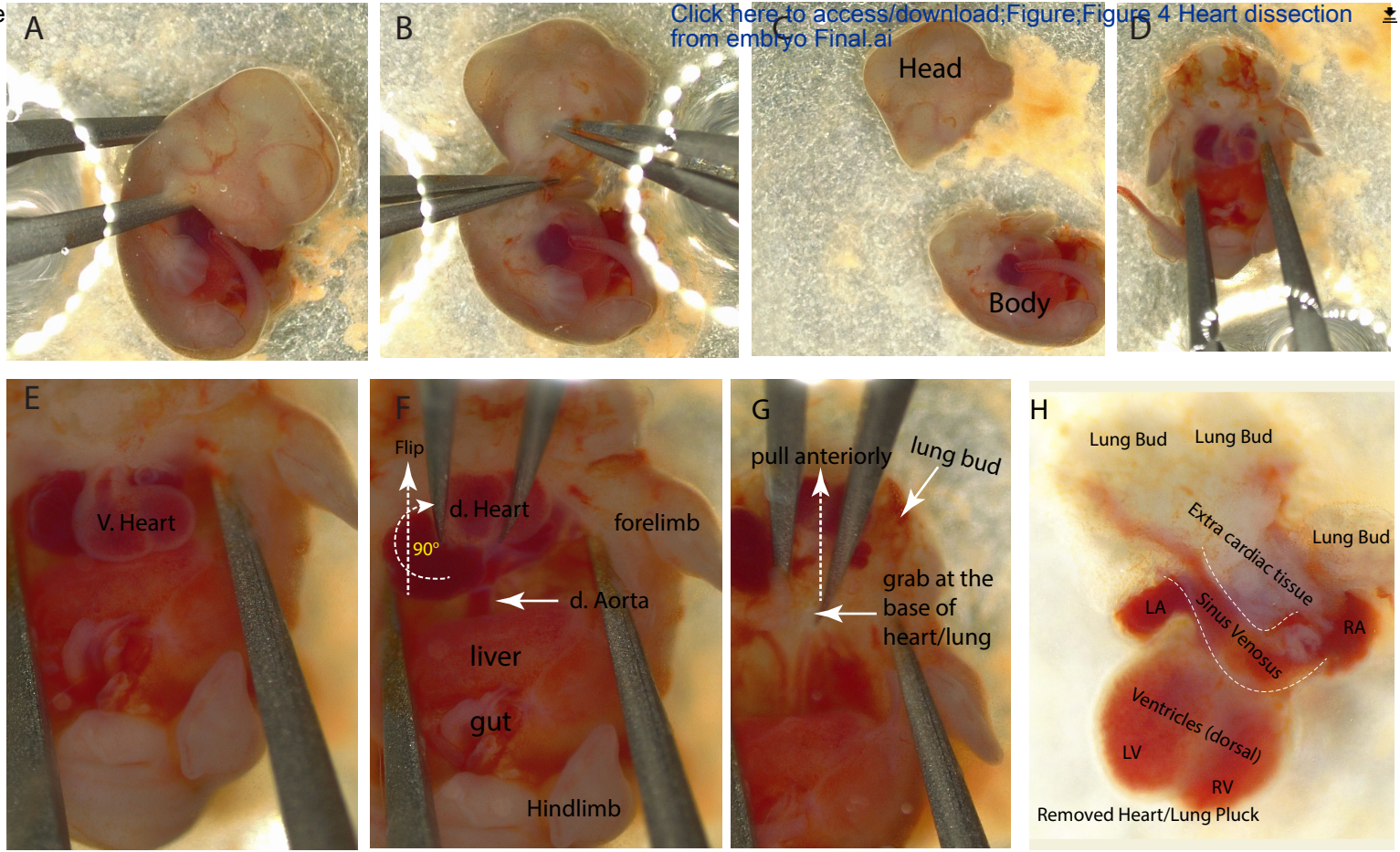

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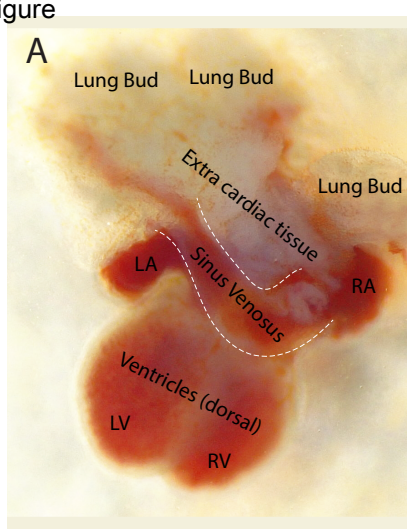

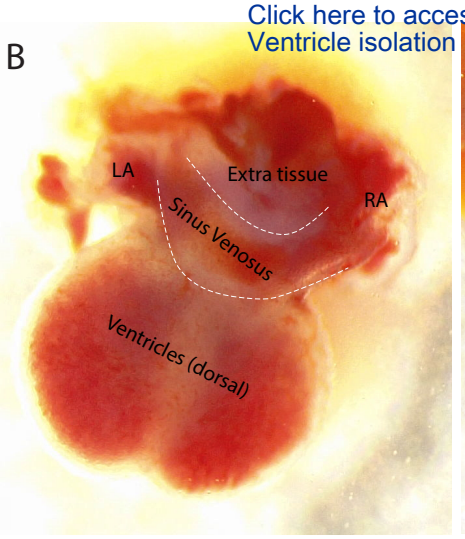

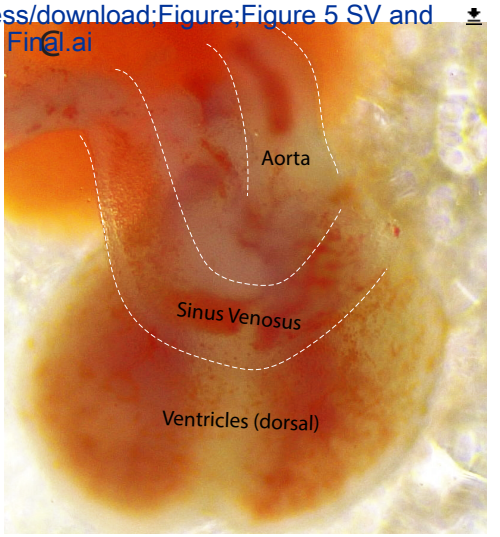

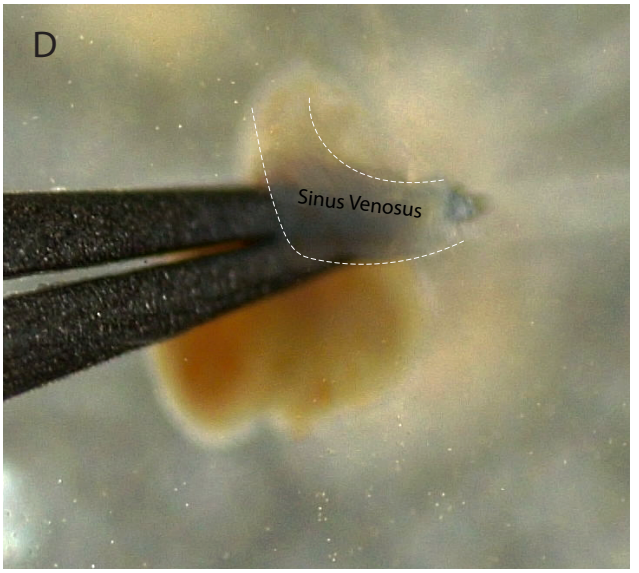

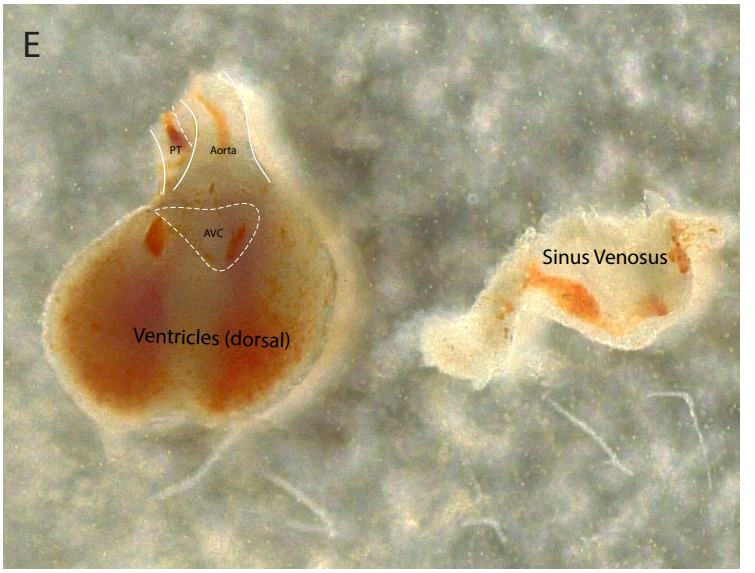

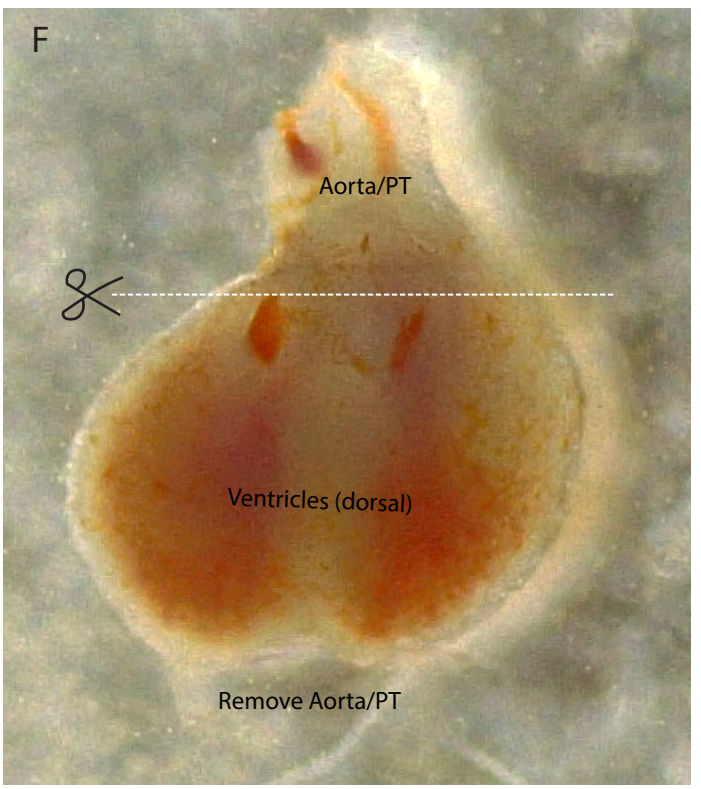

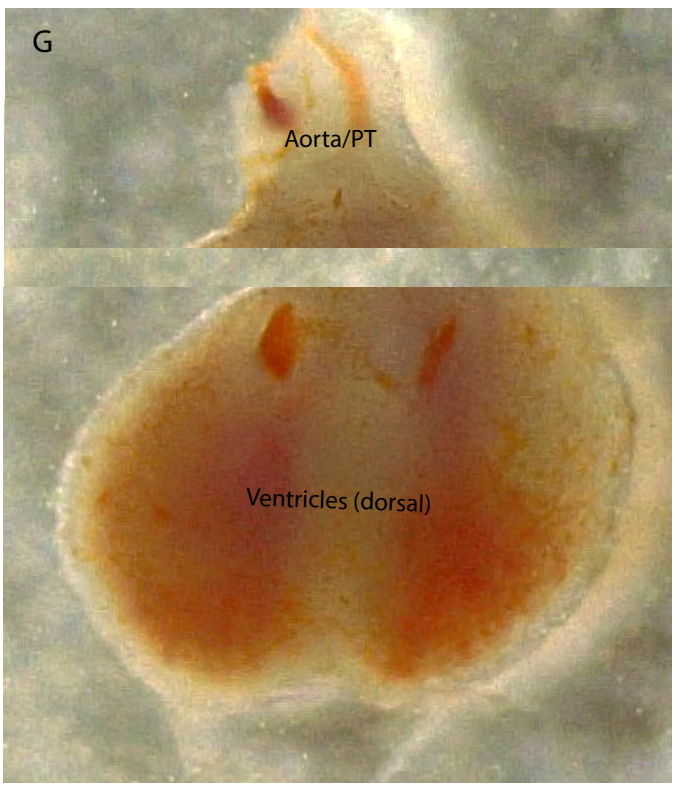

Figure 6

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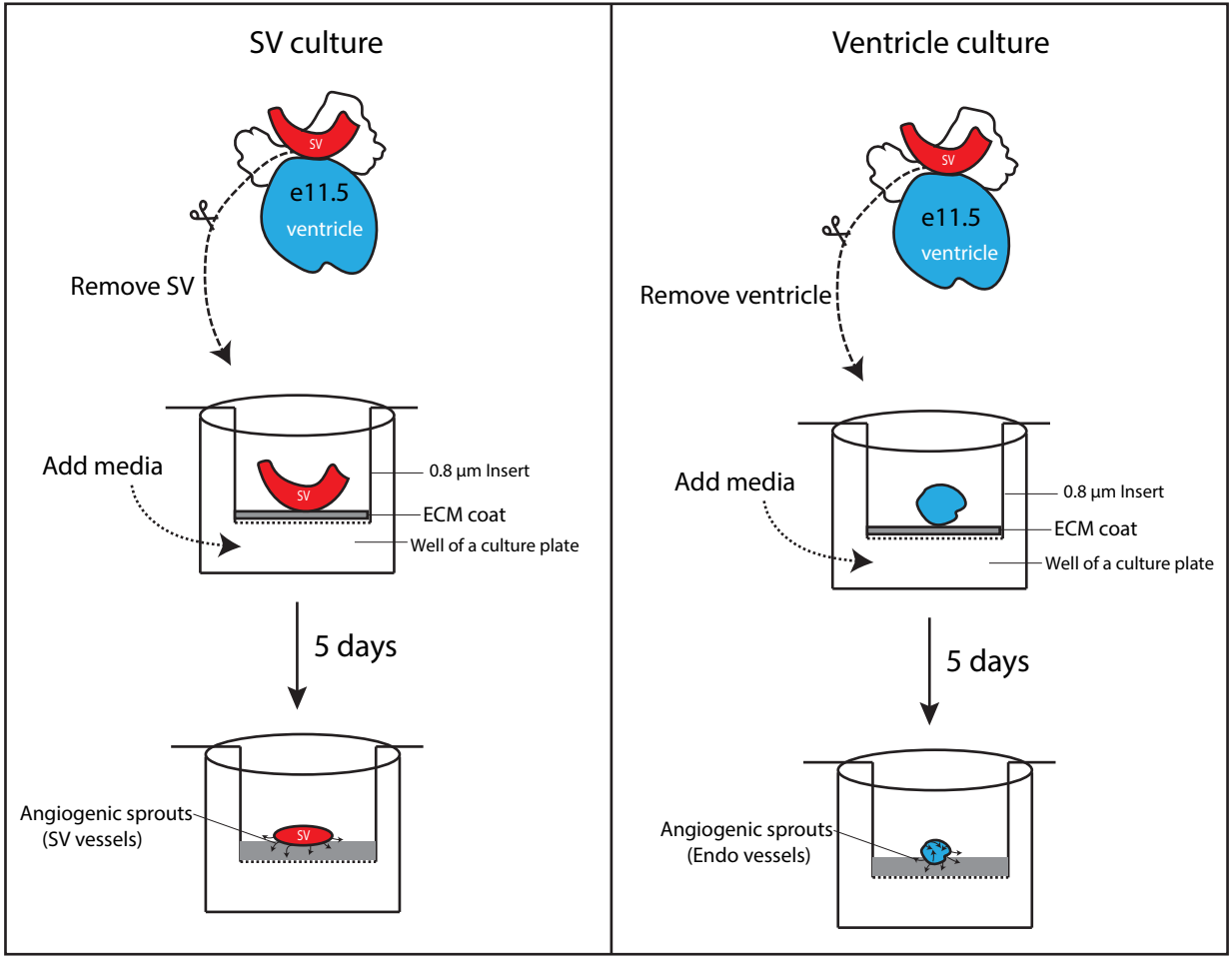

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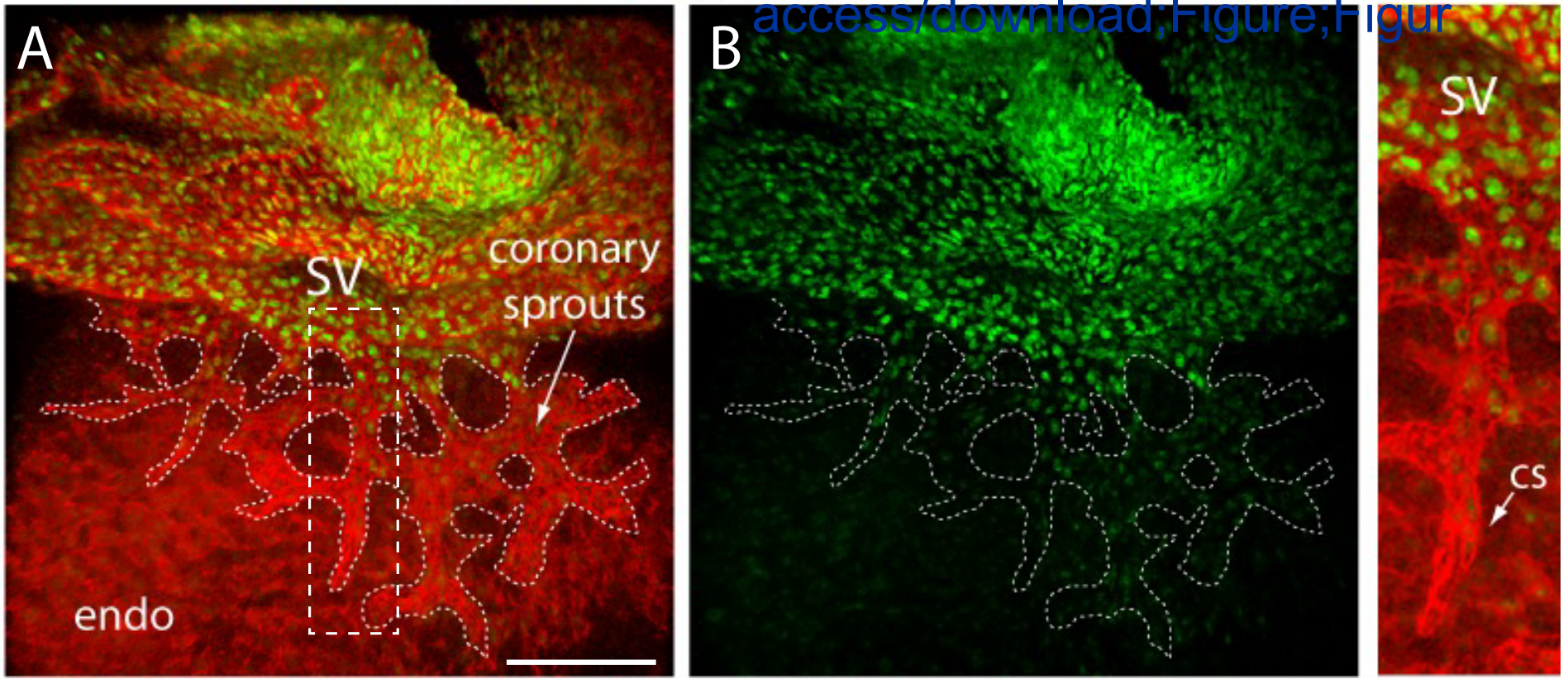
VE-cadherin (endothelial)
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boxed region
A
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coronary
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VE-cadherin (endothelial cells) Click here to access/download Figure Figure 8. in vitro venous reprogramming Final.ai

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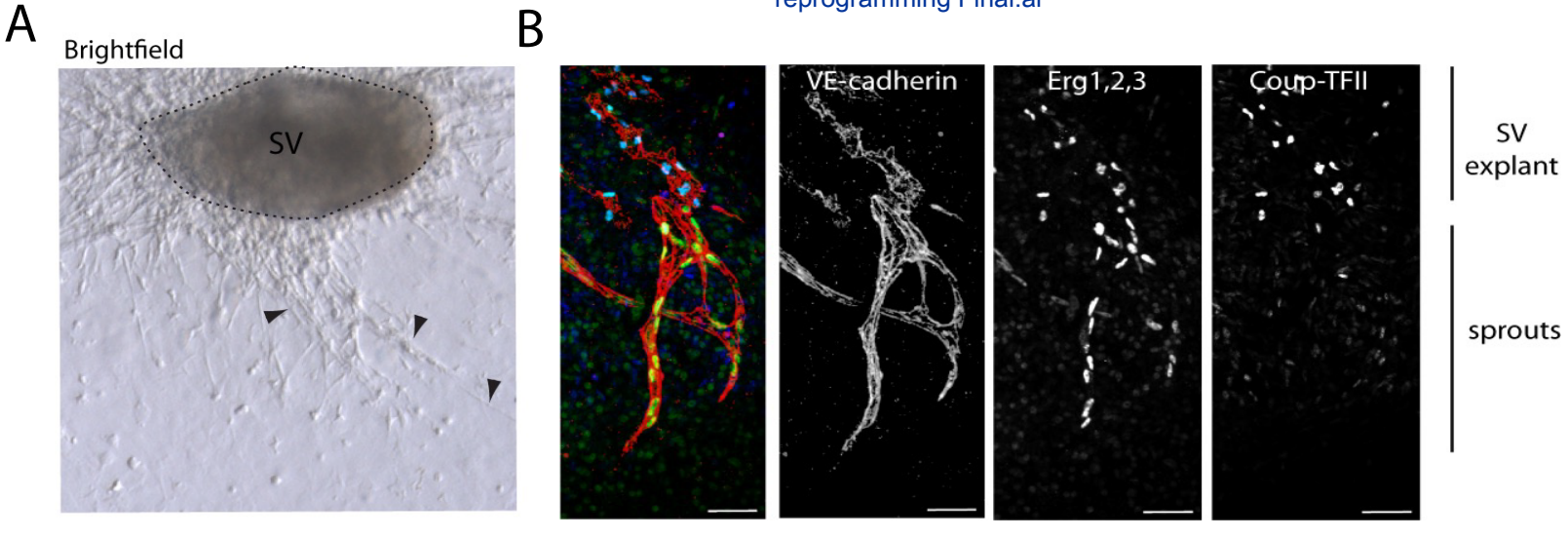

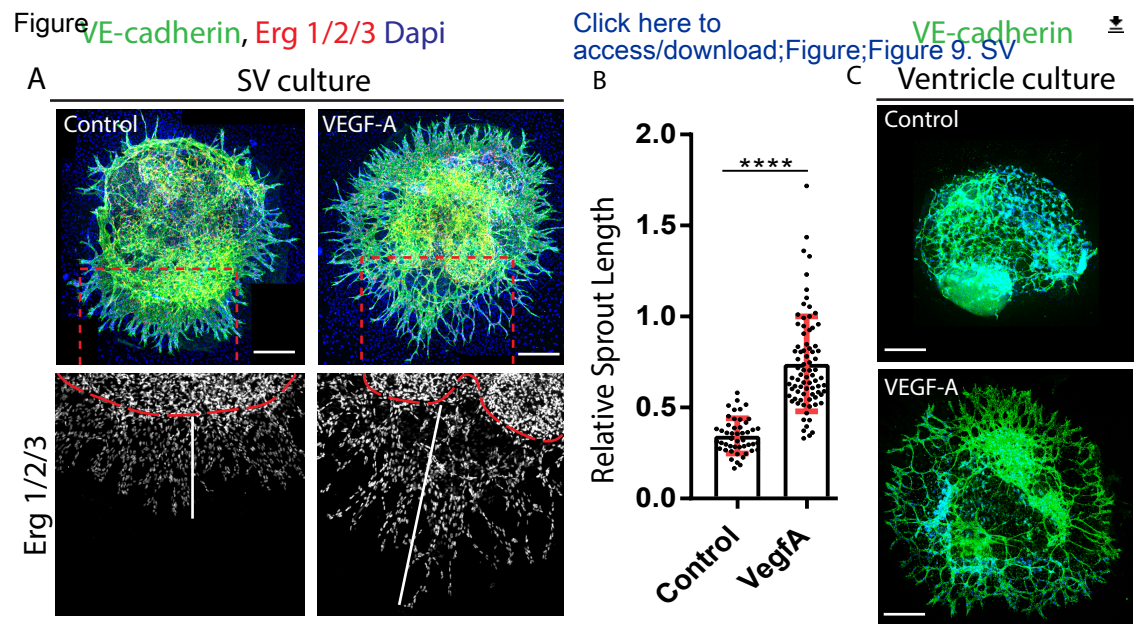

Name of Material/Equipment	Company	Catalog Number
100 x 20 MM Tissue Culture Dish	Fisher Scientific	877222
24-well plates	Fisher Scientific	08-772-51
8.0 uM PET membrane culture inserts	Millipore Sigma	MCEP24H48
Alexa Fluor Donkey anti-rabbit 555	Fisher Scientific	A31572
Alexa Fluor Donkey anti-rat 488	Fisher Scientific	A21206
Angled Metal Probe	Fine science tools	10088-15
Anti- ERG 1/2/3 antibody	Abcam	Ab92513
Anti- VE-Cadherin antibody	Fisher Scientific	BDB550548
CO2 gas tank	Various suppliers	N/A
CO_2 Incubator	Fisher Scientific	13998223
Dissection stereomicrosope	Leica	S9i
EBM-2 basal media	Lonza	CC-3156
ECM solution	Corning	354230
EGM-2 MV Singlequots Kit	Lonza	CC-4147
Fetal Bovine Serum (FBS)	Fisher Scientific	SH3007003IR
FiJi	NIH	NA
Fine Forceps	Fine science tools	11412-11
Fisherbrand Straight-Blade operating scissors	Fisher Scientific	13-808-4
Hyclone Phosphate Buffered Saline (1X)	Fisher Scientific	SH-302-5601LR
Laminar flow tissue culture hood	Fisher Scientific	various models available
Mounting Medium	Vector Laboratories	H-1200
Paraformaldehyde (PFA)	Electron Microscopy/Fish	50-980-494
Perforated spoon	Fine science tools	10370-18
Recombinant Murine VEGF-A 165	PeproTech	450-32
Standard forceps, Dumont #5	Fine science tools	11251-30
Sure-Seal Mouse/Rat chamber	Easysysteminc	EZ-1785

Comments/Description
Referred in the protocol as Petri dish
Secondary antibody
Secondary antibody
Angled 45 degree, used for detecting deep plugs
Primary antibody
Primary antibody, manufacturer BD BioSciences
For 37 ℃, 5% CO2 incubation
Leica S9i Stereomicroscope
Endothelial cell growth basal media
Commercially known as Matrigel
Microvascular endothelial cell supplement kit; This is mixed into the EBM-2 to make the EGM-2 complete media
Image processing software (https://imagej.net/Fiji/Downloads)
Used for embryo dissection
Vectashield with DAPI
This is available at 32%; needs to be diluted to 4%
Useful in removing embryo/tissues from a solution
Euthanasia chamber

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Date:	7/11/2019

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Response to Editorial comments:
Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We proofread the manuscript with great details. We have corrected spelling and grammar issues that were indicated by the reviewers and other errors that were identified.

2. Keywords: Please provide at least 6 keywords or phrases.

New keywords added. Now, total keywords provided is 6.

3. Summary: Please shorten it to no more than 50 words.

The summary is shortened to contain no more than 50 words.

4. Please define acronyms/abbreviations upon first use in the main text.

Acronyms/abbreviations are defined when mentioned first in the main text.

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.

The abbreviations for all units are corrected as suggested.

6. Please include a space between all numbers and the corresponding unit: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc.

This is corrected in the revised manuscript.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: LONZA, Matrigel, Charles, Vectashield, Vector laboratories, Photoshop, etc.

All the commercial languages have been removed and all the products are referenced in the Table of Materials.

8. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

An ethics statement is added.

9. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly, and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

This is corrected in the revised manuscript as much as possible. However, some of the steps required additional description to help follow the action points. But this is provided at NOTE as suggested.

10. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Sub-steps added for some steps in the revised manuscript.

11. Line 176: Step 3.10 does not exist. Please revise.

This is revised correctly.

12. Line 198: Step 4.7 does not exist. Please revise.

This is revised correctly.

13. 9.4: Please describe how analysis/measurement is done. For actions involving software usage, please provide all specific details (e.g., button clicks, software commands, any user inputs, etc.) needed to execute the actions.

Additional description is added for analysis/measurement. Specific details regarding the use of the software is added as suggested in the revised manuscript.

14. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Protocol text upto 2.75 pages to be featured in the video have been highlighted in yellow.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Highlighted the text as suggested above.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Relevant details are included in the highlights.

17. Please remove the titles and figure legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

Titles and figure legends are removed from the figures.

18. Please include a scale bar, ideally at the lower right corner, for all microscopic images to provide context to the magnification used. Define the scale in the appropriate figure Legend.

The scale bars are included for the appropriate microscopic images. However, we were unable to determine the correct scales for the images taken by the dissection microscope and a mobile phone due to the lack of exact micron/pixel information on the images taken.

19. Figure 6: Please use the micro symbol µ instead of u (µm instead of um). Please replace Matrigel with a generic term.

This is corrected in the revised manuscript.

20. Figure 9: Please define error bars in the figure legend.

Error bars are defined in the revised manuscript.

21. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

Information on additional supplies, reagents, equipment, and software is added and the materials are sorted alphabetically as suggested.

22. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate

statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

The in-text reference is corrected as suggested and the references are numbered in order of appearance.

23. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

We have revised the reference citation to appear as suggested above.

Response to Reviewers' comments:

We thank the reviewers for their insightful and helpful comments, which helped clarify several points and significantly improved the manuscript. Below are reviewers' comments followed by our response in bold.

Reviewer #1:
Manuscript Summary:
The protocol by Large and colleagues describes an in vitro model for the culture of explants and outgrowth of vessels from the major embryonic sources, namely sinus venosus and endocardium. This would provide an invaluable resource for the community, to assay the mechanisms of sprouting and to screen for novel pro-angiogenic molecules for regeneration.
Overall, the procedure is clearly explained, with the most critical steps highlighted. Anticipated results and examples of the utility of the method are described. Helpful figures, mostly images of key protocol steps, along with a schematic of the workflow and an image of the resulting vessel sprouts, are provided. Some specific points for clarification are identified below. In general, a thorough grammatical/language review is also required.

Major Concerns:
None

Minor Concerns:
1. Step 1.1: the authors advise using CD1 mice. Have other strains been tested? A comment on the use of these should be provided, as users may wish to test genetically altered mice which would typically be on a C57Bl6 background.

CD1 mice are generally easy to breed and yield high number of embryos. Therefore, we suggested CD1 for wild type use. However, we have also performed these experiments with genetically altered mice on C57Bl6. To avoid confusion, we removed CD1 and suggested use of any strain of mice.

2. Abbreviations defined in the abstract are not defined upon first mention in the main text but should be.

Abbreviations are defined in the main text in the revised manuscript.

3. Although keeping tissues cold is advised in the Discussion, it would be helpful in step 3, to advise keeping the Petri dish containing remaining embryos on ice, whilst the earlier ones are being dissected.

The embryos are suggested to keep on ice in a Petri dish in step 2.3. Embryos are transferred on at a time, while keeping the remaining embryos on ice, during dissection in step 3.

4. Step 3.2: Two instances of "First…."

This error is corrected in the revised manuscript.

5. Step 4.1: As written, "extra cardiac tissue" is ambiguous and would be variably interpreted as either non-cardiac tissue or additional cardiac tissue. Please clarify. For the former, extracardiac (one word) is usual.

We have used "adjacent tissue surrounding the SV" instead of extra cardiac tissue to clarify this in step 4.2.2.

6. Step 4.2: although the figure is helpful, it would also be useful to describe in words how a non-expert might identify the SV.

We have added a note describing SV as "SV is an inflow organ of an embryonic heart that lies in between the atria on the dorsal side of the heart."

7. Step 4.3: "Repeat 4.2 to 4.7". Should this be "4.2 to 4.3"? There are no steps 4.4-4.7 and, as written, it does not appear to relate to panels of Figure 4. Regarding panel figures, numbering them is confusing, as the number could refer to either the figure or protocol step. Is this a journal format or could the figure panels be labelled A-Z instead? Indeed, panels in Figure 1 are (inconsistently) labelled A and B.

We have fixed these errors. We have also changed the labelling of our panel figures from numbering to A-Z format and kept consistent throughout the revised manuscript. We have also carefully matched the in-text citation of these figure panels.

8. Steps 5-9: it would help to list the materials required before beginning the protocol (as for Step 4).

We have listed the materials before beginning protocol in steps 5-9 as suggested.

9. Step 5.2: the authors may wish to comment on the choice of PET membranes - could other membranes be used?

We have only used PET membranes. However, we do not anticipate having any problems with other membranes since the membranes are coated with matrigel for attachment in our protocol.

10. Step 5.3: it may be advisable to include a cautionary note to remind users to keep Matrigel on ice to avoid solidification.

We have added a cautionary note as "Keep the ECM on ice to avoid solidification" in step 5.1.

11. Step 6: are media changes required over the course of the 5 days? State either way.

Media is changed on day 2 and day 3 (media containing treatments). There is no need for media change from day 4 to 6.

12. Step 7.2 states to starve cultures for at least 24 hours. Presumably there is a maximum length of time and it would be advisable to suggest a defined period.

We have removed "at least" and corrected the statement as, "starve the cultures for 24 h).

13. In step 7.2, to avoid ambiguity, please clarify if the additional 100 ul directly into the inserts is of the control/VEGFA medium, for the respective condition.

We thank the reviewer for noting this. We have clarified that 100 ul is added directly into the insert for control/VEGFA medium for the respective condition in step 7.4.

14. Step 8.1: Are the cultures washed in PBS prior to fixation? Confirm if all this step is completed at room temperature.

Yes. The cultures are washed prior to fixation and we have added this in step 8.1.

15. Step 8.2: It is rather surprising that no blocking step/agent is included. Is this correct? Perhaps the authors may wish to comment on this. It may likely be required for other antibodies, even if not for those in the given example.

The antibodies that is mentioned in the protocol works well without the blocking solution. However, we have experienced better results with blocking solution for other antibodies. Therefore, we have suggested diluting antibodies in blocking solution (5% donkey serum, 0.5% Triton X-100 in PBS) in steps 8.2 and 8.4.

16. No details of primary/secondary antibodies are provided. Although the choice of antibodies will be varied by users of the protocol, many will wish to replicate the representative results in their assays and will require details of tried and tested antibodies.

We have provided the details of both primary and secondary antibodies in step 8.2 and 8.4 in the revised manuscript.

17. Figure 7: vein trx factor: define trx in legend or preferably write in full as transcription factor.

Trx is defined as "transcription factor" in the figure legend.

18. Figure 8: As recombinant protein was used, VEGF-A, not Vegf-A, is correct. In fact, it should be specified, in the method and figure, that isoform 165 was used.
Vegf-A is changed to VEGF-A throughout the text in the revised manuscript. VEGF-A 165 is specified in Table of Materials.

19. As mentioned above, the grammar requires attention. Some specific points to consider:
- "In vivo studies are wonderful" - useful or valuable would be more scientific.
- Frequent references to "grabbing" e.g. the mouse, embryo, tissue.
- "media" (plural) referred to, when singular form "medium" would be more appropriate.
- "scrapping" should be "scraping".

We corrected all the grammatical errors pointed by the reviewer. Furthermore, we proofread the manuscript thoroughly and corrected errors. In addition, we are thankful to Drs. Philip J. Smaldino and Carolyn Vann (both are acknowledged) for thoroughly proofreading our manuscript and providing helpful comments, which significantly improved the revised manuscript in terms of grammatical errors.

Reviewer #2:
The protocol by Sharma et al. describes a model for studying coronary angiogenesis in vitro. It is well described and along with a video it will allow scientist to reproduce this technique successfully.

Minor Concerns:
Details about the used antibodies should be included into the material list.

We have included the details of antibodies used both in-text and in the "Table of Materials."

Reviewer #3:
Manuscript Summary:
The manuscript describes methods for an angiogenesis model involving different embryonic heart tissues; specificaly the sinus venosus (SV) and endocardium (Endo), the two progenitor tissues from which much of the coronary vessels arise. The motivation is to create an in vitro culture system to successfully grow coronary vessels as a model of coronary vascular development. In general, the methods are well described. However, additional details are required to be able to fully replicate the model.

Major Concerns:
1. Presumably, the instruments are sterilized (e.g. autoclaved) before use. If so, state the method used. If not sterilized, provide a statement as such.

2. Line 135: provide CO2 flow rate.

CO_2 flow rate is set as 10-30% displacement per minute. This is included in step 2.1.

3. Line 142: should mention that the embryos are still within the uterine horn and that the horn + embryos are removed.

We thank the reviewer for providing this clarification. Indeed, the embryos are still within the uterine horn and that the horn+embryos are removed. We have included this in step 2.2.

4. Line 157: What is "Step 2.8" referring to? There is no step 2.8 in the document. Perhaps this is referring to step 6 of Figure 3 in 2.3?

This error is corrected.

5. Line 175: Similarly, there is reference to steps that are not listed in section 3.

This error is corrected.

6. Line 205: Provide a description of the PET inserts. Can any PET insert be used? Does membrane thickness matter? Must it be PET?

Description of the PET insert is added in step 5.2. We have only used the kind described and we do not have any knowledge on whether the other insert type would work or not. However, we do not anticipate any problems with other kinds of PET or a different porous membrane system since the membrane are coated with ECM for cellular attachment.

7. Line 214: It would be helpful to expand the calculation to state "100 µl/insert x number of inserts".

We have expanded the calculations as suggested in steps 5.3, 7.1.1, and 7.1.2.

8. Section 7.1: Would it not be easier to simply transfer the insert to a new plate for the washing step?

Yes. However, to minimize the number of plates used, we have washed in the same plate.

9. Line 264: Is the media to be removed prior to adding the PFA? If it is removed or it remains should be stated.

We thank the reviewer for noting this. Yes, the media is removed as washed with PBS before adding PFA. This is included in step 8.1.

10. Line 326: This is the first time an air-liquid interface is mentioned. As an apparent key element of the methods, this interface and how to assess that the explant is positioned properly at the interface should be described in the procedure sections.

We thank the reviewer for this helpful comment. We have provided brief description in step 6.4 and described it in the discussion as well.

Minor Concerns:
1. Define SV and Endo in the Introduction.

We have included definition of SV and Endo in the introduction.

2. Line 220: Technically, 70% EtOH is not a sterilant. Perhaps simply indicating the base is cleaned with 70% EtOH should be stated.

We agree with the reviewer and we corrected the statement as suggested by the reviewer.

3. There are numerous grammatical errors.

We have diligently proofread the manuscript and corrected grammatical errors. In addition, we also had our manuscript proofread by two of our colleagues (who are native English speakers) and incorporated their helpful comments. We have corrected numerous grammatical errors in our revised manuscript.