FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 62419
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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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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   Yes

Authors: Please use your microscope camera to film the SCOPE shots and upload the video files to your project page: **https://www.jove.com/account/file-uploader?src=19037843**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 16
Number of Shots: 34

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Rihab Bouchareb:** The use of isolated mouse valve cells is essential to investigate the signaling pathway leading to valve calcification and the use of genetically modified cells from transgenic mice.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.10.2 for 'mouse valve cells'*

1.2. **Rihab Bouchareb:** The two steps digestion is a quick and efficient method.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Rihab Bouchareb:** Isolated cells can be used to test pharmacological targets to inhibit or decelerate the mineralization of the aortic valve.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B-roll: 3.4.2 'mineralization of aortic valve'*

1.4. **Rihab Bouchareb:** The most challenging part of this protocol is the isolation of the aortic valve from 8 weeks mice. It is better to practice on older mice to visualize the aortic valve better.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.4.5 for 'isolation of the aortic valve'*

Ethics Title Card

1.5. Procedures involving animal subjects have been approved by Icahn School of Medicine at Mount Sinai institutional care and use committee.

Protocol

2. Isolation of valve cells

2.1. Before starting the experiment, clean and sterilize all the surgical instruments and workspace with 70% ethanol **[1]** and autoclave the surgical instruments for 30 minutes **[2]**.

2.1.1. WIDE: Establishing shot of talent sterilizing surgical instruments and workplace.

2.1.2. Talent placing instruments into an autoclave.

2.2. When the initial setup is ready, start with cleaning the chest and the abdomen region of an 8-week-old euthanized mouse using ethanol **[1]**. Using scissors, open the abdomen and the chest of the mouse **[2]**, then cut between the left atrium and the left ventricle with small surgical scissors **[3]**.

2.2.1. Talent cleaning abdomen of mouse.

2.2.2. Talent cut opens the abdomen and the chest of the mouse.

2.2.3. Talent giving a cut in the heart.

2.3. Remove blood from the heart by perfusing 10 milliliters of cold PBS **[1]** and cut the heart, keeping 3 millimeters of the ascending aorta **[2]**.

2.3.1. Talent perfusing the heart with PBS.

2.3.2. Talent cutting the heart.

2.4. Under a stereomicroscope **[1]**, dissect the aortic valve by cutting the heart horizontally in the middle of the ventricles **[2]** and cutting the left ventricle towards the aorta **[3]**, then carefully dissect the aortic valve **[4]**. Pool the valves together in a small 35 millimeter-tissue culture dish **[5]**.

2.4.1. Talent working with a stereomicroscope.

2.4.2. SCOPE: Talent cutting the heart. NOTE: Authors were supposed to upload the SCOPE shots but haven't at the time of postshoot processing. They were reminded.

2.4.3. SCOPE: Talent cutting left ventricle.

2.4.4. SCOPE: Talent dissecting the aortic valve.

2.4.5. Talent placing valves in culture dish. *Videographer: This step is important!*

2.5. Wash the isolated valves in a 75-millimeter cell culture dish with 5 milliliters of freshly prepared 10-millimolar cold HEPES supplemented with antibiotics **[1]** and incubate in 5 milliliters of collagenase type one for 30 minutes at 37 degrees Celsius with continuous shaking **[2-TXT]**. *Videographer: This step is important!*

2.5.1. Talent washing the valves.

2.5.2. Talent placing tubes on incubator-shaker. **TEXT: Collagenase 1 mg/mL** NOTE: Split into 2 shots A/B

2.6. After incubation, centrifuge the tube for 5 minutes at 150 × *g* **[1]** and wash the pellet once with 2 milliliters of 10 millimolar HEPES with vortexing at high speed for 30 seconds **[2]**.

2.6.1. Talent placing tube in centrifuge and closing the lid. Videographer NOTE: 2.6.1 and 2.6.3 were filmed one after each other as I did multiple takes at the centrifuge

2.6.2. Talent adding HEPES to pellet and vortexing the tube. *Videographer: This step is important!*

2.7. Collect the resultant suspension from the tube into a 35-millimeter culture dish **[1]** and use thin tweezers to carefully transfer the tissue fragments into a fresh tube **[2]**.

2.7.1. Talent collecting re-suspended pellet into culture dish.

2.7.2. Talent transferring tissue fragments using tweezers.

2.8. Then, incubate the pellet in a 15-milliliter tube with 5 milliliters of collagenase type one at 37 degrees Celsius under continuous agitation for 35 minutes **[1-TXT]**. Separate the cells by re-suspending with a 1-milliliter pipette **[2]** and centrifuge at 150 × *g* for 5 minutes at 4 degrees Celsius **[3]**.

2.8.1. Talent placing the tube on a shaker. **TEXT: Collagenase type I 4.5 mg/mL**

2.8.2. Talent re-suspending cells.

2.8.3. Talent placing tubes in centrifuge and closing the lid.

2.9. Clean the cells twice by re-suspending the separated pellet in 2 milliliters of complete DMEM and centrifuging at 150 × *g* for 5 minutes at 4 degrees Celsius **[1]**.

2.9.1. Talent re-suspending the pellet in DMEM.

2.10. For plating, re-suspend the pellet in 1 milliliter of complete medium **[1]** and add it to one well of a 6-well cell culture dish in a minimum amount of medium **[2]**. Keep the plates undisturbed at 37 degrees Celsius with 5% carbon dioxide **[3]**.

2.10.1. Talent re-suspending pellet in 1mL medium.

2.10.2. Talent adding cell suspension to well.

2.10.3. Talent placing plates in an incubator.

2.11. After 3 days of incubation, confirm growth close to the tissue debris by observing the cells under the microscope **[1]**. Once 1,000 cells are visible, carefully remove the tissue debris with autoclaved tweezers and change the medium **[2]**.

2.11.1. Talent observing culture under the microscope.

2.11.2. Talent removing tissue debris.

2.12. Trypsinize the cells after reaching 70% confluency **[1]** and transfer them to a 75-milliliter tissue culture dish **[2]**.

2.12.1. Talent adding trypsin to the cells.

2.12.2. Talent transferring cells to the culture dish.

3. In-vitro Calcification Assay

3.1. Before beginning the assay, clean the biosafety hood with 70% ethanol **[1]** and warm the DMEM medium to 37 degrees Celsius **[2]**.

3.1.1. Talent cleaning the hood.

3.1.2. Shot of DMEM in a suitable container.

3.2. Next, seed 100,000 cells per condition into 6-well plates in complete DMEM and culture at 37 degrees Celsius **[1]**.

3.2.1. Talent seeding cells into wells.

3.3. After 24 hours, replace the supernatant medium with the calcifying medium **[1]** and incubate the cells for seven days at 37 degrees Celsius, replacing the medium on the third day **[2]**.

3.3.1. Talent adding calcifying medium to plates.

3.3.2. Talent placing culture plates in an incubator.

3.4. After 7 days, treat the cells with Arsenazo III *('three')* reagent in a 96-well plate **[1]** and record the absorbance of the plate at 650 nanometers to calculate calcium concentration **[2]**.

3.4.1. Talent adding the reagent to the samples in a clear 96 well plate.

3.4.2. Talent recording readings.

Results

4. Results: Identification of Mouse Valve Cell Phenotype and Calcification Assay

4.1. In the representative analysis, various valve cell phenotypes were identified with Immunofluorescence staining **[1]** after 3 to 5 days of culture **[2]**.

4.1.1. LAB MEDIA: Figure 3.

4.1.2. LAB MEDIA: Figure 3 A.

4.2. Mouse VICs expressed vimentin **[1-TXT]** and alpha-SMA, the significant markers of valve cells **[2]**. Additionally, CD31 immunofluorescence staining verified no contamination from endothelial cells in VIC culture **[3]**.

4.2.1. LAB MEDIA: Figure 3 B. **TEXT: VIC: Valvular interstitial cells**

4.2.2. LAB MEDIA: Figure 3 C.

4.2.3. LAB MEDIA: Figure 3 D.

4.3. Culturing VICs with phosphate-rich calcifying medium stimulated calcification in cells **[1]**, further confirmed with the red positive staining for calcium nodes **[2]**.

4.3.1. LAB MEDIA: Figure 4 A.

4.3.2. LAB MEDIA: Figure 4 A, C. *Video Editor: emphasize black arrow from images in the right panel*

Conclusion

5. Conclusion Interview Statements

5.1. **Rihab Bouchareb:** The protocol is based on a pool of 3 to 5 valves from different mice. The use of littermates and three different biological replicates is important to validate the findings.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.1 for 'valves from different mice'*

5.2. **Rihab Bouchareb:** The use of mouse valve cells is vital to understand the molecular pathway leading to aortic stenosis by isolating cells from transgenic mice. This protocol has been used previously to investigate the implication of P2Y2R in mineral regression of calcified aortic valve.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.4.2 for 'mouse valve cells' and 2.4.4 for 'aortic valve'*