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TITLE:

Isolation of Human Primary Valve Cells for In Vitro Disease Modeling

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SUMMARY:

This protocol describes the collection of human aortic valves extracted during surgical aortic valve replacement procedures or from cadaveric tissue, and the subsequent isolation, expansion, and characterization of patient specific primary valve endothelial and interstitial cells. Included are important details regarding the processes needed to ensure cell viability and phenotype specificity.

ABSTRACT:

Calcific aortic valve disease (CAVD) is present in nearly a third of the elderly population. Thickening, stiffening, and calcification of the aortic valve causes aortic stenosis and contributes to heart failure and stroke. Disease pathogenesis is multifactorial, and stresses such as inflammation, extracellular matrix remodeling, turbulent flow, and mechanical stress and strain contribute to the osteogenic differentiation of valve endothelial and valve interstitial cells. However, the precise initiating factors that drive the osteogenic transition of a healthy cell into a calcifying cell are not fully defined. Further, the only current therapy for CAVD-induced aortic stenosis is aortic valve replacement, whereby the native valve is removed (surgical aortic valve replacement, SAVR) or a fully collapsible replacement valve is inserted via a catheter (transcatheter aortic valve replacement, TAVR). These surgical procedures come at a high cost and with serious risks; thus, identifying novel therapeutic targets for drug discovery is imperative. To that end, the present study develops a workflow where surgically removed tissues from patients and donor cadaver tissues are used to create patient-specific primary lines of valvular

cells for in vitro disease modeling. This protocol introduces the utilization of a cold storage solution, commonly utilized in organ transplant, to reduce the damage caused by the often-lengthy procurement time between tissue excision and laboratory processing with the benefit of greatly stabilizing cells of the excised tissue. The results of the present study demonstrate that isolated valve cells retain their proliferative capacity and endothelial and interstitial phenotypes in culture upwards of several days after valve removal from the donor. Using these materials allows for the collection of control and CAVD cells, from which both control and disease cell lines are established.

INTRODUCTION:

Calcific aortic valve disease (CAVD) is a chronic pathology characterized by inflammation, fibrosis, and macrocalcification of aortic valve leaflets. Progressive remodeling and calcification of the leaflets (termed aortic sclerosis) can lead to the obstruction of blood flow (aortic stenosis) which contributes to stroke and leads to heart failure. Currently the only treatment for CAVD is surgical or transcatheter aortic valve replacement (SAVR and TAVR, respectively). There is no non-surgical option to halt or reverse CAVD progression, and without valve replacement, mortality rates approach 50% within 2-3 years[1-3]. Defining the underlying mechanisms driving this pathology will identify potential novel therapeutic approaches.

In a healthy adult, aortic valve leaflets are approximately one millimeter thick, and their main function is to maintain the unidirectional flow of blood out of the left ventricle[4]. Each of the three leaflets is comprised of a layer of valve endothelial cells (VECs) that lines the outer surface of the leaflet and functions as a barrier. VECs maintain valve homeostasis by regulating permeability, inflammatory cell adhesion, and paracrine signaling[5-7]. Valve interstitial cells (VICs) comprise the majority of cells within the valve leaflet[8]. VICs are arranged in three distinctive layers in the leaflet. These layers are known as the ventricularis, the spongiosa, and the fibrosa[9]. The ventricularis faces the left ventricle and contains collagen and elastin fibers. The middle layer, the spongiosa, contains high proteoglycan content that provides shear flexibility during the cardiac cycle. The outer fibrosa layer is located close to the outflow surface on the aortic side and is rich in Type I and Type III fibrillar collagen which provide strength to maintain coaptation during diastole[10-12]. VICs reside in a quiescent state, however, factors such as inflammation, remodeling of the extracellular matrix (ECM), and mechanical stress may disrupt VIC homeostasis[8,9,13-16]. With loss of homeostasis, VICs activate and acquire a myofibroblast-like phenotype capable of proliferation, contraction, and secretion of proteins that remodel the extracellular millieu[17]. Activated VICs can transition into calcifying cells which is reminiscent of the differentiation of a mesenchymal stem cell (MSC) into an osteoblast[15,17-25].

Calcification appears to initiate in the collagen-rich fibrosa layer from contributions of both VECs and VICs but expands and invades the other layers of the leaflet[8]. Thus, it is clear that both VECs and VICs respond to stimuli to upregulate the expression of osteogenic genes, however, the precise events driving the activation of osteogenic genes, as well as the complex interplay between the cells and the extracellular matrix of the leaflet, remain ill-defined. Murine models are not an ideal source to study non-genetic drivers of CAVD pathogenesis, as mice do not develop CAVD de novo[26,27], hence the use of primary human tissues and the primary cell lines

isolated from these tissues is necessary. In particular, obtaining these cells in high numbers and good quality is imperative, as the field of 3D cell cultures and organoid modeling is expanding and is likely to become an ex vivo human-based alternative to murine models.

The purpose of the present method is to share a workflow that has established the conditions to efficiently isolate and grow VECs and VICs obtained from surgically removed valves from human donors. Previous studies have showed successful isolation of VECs and VICs from porcine[28] and murine valves[29], to our knowledge this is the first to describe the isolation of these cells in human tissues. The protocol described here is applicable to human excised valves and greatly circumvents and improves the damage caused by the often-lengthy procurement time between tissue excision and laboratory processing by introducing the utilization of a cold storage solution, a buffered solution clinically utilized in organ transplants that greatly stabilizes cells of the excised tissue. The protocol described here also shows how to determine cell phenotype and guarantee high efficiency of cell survival with minimal cell cross-contamination.

PROTOCOL:

All patient samples are collected from individuals enrolled in studies approved by the institutional review board of the University of Pittsburgh in accordance with the Declaration of Helsinki. Cadaveric tissues obtained via the Center for Organ Recovery and Education (CORE) were approved by the University of Pittsburgh Committee for Oversight of Research and Clinical Training Involving Decedents (CORID).

1. Approval and safety

1.1. Obtain Institutional Review Board (IRB) approval or an exempt memo for any collection of patient samples or cadaveric tissues in accordance with the Declaration of Helsinki.

1.2. Take required institutional training to work with human tissues such as Bloodborne Pathogens Training, Biomedical Human Subjects Research, Privacy and Information Security, and Transportation and Shipping of Biological Materials.

2. Logistics and preparation

2.1. Surgical Samples

2.1.1. Ensure that a refrigerator is available and located near the operating room. Keep 50 mL conical tubes pre-labeled with de-identified nomenclature containing 40 mL of sterile aliquots of cold storage solution in this fridge for use by surgical staff. These tubes are stable at 4 °C until expiration date on the original package.

2.1.2. Upon extraction, place valve tissue in these tubes. Place the tubes in a sealed secondary container such as a leak-proof plastic bag or a plastic container that is labeled with a biohazard label. Tissues are picked up and transported on ice according to institutional protocols for transporting biohazards.

2.2. Cadaveric samples

2.2.1. Submerge recovered organs in cold storage solution, place in a sealed secondary containment vessel, and transport on ice according to institutional protocols for transporting biohazards.

3. Reagents preparation

3.1. Make collagen-coated plates at least the day before.

3.1.1. In a 50 mL conical tube, mix 5 mL of isopropyl alcohol, 8.7 mL of acetic acid, and 0.5 µg of collagen I powder. Bring up to 50 mL with sterile water. Mix and filter through a 0.45 µm filter.

3.1.2. Under a sterile cell culture hood, add enough collagen solution to 6 well plates and 10 cm dishes to just cover the entire bottom. Cover the plate and let sit for 4-6 h at room temperature. Remove the excess solution with a sterile pipette, place in a new sterile 50 mL conical tube, and save at 4 °C to make additional plates. Solution can be stored at 4 °C for several months.

3.1.3. Dry the plates in a 37 °C incubator overnight, and subsequently store them in a resealable bag at 4 °C. Collagen-coated plates and dishes can be stored for several months.

3.2. Autoclave the following items: tissue forceps, tissue scissors, cotton swabs and gauze pads.

3.3. VEC growth media: Prepare and use endothelial cell growth medium according to manufacturer's protocols. Store in the dark at 4 °C. Warm to 37 °C just before use on cells, do not leave media in warming bath longer than necessary (10-15 min is sufficient). Use the media within one month of preparation.

3.4. VIC growth media: Supplement DMEM base medium (4.5 g/L glucose, L-glutamine supplement, 110 mg/L sodium pyruvate)[30] with 10% heat-inactivated FBS and 100 I.U./mL penicillin and 100 µg/mL streptomycin. Store in the dark at 4 °C for up to 3 months. Warm to 37 °C just before use on cells, do not leave media in warming bath longer than necessary (10-15 min is sufficient).

3.5. Make sterile rinsing solution just before use. Supplement sterile PBS with 2.5 µg/mL fungicide, 0.05 mg/mL gentamicin and 5 µg/mL bactericide.

3.6. Make sterile collagenase solution just before use. Add 5 mg of collagenase II to 5 mL of sterile fresh DMEM base medium. Mix well and sterilize the solution by passing through a 0.45 µm filter. Keep on ice until use.

4. Tissue preparation and processing

NOTE: Institutional approval for use of human tissues must be obtained prior to beginning work. While handling tissues, the following personal protective equipment (PPE) must be worn: a disposable liquid barrier wrap-around gown, or a dedicated button front lab coat with a liquid-barrier wrap around apron and disposable sleeve clovers; a full face shield, or safety glasses with a surgical mask; double gloves; close-toed shoes; and clothing to cover the legs. Comprehensive workflow diagrams of the tissue preparation for calcification assessment (Section 5) and cell isolation (Section 6 and 7) are illustrated in **Figure 1A,B** respectively.

4.1. Upon receipt of cadaveric organ specimen, excise the aortic root and submerge in a 50 mL conical tube of sterile rinsing solution. Upon receipt of surgical specimen, remove from transport vessels and submerge in a 50 mL conical tube of sterile rinsing solution. Place tube containing the tissue in ice bucket on rocker and mix for 10 min at room temperature (RT).

NOTE: Processing tissues as close as possible to the time of extraction will yield the best cell recovery, however viable cells can be collected upwards of 61 h post excision, and data show that VICs are more robust than VECs as time increases. If tissue cannot be processed immediately, when possible, remove tissue, perform step 4.1, and then put the tissue back in fresh sterile cold storage solution and keep at 4 °C until ready to proceed. Valves collected during night or weekend surgeries can be stored in 40 mL cold storage solution at 4 °C and still are able to yield viable cells more than 2 days post extraction.

4.2. Spray down tubes with 70% ethanol and move to a sterile hood. Remove tissue and excise two valve leaflets (**Figure 2A**). Place one leaflet in a cryogenic vial (or 2-3 vials if several smaller pieces are needed for future analysis) and snap freeze by dropping in liquid nitrogen then store at -80 °C.

4.2.1. If the valve is bicuspid, excise only one leaflet. Using scissors, cut the leaflet in half from the nodule to the hinge. Use one half of the leaflet for snap freezing and the other half for step.

4.3. Process the second leaflet for paraffin embedding to assess calcification content by cutting it in half from nodule to hinge. Place both pieces in a cassette that is submerged in 4% paraformaldehyde (PFA), then place on a rocker at RT for a minimum of 2 h but no more than 4 h.

NOTE: Longer fixation times create more background with immunofluorescent staining. Once steps 4.2 and 4.3 are performed, move immediately to Section 6 and come back to step 4.4 after step 6.12.

4.4. After fixation, wash the tissues by submerging in fresh PBS for 1 h 3-4 times. After these washes, samples can stay in PBS at 4 °C for several months if needed. Proceed to next step just before embedding.

NOTE: The protocol workflow presented here unbiasedly selects one leaflet for VECs and VICs

isolation, while the remaining leaflets are utilized for Von Kossa staining (Section 5) and snap freezing.

4.5. Gradually change from PBS to 70% ethanol. Wash 30-60 min each step with 1:4 70% ethanol:PBS; 1:1 70% ethanol:PBS, 4:1 70% ethanol:PBS; 70% ethanol.

4.6. Embed the tissue such that sections will reveal the three layers of the leaflet (**Figure 1A**) according to established protocols[31]. Alternatively, and if available, bring the tissue to a pathology core for embedding and cutting.

4.7. After handling tissues, dispose or store PPE as appropriate and wash hands immediately. Decontaminate all equipment, surfaces, and solid and liquid wastes with a 1:10 dilution of bleach or detergent disinfectant. Allow 20 min for decontamination, then follow with a 70% ethanol rinse. Treat the cell culture hood with mycoplasma spray according to manufacturer's instructions.

5. Von Kossa staining for calcium content

NOTE: This can be done well after cell isolation and line establishment but be sure to link the calcification level of the tissue to documents pertaining to the primary cell line established.

5.1. Cut 5 or 10 µm thick paraffin slices onto glass slides and bake slides at 65 °C for 1 h, then cool to RT.

5.2. Using fresh solutions, deparaffinize slides by submerging as following: 100% xylene for 30 min, 2x; 100% ethanol for 3 min, 2x; 100% ethanol for 3 min; 90% ethanol for 3 min; 80% ethanol for 3 min; 70% ethanol for 3 min; 50% ethanol for 3 min; ultrapure water for 3 min; keep in ultrapure water until next steps.

NOTE: Times above are minimum, each step may go longer.

5.3. Proceed with Von Kossa staining according to manufacturer's protocols.

5.4. Assess full valve area microscopically and assign a control tissue (no sign of calcification) or CAVD (any evidence of calcification, **Figure 2B**).

6. Valve Endothelial Cell (VEC) isolation, expansion, and confirmation

6.1. In a sterile cell culture hood, open the conical tube containing the remaining valve leaflet and place the leaflet in a new 50 mL conical tube filled with ice cold PBS. Cap tube and gently invert or place on a rocker for 2 min at RT.

6.2. Remove tissue to a 60 mm dish filled with 5-7 mL of cold collagenase solution. Using forceps, dip both sides of the leaflet in the solution 3-4 times. Incubate the tissue for 5-10 min in

the cell culture incubator at 37 °C, rocking gently 3-4 times tissue every 2 min.

6.3. Remove 2 mL of the solution from the dish and place in a sterile 15 mL conical tube. Place forceps at the nodule and use a dry sterile cotton swab to swipe from the forceps to the hinge, twirling the swab while moving it along the leaflet. Between each swipe, swish the swab in the solution in the 15 mL conical tube to remove the cells. Repeat to fully swab the surface of the tissue, then flip over and repeat on the other side.

6.4. Holding the valve leaflet with forceps in one hand, and a 1 mL pipette in the other, rinse the surfaces of the leaflet with the solution in the dish. Once rinsed, transfer all the solution containing the VECs in the dish into the same 15 mL conical tube with the cells from the swab and proceed to Step 6.5. Place the remaining valve tissue in a new 15 mL conical tube with 7 mL of sterile collagenase solution and proceed to Step 7.1.

6.5. Centrifuge the tube containing the VECs at 180 x *g* for 5 min to pellet the isolated VECs. Aspirate the supernatant and resuspend in 3 mL of VEC growth media. Centrifuge one more time and remove the supernatant. Resuspend the cells in 1 mL of growth media and determine the number of cells using a hemocytometer and trypan blue.

6.6. Resuspend VEC pellet in 2 mL of VEC growth media and plate the cells in a collagen pre-coated well of a 6 well plate at approximately 5 x 10^5 cells/cm^2. Let the cells grow at least 3-4 days, then remove media and replenish with fresh media. Repeat media changing every 4 days. VECs will grow in cobblestone shaped patches (**Figure 3B**, left panels).

6.7. When VEC patches cover >80% of the plate, split cells at about 1.3 x 10^4 cells/cm^2 depending on how fast they grow (if they reach 80% in less than 1 week split a slightly lower number of cells/cm^2).

6.8. To split, wash cells two times with 2 mL DPBS then add enough dissociation reagent to just cover the surface of the cells. Incubate for 2-3 min at 37 °C, checking to make sure cells are not over incubated. Stop digestion by adding equal volume of VEC growth media and transfer liquid to a 15 mL tube. Centrifuge at 180 x *g* for 5 min, remove supernatant, and resuspend in appropriate volume of media for number of wells/plates needed.

NOTE: Once expanded into a 10 cm plate VECs may sometimes lose their morphology and change phenotype as they proliferate. This tends occurs when VECs are seeded at a low confluency during the establishment and expansion of the cell line.

6.9. To guarantee a pure culture consider utilizing CD31+ superparamagnetic beads with every splitting.

6.10. For 1 x 10^8 or fewer cells (one 10 cm dish or less), prepare 25 µL of superparamagnetic beads by washing according to manufacturer's protocols.

NOTE: Step 6.10 is recommended to be performed before trypsinization of VECs.

6.11. Detach cells as in step 6.8 but resuspend in 500 µL of PBS with 0.1% BSA, pH 7.4, and place in a 2 mL centrifuge tube. Add 500 µL of washed and resuspended beads to the 2 mL tube of cells and incubate on a rotator for 20 min at 4 °C.

6.12. Place tubes on the magnet for 2 min. While tube is still in the magnet, carefully remove supernatant. Remove tube from magnet, add 1 mL fresh PBS with 0.1% BSA, pipette gently 2-3 times, then place back on magnet for 2 min. Repeat 2 more times. After final removal of buffer, resuspend cells in growth media in volume needed for replating.

NOTE: Cells will still have beads, but these will not affect growth and will be removed in subsequent passages.

6.13. Once cells have been expanded, in addition to morphology, confirm VEC phenotype by positive staining for immunofluorescent markers such as von Willebrand factor (vWF), cadherin 5 (CDH5), or PECAM-1/CD31, and negative to VIC markers such as calponin 1 (CNN) or alpha-2 smooth muscle actin (αSMA, **Figure 4,** left panels).

7. Valve Interstitial Cell (VIC) isolation, expansion, and storage

7.1. As stated in step 6.4, after removing VECs from the valve tissue, the leaflet in placed in a 15 mL conical tube with 7 mL collagenase solution. Incubate for 12 h in the cell culture incubator with the cap slightly open to allow gas exchange. Successful isolation of VICs can still occur with up to 18 h in collagenase solution.

7.2. After incubation, in a sterile cell culture hood, mix the tissue gently by pipetting with a serological pipette to ensure the release of VICs from the leaflet tissue.

7.3. Remove cell suspension and pass through a 0.70 µm filter into a 50 mL conical tube.

7.4. Add 7 mL of VIC growth medium to the 50 mL tube and centrifuge at 180 x *g* for 5 min. Aspirate supernatant and resuspend cell pellet in 1 mL of VIC growth media and determine the cell number. Plate the VICs in a 60 mm tissue-culture treated dish at 1.3 x 10^4 cells/cm^2.

7.5. Let the cells grow at least 1-2 days before replacing media. Remove media and wash twice with PBS to remove residual debris and replace with fresh growth medium. Replenish medium every 2-3 days. VICs will grow in fibroblast shape (**Figure 3B**, right panels).

7.6. When VICs reach a confluency of >90%, wash twice with DPBS to remove the excess media and detach the cells by adding appropriate volume of pre-warmed dissociation reagent to cover the plate (i.e., 2-3 mL per 10 cm dish). Incubate the dish in a 37 °C incubator. VICs will detach from the dish after 2-3 min of incubation. Add 4-6 mL of pre-warmed media.

NOTE: If cells take longer than 3 min to lift off the plate, the dissociation reagent may have lost potency. If needed, a cell scraper may be used to gently lift the cells.

7.7. Transfer the cell suspension to a tube and gently centrifuge at 180 x *g* for 5 min. After removing the supernatant, gently resuspend the cell pellet in pre-warmed VIC growth media and determine the number of viable cells by using a hemocytometer and trypan blue. Seed the viable cells at a 1:2 density of the original dish (i.e., ~ 1×10^6 cells per 10 cm dish or 1.3×10^4 cells/cm^2).

7.8. Assess VIC phenotype by positive staining for immunofluorescent markers such as αSMA, CNN, or SM22α and negative staining for VEC markers such as CD31, CDH5, or vWF (**Figure 4**, right panels).

8. Long-term cell storage

8.1. Once expanded, freeze cells down for long-term storage. Wash cells twice with 2-5 mL DPBS then add enough dissociation reagent to detach cells as in steps 6.8 and 7.6. Stop digestion by adding equal volume of VEC or VIC growth media and transfer cell suspension to a 15 mL tube.

8.2. Determine the number of viable cells by using a hemocytometer and trypan blue.

8.3. Centrifuge at 180 x *g* for 5 min.

8.4. Resuspend cells in chilled conditioned growth media to a cell density of ~ 3×10^6 cells/mL.

8.5. Gently with swirling, add an equal volume of chilled 2x cryopreservation medium. This will bring cell concentration to ~ 1.5×10^6 cells/mL.

8.6. Aliquot 1 mL into cryopreservation vials. Place vials in a cell freezing container, close, and invert 5-6 times to ensure cells remain suspended. Place container at -80 °C for 6-72 h or according to freezing container protocol. Remove vials from -80 °C and transfer to liquid nitrogen for long term storage.

REPRESENTATIVE RESULTS:

The above protocol outlines the steps necessary for the handling of human valve tissues and the isolation and establishment of viable cell lines from these tissues. Leaflets of the aortic valve are processed for paraffin embedding, snap frozen for long term storage for biochemical or genetic analysis and digested for the isolation of VECs and VICs (**Figure 1**). While surgical specimens will likely have a clinical diagnosis of aortic stenosis and may exhibit heavy nodules of calcification that can be visible with the naked eye, aortic valve calcification is present in a significant number of elderly (>65 years old) individuals[32], and because of this prevalence all tissues – surgical and cadaveric – are subjected to Von Kossa staining or similar procedure to assess whether calcification is present (**Figure 2**).

It was found that use of cold storage solution greatly stabilized the cells of the excised valve

tissue. Cold storage solution is used in live organ transplants. It is flushed through organs either before or after removal from the donor and left in the vasculature of that organ during transportation on ice. It was observed that VEC lines were more readily established from donor specimens than surgical tissues, and the donor lines were more likely to retain their endothelial cell morphology for more passages. This was perplexing, as donor tissues would often not reach the lab for 12-24 hours postmortem while surgical tissue was obtained between 2 h and 4 h. Upon packing the surgical specimen tubes with cold storage solution instead of PBS, cell recovery greatly increased. As seen in **Figure 3**, both viable VECs and VICs can be obtained upwards of 61 hours post valve extraction. While a slightly higher percentage of live VICs are obtained than VECs when cells are isolated up to a day after valve excision, cells remain viable after 48 hours post excision. Up to 40% of the total recovered cells correspond to alive cells and we have observed consistent figures between biological replicates. Further, morphology inspection also confirms cell identity; while VECs appear as packed, cobblestone-like and growth contact-inhibited cells, VIC morphology is similar to myofibroblasts with a spindle shape. Immunostaining of the specimens confirmed that 91.8% ± 1.8 (n = 3) of expanded VECs were positive for the endothelial marker vWF whereas 92.0% ± 5.0 (n = 3) of expanded VICs were positive for the interstitial cell marker αSMA (**Figure 4**). These figures are in line with results reported previously[33].

FIGURE AND TABLE LEGENDS:

Figure 1: Valve tissue processing and cell isolation from human control and CAVD valves. (**A**) Schematic representation of tissue processing for assessment of calcification levels of the valves. (**B**) Schematic representation of the time course and steps for the isolation and characterization of human valve endothelial cells (VECs) and valve interstitial cells (VICs) from control and CAVD tissues.

Figure 2: Assessment of Calcification. (**A**) Representative image of control (top) and calcified (bottom) valve tissues from human donors. Note the calcification nodules of the CAVD tissue can severely alter morphology and the ability to cleanly excise the leaflets. (**B**) Representative Von Kossa staining of control (left) and CAVD (right) valve tissue after fixation and further processing of the human valve tissue. Note calcification is revealed by the presence of dark precipitation in the leaflet tissue. Valve images were capture with a lab camera. Von Kossa stained valves were captured with a 10x objective, scale bar 200 μm.

Figure 3: Assessment of Cell Survival (**A**) VECs and VICs survival curves. Three leaflets were obtained from five valve specimens. The first leaflet from each valve was processed immediately (3-12 h post extraction), the second leaflet was processed approximately 24 h later (22-35 h), and the third leaflet was processed approximately 48 hours after obtaining the tissue (45-61 h). Valves leaflets were kept in cold storage solution at 4 °C until they were processed. The y-axis represents the percentage of live cells. (**B**) Morphology of healthy cultures of VECs (left panels) and VICs (right panels). Graphs show mean ± SD live cell proportion of cells isolated from n = 5 valve tissues. Images were captured with a 4x and 10x objective, scale bars 200 μm and 100 μm, respectively.

Figure 4: Representative immunofluorescent staining on VECs and VICs. VECs are positive for the endothelial marker von Willebrand Factor (vWF, left panels) whereas VICs are positive for the interstitial marker αSMA. Note our isolation protocol guarantees a high isolation efficiency; no cross-contamination between VECs and VICs is detected. Images were captured with a 10x objective, scale bar 100 µm.

DISCUSSION:

Obtaining control and disease tissues from humans is critical for in vitro and ex vivo disease modeling; however, while one often speaks about the challenges of bridging the gap between bench to bedside, the reverse order – going from the surgical suite to the bench – is often just as daunting a gap. Essential for a basic scientist to obtain primary human tissue specimens is a collaboration with an invested surgeon scientist who has a team of nurses, surgical technicians, physician assistants, medical students and residents, and clinical protocol managers who can enroll and consent patients, participate and assist in the proper handling of excised tissues, and coordinate the logistics required for tissue pickup. Without the utmost effort from everyone involved to reduce the time from excision to cell isolation, vital cellular material and the information it contains will be irreparably altered or lost.

Critical to maintaining viability of the tissue specimens is the use of cold storage solution. This is the same solution used by the organ transplant teams at UPMC and other transplant medical centers. Better cell yield was obtained from cadaveric tissues that had been excised from the donor many hours beforehand than from tissues obtained more quickly from the operating room but kept in cold PBS. This accidental discovery has been essential for cell procurement from human tissues. Procurement time from tissue excision to delivery in the laboratory ranges from 1-5 hours for surgical tissue and upwards of 24 hours for cadaveric tissue. In comparison, procurement and processing of animal tissues can often be done within minutes of euthanasia, which is ideal for cell viability. In the absence of cold storage solution, it is likely that a medium suitable for the culturing of cells could also perform better than PBS, however this medium was not tested herein due to the success of the cold storage solution in live organ transplantation and to the receipt of cadaveric tissues in this solution. The solution is shelf-stable which is ideal for storage in operating rooms, and specific ingredients such as adenosine are known to promote beneficial responses to cellular stresses such as ischemia/hypoxia[21,34].

Another essential step to obtaining viable cells is washing the tissues with fungicide, gentamicin, and bactericide. This short rinse helps to ensure that cells remain uncontaminated by bacteria and fungus. Equally critical are the steps to digest VECs out of the valve tissue, where in the span of just a few minutes, the VECs are detached and then swabbed off the surface of the valve leaflets. The subsequent digestion of the VICs that reside on the dense extracellular matrix of the leaflet has much more wiggle room for duration and strength. The unbiased tissue treatment described in this protocol allows the isolation of the main two cell populations present in the valve leaflet, VECs and VICs. Although a recent single cell transcriptome analysis has shown the co-existence of at least fourteen different cell subtypes residing in the human valve, including six non-valve derived stromal cells in CAVD tissue[35], this diversity may represent variations due

effects from processing and digesting these hardened tissues, or it may be due to different microenvironments to which the leaflet cells are exposed: VECs are exposed to two different blood flows while VICs are embedded in three different extracellular matrix stratums[8,9]. The large-scale isolation protocol and analysis described herein ensure that over 90% of VECs and VICs correspond to their main phenotype. Although a degree of heterogenicity may be found, it does not affect the general outcomes of the study of VEC and VIC homeostasis[8,9,13-16].

It is also important to note that patients and their valve tissues, and thus the cell lines procured from them, are not identical. Genetics, co-morbidities, handling during surgery and processing, and freshness of digestion solution ingredients may affect the growth rate and even perhaps the behavior or phenotype of the cells isolated. While the present study demonstrates the ability to yield a sufficient number of viable cells with this procedure, there may be innate or induced differences in these cell lines that may impact downstream experimentation. It is often difficult to know precisely the time since the tissue has been removed from the patient or donor, and particularly in the case of the latter, the time since circulation has stopped. Further, there is inherent variability among tissues regarding the number of viable valve cells obtained, the proliferative capacity of the cells, and the retention of cell phenotype. Cell lines may harbor genetic mutations – either congenital or somatic – of which the physician and research team are unaware, and the remodeling and subsequent handling of the tissues may also modify the cell phenotype or even epigenetics. As such, for all experiments in which these primary human cells are used, it is absolutely essential that biological replicates – i.e., cell lines obtained from different patients – be used, despite the substantial time and cost they incur. This helps ensure that any results are not due to confounding effects from the procurement and processing of the tissue. The variable proliferation rate of different cell lines can be adjusted for by either collecting cells in experiments at different times or seeding cells for experiments at different densities; no one answer is best for all experimental designs. While the complications are not insignificant, the use of primary human control and CAVD tissue-derived cell lines for in vitro and ex vivo experimental models is essential for defining the initiating factors and propagating processes that drive CAVD pathogenesis.

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We would like to thank Jason Dobbins for insightful discussion and critical reading of this manuscript. We would like to acknowledge the Center for Organ Recovery and Education for their help and support and thank tissue donors and their families for making this study possible. All patient samples are collected from individuals enrolled in studies approved by the institutional review board of the University of Pittsburgh in accordance with the Declaration of Helsinki. Cadaveric tissues obtained via the Center for Organ Recovery and Education (CORE) were approved by the University of Pittsburgh Committee for Oversight of Research and Clinical Training Involving Decedents (CORID).

Some figures created with Biorender.com.

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DISCLOSURES:

IS receives institutional research support from Atricure and Medtronic and serves as a consultant for Medtronic Vascular. None of these conflicts are related to this work. All other Authors have nothing to disclose.

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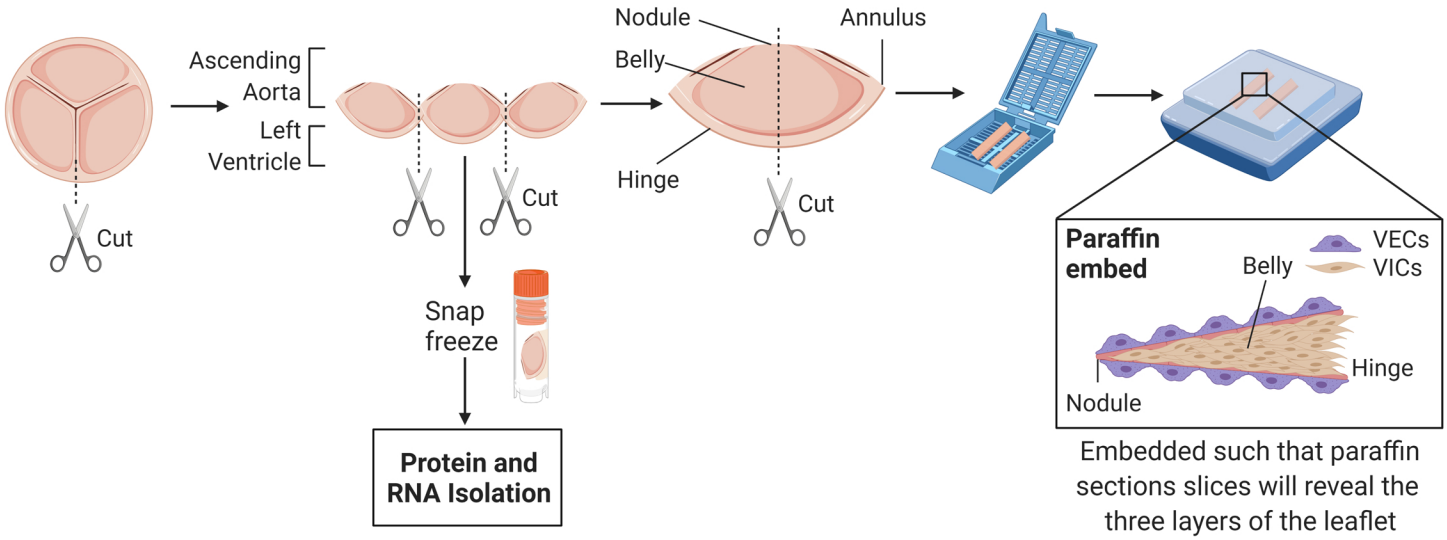
A

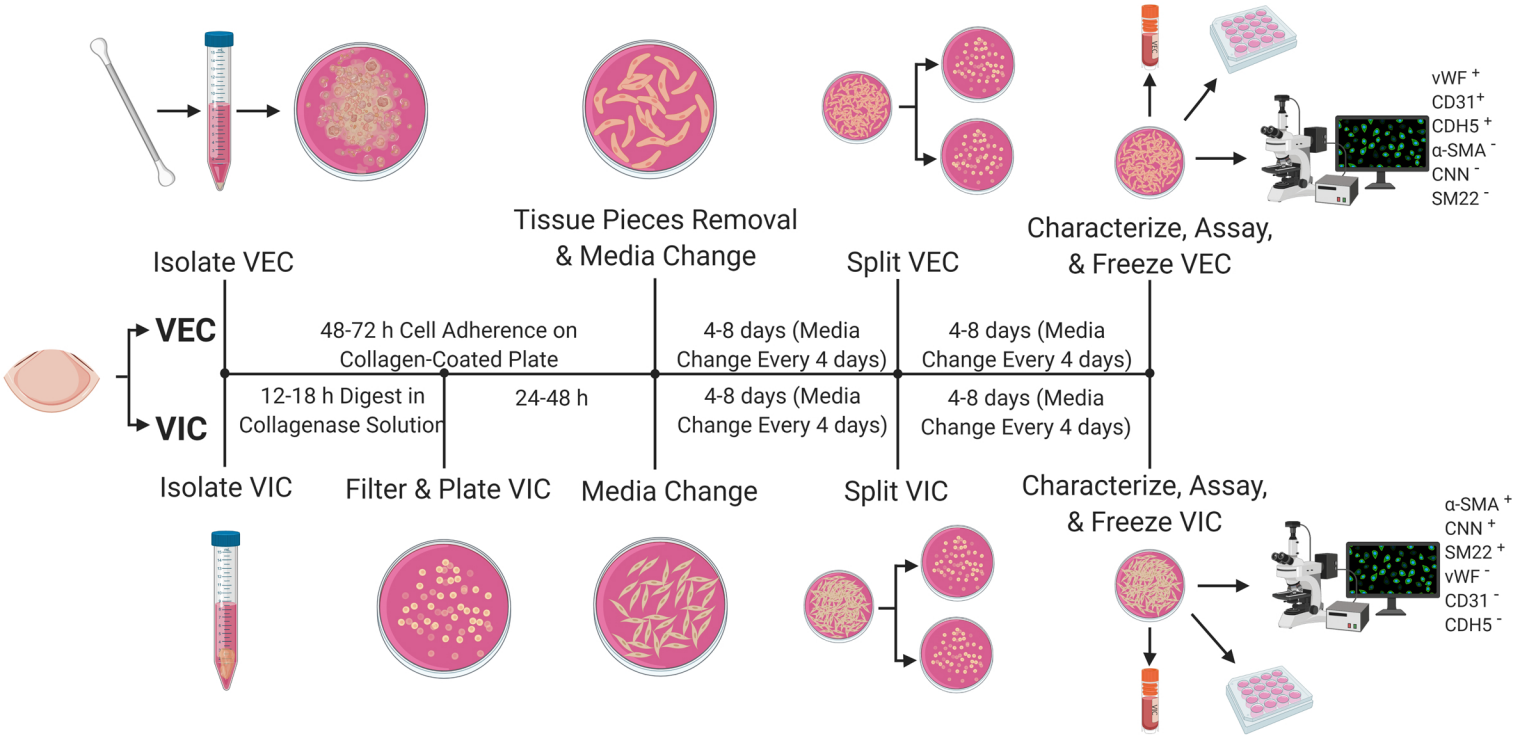
Tissue processing for valve calcification assesment

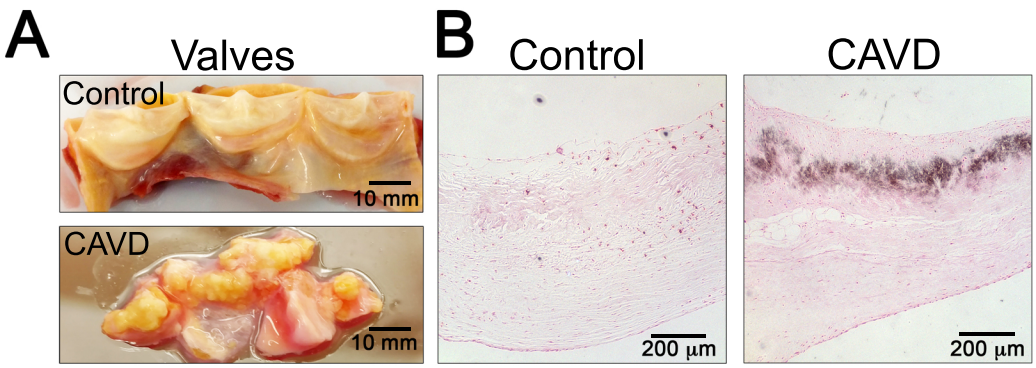


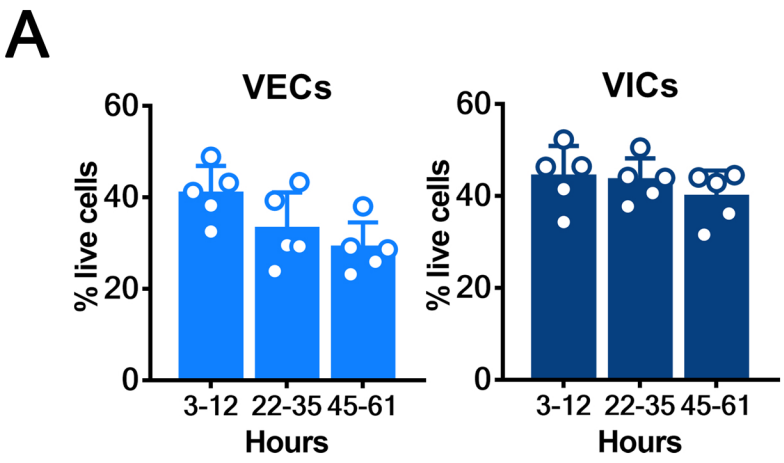
Embedded such that paraffin sections slices will reveal the three layers of the leaflet

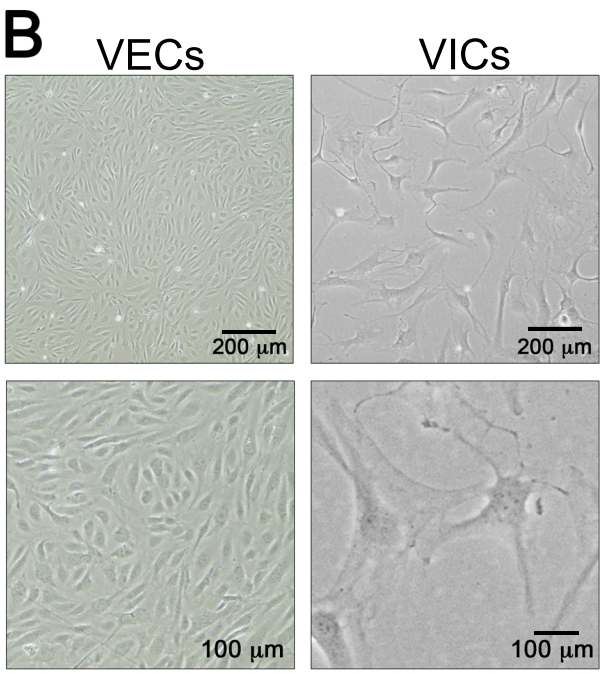
B

Tissue processing and cells isolation procedure









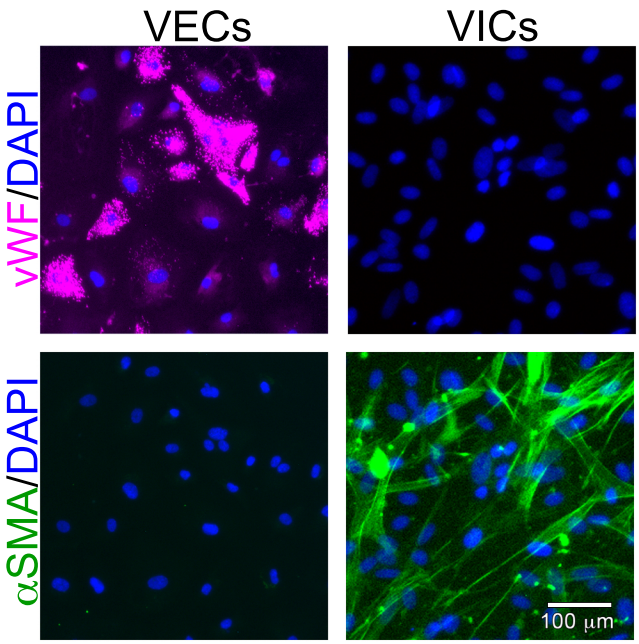


TABLE 1: List of Material

Name of Material/ Equipment
0.45 µm filter
10 cm cell culture plate
10 mL serological pipet
1000 µL filter tips
10XL filter tips
15 mL conical tubes
16% paraformaldehyde aqueous solution
190 proof ethanol
1x DPBS: no calcium, no magnesium
1x PBS
20 µL filter tips
200 proof ethanol
2-propanol
5 mL serological pipet
50 mL conical tubes
60 mm dish
6-well cell culture plate
Acetic acid, glacial
AlexaFluor 488 phalloidin
Belzer UW Cold Storage Transplant Solution
Bovine Serum Albumin, Fraction V - Fatty Acid Free 25g
Calponin 1 antibody
CD31 (PECAM-1) (89C2)
CD31+ Dynabeads
CDH5
Cell strainer with 0.70 µm pores
Collagen 1, rat tail protein

Collagenase II
Conflikt Ready-to-use Disinfectant Spray
Countess II Automated Cell Counter
Countess II reusable slide coverslips
Coverslips
Cryogenic vials
Disinfecting Bleach with CLOROMAX - Concentrated Formula
DMEM
EGM - Endothelial Cell Medium, Basal Medium, Phenol Red free 500
EGM-2 Endothelial Cell Medium-2 - 1 kit SingleQuot Kit
EVOS FL Microscope
EVOS XL Microscope
Fetal Bovine Serum - Premium Select
Fine scissors
Fisherbrand Cell Scrapers
Fungizone
Gentamicin
Glass slides
Goat anti-Mouse 488
Goat anti-Mouse 594
Goat anti-Rabbit 488
Goat anti-Rabbit 594
Invitrogen Countess II FL Reusable Slide
Invitrogen NucBlue Fixed Cell ReadyProbes Reagent (DAPI)
LM-HyCryo-STEM - 2X Cryopreservation media for stem cells
Mounting Medium
Mr. Frosty freezing container
Mycoplasma-ExS Spray
Penicillin-Streptomycin

Plasmocin
SM22a antibody
Sstandard pattern scissors
Sterile cotton swab
Swingsette human tissue cassette
Taylor Forceps (17cm)
Trypan Blue Solution, 0.4%
TrypLE Express Enzyme
Von Kossa kit
von Willebrand factor antibody
Xylenes
αSMA antibody

Company	Catalog Number
Thermo Scientific	7211345
Greiner Bio-One	664160
Fisher	14955234
VWR	76322-154
VWR	76322-132
Thermo Scientific	339650
Electron Microscopy Sciences	15710S
Decon	2801
Gibco	14190250
Fisher	BP2944100
VWR	76322-134
Decon	2701
Fisher	A416P 4
Fisher	14955233
Thermo Scientific	339652
GenClone	25-260
Corning	3516
Fisher	BP2401 500
Invitrogen	A12379
Bridge to Life	BUW0011L
Bioworld	220700233
Abcam	ab46794
Cell Signaling	3528
Invitrogen	11155D
Cell Signaling	2500
Corning	431751
Gibco	A1048301

Worthington Biochemical Corporation	LS004176
Decon	4101
Invitrogen	A27977
Invitrogen	2026h
Fisher	125485E
Olympus Plastics	24-202
Clorox	N/A
Gibco	10569044
Lonza Walkersville	CC3129
Lonza Walkersville	CC4176
Life Technologies	Model Number: AME3300
Life Technologies	AMEX1000
R&D Systems	S11550
Fine Science Tools	14088-10
Fisher	08-100-241
Gibco	15290-026
Gibco	15710-064
Globe Scientific Inc	1358L
Invitrogen	A11001
Invitrogen	A11005
Invitrogen	A11008
Invitrogen	A11012
Invitrogen	A25750
Invitrogen	R37606
HyClone Laboratories, Inc.	SR30002
Fisher Chemical Permount	SP15-100
Nalgene	51000001
PromoCell	PK-CC91-5051
Gibco	15140163

Invivogen	ANTMPT
Abcam	ab14106
Fine Science Tools	14001-14
Puritan	25806 10WC
Simport Scientific	M515-2
Fine Science Tools	11016-17
Gibco	15250061
Gibco	12604021
Polysciences	246331
Abcam	ab68545
Fisher Chemical	X3S-4
Abcam	ab7817

Comments/Description	Section
Preparing plate with collagen coating	3.1.1
Cell culture/cell line expansion	3.1.2
VEC/VIC isolation, cell culture, cell line expansion	7.2
Cell culture/cell line expansion	3.1.2
Cell culture/cell line expansion	3.1.2
Tissue storage, VIC/VEC isolation	6.3
Tissue and cell fixative	4.4
Disinfection	4
Saline solution. VIC/VEC isolation	6.1
Saline solution. Tissue preparation, VIC/VEC isolation	3.5.1
Cell culture/cell line expansion	3.1.2
Deparaffinizing tissue samples	4.3
Making collagen coated plates	3.1.1
VEC/VIC isolation, cell culture, cell line expansion	7.2
Tissue storage, VIC/VEC isolation	2.1.1
VEC isolation	6.2
Cell culture/cell line expansion	3.1.2
Making collagen coated plates	3.1.1
Fluorescent f-actin counterstain	7.6
Tissue storage solution	2.1.1
VEC confirmation with CD31+ Dynabeads	6.12
Primary antibody (VIC positive stain)	7.6
Primary antibody (VEC positive stain)	7.6
VEC confirmation with CD31+ Dynabeads	6.1
Primary antibody (VEC positive stain)	7.6
VIC isolation	7.3
Making collagen coated plates	3.1.1

Tissue digestion. Tissue preparation, VIC/VEC isolation	3.6.1
Disinfection	4
Automated cell counter	8.2
Automated cell counter required slide cover	8.2
Mounting valve samples	5.1
Freezing cells/tissue samples	4.3
Disinfection	4
Growth media. VIC expansion	3.4.1
Growth media. VEC expansion	3.3.1
Growth media supplement. VEC expansion	3.3.1
Fluorescent imaging	7.6
Visualizing cells during cell line expansion	7.5
VIC expansion	3.4.1
Tissue preparation, VIC/VEC isolation	3.2.1
VIC expansion	7.5
Antifungal: Tissue preparation, VIC/VEC isolation	3.5.1
Antibiotic: Tissue preparation, VIC/VEC isolation	3.5.1
mounting valve samples	5.1
Fluorescent secondary Antibody	7.6
Fluorescent secondary Antibody	7.6
Fluorescent secondary Antibody	7.6
Fluorescent secondary Antibody	7.6
Automated cell counter required slide	8.2
Fluorescent nucleus counterstain	7.6
Frozen cell storage	8.5
Mounting valve samples	5.1
Container for controlled sample freezing	8.6
Disinfection	4
Antibiotic. VIC expansion	3.4.1

Anti-mycoplasma. VIC/VEC isolation and expansion	3.5.1
Primary antibody (VIC positive stain)	7.6
Tissue preparation, VIC/VEC isolation	3.2.1
VEC isolation	3.2.1
Tissue embedding container	4.4
Tissue preparation, VIC/VEC isolation	3.2.1
cell counting solution	8.2
Splitting VIC/VECs	6.8
Staining paraffin sections of tissues for calcification	5
Primary antibody (VEC positive stain)	7.6
Deparaffinizing tissue samples	5.2.1
Primary antibody (VIC positive stain)	7.6

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February 22, 2021

Dear Dr. Nguyen,

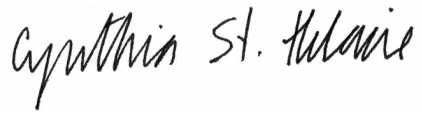
We are pleased to submit revisions to manuscript JoVE62439 entitled, "Isolation of Human Primary Valve Cells for In Vitro Disease Modeling".

We thank you and the reviewers for all the thoughtful comments and suggestions. In this revised manuscript we have addressed all the comments, critiques, and suggestions provided by the reviewers. Importantly, we now provide data clarifying the phenotypic differences between valve endothelial cells (VECs) and valve interstitial cells (VICs) as well as cell survival at varying hours post extraction.

Our responses to comments made by you and the Reviewers are detailed point-by-point below. The location of changes in the text are noted in this response, and in the manuscript, these changes as well as other corrections to text have been marked in red font.

Thank you very much for considering our manuscript for publication in *JoVE*

Sincerely,



Cynthia St. Hilaire, PhD

Editor Comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Completed as requested

2. Please provide an institutional email address for each author.

Response: This information can now be found on the title page of the manuscript.

3. Please revise the following lines to avoid previously published work: 58-60.

Response: We have updated the manuscript and now revised lines 60-63 state: "Currently the only treatment for CAVD is surgical or transcatheter aortic valve replacement (SAVR and TAVR, respectively). There is no non-surgical option to halt or reverse CAVD progression, and without valve replacement, mortality rates approach 50% within 2-3 years[1-3]."

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have rectified and removed all the personal pronouns used in our initial submission.

5. 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.

Response: We have updated the manuscript and all the references to commercial products, including the symbols and company names, have been removed or changed to a generic and descriptive name. All commercial products are now referenced in a separate document which includes the Table of Materials attached to this revision.

6. Line 105-119: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: We have changed the language and now the protocol section is written in the imperative tense as recommended by the editor. Notes have also been simplified for easy

reading. Safety procedures and use of hoods recommendations have been added to Section 4 "Tissue Preparation and Processing".

7. For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2, 5 µm (Line: 125, 126, 197,210, 212, 215, 226, etc.).

Response: We have corrected and updated the format of the units utilized in the revised manuscript. It now follows standard SI abbreviations. These amendments also apply to the updated Table of Materials attached to this revised manuscript.

8. Please use abbreviated forms for durations of less than one day when the time unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks (Line: 131, 135, 168, 170, 183, 190, 200-202, 213, 214, 225, 273, etc.).

Response: We have reviewed and edited the manuscript and every time unit follows the editor's recommendations.

9. Line 168: Please specify the volume of the cold storage solution used.

Response: Thanks to the editor for noticing this omission. We have included the volume of the cold storage solution in the manuscript.

Section 2.1.1: "A refrigerator must be available and located near the operating room. 50 mL conical tubes pre-labeled with de-identified nomenclature containing 40 mL of sterile aliquots of cold storage solution are kept in this fridge for use by surgical staff. These tubes are stable at 4 °C until expiration date on the original package.

Section 4: Valves collected during night or weekend surgeries can be stored in 40 mL cold storage solution at 4 °C and still yield viable cells more than 2 days post extraction."

10. Line 192-193: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. A citation would suffice.

Response: Thanks to the editor for this suggestion. We have included a new reference (PMID 21356832) that describes the method routinely used for embedding tissues in paraffin blocks for subsequent sectioning. The reference is cited in Section 4.6 and illustrated in Figure 1A.

11. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: Thanks to the editor for this recommendation. We have highlighted the pages that we believe are critical for the video. They are selected from the following sections: Section 2.1 Surgical Sample Preparation, Section 3.1 Reagent Preparation, Section 4: Tissue Processing, Section 6: Valve Endothelial Cell Isolation, Expansion, and Confirmation, and Section 7 Valve Interstitial Cell Isolation, Expansion, and Storage

12. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Response: We have removed the Table of Materials originally included in the manuscript submitted for peer-review. It is now attached as a .xlsx file.

13. Please title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Response: We have double checked the references and they adhere to *JoVE* guidelines.

14. Figure 2/ 3B: Please include scale bars in all the images (microscopy) of the panel. Please include the details of the magnifications used in the Figure Legends.

Response: Thanks to the editor for noticing this oversight. Now, all microscopy images are updated with scale bar. We have also included the magnifications at which the images were obtained.

15. Figure 4: Please define the scalebars either in the image or in the Figure Legends. Please provide the details of the magnifications used in the Figure Legends.

Response: Thank you to the editor for this suggestion. Figure 4 has been updated with the scales in a proper size and the Figure Legend also states the magnification used for the capture of those images.

16. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

Response: Thanks to the editor for this recommendation. We have removed all trademark and registered symbols from the Table of Materials.

Reviewer #1:

Manuscript Summary:

This paper describes method for the isolation and culture of human valve interstitial and endothelial cells. It is helpful to document this methodology for researcher interested in heart valve biology and the pathogenesis of valve calcification.

Major Concerns:

1. *The authors comment of the usefulness of Belzer UW Cold Storage Solution in comparison to PBS. However, they should recognize that there are a number of other cell culture media (M199, DMEM, RPMI) that are likely yield greater cell preservation that PBS.*

Response: Thank you for bringing this omission to our attention and we fully agree. Our experience working with healthy and CAVD tissue directed us to find better ways to preserve specimen tissues to increase cell isolation yields. After the introduction of the cold storage solution isolation buffer, we observed better cell yields in our isolation protocol, especially for cells isolated from CAVD tissue, that remain viable even >48 h after valve excision, with consistent yields between specimens. These observations motivated us to write this protocol which builds on previous reports and introduces the utilization of the cold storage buffer. We have adjusted the text in the Discussion to include and expanded explanation of the issue. Lines 451-456 read, "In the absence of cold storage solution, it is likely that a medium suitable for the culturing of cells could also perform better than PBS, however this medium was not tested herein due to the success of the cold storage solution in live organ transplantation and to the receipt of cadaveric tissues in this solution. The solution is shelf-stable which is ideal for storage in operating rooms, and specific ingredients such as adenosine are known to promote beneficial responses to cellular stresses such as ischemia/hypoxia[21,34]."

2. *The method uses high glucose DMEM for the VICs. Would it be considered as hyperglycemic?*

Response: We appreciate the reviewer for highlighting this point. While it is true that this higher glucose DMEM could be considered hyperglycemic, we and others have not observed differences in comparison to lower glucose media. In fact, Selig et al, (PMID 31152868) previously reported the effect of high glucose media on the phenotype of VICs. They concluded that VICs morphology remained unaltered when the cells were treated with hyperglycemic media. Further, the same report showed that the utilization if the high glucose DMEM does not affect the expression of ACTA2 nor SPP1, key molecules in the activation of VICs. We have added this reference to Section 3.4.1. It has been also reported that utilization of low-glucose DMEM (PMID 29282325) produces VICs that exhibit a quiescent phenotype. Low-glucose DMEM is commonly utilized for experimentation when comparing a cell culture phenotype to native valves. Our protocol focusses in cell isolation and expansion and subsequent cell storage.

3. *The immunocytochemical characterization of the cells in figure 4 is brief. Did the VEC staining positively for CD31 or vWF. Were cells isolated from calcified valves phenotypically similar to those from non-calcified valves? The presence of αSMA is indicative of the presence of myofibroblasts - however, the cells in a healthy valve are generally of a fibroblast phenotype. The authors should comment on this.*

Response: We are very happy to now provide additional data. We included an updated Figure 4 where the reviewers may find representative co-immunostaining of VECs and VICs. Both cell lines were co-stained with the endothelial marker von Willebrand Factor (vWF) and the VIC marker alpha-SMA (αSMA). As expected, over 90% of the cells were positive for their respective markers. Please note that we are showing the same cell fields, to highlight the VECs were negative for the VIC marker αSMA while VICs were negative for the marker vWF. Those figures are in agreement with previously reported analysis (PMID: 15473488). We have included this analysis in the Representative Results Section (lines 390-393) as well in the Discussion Section.

VICs are highly sensitive to substrate stiffness as demonstrated previously (PMID 22581728), which is evident by the levels of αSMA showed in Figure 4. Further, VICs acquire an activated myofibroblast-like state in vitro, as observed in Figure 3. This is due in part to the proliferative phenotype acquired in culture, characterized by the elevated levels of αSMA expression as observed during embryonic development (PMID 16645142), in clear contrast to the quiescent phenotype typically observed in adult healthy valves (PMID: 15473488 and our observations).

Reviewer #2:

Manuscript Summary:
The manuscript entitled "Isolation of Human Primary Valve Cells for In Vitro Disease Modeling" and submitted by Cuevas and co-workers describes methods of isolation and culture of valvular cells. The methods summarized here have been largely published in prior work in the same journal as well as elsewhere. Moreover, methodological weakness and formal issues significantly limit the value of the work.

Major Concerns:

1. *The manuscript ignores previous work published in the same journal on isolation of VEC and VIC. In 2010 protocols for VIC and VEC isolation have bene published by J. Butcher (Isolation of valvular endothelial cells. Gould RA, Butcher JT. J Vis Exp. 2010 Dec 29;(46):2158. doi: 10.3791/2158.). in 2014, also published in JOVE, Lincoln and co-workers have published methods of VEC isolation from very small tissue samples from murine donors (Isolation of murine valve endothelial cells. Miller LJ, Lincoln J. J Vis Exp. 2014 Aug 21;(90):51860. doi: 10.3791/51860.). This limits substantially the novelty of the submitted manuscript. Moreover, and irrespective of the novelty momentum, previous work in the same field always deserves to be acknowledged.*

Response: The authors thank the reviewer for commenting on this regrettable oversight. We have updated the Introduction Section and now we acknowledge Drs. Butcher and Lincoln's previous works (see lines 97-99) regarding the isolation of cells from porcine and murine valves. We also state that our work on human valve cell isolation builds on previous protocols, and we clearly state the advantages of our approach and how we have improved valve cell isolation methods by introducing the cold storage solution. We believe that the utilization of this buffer may greatly improve not only human valve cell isolations, but also other systems like valve isolation from murine and porcine valves.

2. *The authors state that the time delay between death of donors or tissue retrieval (in case of intraoperative specimens) until start of isolation procedure (up to 51 hours) has no impact on the outcome of the isolation. This is in clear contrast to the experience of the reviewer. In fact, the authors deliver no analysis to support their statements.*

Response: In an effort to validate our isolation procedures and provide strong evidence that the introduction of the cold storage solution improves cell isolation, we are now showing data from several clinical tissue specimens. Figure 3A shows the total number of alive cells obtained between 3-12, 22-35, or 45-61 hours after extraction, as well as the percentages of alive cells relative to the total cells that our automatized system counts. For this experiment, we collected five valves and took 1 leaflet for processing right away, and then left the other two leaflets in cold storage solution in 4 °C for approximately 24 and 48 hours. The graphs show a 14% loss in cell viability (43% to 29% live cells obtained between the immediate processing and 48 h after that initial processing). For cell counting, we use the Countess II system, which is based on the Trypan Blue exclusion principle and automatically determines the total cell number and the number of dead/cellular debris and alive cells based on cell shape and a proprietary algorithm.

3. *A minimal set of morphological and functional analysis are warranted to allow for the statement of equal outcome irrespective of the time delay. The manuscript contains only one single figure representing one single experiment (n=1) with this respect, which is clearly not sufficient.*

Response: We fully agree and regret this omission from our original submission. We have updated our bright field images of both VECs and VICs to show their respective morphologies. Figure 4 now shows immunostaining of VECs and VICs with the marker von Willebrand Factor and αSMA. The new data is a representative 5 different fields of a group of 3 biological replicates, and show that over 90% of the isolated cells show the expected endothelial or interstitial phenotype, respectively.

4. *Moreover, inter-individual (i.e. biological) variance of cell fate is of particular impact when clinical tissue specimens are used. VIC from different patients show a different susceptibility to biomineralization in vitro. This is also in contrast to the statements in this manuscript. The authors should change the respective sections or alternatively data on the equal outcome (e.g. biomineralization, gene expression, etc.) should be shown.*

Response: We agree with the reviewer that there are both procedural and biological variables that may affect consistency between clinical tissue specimens; it is precisely this reason why we wanted to optimize the tissue procurement process such that once extracted, the tissues are in an environment that promotes cell viability and limits variability. We have specifically highlighted some these confounding effects and emphasize the need for robust characterization of the cells to limit inter-patient cell line variability. We reflect these considerations in our Discussion. Importantly, we emphasize the need for using biological replicates for all experiments (i.e. in biomineralization assays as you suggest) such that inherent inter-cell lines differences do not confound data interpretation.

5. *There seems to be a certain variation in methodology:*
   ** Collagen coating varies between 4 h and over-night incubation?*
   ** Collagenase digestion varies between 12 and 18 hours?*

   Response: Thank you for bringing this to our attention, we have clarified the instructions in section 3.1.2 regarding the coating of collagen plates.

6. *The characterization of isolated cells appears to be very limited. Regarding VIC: What is the level (uncontrolled) of EndoMT? What is the proportion of alpha-sma-pos. cells? Fig. 1 suggests that VECs are isolated from structurally unchanged valves and VIC are isolated from degenerated valves - is that correct? If so, such a pattern of selection should be more clearly stated in the manuscript.*

   Response: We thank the reviewer for bringing these questions up. The isolation of any primary endothelial cells is difficult as the do tend to lose their endothelial phenotype. While we have not quantified a rate or percentage of VECs that lose their phenotype, we have added to Section 6.9 that in order to ensure a consistent culture an individual may use CD31+ bead with every splitting. Additionally, we provide a seeding density that helps to ensure minimal EndMT issues. We have now added data that shows the quantification of the total number expressing αSMA marker. Lines 390-393 state, "Immunostaining of the specimens confirmed that 91.8% ± 1.8 (n = 3) of expanded VECs were positive for the endothelial marker vWF whereas 92.0% ± 5.0 (n = 3) of expanded VICs were positive for the interstitial cell marker **α**SMA (Figure 4)These figures are in line with results reported previously[33]." We have also discussed this analysis in the Discussion Section. Regarding Figure 1, we apologize the original diagram was confusing. We have now adjusted the images depicted to avoid confusion, further, we clearly state that VECs and VICs are collected from both control and CAVD tissues.

7. *Figure 3 misses phase contrast images of isolated cells, which should be added for the respective isolation time points. Moreover, the given cell numbers are not clear: Have the authors counted living AND dead cells? Is there a viability proportion available? Such information should be provided, beyond the mere number of cells. Statements on "cell survival" warrant a clear picture of the relative proportion of living cells (as opposed to dead*

cells) at different time points, e.g., here at time points pre-seeding and post culture of 24 or 48 hours.

Response: Thank you for bringing these oversights to our attention. Figure 3 now includes images of VECs and VICs to highlight cell morphology as well as depicts graphs of total cell numbers and percentage of living cells that were counted with an automated cell counter; we describe in comment 2 above how we performed these analyses.

8. *The section focusing on Belzer storage misses appropriate read-outs to support the statement. Here the impact of post-seeding proliferation is uncontrolled by the given read-outs. Appropriate analyses, e.g. Live/Dead assay, Ki67 staining etc. are needed.*

Response: Thank you for bringing this oversight up. We have now clearly stated the seeding density of cells where appropriate (Section 6.6, 6.7, 7.6, 8.4). Live/Dead analysis has been described in the comments above.

Minor Concerns:

9. *The manuscript has significant need for language editing. This includes spelling and also the use of capital letters. Style and formatting is inconsistent and needs thorough work-up (e.g. colon or no colon after sub-titles).*

Response: Thanks to the reviewer for bringing this important aspect to our attention. We have thoroughly proofread our work and all the suggestion have been included.

10. *Any work including human material should include complete ethical statements, including the acknowledgement of Declaration of Helsinki.*

Response: We agree with the reviewer and the manuscript clearly state full ethical statements in Section 1.1: "Institutional Review Board (IRB) approval or an exempt memo is essential for any collection of patient samples or cadaveric tissues. For this manuscript, all patient samples are collected from individuals enrolled in studies approved by the institutional review board of the University of Pittsburgh in accordance with the Declaration of Helsinki. Cadaveric tissues obtained via the Center for Organ Recovery and Education (CORE) were approved by the University of Pittsburgh Committee for Oversight of Research and Clinical Training Involving Decedents (CORID)."

11. *Figures should be numbered according to their first chronological appearance (figure 3 is mentioned in the text prior to the first reference to figure 1 and 2). Dyanbeads vs. Dynabeads. Inconsistency: "FIGURE", "Fig.", "Figure". Also: Belzer UW® cold storage solution vs. Belzer UW Cold Storage Solution*

Response: Thanks for bringing these issues to our attention. Figures are now numbered according to their appearance in the manuscript and described as main figure or subfigure (i.e.,

1A, 1B left panel, etc.). Also, as requested by the Editor, the references to trademarked and registered reagents have been removed from the manuscript and are now listed in the additional Table of Materials.

12. *Legend of fig. 4 is incomplete. There is an interruption of a sentence. Figure 2B misses scale bar, whereas in 2C the scale bar appears over-dimensional.*

Response: Thank you for this comment and we apologize for any lack of consistency in our figures. All figures now include a properly dimensioned scale bar while the legend of Figure 4 has been updated, "Figure 4: Representative immunofluorescent staining on VECs and VICs. VECs are positive for the endothelial marker von Willebrand Factor (vWF, left panels) whereas VICs are positive for the interstitial marker αSMA. Note our isolation protocol guarantees a high isolation efficiency; no cross-contamination between VECs and VICs is detected. Images were captured with a 10x objective scale bar 100 um."

Reviewer #3:

Manuscript Summary:

This will be helpful to investigators studying heart valve biology and disease. My comments are listed sequentially. The major concern is the lack of characterization of the VECs (points 14-15 of my specific comments).

Major Concerns:

1. *In the Abstract, mention some of the key improvements or critical steps needed for successful valve cell isolation.*

Response: We thank the review for this suggestion and have now mentioned some of the key improvements and critical steps needed for successful valve cell isolation. Lines 45-52 now state: "To that end, the present study develops a workflow where surgically removed tissues from patients and donor cadaver tissues are used to create patient-specific primary lines of valvular cells for in vitro disease modeling. This protocol introduces the utilization of a cold storage solution, commonly utilized in organ transplant, to reduce the damage caused by the often-lengthy procurement time between tissue excision and laboratory processing with the benefit of greatly stabilizing cells of the excised tissue. The results of the present study demonstrate that isolated valve cells retain their proliferative capacity and endothelial and interstitial phenotypes in culture upwards of several days after valve removal from the donor."

2. *How long can collagen coating solution be stored? At what temperature? What is the temperature for collagen coating of culture dishes?*

Response: Thanks to the reviewer for bringing these critical omissions to our attention. Plates and dishes coated with collagen are stored in sealable bags at 4 °C and can be stored for several months in this way. We have updated the protocol manuscript by adding this point

as a note in section 3.1.2: "Under a sterile cell culture hood, add enough collagen solution to 6-well plates and 10 cm dishes to just cover the entire bottom. Cover the plate and let sit for 4-6 h at room temperature. Remove the excess solution with a sterile pipette, place in a new sterile 50 mL conical tube, and save at 4 °C to make additional plates. Solution can be stored at 4 °C for several months. Dry the plates in a 37 °C incubator overnight, and subsequently store them in a resealable bag at 4 °C. Collagen-coated plates and dishes can be stored for several months."

3. *How long can the EGM2 media with bullet kit factors added be stored? i.e., how long do the added factors remain stable and effective for cell expansion?*

Response: We use the medium within in one month of preparation and have updated the protocol to mention it accordingly in see Section 3.3: "Prepare and use endothelial cell growth medium according to manufacturer's protocols. Store in the dark at 4 °C. Warm to 37°C just before use on cells, do not leave media in warming bath longer than necessary (10-15 min should be sufficient). Use the media within one month of preparation."

4. *Section 4.3-4.4 - is there any consideration given to the anatomy of the valve, in terms of which parts to use for cell isolation? Would different types of VEC and VIC be obtained if different cusps are used?*

Response: We thank the reviewer for bringing this point to our attention. We routinely process the three leaflets where 1-2 leaflets are preserved for histology and snap freezing, and the remaining 1-2 are utilized for cell isolation. The classification of control or CAVD is not assigned until we perform a von Kossa stain to assess the presence of calcification. This information is included in an additional note in Section 4.4 "NOTE: Our protocol workflow unbiasedly selects two leaflet for VECs and VICs isolation, while the remaining leaflet is utilized for Von Kossa staining (Section 5) and snap freezing"

Additionally, we have added the following statement to the discussion (lines 463-473):

"The unbiased tissue treatment described in this protocol allows the isolation of the main two cell populations present in the valve leaflet, VECs and VICs. Although a recent single cell transcriptome analysis has shown the co-existence of at least fourteen different cell subtypes residing in the human valve, including six non-valve derived stromal cells in CAVD tissue[35], this diversity may represent variations due to different microenvironments to which the leaflet cells are exposed: VECs are exposed to two different blood flows while VICs are embedded in three different extracellular matrix stratums[8,9]. The large-scale isolation protocol and analysis described herein ensure that over 90% of VECs and VICs correspond to their main phenotype. Although a degree of heterogenicity may be found, it does not affect the general outcomes of the study of VEC and VIC homeostasis[8,9,13-16].

5. *For 6.6 - how many cells are plated in the 6-well. Does cell density matter? It usually does for endothelial cells. How many cells to you typically retrieve from the valve tissue using this method? What percent are viable? 6.7 - 1:2 and 1:3 is vague, better to state the plating density in cells/cm2. A range is acceptable.*

   Response: Thank you for bringing this up, for it is an important clarification to make. Cell density information has been added to Section 6.6, 6.7, 7.6, and 8.4. Additionally, viable cell retrieval data is now included in Figure 3. In data shows the total number of alive cells obtained between 3-12, 22-35, or 45-61 hours after extraction, as well as the percentages of live cells relative to the total cell count that our automatized system determines. For this experiment, we collected five valves and took 1 leaflet for processing right away, and then left the other two leaflets in Cold Storage Solution in 4°C for approximately 24 and 48 hours. The graphs show that there is a 10% loss in cell viability (40 – 30% live cells obtained between the immediate processing and 48hrs after that initial processing).

6. *6.8 - the phenotype and heterogeneity of the culture should be assessed by staining for an endothelial marker and a mesenchymal marker. Co-staining is best - flow cytometry and or immunofluorescence. This is discussed in 6.14 but should be moved up in the manuscript. Also, it is critical to look at endothelial and mesenchymal markers simultaneously as double-positive cells will indicate EMT and cells positive for one or the other will indicate VICs in the endothelial prep.*

   Response: Thank you for this comment. We are very happy to now provide additional data. We included an updated Figure 4 where the reviewers may find representative co-immunostaining of VECs and VICs. Both cell lines were co-stained with the endothelial marker von Willebrand Factor (vWF) and the VIC marker alpha-SMA (αSMA). As expected, over 90% of the cells were positive for their respective markers. Please note that we are showing the same cell fields, to highlight the VECs were negative for the VIC marker αSMA while VICs were negative for the marker vWF. Those figures are in agreement with previously reported analysis (PMID: 15473488). We have included this analysis in the Representative Results Section (lines 390-393) as well in the Discussion Section.

7. *In 6.10 - this sentence is too vague, confusing. "This is usually due to growing at too low confluence."*

   Response: We apologize that our language was confusing, we have now modified it. Section 6.9 now reads, “Once expanded into a 10 cm plate VECs may sometimes lose their morphology and change phenotype as they proliferate. We have observed that is tends to occur when VECs are seeded at a low confluency during the establishment and expansion of the cell line. To guarantee a pure culture one may consider utilizing CD31+ superparamagnetic beads with every splitting.”

8. *In 6.12. Is this correct? " PBS w/0.1% BSA, pH 7" - pH 7 might be too low. pH7.4 is better.*

Response: Thank you for catching this, the correct pH is 7.4 and that has been fixed in the manuscript.

9. *In 8.1 - what are the signs of over incubated - referring to "checking to make sure cells are not over incubated." VECs are often harder to remove from the culture dish than other types of endothelial cells. Is this the case with human aortic VEC?*

   Response: Our language was confusing, and we have modified it to clearly state how long dissociation should take and added a note that if cells take longer than this time it could be because the dissociation reagent is too old and if needed a cell scraper may be used to help lift stubborn cells, see the NOTE after Section 7.6: "If cells take longer than 3 min to lift off the plate, the dissociation reagent may have lost potency. If needed, a cell scraper may be used to gently lift the cells. "

10. *In the Discussion - what does this sentence mean "Cell cultures are started in a single well from a 6 well plate and then expanded according to their growth rate." How is growth rate determined and then how is this information used in the expansion protocol?*

    Response: Our language was imprecise, and we have now modified it. We are not providing growth rate nor doubling time since our protocol focuses on cell isolation. Thus, we removed the statement. Our apologies for the confusion.

11. *Figure 4 shows immunofluorescence staining for aSMA and DAPI staining. How many of the cells in the VIC preparation express aSMA. It appears not all from the images shown. This would be helpful information for the readers. The major concern is that VECs are not shown. Immunofluorescence staining of the VEC obtained by this procedure is critical since more than half of the manuscript is devoted to VECs!*

    Response: Thanks to the reviewer for bringing this important flaw and the suggestion to amend it. As mentioned above, we have corrected this issue by including an updated Figure 4. In it, the reviewer will find a co-immunoassay of both VECs and VICs for the specific markers von Willebrand Factor (vWF) and alpha-SMA (αSMA), respectively. Our isolation protocol shows that over 90% of the cells are positive for their respective markers, which is in agreement with previous isolation protocols and valve staining reports (PMID 15473488). As mentioned above in response 8 to this reviewer, we included this analysis in the Representative Results Section and in the Discussion Section.

12. *I strongly recommend showing both flow cytometry and immunofluorescence staining. Each technique has particular advantages. For flow cytometry, cells can be permeabilized to analyze mesenchymal markers such as αSMA or calponin or SM22, which are intracellular. CD90 is a very helpful marker for distinguishing endothelial from mesenchymal.*

Response: We are now including co-immunostaining of VECs and VICs stained for the endothelial and mesenchymal markers vWF and αSMA. Our staining matches our predictions where VECs stained positive for vWF with no αSMA signal. Likewise, over 90% of the VICs were αSMA positive with no detectable vWF signal. This information is shown in an updated Figure 4 and described in line 390-393. We fully agree with the reviewer regarding the advantages of flow cytometry, however in light of the timeline given to us for this revision we are unable to procure and test new antibodies that work well with flow cytometry.