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TITLE:

Isolation of Mouse Interstitial Valve Cells to Study the Calcification of the Aortic Valve *In Vitro*

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SUMMARY:
This article describes the isolation of mouse aortic valve cells by a two-step collagenase procedure. Isolated mouse valve cells are important for performing different assays, such as this in *vitro* calcification assay, and for investigating the molecular pathways leading to aortic valve mineralization.

ABSTRACT:
The calcification of aortic valve cells is the hallmark of aortic stenosis and is associated with valve cusp fibrosis. Valve interstitial cells (VICs) play an important role in the calcification process in aortic stenosis through the activation of their dedifferentiation program to osteoblast-like cells. Mouse VICs are a good *in vitro* tool for the elucidation of the signaling pathways driving the mineralization of the aortic valve cell. The method described herein, successfully used by these authors, explains how to obtain freshly isolated cells. A two-step collagenase procedure was performed with 1 mg/mL and 4.5 mg/mL. The first step is crucial to remove the endothelial cell layer and avoid any contamination. The second collagenase incubation is to facilitate the migration of VICs from the tissue to the plate. In addition, an immunofluorescence staining procedure for the phenotype characterization of the isolated mouse valve cells is discussed. Furthermore, the calcification assay was performed *in vitro* by using the calcium reagent measurement procedure and alizarin red staining. The use of mouse valve cell primary culture is essential for testing new pharmacological targets to inhibit cell mineralization *in vitro.*

INTRODUCTION:

Calcified aortic valve disease (CAVD) is the most prevalent valvular heart disease in western populations, affecting nearly 2.5% of elderly individuals over 65 years of age[1]. CAVD affects over six million Americans and is associated with changes in the mechanical properties of the leaflets that impair normal blood flow-through[1,2]. Currently, there is no pharmacological treatment to stop the progression of the disease or to activate mineral regression. The only effective therapy

to treat CAVD is aortic valve replacement by surgery or transcatheter aortic valve replacement[3]. It is therefore imperative to investigate the molecular mechanisms leading to valve mineralization to identify new pharmacological targets. Indeed, non-treated aortic stenosis has several adverse consequences such as left ventricle dysfunction and heart failure[4].

The aortic valve consists of three layers known as fibrosa, spongiosa, and ventricularis, which contain VICs as the predominant cell type[5]. The fibrosa and the ventricularis are covered by a layer of vascular endothelial cells (VECs)[5]. The VECs regulate the permeability of inflammatory cells as well as paracrine signals. Increased mechanical stress may affect the integrity of the VECs and disturb the homeostasis of the aortic valve, leading to inflammatory cell invasion[6]. Scanning electron microscopy analyses showed disrupted endothelium in a human calcified aortic valve[7].

Histological analyses of calcified tissue reveal the presence of osteoblasts and osteoclasts. Furthermore, osteogenic differentiation of VICs was observed both *in vitro* and in human valve tissue[8]. This process is mainly orchestrated by the Runt-related transcription factor 2 (Runx2) and the bone morphogenetic proteins (BMPs)[8,9].

PROTOCOL:

NOTE: All animal procedures described here have been approved by Icahn School of Medicine at Mount Sinai institutional core and use committee.

1. Preparation before valve cell isolation from adult mice

1.1. Clean and sterilize all the surgical instruments shown in **Figure 1A** by using 70% v/v ethanol and subsequently autoclaving them for 30 min. clean the surgical workspace with 70% ethanol.

1.2. Add 500 µL of penicillin-streptomycin to 50 mL of 10 mm HEPES. Prepare an aliquot of 50 mL of 1x phosphate-buffered saline (PBS). Keep the solutions on ice.

1.3. Prepare 1 mg/mL and 4.5 mg/mL collagenase solutions, and use 5 mL of each solution in 15 mL tubes to perform the entire procedure. To prepare 5 mL of 1 mg/mL collagenase, mix 5 mg of collagenase with 2.5 mL of Dulbecco's Modified Eagle Medium (DMEM, fetal bovine serum (FBS)-free) and 2.5 mL of 10 mM HEPES supplemented with antibiotics (1% penicillin-streptomycin from step 1.2). Filter the solutions through a 0.22 µm filter to remove any contamination.

NOTE: Keep the solutions on ice to protect the enzymes.

1.4. Warm the DMEM solution to 37 °C before use in all the steps described below. Prepare complete medium by supplementing DMEM with 1% penicillin-streptomycin, 1% sodium pyruvate, 1 mL of mycoplasma elimination reagent (see the **Table of Materials**), and 10% FBS.

2. Isolation of valve cells

2.1. To obtain 10^6 cells for the experiment, use five 8-week-old mice (minimum of three). Place the mouse in an induction chamber along with a small piece of tissue paper soaked with 1 mL of isoflurane, but do not allow contact with the tissue. To confirm that the animal is fully anesthetized; check for toe pinch reflex, and then euthanize the mouse by cervical dislocation. Use isoflurane to alleviate any pain prior to the cervical dislocation as the procedure described below is terminal.

2.2. Place the mouse on a dissecting platform, and fix the paws with cannulas to hold it in place. Clean the chest and the abdomen with ethanol; open the abdomen and the chest with scissors. With small surgical scissors, cut between the left atrium and the left ventricle to exsanguinate the mouse. Perfuse the heart with 10 mL of cold 1x PBS to remove blood from the heart.

2.3. Cut the heart, and keep 3 mm from the ascending aorta as shown in **Figure 1B**. Dissect the aortic valve under a stereomicroscope. Cut the heart horizontally in the middle of the ventricles (**Figure 1C**). Cut the left ventricle toward the aorta, and carefully dissect the aortic valve (**Figure 1D–F**). Pool the valves together in a small 35 mm tissue culture dish.

2.4. Wash the isolated valves in a 75 mm cell culture dish with 5 mL of cold HEPES (10 mM) supplemented with antibiotics (1% penicillin-streptomycin) to remove blood (**Figure 2**). Prepare two 15 mL tubes of collagenase 1 mg/mL and 4.5 mg/mL as described above in step 1.3.

NOTE: After the dissection, manipulate the isolated valves in a sterile biosafety hood to minimize contamination.

2.5. Incubate the valves in collagenase type I (1 mg/mL) for 30 min at 37 °C with continuous shaking (**Figure 2**). Centrifuge the tube for 5 min at 150 × *g*, wash the pellet once with 2 mL of HEPES (10 mM), and vortex for 30 s at high speed. Pour the contents of this tube into a 35 mm culture dish, and carefully transfer the fragments of tissue using thin tweezers into a new tube.

NOTE: At this stage, the VICs are still not dissociated from the tissue, and the pellet contains pieces of tissue. To avoid contamination with endothelial cells, do not centrifuge after vortexing in step 2.5.

2.6. Incubate the pellet in a 15 mL tube with 5 mL of collagenase type I (4.5 mg/mL) at 37 °C under continuous agitation for 35 min. Re-suspend the cells with a 1 mL pipette to separate the cells, and centrifuge at 150 × *g* for 5 min at 4 °C.

2.7. Discard the supernatant, and re-suspend the pellet in 2 mL of complete DMEM. Centrifuge at 150 × *g* for 5 min at 4 °C. Repeat this step twice to clean the cells.

NOTE: The pellet will still have some tissue fragments.

133
134 2.8. Re-suspend the pellet in 1 mL of complete medium, and plate the cells in one well of a 6-
135 well cell culture dish in a minimum amount of medium to facilitate their attachment to the
136 culture dish. Leave the cells, undisturbed, in a 37 °C incubator with 5% carbon dioxide.
137
138 2.9. After 3 days, check the cells under the microscope to verify good growth close to the
139 tissue debris. Once 1,000 cells are visible under the microscope, carefully remove the tissue
140 debris with autoclaved tweezers, and change the medium.
141
142 NOTE: The plate should not be disturbed; if the required number of cells are not observed, place
143 the cell culture dish back in the incubator for another 2 days.
144
145 2.10. When the cells are 70% confluent (2.5×10^5), trypsinize and then transfer them to a 75
146 mm tissue culture dish.
147
148 **3. Analysis of cell identity and morphology**
149
150 NOTE: Immunofluorescence staining was used to study cell morphology and endothelial cell
151 contamination.
152
153 3.1. Clean the hood with 70% v/v/ ethanol. Place sterile coverslips (22 mm x 22 mm) in 6-well
154 plates.
155
156 NOTE: To sterilize the coverslips, wash them with 70% ethanol, and keep them in the hood
157 overnight under ultraviolet light.
158
159 3.2. Seed 100,000 cells per well in a 6-well plate. After 24 h, wash the cells twice in 1x PBS,
160 and fix them in 4% paraformaldehyde (PFA) for 20 min. Wash the cells again twice with 1x PBS.
161
162 NOTE: At this point, the cells could be kept in PBS at 4 °C until the start of the staining procedure.
163
164 3.3. To verify the purity of the VICs, use alpha-smooth muscle actin (αSMA), vimentin, and
165 cluster of differentiation 31 (CD31) to detect contamination with VECs.
166
167 3.4. Prepare an aliquot of blocking buffer by mixing 500 µL of normal serum (the same species
168 as the secondary antibody), 9.5 mL of 1x PBS, and 30 µL of Triton X-100. Incubate the cells in 2
169 mL of the blocking buffer for 1 h.
170
171 3.5. Prepare the antibody dilution buffer containing 30 µL of Triton X-100, 10 mL of 1x PBS,
172 and 0.1 g of bovine serum albumin (BSA).
173
174 3.6. Take an empty tips box, fill half of the box with water to create a humid chamber. Cover
175 the tip holder with a wet tissue and then with a sheet of parafilm.
176

177 3.7. Take 1 µL of the primary antibody, and mix it with 100 µL of the dilution buffer prepared
178 in step 3.5. Place 50 µL of the diluted antibody on the parafilm. Take the coverslips from the wells,
179 flip them over, and place them on the top of the drops of antibody; incubate the cells overnight
180 with the antibody.
181
182 3.8. Add 1 mL of PBS in the 6-well plate. Carefully take out the coverslip from the parafilm, flip
183 it over, and place it in the well. Wash the cells with a continuous gentle agitation for 5 min.
184 Replace the PBS with fresh PBS; wash the cells 3 times.
185
186 3.9. Incubate the cells with the diluted secondary antibody (1/500) (Alexa-488, Alexa-555) for
187 1 h. Add 1 µL of the secondary antibody to 500 µL of the antibody dilution buffer (prepared in
188 step 3.5). Cover the plate with aluminum foil. Wash the cells 3 times with 1 mL of 1x PBS with
189 continuous agitation.
190
191 3.10. Mount the coverslips with 50 µL of 4',6-diamidino-2-phenylindole (DAPI)-mounting
192 medium, and observe the cells under the microscope to analyze the morphology of cells and VEC
193 contamination.
194
195 **4. *In vitro* calcification assay**
196
197 4.1. Clean the hood with 70% ethanol, warm the DMEM medium to 37 °C.
198
199 4.2. Seed 100,000 cells/condition into 6-well plates in complete DMEM, and culture for 24 h
200 at 37 °C.
201
202 4.3. Prepare the calcifying medium by mixing 2 mM of NaH_2PO_4, 10^{-7} M insulin, and 50 µg/mL
203 ascorbic acid in DMEM with 5% FBS. For 93 mL of DMEM, add 5 mL of FBS, 1 mL of antibiotics
204 (final concentration 1%), 1 mL of sodium pyruvate (100 mM), 27.5 mg of NaH_2PO_4, 5.8 µL of
205 insulin, and 5 mg of ascorbic acid.
206
207 NOTE: Filter the solution using a 0.22 µm filter before use.
208
209 4.4. After 24 h, replace the supernatant medium with the calcifying medium. Incubate the
210 cells for 7 days at 37 °C. On the 3rd day, replace with fresh calcifying medium, and place the plate
211 back in the incubator to complete the 7 days of treatment.
212
213 4.5. After 7 days, remove the medium, and wash the cells twice with 2 mL of 1x PBS. Incubate
214 the cells in 1 mL of 0.6 N hydrochloric acid (HCl) for 24 h at 37 °C. Collect the HCl in a 1.5 mL tube,
215 and evaporate it in a rotary evaporator. Re-suspend the contents of all the tubes in 60 µL of HCl.
216
217 NOTE: The drying procedure is important to concentrate the solution and to have the same
218 volume for each condition.
219

220 4.6. Use a 96-well plate to measure calcium concentration by using Arsenazo III reagent,
221 available in a ready-to-use kit (see the **Table of Materials** for more details).
222
223 4.7. Prepare a calcium standard solution of 10 mg/dL concentration. Weigh 10 mg of calcium
224 hydroxide ($Ca(OH)_2$) and dissolve in 100 mL of distilled water.
225
226 4.8. In a clear 96 well plate, pipet 2 µL of blank solution (HCl, 0.6 N), the standard solution, the
227 sample per well (10 mg/dL), and the samples. Perform the experiment in triplicate to verify the
228 pipetting variability. Add 200 µL of the reagent for each condition.
229
230 NOTE: Samples above 15 mg/dL should be diluted 1:1 with saline, re-assayed, and the result
231 multiplied by two.
232
233 4.9. Incubate the reaction for 15 min at room temperature.
234
235 NOTE: The reaction is stable for 60 min.
236
237 4.10. Read and record the absorbance of the plate at 650 nm. Use the following formula to
238 calculate the amount of calcium in the samples:
239
240 Calcium (mg/mL) = (Absorbance of sample/absorbance of standard) × Concentration of standard
241
242 **Representative Results**
243 As murine aortic valves are typically 1 mm in diameter, at least three valves must be pooled to
244 collect a million viable cells for different experimental procedures. The different steps of the VIC
245 isolation process are shown in **Figure 1** and **Figure 2**. As it is difficult to manually scrape the valve
246 tissue, it is preferable to use shear stress created by vortexing to remove the VECs. Indeed, the
247 CD31 immunofluorescence staining results showed the absence of endothelial cells
248 contamination (**Figure 3D**). In addition, mouse VICs express vimentin and α-SMA, which are the
249 major markers of valve cells (**Figure 3B,C**).
250
251 **Cell mineralization *in vitro***
252 A calcium reagent kit was used to measure the calcium concentration; cells treated with calcifying
253 medium have higher calcium concentration compared to non-treated cells (**Figure 4A**). The
254 concentration of calcium was normalized with the total protein concentration. Alizarin red
255 staining confirmed the calcium-reagent kit measurements by showing red positive calcium nodes
256 (**Figure 4B**).
257
258 **FIGURE AND TABLE LEGENDS:**
259 **Figure 1: Description of valve dissection.** (**A**) Representative image of all the surgical instruments
260 needed for the dissection, scissors 2 is needed to open the skin of the mouse and scissors 3 to
261 open the chest. Tweezers 5 and 6 are needed to hold the skin and open the chest. (**B**) Leave 3
262 mm of tissue from the aorta (black arrow). (**C**) Cut the heart in the middle of the ventricles with
263 scissors number 4. (**D**) Open the heart toward the aortic valve with scissors 3. Use the thin

264 tweezers 7 and 8 to carefully dissect the aortic valve. The valve is visible and has some black dots
265 that are characteristic of mice valve tissue (blue arrow). (**E**) Increase the magnification to better
266 visualize the aortic valve. Isolate the valve with the small scissors 4; (**F**) maintain the tissue with
267 tweezers 7.
268
269 **Figure 2**: **Representative description of mouse valve cell isolation**. Abbreviations: HEPES = 4-(2-
270 hydroxyethyl)-1-piperazineethanesulfonic acid; RT = room temperature; DMEM = Dulbecco's
271 modified Eagle medium; FBS = fetal bovine serum.
272
273 **Figure 3**: **Mouse valve cell phenotype**. Microscopic view of (**A**) freshly isolated valve cells.
274 Immunofluorescence staining showing (**B**) vimentin-positive cells and (**C**) α-SMA. Cells are
275 negative for (**D**) CD31 staining. Scale bars = 200 µm. Abbreviations: DAPI = 4′,6-diamidino-2-
276 phenylindole; CD31 = cluster of differentiation 31; α-SMA = alpha-smooth muscle actin.
277
278 **Figure 4**: ***In vitro*** **calcification assay**. (**A**) Phosphate-rich calcifying medium induced VIC
279 calcification *in vitro*, which was measured with a reagent kit. (**B**) Microscopic image showing red
280 positive staining (right) for calcium nodes. (**C**) Alizarin red staining showed positive calcium nodes
281 (black arrow) of VICs in response to calcifying medium. Scale bars = 100 µm. Abbreviations: CTL-
282 = Control; mVICs = mouse valvular interstitial cells.
283
284 **DISCUSSION:**
285 This article presents a detailed protocol of mouse valve cell isolation for primary culture. Three
286 aortic valves from 8-week-old mice were pooled to obtain an adequate number of cells. In
287 addition, this protocol describes the characterization of VIC phenotype and the *in vitro*
288 mineralization assay. The method was adapted from the previously described protocol from
289 Mathieu et al.[7].
290
291 During the isolation of aortic valves, care must be taken to avoid all sources of possible contagion
292 to protect the cells from bacterial or mycoplasma contamination. Indeed, it is crucial to autoclave
293 all the surgical tools prior to starting the experiments. The HEPES solution should be
294 supplemented with 1% antibiotics to minimize bacterial infection. Furthermore, mycoplasma
295 may cause cytopathology and consequently interfere with every parameter measured in cell
296 culture[10].
297
298 Plating cells in small culture dishes with lower volume of culture medium is critical for VIC growth
299 and proliferation. Letting the tissue settle and adhere to the cell culture dish permits cell
300 migration from the tissue to the dish wall. Given that isolated cells from young mice proliferate
301 faster, it is recommended to transfer cells to a larger culture dish of 75 cm^2 after 5 days of culture.
302 Maintaining cells to 80% confluence is crucial to minimize the differentiation of VICs to a
303 myofibroblast phenotype[8].
304
305 As shown by immunofluorescence imaging, the isolated valve cells show a fibroblast-like
306 phenotype. VICs have an elongated cytoplasm and express both vimentin and αSMA as described
307 by previous studies. The present work confirmed that the mouse VIC phenotype is similar to that

308 previously described for porcine VICs[11] and human VICs[12]. Most *in vitro* studies on aortic stenosis
309 are performed on cells from large animals[8,11]. The key disadvantage of porcine VICs is their
310 spontaneous differentiation to an osteoblast phenotype i*n vitro* even in normal media[13].
311 However, mouse VICs do not calcify spontaneously even at higher passages.
312
313 Mouse VICs differentiate to the osteoblast phenotype in response to calcifying medium using
314 ascorbic acid, insulin, and phosphate stimulation. This article describes a quantitative method of
315 calcium measurement using a kit and a qualitative method using Alizarin red staining. Both
316 methods showed significant increase of calcification in response to calcifying medium treatment.
317 The calcium measurement kit is the gold standard method, which offers an exact quantitative
318 calcium measurement[14].
319
320 In the Arsenazo III reagent, magnesium interference is prevented by the inclusion of 8-
321 hydroxyquinoline sulfonate. Calcium reacts with the reagent to form a purple-colored complex,
322 which absorbs at 650 nm. The intensity of the color is proportional to the calcium concentration.
323 The accuracy of the Arsenazo-III reagent was previously validated with atomic absorption
324 spectrophotometry. The same method is used in clinical laboratories to measure total calcium
325 concentration in biological fluids[14]. The calcification in aortic stenosis is mainly hydroxyapatite,
326 as shown with dispersive x-ray energy scanning electron microscopy analysis[7,12,15]. Indeed, it is
327 important to analyze the calcification of the cell membrane rather than free calcium to more
328 accurately mimic the calcification of the aortic valve tissue.
329
330 Mice represent a good source of VICs for the study of molecular mechanisms leading to aortic
331 valve calcification. However, keep in mind that VICs *in vitro* are not similar to VICs in living valves.
332 Another limitation is the fact that a pool of valves from 3–5 mice is needed to make a single cell
333 culture. The pool should be from littermate mice to minimize variations. In addition, experiments
334 should be performed in triplicate to confirm all findings. However, the use of the entire aortic
335 valve in the culture can alleviate this limitation. Nevertheless, these *in vitro* studies must be
336 validated in human tissue to strengthen the findings.
337
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377
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382

Figure

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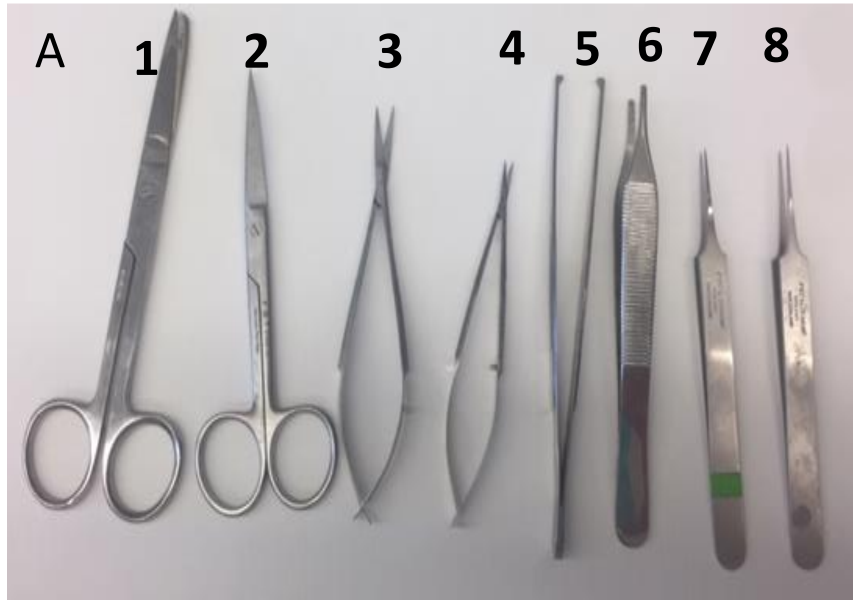
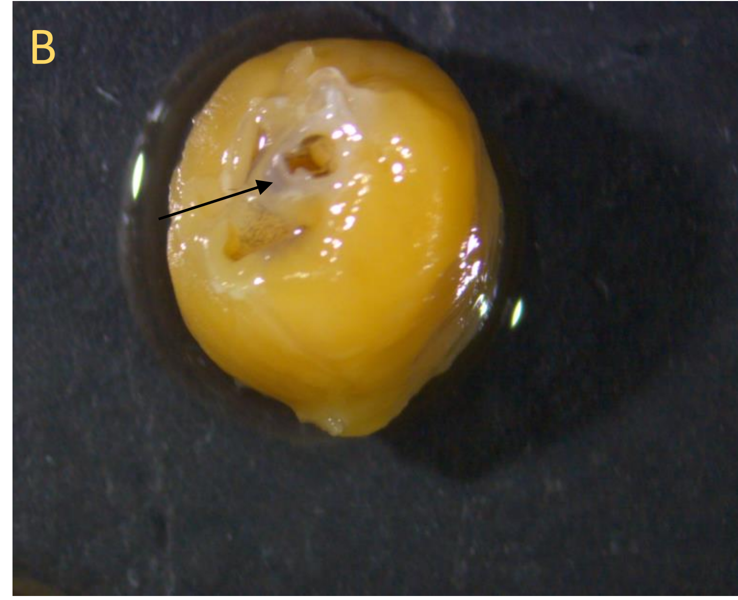
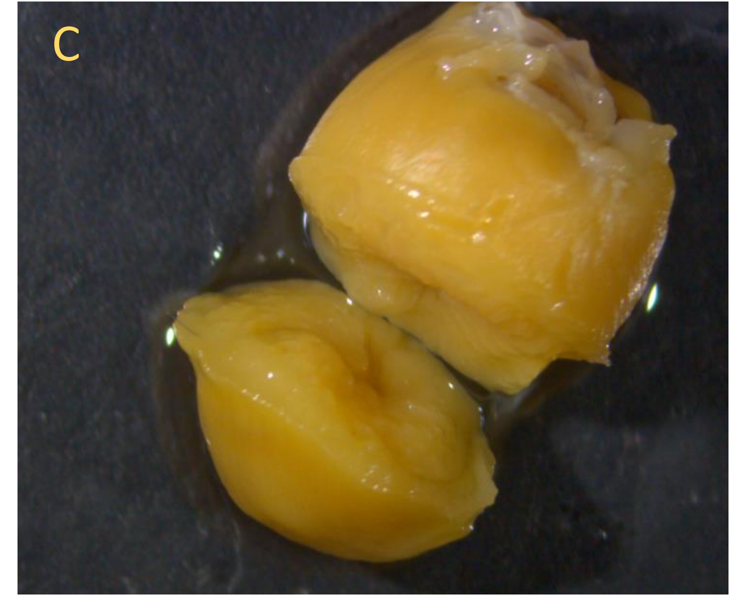
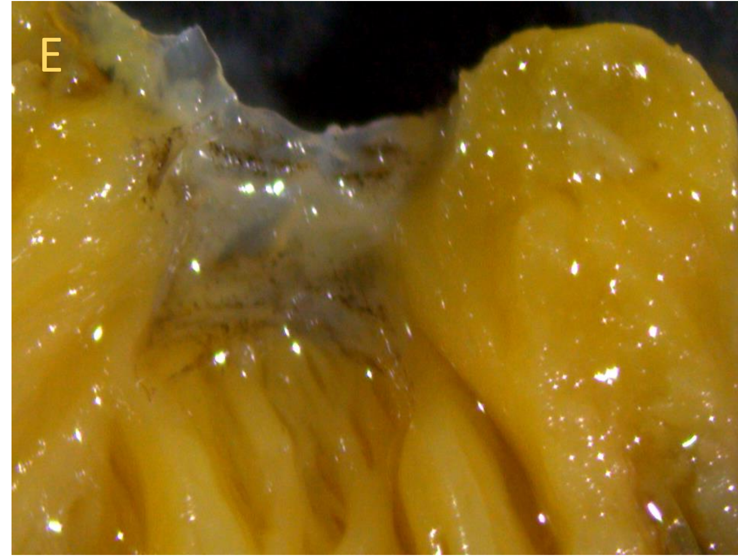
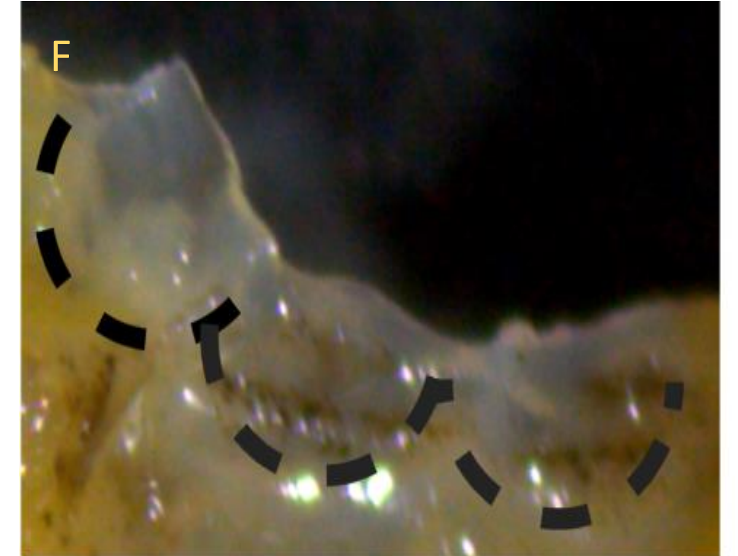
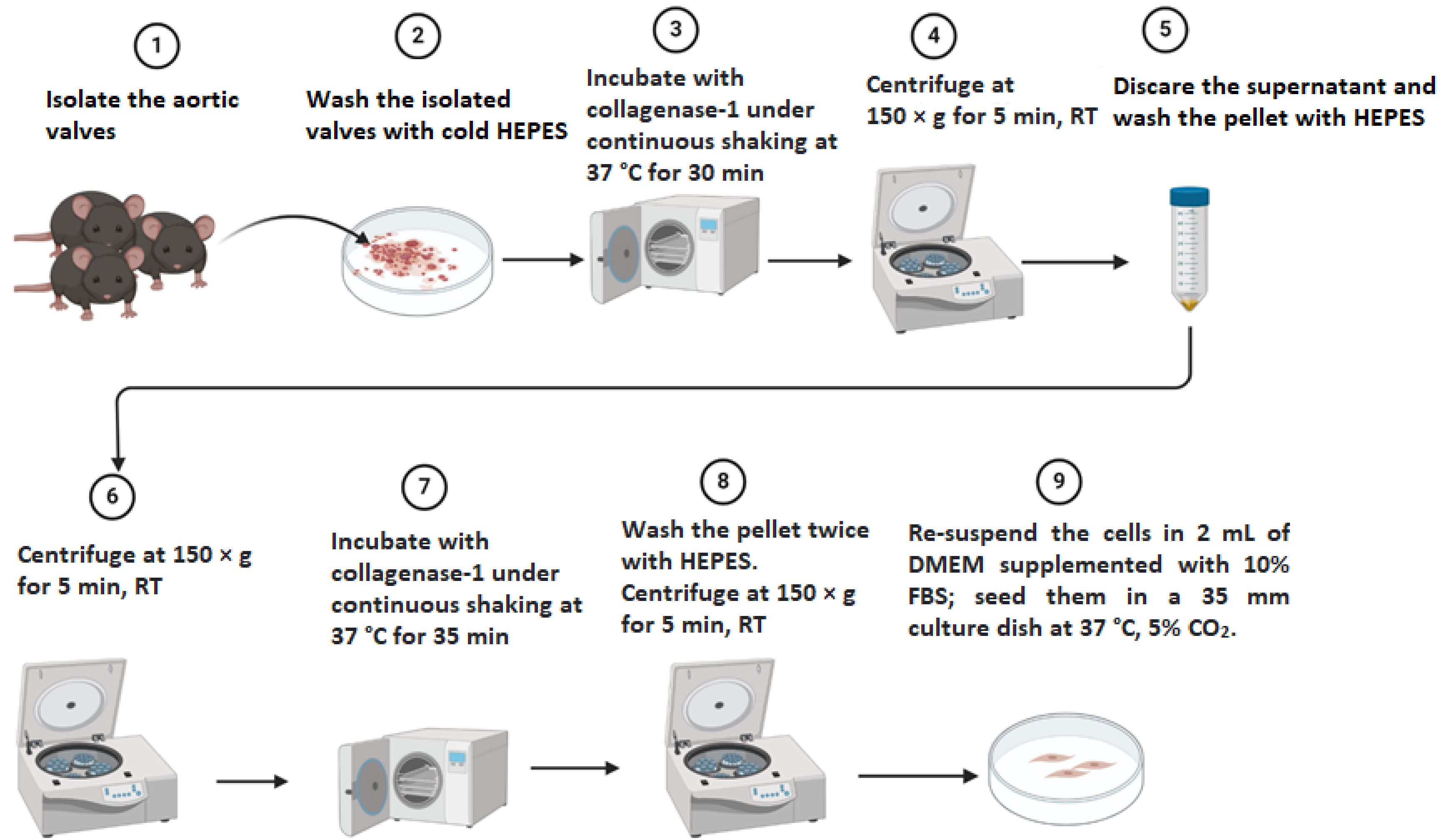




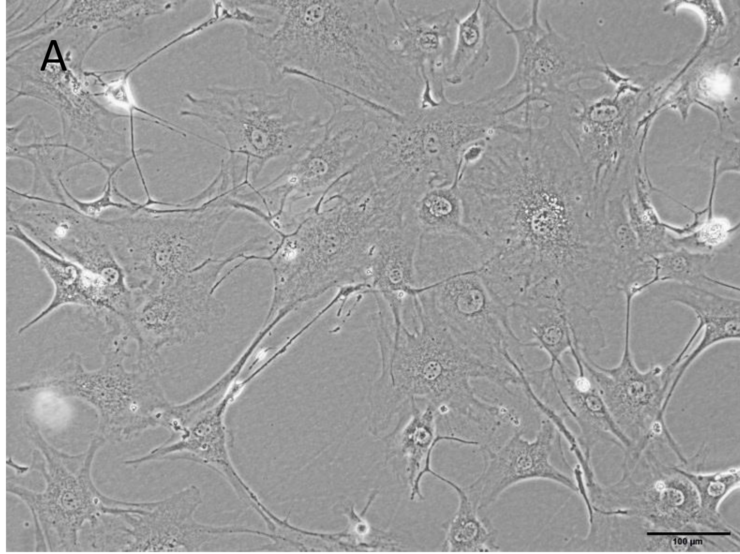








1
Isolate the aortic valves
2
Wash the isolated valves with cold HEPES
3
Incubate with collagenase-1 under continuous shaking at 37 °C for 30 min
4
Centrifuge at 150 × g for 5 min, RT
5
Discare the supernatant and wash the pellet with HEPES
6
Centrifuge at 150 × g for 5 min, RT
7
Incubate with collagenase-1 under continuous shaking at 37 °C for 35 min
8
Wash the pellet twice with HEPES. Centrifuge at 150 × g for 5 min, RT
9
Re-suspend the cells in 2 mL of DMEM supplemented with 10% FBS; seed them in a 35 mm culture dish at 37 °C, 5% CO_2.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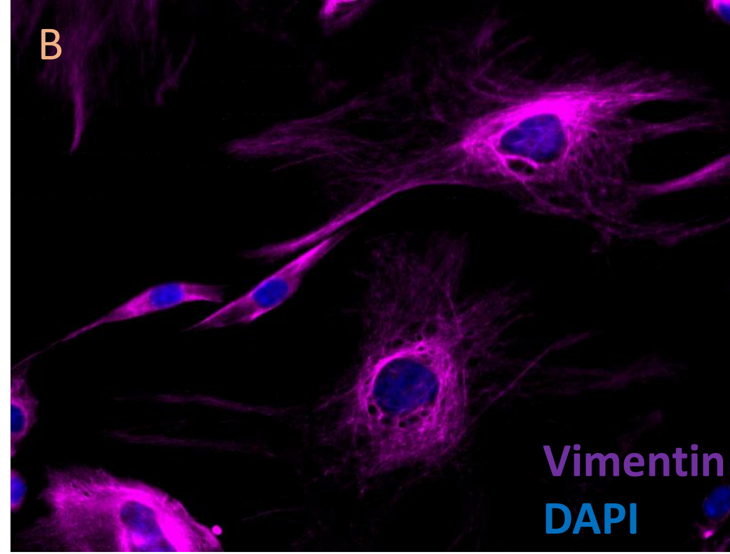
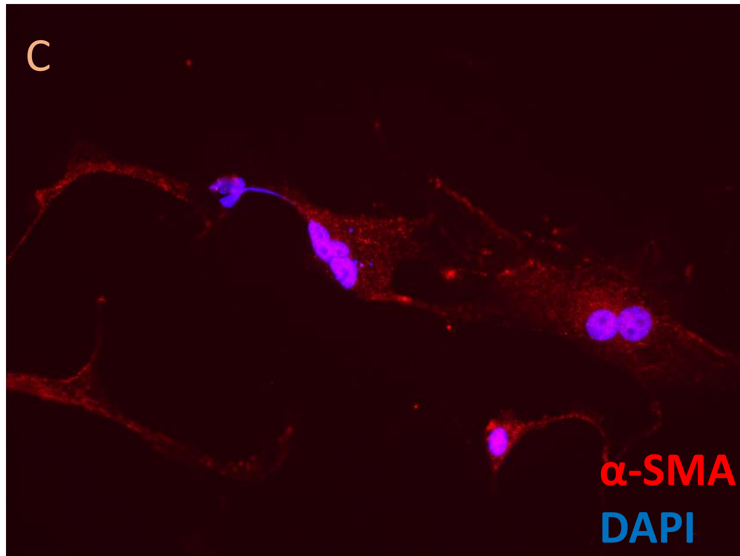
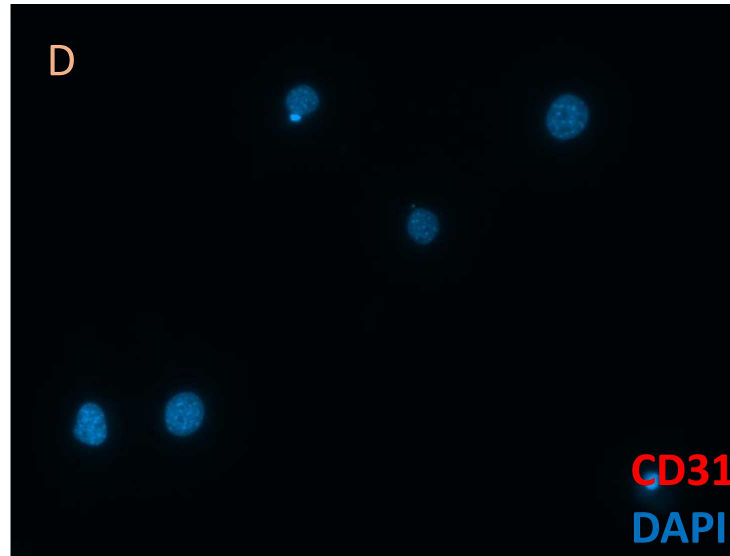
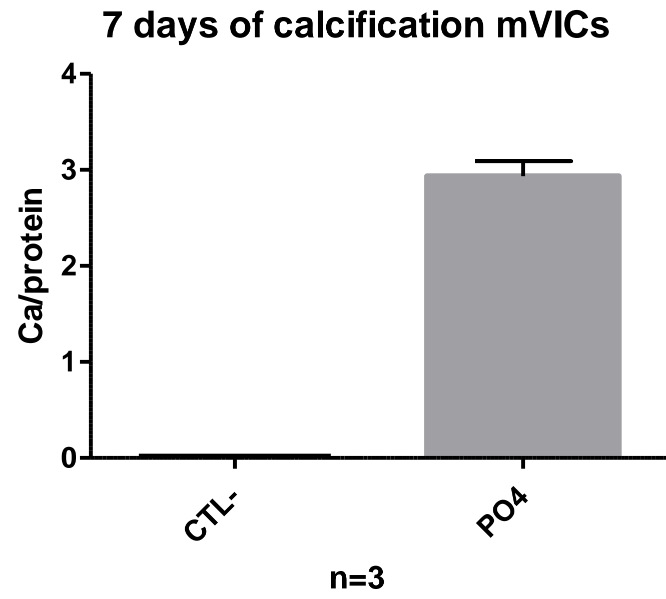

B
Vimentin
DAPI

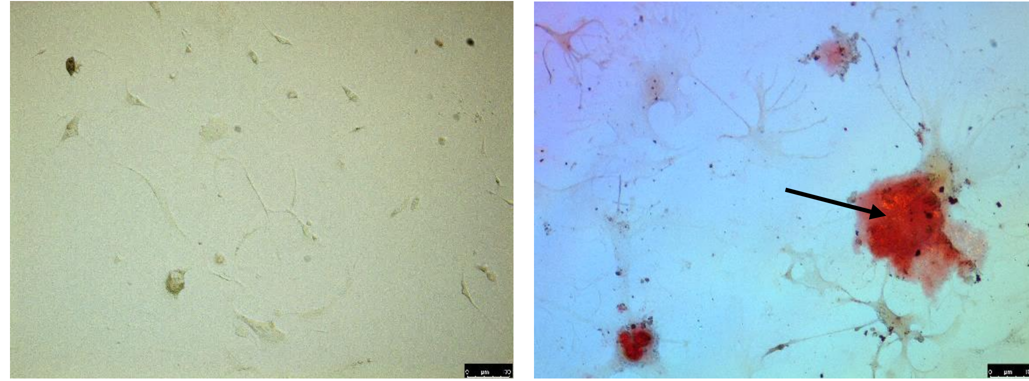

C
α-SMA
DAPI


D
CD31
DAPI

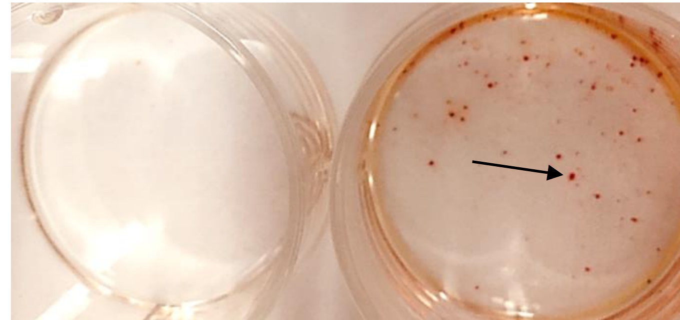
A



B



C



Click here to access/download;Table of Materials;62419 Table of Materials.xlsx

Equipment/reagent used	Brand name
3 mm cutting edge scissors	F.S.T
Anti-alpha smooth muscle Actin antibody	abcam
Anti-mouse, Alexa Fluor 488 conjugate	Cell Signaling
Arsenazo-III reagent set	POINT SCIENTIFIC
Bonn Scissors	F.S.T
Calcium hydroxide	SIGMA -Aldrich 31219
CD31	Novusbio
Collagenase type I (125 units/mg)	Thermofisher Scientific
DMEM	Tthermofisher
Extra fine graefe forceps	F.S.T
FBS	Gibco 16000044
Fine forceps	F.S.T Dumont
HCl	SIGMA-ALDRICH
HEPES 1 M solution	STEMCELLS TECHNOLOGIES
L-Glutamine 100x	Thermofisher Scientific
Mycozap	Lanza
PBS 10x	SIGMA-ALDRICH
penecillin streptomycin 100x	Thermofisher Scientific
Sodium Pyruvate 100 mM	Thermofisher Scientific
Standard pattern forceps	F.S.T
Surgical Scissors - Sharp-Blunt	F.S.T
Trypsin 0.05%	Thermofisher Scientific
Vimentin	abcam

catalog number	Comments/description
15000-00	
4412	
C7529-500	a Kit to measure the concentration of calcium
14184-09	
31219	
17018029	
11965092	
11150-10	
H1758	
25030081	
VZA-2011	Mycoplasma elimination reagent
10378016	
11360070	
11000-12	
14008-14	
25300054	

rebuttal

Click here to access/download;Rebuttal Letter;Editorial comments v2.pdf

We thank you very much for the interest you gave to our manuscript and for the interesting comments you provided. We have answered all the comments; the modifications in the text are highlighted in yellow.

The important sections for the video are in red.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise the following lines to avoid previously published work: 35-37, 44-45, 137-141. The sections were reviewed and I added the references.
3. Please provide an institutional email address for each author. Please revise the title to make it concise without any punctuation marks. We have adjusted the title accordingly
4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). all the personal pronouns were removed from the manuscript
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. I removed the commercial symbols from the text
For example: Zeiss, Eppendorf, Arsenazo-III, POINT SCIENTIFIC, etc. Arsenazo is a common name for a chemical and not commercial
6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. I added a statement about the ethic before starting the description of the protocol
7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. I adjusted the numbering as required
8. Line 57-61: 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. (Examples: What is the volume or concentration of isoflurane used. Is the animal revived after the surgery? If not, why is the animal anesthetized and not euthanized?) I added Figure 1 with image description for valve dissection. In the main manuscript more details were added and they are highlighted in yellow.
9. Line 68/76/81: 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 (Line: 68,76,81,109, 108,110, 123, 142, etc.). I corrected the unites as described
10. Line 81/101: For time units, please use abbreviated forms for durations of less than one day when the 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. The units were corrected as required
11. Line 101-104/114: Please provide all the details necessary to replicate the experiment. What is the volume of PBS used for washing? I added the washing volume in each step.
12. Line 113/115: Please specify the volume of the primary and the secondary antibodies used. I used 50 ul of diluted antibody per sample. The modification is in the text as well.
13. Line 136-141: 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.
14. Please include a one-line space between each protocol step and 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. The important steps for the video are highlighted in red.
15. Please move the Figure Legends section to the end of the Representative Results Section. Please upload each figure individually to your editorial manager account. Modifications were performed
16. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. I changed the structure of the bibliography to meet the requirement of eh journal.
17. Figure 2: Please revise the figure to include figure labels to make the figure more informative. Please ensure that the description in the figure legend is based on the figure label. Please include scale bars in all the figures of the panel. Please define the scale bar and provide the details of the magnification in Figure Legends. Scale bar is 200 um. I added the details in the legend
18. Figure 3: Does the Y-axis in Figure 2A require units? If so, please include the units within the parenthesis. Please capitalize the Figure labels. There is no unit, because it is a ratio of mg/ml of calcium by mg of proteins
19. Please sort the Table of Materials in alphabetical order.

____

Reviewers' comments:

Reviewer #1:

We thank you for the comments that will help the reader to better understand the protocol.

Manuscript Summary:
The authors describe a method to isolate murine valvular interstitial cells, propagate them under cell culture conditions, and characterize them by immunocytochemistry and in an in vitro calcification assay.

Major Concerns:
1. Please start section A of the Protocol part (line 56) with a list of solutions that should be prepared before the procedure starts. Specify the precise composition of all ingredients (as absolute concentration or percentage) and the volume needed for the entire procedure as well as the use of the solutions (wash, culture, etc.). Define a name for each solution and use the name throughout the manuscript. Indeed, I added a section with the name of the solutions and I highlighted them in yellow. This will improve the precision of the technical language and help to make the protocol easier to understand. Do you use HEPES as 1 M solution as stated in the materials list on the last page of the manuscript or at a dilution? I thank you for this important question; yes I forgot to put the dilution. Now I corrected. The HEPES should be diluted to have a final concentration of 10mM

2. Lines 70-73: This step is critical for the procedure. It would be helpful to prepare a set of graphical delineations as Figure 1B (with the present Figure 1 of the workflow as subfigure 1A) to explain where and in which order the cuts of the heart should be set. Give approximate dimensions on the delineations. Thank for the interesting comment, I added new figure 1 with valve dissection details.
3. Line 76: It is not clear how the collagenase solutions at different concentrations should be prepared. "1 mg/ml and 4.5 mg/ml in 5 ml DMEM and 5 ml HEPES" could mean a total volume of 5 ml each in the respective solvents or a total volume of 10 ml each in a 1:1 mix. See comments under point 1. Also, the Notes in lines 77-78, lines 83-84, and lines 98-99 can be avoided with a proper materials list. Each solution is a mixture of 2.5 ml of HEPES and 2.5ml of DMEM. More details were added to main manuscript.
4. Lines 85-86: Does this step require one wash only or one wash, centrifugation, a second wash, and another centrifugation? Please specify what to expect in the solution and pellet, respectively (tissue/endothelial cells). It's only one wash, please see the main manuscript with the required modifications.
5. Lines 87-88: Please specify the time of incubation. What do you mean by "under continuous agitation"? Please specify the shaker and the detailed settings used. Is agitation also needed during the first collagenase digest (lines81-82)? Is a centrifugation step needed at the end, as the next step starts with a re-suspension. The agitation is needed during the incubation of both collagenase solutions. Since this article will be accompanied with a video, the reader will better understand this step.
6. Line 91: Please specify whether the pellet should contain pieces of tissue or cells. at this stage the pellet will have both tissue and cells
7. Instead of using weak phrases like "good number of cells" (line 57) or "Murine aortic valve are tiny" (line 155), please use precise technical language and specify the approximate size of a murine aortic valve cusp. Also, please specify the approximate number of cells that you usually obtain after the 2-day incubation (line 93) and at 70% confluency (line 96). This will help users to troubleshoot if problems may occur. We adjusted the number of cells. However, it's just indicative because it may vary from a user to another one and it is also dependent on the number of mice used for the cell culture.
8. Line 100: Please specify that you used immunocytochemistry as a method to characterize the cells. Also, as compared to e.g. flow cytometry, this method is not so well suited to characterize the "purity" (lines 100, 105) of the cells but rather characterizes the morphology and identity of the cells. we thank you for the comment, in this paper I described mainly cell isolation protocol. Aortic valve tissue is mainly endothelial layer and interstitial cells. We use both western blotting and immunocytochemistry to identify the cells. However, the FACS could be used to purify a specific cells.
9. Lines 105-106: Do you use antibody against CD34 as stated or against CD31 as described in the materials list on the last page of the manuscript? Please specify at which concentration you used the respective antibodies. CD31 was the antibody used.
10. The calcification assay is a very basic method used in the field of aortic valve research. In section C of the Protocol part (line 122) you describe in detail the individual steps of a calcification assay from a specific company (Point Scientific). Similar calcium assays are available from other companies (e.g. Calcium Colorimetric Assay from Sigma Aldrich, #MAK022-1KT). Therefore, I don't see the rationale as to why promoting this specific product. Furthermore, a wide array of calcification assays has been described by now using different detection methods (vis microscopy, absorbance, fluorescence, near infrared fluorescence, etc.) and target analytes (free calcium vs. hydroxyapatite).

It would be more beneficial for this manuscript and helpful for the field to give here an overview of the different assay variants with the respective references and advantages/disadvantages of the respective methods. This may be provided instead of or in addition to the method and results described. We wanted just to clarify that we don't have any specific preference for the company. The Arsenazo III could be prepared in the lab. We are interested to the calcification of the cell membrane as a mimic to the in vivo calcification of the artic valve. As described in the protocol, this reagent doesn't interfere with magnesium. We have tried several method and we figure out that Arsenazo is the most accurate method.

11. In the Discussion part of the manuscript you should elaborate more on species specific characteristics of VICs (as already started with lines 191-194), stressing in particular the advantages and disadvantages of murine VICs in the study of human disease. Furthermore, why do you use and describe here a tissue culture and "out-growth" method instead of a full tissue digestion and straining of cells as used e.g. for the isolation of human VICs? Mouse cells are more sensitive to the collagenase; longer exposer might affect the cells. After 35 minutes we will have isolated cells, we keep the issue to ensure isolating all the cells.

Reviewer #2:

Manuscript Summary:

This method is of potential used by interested audience and worth publishing.

Major Concerns:

As the tile states, the method should focused on the isolation of mouse interstitial valve cells. We thank you for you interest to our paper and for all your comments. We have corrected the title accordingly

Minor Concerns:

The late steps of the isolation of interstitial valve cells need more detailed information such as the culture volume, anticipated initial cell density in 35 mm dish and expansion ratio, etc.

Figure 1 should eliminate the pictures of instruments and instead make a detailed flowchart for the steps of isolation. Adding photos and microscopic images of the final dissection of valve tissue and low magnification images of good quality fresh isolated cells and after passage will be helpful.

We have added a new figure 1 with valve dissection details. In addition, we will record a video of all the steps of the isolation.