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Dear Dr. Nguyen,

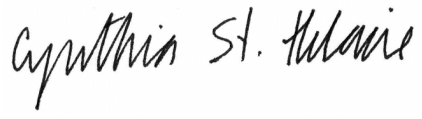
We are pleased to submit revisions to manuscript JoVE62439 entitled, "Isolation of Human Primary Valve Cells for In Vitro Disease Modeling".

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.