Isolating Smooth Muscle Cells from Mouse Aorta

Adapted from protocol provided by Rupande Tripathi (rnt4h@cms.mail.virgina.edu) in Dr. Gary Owens Lab

Reagents

1. Collagenase Type II (Worthington Biochemical LS004174)
2. Elastase (Worthington Biochemical LS002279)
3. Soybean Trypsin Inhibitor (Worthington Biochemical LS003570)
4. HBSS (with calcium and magnesium)
5. DMEM/F12 medium (Gibco 11320)
6. Penicillin/Streptomycin
7. Fetal Bovine Serum

Enzyme Solution [make fresh the day of isolation]

1. 50 mg collagenase to a final concentration of 1 mg/ml
2. 50 mg soybean trypsin inhibitor to a final concentration of 1 mg/ml
3. 50 ml HBSS
4. ~0.354 ml elastase to a final concentration of 0.744 units/ml
5. 500 ul pen/strep to a final concentration of 1%
6. Mix well by pipetting 4-5 times
7. Filter with a low protein binding syringe filter

Media

1. 1X DMEM/F12: 400 ml
2. Penicillin/Streptomycin: 5 ml (1% final)
3. Fetal Bovine Serum (bought heat inactivated): 100 ml (20 % final)
4. Filter

Set Up

1. Generally the isolation is more successful if the mice are < 10 weeks old [3-4 week mice are preferred by Owens lab]
2. Put 4 ml enzyme solution into each of 2 60 mm tissue culture dishes. 2 aortas can go into a single dish. Warm and equilibrate with 5% CO_2 at 37^{o}C in the incubator.
3. Put 4 ml of 20% DMEM/F12 medium into each of 1 60 mm tissue culture dishes/aorta set. Warm and equilibrate with 5% CO_2 at 37^{o}C in the incubator.
4. 5 ml of HBSS into 1 15 ml conical tube/ 2 aortas

Aortic Retrieval

5. Isolation is most successful if the aortas are retrieved from 2 mice.
6. Asphyxiate mouse with CO_2 for 2 min
7. Spray the mouse with 70% ethanol
8. The surgery is done under the dissecting lamp with a magnifier.
    a. Open the mouse without cutting any blood vessels to avoid bleeding.
    b. Collect the blood with a 1 ml syringe with 26 G⅜ needle through a cardiac puncture.
    c. Perfuse the heart through the apex with 1 ml 1X sterile HBSS.
    d. Remove the organs, except heart, to allow a clear view of the aorta

e. Move to microscope
f. Remove the fat tissue around the aortic region
g. When the vessel is free of the fibrous material and fat make two cuts, one below the arch, and one at the proximity of the diaphragm.
h. Place the aortas into the HBSS in 15 ml conical and keep them in a cool place.

Removal of adventia and endothelial layer

9. Remove aortas from 15 ml conical.
10. Rinse quickly in clean HBSS.
11. Place the 2 aortas into a dish with enzyme solution.
12. Incubate 37°C for 8-10 minutes.
13. Remove the aortas from the enzyme solution and place into a 60 mm dish with equilibrated DMEM/F12 media to wash off the enzymes.
14. Strip off the adventitia under the dissection microscope by holding one end of the media with one forceps and strip off the adventitia with another forceps. It will unroll like a sock**.**
15. Open the aorta longitudinally with scissors
16. Remove any blood clots
17. Remove the endothelial cell layer by gently scrapping the inside of the vessel with a forceps.
18. Place cleaned vessel into second 60 mm dish with equilibrated DMEM/F12 while working on the remaining aortas

Final Digestion

19. Place aortas into a new dish of enzyme solution and incubate at 37^{o}C in 5% CO_2 in the incubator for about an hour, or until the vessels look like they are dissolved and cells seems to be floating.
20. After an hour the triturate the cells with a fire polished Pasteur pipette (15x)
21. Wash plate with 4 ml warmed DMEM/F12 media
22. Collects cells 1.5 RPM 5 min
23. Carefully remove all but 1 ml media
24. Wash cells with 5 ml warmed DMEM/F12 media
25. Collects cells 1.5 RPM 5 min
26. Remove all but 1 ml media
27. Add 1.5 ml equilibrated DMEM/F12 media
28. Plate 2 aortas into 3 wells of a 48 well dish (0.75 ml per well)
29. Leave undisturbed for 1 week and then replace media
30. Cells are weaned into 10% FBS media after passage 3-5 depending on their viability

Subcultivation

31. Ideally cells should be split when ~95 % confluent.
32. Remove cell culture medium.
33. Wash cells twice with warm 1X PBS.
34. Add 0.1 ml trypsin to each well of a 48 well plate
    [0.05% trypsin-0.53 mM EDTA in HBSS (Cellgro 25-052-Cl)]
35. Incubate for ~ 1 min at 37°C.

36. The older and more confluent the SMCs are the longer one needs to incubate them with trypsin to get efficient detachment. Thus, it is important to microscopically monitor cell detachment (~ 80 % of cells should be detached) and increase incubation time if necessary.
37. Mechanically detach remaining cells by rapping the plate against the palm of your hand.
38. Add 0.35 ml DMEM/F12 media containing 10% FBS (at least an equal amount of cell culture medium containing 10% FBS is required to neutralize the Trypsin-EDTA).
39. Resuspend cells by gently pippeting and pool all wells together. If necessary wash each well with 0.25 ml DMEM/F12 media.
40. Count and plate cells as desired.
41. Leave undisturbed for 2 days and then replace the media.
42. Change media at least twice a week.

Passaging

43. Plate freshly isolated cells into 3 wells of a 48 well plate
44. P1 = split into 6 wells of a 48 well plate
45. P2 = split into 12 wells of a 48 well plate
46. P3 = split into 24 wells of a 48 well plate divided onto 3 plates
47. P4 = split into 60 wells of a 48 well plate divided onto 6 plates
48. P5 = at this point you usually have enough cells to count and do experiments
49. In order to get the most use out of your explants divide each into two waves and plate such that they are ready to use on opposite weeks:
    - To use the cells in 1 week plate 30,000 cell per well of 48 well plate
    - To use the cells in 2 weeks plate 15,000 cells per well of 48 well plate
    - To use the cells in 1 week plate 500,000-1,000,000 cells onto a 100 mm dish
    - To use the cells in 2 week plate 200,000-500,000 cells onto a 100 mm dish
    - This of course in a generalized count, each explant will grow differently so you will need to determine the exact number to plate empirically
50. Always maintain a portion of the cells on 48 well plates even if the majority of the cells are on 100 mm dishes because the large dishes are significantly more prone to contamination than the small cluster well plates.
51. Cells can be used until P10-12. You can tell when they are no longer usable because the growth slows significantly and they become more spread out.