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Isolation and Culture of Human Endothelial Cells from Micro- and Macro-vessels

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Methods in Molecular Medicine

Angiogenesis Protocols III

Isolation and culture of human endothelial cells from micro- and macro-vessels

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ABSTRACT

The endothelium from different vascular beds exhibits a high degree of phenotypic heterogeneity. Endothelial cells (EC) can be easily harvested from large vessels by mechanical removal or collagenase digestion. In particular, the human umbilical vein has been used due to its wide availability, and the study of ECs derived from it has undoubtedly greatly advanced our knowledge of vascular biology. However, the majority of the body's endothelium (>95%) forms the microvasculature and it is these cells providing the interface between the blood and tissues that play a critical role in the development of new blood vessels. This has led to the establishment of techniques for the isolation of microvascular ECs (MEC) from different tissues to provide more physiologically relevant *in vitro* models of angiogenesis and EC function.

In this chapter the use of superparamagnetic beads (Dynabeads) coated with anti-PECAM-1 (CD31) antibodies ('PECA-beads') to culture MECs from human adipose tissue is described along with the standard methods used to characterise them. Adipose tissue is an ideal source of MECs as it is composed mainly of adipocytes with a very rich microvasculature, and is easy to disaggregate. Furthermore, it can be obtained in large quantities during plastic surgery procedures. Adipose obtained at reduction mammoplasty or abdominoplasty is first dissected free of connective tissue, minced finely and subjected to collagenase type-II digestion. The adipocytes are removed by centrifugation to obtain a microvessel rich pellet, which is further disaggregated with trypsin/EDTA solution. Following filtration to remove fragments of

connective tissue the pellet is incubated with PECA-beads and microvessel fragments/ECs washed and harvested using a magnet. In addition, the adaptation of this basic technique for the isolation of human lung and stomach MECs is also described along with common methods for the preparation of large vessel endothelial cells.

Key words: endothelial cells, microvascular, adipose, Dynabeads, PECAM-1/CD31, von Willebrand Factor, E-selectin.

1. Introduction

Human ECs derived from large vessels including the aorta, and umbilical (1) and saphenous veins have proven an abundant and convenient tool for the investigation of many aspects of endothelial biology. However, the endothelium demonstrates a high degree of functional, morphological, biochemical and molecular diversity between organs, and within the different vascular beds of a given organ (2-6). This phenotypic heterogeneity has highlighted the need for reliable techniques for MEC isolation and culture from a variety of tissues in order to establish more realistic *in vitro* models.

Many techniques have been developed to enrich ECs from tissue homogenates, either directly or after a period in culture (6). Most methods use as their starting point tissue homogenisation and digestion and are often hampered by low EC yield and problems of contaminating cell populations that readily adapt to culture. Some tissues are inherently better suited to MEC isolation such as brain and adipose, which have high microvascular densities, and can be disaggregated easily (2, 6-9). Wagner and Matthews (7) were the first to utilise adipose tissue from the rat epididymal fat pad for the isolation of microvascular endothelium. The major advantage of this tissue is the difference in buoyant densities between the adipocytes and stromal component allowing their separation by centrifugation.

The development of superparamagnetic beads (Dynabeads™) coupled to

endothelial-specific ligands represented a major advance in the purification of MECs from mixed cell populations. The original technique described by Jackson and colleagues (10) employed the lectin *Ulex europaeus* agglutinin-1 (*UEA-1*), which binds specifically to α -fucosyl residues of EC glycoproteins. However, *UEA-1* also binds to some epithelial and mesothelial cells (1,11). We refined this technique by coupling antibodies raised against platelet EC adhesion molecule-1 (PECAM-1/CD31) (12), a pan-endothelial marker (3,4), to Dynabeads ('PECA-beads'), and have used these to prepare MECs from various human tissues (13,14). Similarly, other endothelial markers, including CD34 have been used to isolate MECs. However, the majority of EC markers, including PECAM-1 and CD34 cross-react with subpopulations of haematopoietic cells which share common developmental origins. However, haematopoietic cells do not usually represent a significant problem for MEC isolation due to their limited viability in culture. Mesothelial cells exhibit similar morphology and share common markers with ECs and so represent a potentially difficult contaminant of EC cultures isolated from tissues surrounded by a serosal layer, such as omentum and lung. (6,11). However, the absence of constitutive PECAM-1 expression in these cells means that use of PECA-bead selection should eliminate mesothelial cell contamination of endothelial isolates (11).

In this chapter we describe in detail the use of PECA-beads to isolate MECs from human adipose tissue and methods for the routine culture of these cells. In addition, the adaptation of this purification technique for the culture of MECs from

human lung and stomach (13,14) is provided alongside routine methods for the preparation of large vessel endothelial cells from the umbilical vein and aorta.

2. Materials

2.1. Solutions for MEC isolation and culture

All solutions should be warmed to 37°C prior to use.

1. *10% BSA solution*: Dissolve 10 g of bovine serum albumin (BSA) in 100 ml of calcium-magnesium free Dulbecco's phosphate buffered saline (PBS/A), 0.22 µm filter sterilise and store at 4°C.
2. *Antibiotic/antimycotic solution*: Dilute 100x antibiotic/antimycotic solution (Sigma) in PBS/A to give final concentration of 100 U/ml Penicillin, 0.1 mg/ml Streptomycin and 0.25 µg/ml Amphotericin B. Aliquot and store the 100x stock solution at -20°C.
3. *Collagenase solution*: Dissolve type-II collagenase (Sigma) at 2000 U/ml in Hank's balanced salt solution (HBSS) containing 0.5 % (w/v) BSA, 0.22 µm filter sterilise, aliquot and store at -20°C.
4. *Trypsin/EDTA solution*: Dilute 10x stock solution of porcine trypsin (2.5%) in PBS/A, and add 1 mM (0.372 g/L) ethylenediaminetetraacetic acid (EDTA). 0.22 µm filter sterilise, aliquot and store at -20°C.
5. *Gelatin solution*: Dilute stock (2%) porcine gelatin solution in PBS/A to give

- a 0.2% (v/v) solution and store at 4°C. To coat tissue culture dishes add 0.2% gelatin solution and incubate for 1 h at 37°C or overnight at 4°C. Remove the gelatin solution from the flasks immediately prior to plating the cells.
6. *Growth medium:* Many different growth media have been described for maintaining EC in culture (see **Note 1**). Supplement M199 (with Earle's salts) with 14 ml/l of 1 M N-[2-hydroxyethyl] piperazine-N'-[2-hydroxypropane] sulphonic acid (HEPES) solution, 20 ml/L of 7.5% sodium hydrogen carbonate solution, 20 ml/L 200 mM L-glutamine solution and mix 1:1 with Ham F12 nutrient mix. To 680 ml of medium M199/Ham F12 solution add 20 ml of Penicillin (100 U/ml) / Streptomycin (100 µg/ml) solution, 1500 U/L of heparin, 300 ml of iron-supplemented calf serum (CS) (see **Note 2**), 1 µg/ml hydrocortisone, 5 ng/ml basic fibroblast growth factor (bFGF/FGF-2) and 20 ng/ml epithelial growth factor (EGF) (PeproTech EC Ltd, London, UK) (see **Note 3**). Store growth medium at 4°C.
 7. *Cryopreservation Medium:* Growth medium containing 10% (v/v) tissue culture grade dimethyl sulphoxide.
 8. *Dispase solution:* Dissolve 2/U ml dispase in Medium M199 containing 20% CS, 0.22 µm filter sterilise, aliquot and store at -20°C. *Note:* This is only required for the isolation of human lung MEC.

2.2. Preparation of PECA beads.

Mix 0.1-0.2 mg of mouse anti-PECAM-1 monoclonal antibody (e.g. clone 9G11 R&D Systems) in sterile PBS/A containing 0.1% BSA (PBS/A+0.1% BSA) per 10 mg of Dynabeads-M450 (Invitrogen Dynal AS - see **Note 4**) pre-coated with pan anti-mouse IgG₂ (see **Note 5**). Incubate on a rotary stirrer for 16 h at 4°C. Remove free antibody by washing four times for 10 min, and then overnight in PBS/A+0.1% BSA. PECA-beads maintain their activity for more than 6 months if sterile and stored at 4°C. However, it is necessary to wash the beads with PBS/A + 0.1% BSA to remove any free antibody prior to use.

2.3. Equipment for EC isolation

A class II laminar flow cabinet is essential for all procedures involving the handling of tissue and cultured cells to maintain sterility and protect the operator. Scalpels, scissors and forceps are required for the isolation procedures and should be sterilised by autoclaving at 121°C for 30 min.

100 µm nylon filters: Cover the top of a polypropylene funnel ~ 10 cm with 100 µm nylon mesh and sterilise by autoclaving (see **Note 6**).

Magnet: A suitable magnet is required for the magnetic cell selection system employed which accepts 15 ml tubes (see **Note 7**).

Disposable sterile plastics

1. 25 and 75 cm² tissue culture flasks
2. Large plastic dishes (e.g. Bioassay dishes, Nunc, Naperville, IL, USA)
3. 30 ml universal tubes
4. 50 ml centrifuge tubes
5. Lab-Tek multiwell glass chamber slides (Nunc, Naperville, IL, USA)
6. 20 ml luer-lock syringes.
7. luer-lock three-way stopcocks

2.4. *Antibodies for EC characterisation*

There are many commercial antibodies available against endothelial markers.

1. Monoclonal antibodies against human PECAM-1, E-selectin and vWF (e.g. clone F8/86; Dako, High Wycombe, Bucks, UK).
2. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibodies.
3. *Nuclear counterstain*: Dissolve Hoescht 33342 (Sigma) in PBS/A 10 µg/ml to give a 10 µg/ml solution, aliquot and store at -20°C.

3. Methods

3.1. *Isolation and culture of human adipose MECs*

3.1.1. Collection of tissue

A suitable large sterile container is required for the collection of adipose tissue

obtained during breast or abdominal reductive surgery (see **Note 8**). The fat can be processed immediately or stored for up to 48 h at 4°C.

3.1.2. *Isolation of adipose MECs*

1. Working under sterile conditions in a class II cabinet, place the tissue on a large sterile dish (e.g. Bioassay dish, Nunc) and wash with 2% antibiotic/antimycotic solution. Avoiding areas of dense (white) connective tissue (that are often prevalent in breast tissue) and visible blood vessels, scrape the fat free from the connective tissue with two scalpel blades.
2. Chop the fat up finely and aliquot 10-20 g into sterile 50 ml centrifuge tubes. Add 10 ml of PBS/A, and 5-10 ml of the type-II collagenase solution. Shake the tubes vigorously to further break up the fat and incubate with end-over-end mixing on a rotary stirrer at 37°C for approximately 1 h. Following digestion the fat should have broken down and no spicules should be evident.
3. Centrifuge the digests at 500 g for 5 min, discard the fatty (top) layer and retain the cell pellet with some of the lower (aqueous) layer. Add PBS/A, and re-centrifuge 500 g for 5 min.
4. Re-suspend the cell pellet in 10% BSA solution and centrifuge (200 g, 10

- min). Discard the supernatant and repeat the centrifugation in 10% BSA solution. Wash the pellet with 50 ml of PBS/A. Viewed under the light microscope the tissue digest should contain obvious microvessel fragments in addition to single cells and debris.
5. Re-suspend the pellet obtained in 5 ml of trypsin/EDTA solution and incubate for 10-15 min with occasional agitation at 37°C. Add 20 ml HBSS containing 5% CS (HBSS+5%CS) and mix thoroughly to neutralise the trypsin. We have found it advantageous to break up the microvessel fragments and cell clumps further with trypsin/EDTA as this reduces the number of contaminating cells co-isolated with the ECs during PECA-bead purification.
 6. Filter the suspension through 100 µm nylon mesh to remove fragments of sticky connective tissue. Centrifuge the filtrate at 700 g, for 5 min and re-suspend the resulting pellet in ~1-2 ml of ice-cold HBSS+5%CS.
 7. Add approximately 50 µl of PECA-beads and incubate for ~20 min at 4°C with occasional agitation (see **Note 9**). Add HBSS+5%CS to a final volume of ~12 ml, mix thoroughly, and select the microvessel fragments / ECs using a suitable magnet (see **Note 7**) for 3 min. Repeat the cell selection process a further 3-5 times by washing the magnetically separated material in ~12 ml of HBSS+5%CS and reselecting the

microvessel fragments with the magnet.

8. Suspend the magnetically separated cells in growth medium (see **Note 10**) and seed at high density onto 0.2% gelatin-coated 25 cm² tissue culture flasks and incubated at 37°C in a humidified atmosphere of 5% CO₂.

3.1.3. Adipose MECs in culture

Following the PECA-bead selection procedure small microvessel fragments and single cells coated with Dynabeads should be evident under light microscopy (see **Note 11**). After 24 h the cells adhere to the flasks and start to grow out from the microvessel fragments present to form distinct colonies. Human mammary microvessel EC (HuMMEC) isolated using this technique grow to confluence forming contact-inhibited cobblestone-like monolayers within 10-14 days depending on the initial seeding density. We have successfully cultured these cells to passage 8 without observable change in their morphology, but routinely use them in experiments between passages 3 to 6.

3.1.4. Subculture of adipose MEC

Maintain the MECs at 37°C, 5% CO₂ changing the medium every 3-4 days. When confluent EC can be passaged using trypsin/EDTA solution, onto 0.2% gelatin-coated dishes at a split ratio of 1:4 as follows.

1. Discard the old medium and wash the cell monolayer twice with 5-10 ml of PBS/A. Add a few ml of trypsin/EDTA solution, wash it over the cell monolayer, remove the surplus leaving the cells just covered and incubate at 37°C for 1-2 min. Monitor the cells regularly under the microscope until they round up and detach (see **Note 12**). Striking the flask sharply helps to dislodge the cells and break up cell aggregates.
2. Add sufficient growth medium to achieve a split ratio of ~1:4 and plate the cells onto gelatin-coated flasks.

3.1.5. *Microcarrier beads*

Microcarrier beads can be used to continuously culture EC without the need to use trypsin. Following hydration and sterilisation according to the manufacturer's instructions add the gelatin-coated Cytodex 3™ microcarrier beads (Sigma) to the medium and allow the EC to crawl onto and attach to the microcarriers. Agitate the flasks occasionally to facilitate seeding carriers over a period of 2-3 days. Remove the beads and place into a fresh gelatin-coated flasks containing growth medium and allow the cells to attach to the flask agitating occasionally to ensure good distribution of the cells. Once sufficient cells have attached the beads may be removed and placed into a fresh flask and the process is repeated.

3.1.6. *Cryopreservation of EC*: Following trypsinization (see **Subheading 3.1.4.**) Suspend ECs at ~ 2x10⁶/ml in the cryopreservation medium and dispense into

suitable cryovials. Cool the vials to -80°C at 1°C/min and store under liquid nitrogen.

3.1.7. Maintaining the purity of MEC cultures

It may be necessary to reselect the ECs with PECA-beads and/or perform minor manual 'weeding' to maintain the purity of cultures. Reselection with PECA-beads can be performed as described above (see **Subheading 3.1.2. Item 7**) following removal of the cells from flasks using trypsin/EDTA solution (see **Section 3.1.4.**). Provided that there are clear morphological differences between contaminating cell populations and the ECs (see **Note 13**) it is relatively straightforward, although time consuming, to physically remove them. Manual weeding should be performed with the stage of a phase contrast microscope within a class II cabinet to ensure sterile conditions. A needle or Pasteur pipette is used to carefully remove contaminating cells from around EC colonies. The medium is discarded and the adherent cells washed with several changes of sterile PBS/A to remove the dislodged contaminating cells.

3.2. Characterisation of ECs

3.2.1. EC morphology in culture

Cobblestone morphology is very typical of ECs derived from many tissues and they are usually readily distinguished from the typical fibroblastoid contaminating cell populations. However, a more elongated morphology has been reported for

human MEC derived from some tissues, and similar elongated phenotypes forming 'swirling' monolayers are often observed following stimulation of ECs with growth factors. EC will form "capillary-like" tube networks within few hours of plating on matrices such as growth factor-reduced Matrigel™. This phenomenon also occurs in many types of MECs if cultures are left for several days at confluence (**Figure 1**). However, the formation of "capillary-like" structures is not an exclusive property of ECs in culture.

3.2.2. Key EC markers

There are many criteria on which EC identification may be based which have been reviewed extensively (see 2-4,6). Many endothelial markers/properties are not unique to ECs and several may be required to confirm endothelial identity. ECs isolated from different vascular beds may also display phenotypic heterogeneity and lack of a particular marker may not preclude the endothelial origin of isolates (2). It is often useful to demonstrate the absence of markers characteristic of potential contaminating cell populations such as smooth muscle α -actin positive stress fibres and the intermediate filament protein, desmin, which are expressed by smooth muscle cells and pericytes (15).

A number of good endothelial markers have been identified, including endothelial cell adhesion molecule (ICAM-2 / CD102), endothelial cell-selective adhesion molecule (ESAM) and vascular endothelial cadherin (VE cadherin) (4). Here we focus on von Willebrand Factor (vWF), PECAM-1 (12), and E-selectin

(endothelial-leukocyte adhesion molecule-1 / CD62E) (16) that we believe to be useful for the rapid identification of ECs.

vWF is only expressed at significant levels in ECs, platelets, megakaryocytes, and the syncytiotrophoblast of the placenta. In ECs it is stored in the rod-shaped Weibel-Palade bodies, which produce characteristic punctate perinuclear staining. These organelles are present in large vessel EC but have been reported to be scarce or absent in the capillary endothelium of various species (1-4,6). However, typical granular perinuclear staining for *vWF* has been reported in cultured human kidney, dermis (10), synovium, lung (13), stomach (14), decidua, heart, adipose (9,13) and brain (8) MECs.

PECAM-1 is constitutively expressed on the surface of ECs ($>10^6$ molecules/cell), and to a lesser extent in platelets, granulocytes and a subpopulation of CD8+ lymphocytes (6,12). *PECAM-1* staining of ECs *in vitro* is characterised by typical intense membrane fluorescence at points of cell-cell contact (see **Figure 2**).

E-selectin: Strong expression of E-selectin following stimulation with pro-inflammatory cytokines appears to be a unique characteristic of ECs (13). It is not expressed constitutively by the majority of ECs, but stimulation with tumour necrosis factor- α (TNF α), or interleukin-1 β (IL-1 β) leads to intense E-selectin staining of the EC plasma membrane reaching a maximum after 4-8 hours (see **Figure 3**).

3.2.3. Immunocytofluorescent characterisation of ECs

Outlined below is a simple protocol for the immunofluorescent detection of EC markers.

1. *Preparation of ECs on glass slides.* Multiwell glass chamber slides are extremely useful for this purpose as multiple tests can be performed on the same slide conserving both reagents and cells. ECs are cultured on chamber slides that have been pre-treated for 1 h with 5 $\mu\text{g}/\text{cm}^2$ bovine fibronectin (Sigma) in PBS/A or 0.2 % gelatin. When sufficient cells are present discard the medium and wash twice with PBS/A prior to fixation. Different fixatives can be employed depending on the activity of the antibody used. Acetone fixation is suitable for most antibodies: Place the slides (see **Note 14**) in cold acetone (-20°C , 10 min), air dry and store frozen at -80°C . Alternatively, fix cells in 3.7 % formaldehyde solution for 30 min at room temperature. Formaldehyde does not permeabilise the plasma membrane and further treatment with 0.1 % Nonidet P-40 or Triton X-100 is required to detect cytoplasmic / nuclear antigens.
2. *Immunocytofluorescent staining.* Warm up slides to room temperature and wash with PBS/A (2 x 5 min). Block slides for 20 min with 10 % normal serum from the species in which the secondary antibody was raised to prevent non-specific binding of the secondary antibody.

3. Incubate slides with a predetermined or the manufacturer's recommended concentration of primary antibody in PBS/A for 60 min at room temperature.
4. Wash slides with PBS/A (3 x 5 min) and incubate with the appropriate FITC-labelled secondary antibody at 1:50 dilution in PBS/A for 30 min to 1 h at room temperature and protect direct light.
5. Counterstain cells with Hoescht 33342 (10 μ g/ml) in PBS/A for 10 mins to facilitate assessment of EC purity.
6. Wash slides in PBS/A (3 x 5 min), mount in 50 % (v/v) glycerol in PBS/A. Stained slides can be stored for several months in the dark at 4°C.

Controls: To avoid false positives generated by non-specific binding of secondary antibodies it is essential to include negative controls of cells treated as described above, but with an isotype-matched control antibody and/or PBS/A substituted for the primary antibody. It is also useful to include as controls other cell types such as fibroblasts, smooth muscle cells and previously characterised ECs to act as negative and positive control cells respectively.

E-selectin: Cells are incubated with 1-10 ng/ml TNF α or IL-1 β in growth medium for 4-6 h prior to fixation to induce E-selectin expression. Unstimulated cells

should be used as controls.

3.2.4. Other properties of human adipose MECs

These cells possess typical EC characteristics including scavenger receptors for acetylated low-density lipoprotein, expression of the transforming growth factor- β co-receptor, endoglin (CD105), and high levels of angiotensin-converting enzyme activity. All the EC types that we have cultured also express the vascular endothelial cell growth factor (VEGF) receptors Flt-1, Flt-4 and KDR/FIk-1 (15), and proliferate and express tissue factor in response to VEGF. Similarly, the angiotensin receptor, Tie-2/Tek and Tie-1 and are also expressed by these cells.

3.3. Isolation of MECs from other vascular beds

We have adapted the basic method for the selection of adipose MECs to isolate ECs from other tissues. Here we outline briefly the modifications that have been made for the isolation of human lung (12) and stomach MECs (18).

3.3.1. MECs from human lung

Although it has a high microvascular density, lung is composed of many diverse cell types that readily adapt to culture, and is generally more difficult to obtain than adipose tissue. We have successfully isolated EC from normal lung from transplant donors and diseased tissue from transplant recipients (13). To ensure that MECs are harvested a thin strip of tissue at the periphery of the lung is used. As the amount of tissue available is usually limited, and we have found that the

yield of cells following direct Dynabead selection is low, it is better to allow the cells to proliferate in culture for a few days and then perform the magnetic purification with PECA-beads before they became overgrown with contaminating cells.

1. Cut small peripheral sections of lung (3-5 cm long ~ 1 cm from the periphery) and wash in antibiotic/antimycotic solution.
2. Dissect the underlying tissue from the pleura and chop it up very finely between scalpel blades, or by using a tissue chopper.
3. Wash the minced tissue above sterile 20 μm nylon mesh (prepared as described under **Subheading 2.3.** - see **Note 6**) to filter out blood cells and fine debris.
4. Incubate the retained material overnight in dispase solution on a rotary stirrer at 37°C overnight.
5. Pellet the digest, resuspended in ~5 ml of trypsin/EDTA solution and incubate at 37°C for 15 min.
6. Add growth medium and remove fragments of undigested tissue by filtration through 100 μm nylon mesh.

7. Pellet and resuspended the cells in growth medium and plate onto gelatin-coated dishes.
8. Monitor the cultures daily, trypsinize and select the ECs using PECA-beads before they became overgrown by contaminating cells (see **Subheading 3.1.7.**).

3.3.2 *Human stomach MECs*

MECs can be cultured from stomach mucosa obtained from biopsies or organ donors (14).

1. Expose the stomach mucosa wash with antibiotic/antimycotic solution.
2. Dissect the mucosa from the from the underlying muscle, chop into 2-3 mm pieces, and incubate in 1 mM EDTA in HBSS at 37°C in a shaking water bath for 30 min.
3. Transfer the pieces of mucosa to collagenase type-II solution for 60 min, and then trypsin/EDTA solution for 15 min, in a shaking water bath at 37°C.
4. Using a blunt dissecting tool scrape the mucosa and submucosa from white fibrous tissue.

5. Suspend the mucosal tissue in HBSS+20% CS and wash through 100 μ m nylon mesh.
6. Centrifuge the filtrate (700 g, 5 min) and re-suspend the pellet in ~12 ml of HBSS+5%CS. Proceed with PECA-bead selection (see **Subheading 3.1.2. - Item 7**).

3.4. Human large vessel EC isolation

3.4.1. Preparation of HUVEC

The umbilical vein provides an abundant source of large vessel EC, which have been used extensively due to the availability of this post-natally redundant tissue and ease with which pure HUVEC preparations can be obtained. The procedure given below has been adapted from the method originally reported by Jaffe and colleagues (1).

1. Collect umbilical cords in a suitable covered container and store at 4°C (see **Note 15**).
2. Working over a large dish or tray to contain any spills, wash off any blood from the surface of the cord with antibiotic/antimycotic solution and inspect the cord very carefully for the presence of clamp marks and damaged areas, which must be avoided to prevent smooth muscle cell contamination. Cut a length (>20 cm) of cord free of clamp marks using cord scissors (see **Note 16**).

3. Cannulate the umbilical vein using a three-way disposable luer-lock stop-cock and secure using ligature. The umbilical vein with its larger and thinner walls is relatively easy to distinguish from the two narrow umbilical arteries surrounded by smooth muscle.
4. Attach a syringe to the stop-cock and wash the cord through gently with ~30 ml of PBS/A until all the residual blood is removed.
5. Perfuse the cord with ~5-10 ml of 0.1 % (v/v) type-I collagenase (e.g. CLS-Collagenase, Worthington) solution in HBSS (prepared as described for type-II collagenase – see **Subheading 2.1.**) carefully displacing any residual air, and clamp off the free-end.
6. Place the cord on a large plastic tray (e.g. Nunc bioassay dish) cover and place in a humidified incubator at 37°C for ~15 min.
7. Gently massage the cord and collect the collagenase solution from the vein into a 30 ml universal tube. Wash through with ~ 20 ml of HBSS+5% CS. Centrifuge at 200 g for 10 min and resuspend the pellet in growth medium (see **Note 1**) and plate cells onto gelatin-coated 25 cm² flasks. Under the phase contrast microscope sheets of ECs in addition to many single cells, red blood cells and debris should be apparent.
8. Allow cells to attach to the flask for at least 4 h, or overnight, and then wash the cells twice with PBS/A to remove non-adherent cells and debris. Add fresh growth medium and incubate the flask at 37°C, in a humidified 5% CO₂ incubator changing the medium every two-to-three days until the cells reach confluence. Passage the cells at a one-to-three ratio as described above (see **Subheading 3.1.4**). HUVEC de-differentiate quite rapidly in culture and we do not routinely use them beyond passage 4.

3.4.2. *Human aortic ECs*

A relatively simple method for the isolation of human aortic ECs (HAEC) is outlined below as described previously (18). It is also possible to physically remove the EC by gently scraping of the luminal surface of the aorta rather using collagenase digestion (see **Note 17**). This method can be directly applied to isolate EC from the aortas of other large mammals.

1. Wash sections of human thoracic aorta obtained at post-mortem are thoroughly in antibiotic/antimycotic solution.
2. Cut the vessel open longitudinally to expose the surface of the luminal endothelium and lay flat on a sterile petri dish. Add collagenase solution (see **Note 17**) over the exposed luminal surface, cover with lid, and incubate at 37°C for ~15 min. in a humidified incubator.
3. Wash the surface of the aorta with ~ 5 ml of HBSS+5% CS, drain and collect into a 30 ml universal tube. Centrifuge at 200 g for 10 min, resuspend the cell pellet in ~ 5 ml of growth medium and plate onto gelatin-coated T25 flasks and incubate in a 37°C, in a humidified 5% CO₂ incubator.
4. After 12-24 h discard the medium, wash twice with PBS/A (10 ml), and add fresh medium and subculture the cells as described above passaging with trypsin at confluence (see **Subheading 3.1.4.**).

4. Notes

1. Many different growth media have been described for the culture of ECs. Some MECs have very specific requirements such as the presence of

human serum, while large vessel ECs tend to be far less fastidious in their growth requirements. This medium M199/Ham F12 nutrient mix based recipe is relatively inexpensive and works well for a range of ECs, but researchers may wish to optimise their media further. MCDB 131 (19) containing the supplements described (**Subheading 2.1. - Item 6**) represents an excellent alternative and can be used with lower concentrations of serum. There are now also several commercial sources of optimised EC media that are based on MCDB 131. For example EGM™-1/2 and EGM™-MV-2 from Lonza (Lonza, San Diego, CA, USA) and although more expensive, they are very convenient and provide reliable support.

2. Iron-supplemented bovine calf serum (CS) provides an economical alternative to FBS and in our hands supports the proliferation and survival of the EC well (see **Note 1**). However, it is necessary to batch test all bovine sera to ensure optimal growth of ECs – some MECs such as those isolated from decidualized endometrium are reported to require the presence of human serum or specific growth factors such as VEGF (20).
3. We use recombinant FGF-2 and EGF routinely, but VEGF (5-10 ng/ml) can also be added and provides excellent support for the majority of cultured EC, but is more expensive. These growth factors may also be substituted with EC growth supplement (ECGS) derived from bovine brain, which is rich in acidic and basic FGF and can be obtained from

various suppliers.

4. There are alternatives to the use of Dynabeads, for example the Miltenyi Biotech MACS system. These use composite iron oxide and polysaccharide beads (50 nm diameter) and require a MACS separation system comprising a disposable filtration column which is placed in a magnetic field. They offer the advantage that they are small and so do not interfere with cell attachment and subsequent use of the cells in procedures such as flow cytometry.
5. Precoated Dynabeads (and CELLlection™ beads - see **Note 10**) carrying various secondary antibodies (e.g. pan anti-mouse) are available from Dynal and are very convenient. However, anti-immunoglobulin-coated beads can be prepared as follows: Incubate the secondary antibody (150 µg/ml) in 0.17 M sodium tetraborate buffer (pH 9.5; 0.22 µm sterile filtered) with tosyl-activated Dynabeads-M450 for 24 h on a rotary stirrer at room temperature. Wash the beads 4x for 10 min and then overnight in PBS/A+0.1% BSA on a rotary stirrer at 4°C before proceeding to coat them with the primary antibody as described (see **Subheading 2.2.**). Although we have found PECA-beads to be more reliable for purification of ECs (13) than using tosyl-activated Dynabeads directly coated with *UEA-1* (9).
6. There are now several commercial suppliers of sterile disposal cell strainers that provide a very convenient alternative.

7. The MPC-1 magnet that we have used for many years is no longer manufactured, but suitable alternatives are available from Invitrogen Dynal AS in the DynMag range which will accept 15 ml a to 50 ml tubes.
8. Omental adipose tissue obtained through general abdominal surgery can also be used. However, care should be taken to remove the fat from the omental membranes that are covered with a layer of mesothelium prior to dissection. Using PECA-bead selection we have not found mesothelial cell contamination of MEC cultures to be a problem.
9. The cell PECA-bead suspension is incubated at 4°C during the purification steps to minimise non-specific phagocytosis of Dynabeads.
10. We do not routinely remove Dynabeads following cell selection. However, it may be necessary to remove the Dynabeads if you are using the cells to perform techniques such as flow cytometry. CELLelection™ Dynabeads (Dynal) coated with the anti-PECAM-1 antibody can be used to select the MECs. In this system antibodies are conjugated to the Dynabeads via a DNA linker that can be cleaved with DNase-1 to release the beads from the cells following selection.
11. Dynabeads are internalised within ~24 h of selection and are diluted to negligible numbers/cell by the first passage, through cell division. Consistent with the original observations of Jackson and colleagues (10) using UEA-I-coated Dynabeads, we have not observed any adverse

effects on the adherence, proliferation or morphology of EC following PECA-bead selection (12).

12. To maintain cell viability it is important to rapidly remove the endothelial cells from the flasks as they are very sensitive to trypsin exposure. The use of a trypsin inhibitor to neutralise the tryptic activity immediately following detachment from the flask has been reported to prolong the viability of EC cultures. Mung bean trypsin inhibitor (Sigma) can be for this purpose, although we have not assessed its effect on endothelial viability.
13. The major contaminating cell population observed in unselected adipose MEC cultures demonstrate a distinct fibroblastic morphology.
14. The plastic wells must be removed from the multiwell chamber slides as the acetone will rapidly dissolve the plastic. Depending on the type used it may be possible to retain the gasket to make it easier to keep reagents on individual wells separate during the staining procedure.
15. In our hands storage of the umbilical cords at 4°C for up to 48 h after delivery does not appear to adversely affect the yield, or viability of isolated cells.
16. Specialised umbilical cord scissors can be obtained cheaply from a number of suppliers and make the task of cutting lengths of cord a lot easier and safer than using scalpels.
17. The luminal surface of the aorta can be simply scraped very gently to

remove the aortic EC. However, we have observed greater contamination of cultures with smooth muscle cells from the underlying intima using this approach compared with collagenase digestion. Following isolation of EC smooth muscle cells can be explanted from the aortic segments.

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Figure Legends

Figure 1 Photomicrograph of MEC isolated from human mammary adipose (at passage 2) demonstrating typical cobblestone morphology and tube formation on the surface of the post-confluent EC monolayer.

Figure 2 Immunofluorescent staining of platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) in human mammary adipose MEC.

Figure 3 Intense immunofluorescent staining of E-selectin (CD62E) detected in human mammary microvessel MEC following 6 h exposure to 10 ng/ml Tumour necrosis factor- α (TNF α), which is absent in unstimulated control cells (*inset*).