# Isolation of mouse primary aortic endothelial cells by selection with specific antibodies

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Running head: Mouse endothelial cell culture.

**Summary**

Endothelial cells (ECs) are key blood-vessel-wall components that play critical roles in the regulation of many physiological processes, including angiogenesis, coagulation and vascular tone control, and in pathological events such as vessel inflammation and leukocyte infiltration. EC dysfunction is one of the first events associated with the development of atherosclerosis and is sustained throughout progression of the disease. The study of ECs *in vitro* has become an invaluable tool for investigating these vascular processes at the molecular level, and is widely used in the search for therapeutic targets and strategies. This chapter describes a protocol for the isolation and culture of primary mouse aortic ECs based on antibody-mediated EC selection.

**Key words**

Endothelial cells, isolation, culture, antibody-mediated selection, mouse aorta, atherosclerosis.

## 1. Introduction

The endothelium is the thin cell layer that faces the blood-vessel lumen and is in direct contact with flowing blood. The key functional components of any vascular endothelium are the endothelial cells (ECs). First described as a simple passive barrier, the endothelium is now viewed as a complex and active participant in the regulation of diverse vascular processes, including inflammation and leukocyte infiltration *(1)*

vascular tone *(2)*, and coagulation *(3, 4)*. In the particular case of atherosclerosis, risk factors such as hypercholesterolemia, diabetes and smoking trigger an inflammatory response that causes the accumulation of dysfunctional ECs, characterized by the expression of adhesion molecules that promote leukocyte recruitment to the artery wall *(5)*. This endothelial dysfunction is the first step in atherogenesis, preceding the formation of fatty streaks, and is sustained throughout disease progression, thus contributing to the eventual generation of gross atheromatous plaques *(6)*. Since the endothelium is also involved in the regulation of vascular tone, EC dysfunction may contribute to atherosclerosis development by increasing blood pressure, a well-known proatherogenic risk factor.

Today, a variety of approaches for studying EC function and its relation to vascular disease are available, ranging from *ex-vivo* systems such as wire and pressure myography *(7)* to highly sophisticated genetically-modified mouse models in which gene expression in the endothelium is specifically disrupted or augmented *(8-13)*. However, *in vitro* studies with primary EC cultures remain an essential tool for investigating critical processes in EC pathobiology at the molecular level (e.g. proliferation, migration, apoptosis, angiogenesis, release of vasoactive molecules, expression of adhesion molecules, etc). Here we describe a simple procedure for obtaining ECs from mouse aorta based on selection for specific markers.

## 2. **Materials**

1. Young mice (8-12 weeks old) (e.g. C57BL/6J).
2. Surgical material: fine forceps (e.g. Dumont Medical Biology Forceps, FST Cat. No. 11254-20) and scissors (e.g. Extra Fine Bonn Scissors, FST Cat. No. 14084-08).

3. Anesthetics: Ketamine (e.g. Imalgene 1000, Merial) and Medetomidine (e.g. Medeson, Urano). Alternatively, $CO_2$ chamber.
4. Phosphate-buffered saline (PBS): 8.0 g/L NaCl, 0.2 g/L KCl, 1.44.g/L $Na_2HPO_4$ in distilled water.
5. Saline solution: 0.90% (w/v) NaCl in distilled water.
6. Stereo microscope (e.g. Olympus SZX10).
7. DMEM-F12 medium (e.g. Lonza, Cat. No. BE04-687Q).
8. Heparin sodium salt (heparin) from porcine intestinal mucosa (e.g. Sigma, Cat. No. 9041-08-1).
9. Fetal bovine serum (FBS, e.g. Thermoscientific, Cat. No. SV30160.03) inactivated by heating at 56ºC for 30 minutes.
10. Penicillin and streptomycin (Pen/Strep) solution for culture media (e.g. Pen/Strep stock 10K/10K, Lonza, Cat. No. DE 17-602E).
11. L-glutamine (e.g. Lonza, Cat. 17-605C).
12. EC growth factor (ECGF) (e.g. Sigma, Cat. No. E2759).
13. EC medium: DMEM-F12 supplemented with 1% Pen/Strep, 0.4mM L-glutamine, 10 mM heparin and 50 μg/mL ECGF.
14. HEPES (1M stock in normal saline; e.g. Lonza, Cat. No. 17-737E).
15. 0.25% Trypsin/EDTA 1X (e.g. Gibco, Cat. No. 25200-072).
16. Anti-mouse-CD102 antibody (e.g. rat anti-mouse-CD102, BD Pharmigen, Cat. No.553326).
17. Secondary antibody bound to magnetic beads (e.g. Dynabeads® sheep anti-rat IgG, Life Technologies, Cat. No. 110.35).
18. Magnet (e.g. DynaMag™-15 Magnet, Life Technologies, Cat. No 12301D).
19. 24-well culture plates.

20. Matrigel (e.g. Corning, Cat. No 354234).
21. Gelatin (e.g. Sigma, Cat. No. G9382).
22. Collagen (e.g Sigma, Cat. No C8919).
23. Gelatin coating solution: PBS supplemented with 0.5% gelatin and 0.1mg/ml collagen.
24. Ice, fridge, or cold-room.
25. Three-dimensional laboratory shaker.

## 3. Method

1. Euthanize five to eight mice using any ethically approved method (see Note 1).
2. Place the mice in *decubitus supine* position and dissect them to remove the thoracic aorta (see Note 2 and Figures 1A and 1B).
3. Place the aortas in cold PBS or saline solution (see Note 3 and Figure 1C).
4. Under a stereo microscope, use fine forceps to carefully remove all fat tissue from the aorta (see Note 4 and Figure 1D).
5. Dissect aortas longitudinally with a scalpel to obtain 0.5-0.8-mm-wide strips (see Figure 1E).
6. In sterile conditions (see Note 5) place the aortic strips lumen-face down in the wells of a 24-well culture plate coated with unset Matrigel. Incubate the plates in a humidified cell culture incubator at 37ºC until the Matrigel solidifies (at least 2 h) (see Notes 6 and 7).
7. Cover the wells with 2 mL of EC medium and culture the aortic strips for a minimum of one week (see Note 8 and Figure 1F). When cell colonies are formed (see Figure

1G), remove the remaining aortic tissue from the well and collect the cells as follows:

- Wash the attached cells with PBS at least three times.
- Add trypsin and incubate at 37ºC to detach the cells (usually 5 min).
- Block trypsin activity by adding EC medium supplemented with 10% FBS, and pellet the cells by centrifugation at approximately 200xg for 5 min.

8. Resuspend the cells in EC medium containing 10% of FBS and plate them in gelatin-coated plates (see Note 9). Incubate the cells to expand the culture (see Note 10).
9. Select the ECs from the culture as follows:

- To each well add anti-CD102 antibody (3.5 µL/mL EC medium) and incubate for 30 min at 4ºC in in a shaker set at low velocity (see Notes 11 and 12).
- Remove the medium containing the anti-CD102 antibody and wash the cells 2-3 times with PBS.

10. To each well add EC medium containing an appropriate secondary antibody linked to magnetic beads (e.g., anti-rat IgG if using rat IgG anti-mouse CD102 as primary antibody). Secondary antibody should be diluted 3.5 µL/mL EC medium. Incubate for 30 min at 4º C (see Note 13).
11. Wash the cells with PBS (2-3 times) and detach them with trypsin (as described in step 7).
12. Use a magnet to retrieve cells bound to magnetic beads.
13. Wash the ECs retained on the magnet with PBS and culture them in EC medium in gelatin-coated plates (as described in step 8).
14. Expand the ECs culture as necessary (see Note 14).

## 4. Notes

1. For this procedure several euthanasia methods can be used, for example, $CO_2$ inhalation or chemical anaesthetics overdose. Cervical dislocation carries a significant risk of damaging the aorta and therefore of losing useful material for EC extraction.

2. The abdominal aorta can also be used for endothelial cell isolation, but its extraction and cleaning requires additional time that prolongs the protocol. The duration of the procedure strongly influences extraction efficiency and EC viability, and therefore the shorter the dissection and cleaning time the better the result.

3. In many dissection procedures mice are transcardially perfused with buffer to flush out blood from organs and tissues. However, this procedure can damage or even remove the endothelium, and therefore for EC extraction this step should either be omitted or performed with extreme care.

4. For this procedure, it is important that the aorta be dissected as cleanly as possible from the beginning; this avoids excessive handling in subsequent steps and thus increases final cell viability. To maintain endothelium integrity during cleaning, it is important to avoid pinching the aorta as much as possible.

5. Working in sterile conditions is recommended throughout the procedure in order to avoid contamination of the cell culture. Unfortunately, sometimes this is not possible due to technical limitations, especially during aorta isolation and cleaning.

6. Prepare the coated plates ahead of time by chilling them on ice and covering the wells with a solution of 200 μg/ml of cold Matrigel; this will solidify upon incubation at 37ºC in the cell culture incubator once the aortic strips are added.

7. Usually the strips derived from 2-3 aortas are pooled together in one well. Correct attachment of the aorta strips to the well surface is critical for the success of the procedure. Therefore try not to shake the culture plates accidentally when placing them in the incubator or transporting them back to the culture hood.

8. EC medium should be changed at least once every 3 days and always with extreme care to avoid detaching the artery explants. Cell colonies should start to appear before day 7; however, due to technical variability, this can take up to 2 weeks.

9. ECs need to be cultured on gelatin-coated plates. The plates can be prepared as follows: pour gelatin coating solution (see item # 23 in Materials) into each well to cover the surface (0.5 ml per well for a 24-weel plate), place the plates for at least 1 hour at 37ºC in a cell culture incubator, and remove the leftover of gelatin by aspiration just before using the plates.

10. ECs are not the most abundant cell type in the aorta, and their proliferation rate is relatively low compared with other cells from the same blood vessel. To ensure a

reasonable yield of ECs at the end of the procedure, it is advisable to expand the culture as much as possible before selection. However, it is also important to consider that the other cell types present in the culture can colonize the plate before ECs. In our hands, a good quantity of ECs is obtained by expanding the cells until 60-70% confluence before selection.

11. ECs can be selected with different antibodies (e.g. CD102, CD31, CD106, or CD146) with similar results. Before choosing an antibody, check that it is optimal for your particular set of experiments. The procedure described here is based on selection with an antibody to CD102, which is specifically expressed in ECs and leukocytes. However, this antibody may not be appropriate if you do not work in an inflammatory context, because expression of CD102 is very low in aorta in basal conditions.

12. Although low temperatures can be harmful to cultured cells, we have found that the final yield of viable ECs increases if the incubation with the specific primary antibody is performed at 4ºC. This temperature improves recognition of the specific antigen at the cell surface and avoids loss of efficiency due to antibody internalization.

13. In addition to selection with magnetic beads as described here, many other systems are available for selection of a specific cell population from a mixture of cell types, for example, use of fluorescent-labeled antibodies followed by flow cytometry sorting.

14. With prolonged time in culture, primary ECs undergo processes of cell senescence and dedifferentiation, and therefore should not be used for in vitro studies after more than 7-8 passages.

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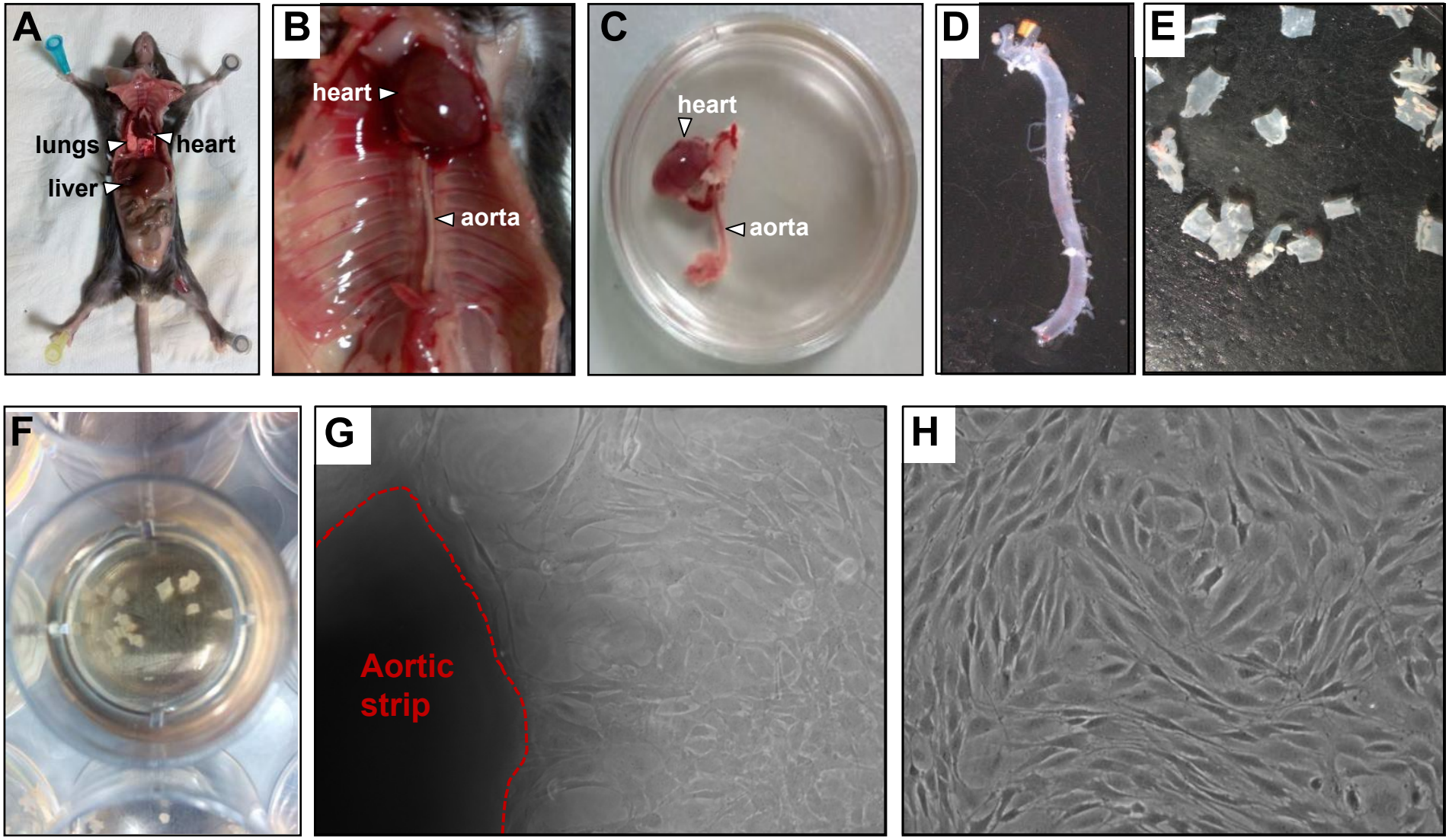
## Figure legends

**Figure 1. Steps in mouse aortic endothelial cell extraction and culture.**

**A.** Dissection of the animal to expose the thoracic and abdominal cavities. **B.** Isolation of the aorta and heart in the thoracic cavity. **C.** Petri dish containing aorta and heart in saline solution or PBS. **D.** Cleaned aorta. **E.** Aortic strips in a Petri dish. **F.** Aortic strips in Matrigel-coated plates. **G.** Migration of primary cell from an aortic strip (outlined by the red dashed line) onto the surface of the culture plate. **H.** Confluent EC monolayer.

**Figure 1**


A
lungs
heart
liver
B
heart
aorta
C
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