Isolation and Primary Culture of Various Mammalian Cells

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Abstract

Isolation and primary culture of four major mammalian cells comprise the major contents of this chapter. The cells described here are endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells. While endothelial cells are present only in the various micro- and macrovascular beds, smooth muscle cells and fibroblasts are present both in the vascular and nonvascular regions of a mammalian body. However, epithelial cells are present in the epithelium, located exclusively in the nonvascular regions. The epithelium is the thin tissue layer or

multiple layers of epithelial cells, forming the inner or outer surface of organs, glands, mouth, nostrils, trachea, alveoli, ducts lumen of mammary glands, the lining of the alimentary canal, and urinary bladder. This chapter focuses on the following basic aspects of the isolation and culture of these mammalian cells: (1) the basic concept of these cells; (2) locations of the body at which these cells are present; (3) various instruments and materials are needed to isolate these cells; (4) various isolation and culture procedures of these cells; (5) phenotypic and protein maker-based identification of these cells; and finally, (6) utility to culture prospects of these cells.

Keywords

Isolation of cells from tissue/organs · Importance of Mammalian cell primary culture · Primary culture of cells · Endothelial cells · Vascular smooth muscle cells · Fibroblasts · Epithelial cells · Phenotypic identification of cells · Marker protein-based identification of cells

1 Introduction

This chapter describes the isolation and primary culture of various mammalian cells. Cells for primary culture originate directly from organs and are recovered either via mechanical disruption or enzymatic dissociation. Access to fresh mammalian organs or organ-derived tissues is required for isolation and primary culture of cells. A major benefit of primary cultures is that the cells maintain most of their innate characteristics and normal physiological functions, particularly during early passages. The basic approach for isolation, characterization, and primary culture of vascular endothelial cells, vascular smooth muscle cells, vascular and nonvascular fibroblasts, and epithelial cells is described in this chapter. While large blood vessels are made up of endothelial cells (in the tunica intima), smooth muscle cells and fibroblasts prevail in the tunica media and tunica adventitia, respectively, epithelial cells are present particularly in the lining organs of mammals. Besides epithelial cells, smooth muscle cells and fibroblasts are exclusively present in the nonvascular regions of the body.

The following points focused on describing the isolation and culture of each of the abovementioned cell types: a basic concept of each of these cells, that is, endothelial, smooth muscle, fibroblasts, and epithelial cells; the major vascular and nonvascular regions of the mammalian body at which these cells are located; various instruments and other materials necessary for the isolation and culture of these cells; phenotypic and other marker protein-dependent identification of these cells; different culture procedures of these cells as described by various scientists; and finally, utility of culturing these cells. The content of this chapter would prove a valuable database for strengthening an understanding of the isolation and culture of mammalian cells, both from vascular and nonvascular origin.

2 Major Types of Mammalian Cells Used in Primary Culture

For isolation of mammalian cells, fresh organs and tissues are collected. Generally, cells are isolated from various tissues and organs either by enzymatic or mechanical dissociation. Following isolation from various tissues and organs, cells are subjected to culture in a CO_2 incubator.

In this chapter, the isolation and (primary) culture of the following cells are discussed:

Isolation and primary culture of vascular endothelial cells
Isolation and primary culture of vascular smooth muscle cells
Isolation and primary culture of vascular and nonvascular fibroblasts
Isolation and primary culture of epithelial cells

3 Isolation and Primary Culture of Vascular Endothelial Cells

3.1 Endothelial Cells

- Endothelial cells are a thin layer of squamous cells forming the interior surface (lumen) of blood vessels (artery–capillary–vein), lymphatic vessels, bone marrow, and the interior surface of the heart (endocardium).
- Endothelial cells originate from epithelial cells, which in turn are of mesodermal origin.
- The term **endothelium** refers to endothelial cells that line the interior surface of blood vessels (vascular endothelial cells) and lymphatic vessels (lymphatic endothelial cells), respectively.
- The mammalian vascular bed consists of **microvessels** and **macrovessels**. While microvessels are located in the capillary bed, macrovessels are the major blood circulation tubes of the mammalian body. The macrovessels are involved in various functions, including the blood–brain barrier, clearance of sinusoidal endothelial cells in the liver, and blood filtration by glomerular vascular segments in the kidney. **The inner lining of both micro- and macrovessels is made up of a single layer of endothelial cells.**
- The entire vascular bed is made up of a single endothelial cell layer.

3.1.1 Locations of Endothelial Cells in Humans

- A generic endothelial cell does not exist.
- Endothelial cells differ in structure and phenotype, depending on the vessel type.
- In microvessels (generally, (3–4) μM in diameter) a single layer of endothelial cells is supported by a basal lamina.
- However, macrovessels (up to 25 mm in diameter) consist of three cell layers, namely,
- **Tunica intima**

  Endothelial and pericytes cell layer lining the inside of vessels.

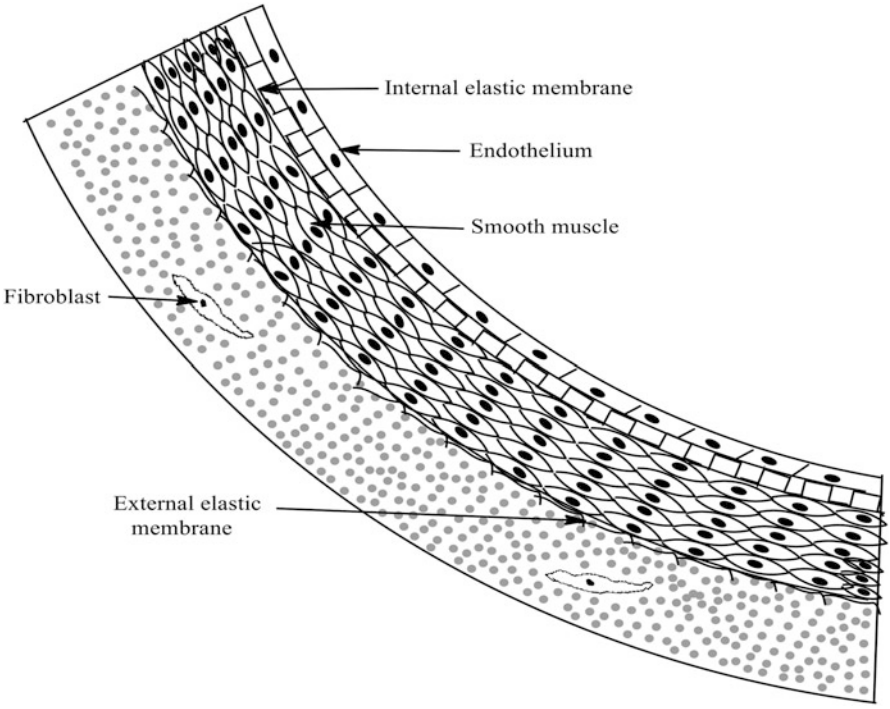

Fig. 1 Pictorial depiction of various layers of a large vessel (e.g., artery)

- **Tunica media**
  The smooth muscle cell layer forms the middle layer of large blood vessel walls.
- **Tunica adventitia**
  The outer thickest layer of large blood vessels is constituted of fibroblasts.
  Other components of the tunica adventitia are mast cells, nerve endings, vasa vasorum, and collagen fiber **(**Fig. 1**)**.

3.2 Dissecting Instruments for Isolating Endothelial Cells

Scissors, forceps, scalpels, syringes (10 and 20 ml), a small cannula or plastic tube, and a 20 cc syringe are needed for dissection and collection of endothelial cells from blood vessels such as an umbilical vein. Before use, the sterility of all materials must be ensured.

3.3 Materials for Culturing Isolated Endothelial Cells

3.3.1 Specimen Collection Container

Sterile plastic containers are used to collect blood vessels/umbilical cords.

3.3.2 Cell Dissociation Enzyme

The freshly prepared enzyme (e.g., 0.1% filter-sterilized collagenase is used to isolate HUVECs).

3.3.3 Buffers

Sterile phosphate-buffered saline (PBS) without Ca^{+2} or Mg^{+2} is used to wash the umbilical cord or the lumens of other blood vessels.

3.3.4 Cell Culture Medium

The culture configuration for endothelial cells: M199 with Earl's salt or Dulbecco's Modified Eagle's Medium (DMEM). To make a complete medium, the following ingredients need to be added: (5–10)% fetal calf serum (FCS) or fetal bovine serum (FBS), 100 $U \cdot ml^{-1}$ penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycins, 100 $\mu g \cdot ml^{-1}$ heparin sodium salt, (10–50) $\mu g \cdot ml^{-1}$ vascular endothelial cell growth factor (VEGF), and 2 mM L-glutamine (for faster growth). Make a complete medium, filter-sterilize the aliquot, and store at 4 °C for use within a week.

3.3.5 Cell Adhesive Agents

Sterile 0.1% gelatin (cheapest adhesive agent). Laminin or poly-L-lysine can also be used.

3.3.6 Cell Culture Containers and Associated Materials

Flasks ((25 or 75) mm) or plates ((30, 60, or 100) mm), (15 and 50) ml centrifuge tubes, and pipettes of various capacities are needed for cell culture. All these materials should be sterile, disposable, of mammalian cell culture grade, and made using polystyrene. Membrane filters having various pore sizes are used for filter sterilization of the culture medium.

3.3.7 Blood Vessels

All blood vessels, macro or micro, can be used for the isolation and culture of endothelial cells.

3.4 Cell Division Capacity and Growth Properties of Endothelial Cells

- Endothelial cells are characteristically "quiescent" as they do not actively proliferate. The average lifespan is >1 year.

- A fine illustration pertains to distinct turnover times in different organs, a couple of months for liver and lung, while for brain and muscle, this duration lasts for years.
- However, in embryonic tissues, damaged blood vessels, and menstruating uterus, the endothelial cell doubling time is only a couple of days. So, it is possible to culture the endothelial cells, in vitro.
- A characteristic of in vitro cultured endothelial cells is the (48–72) hour doubling time during the logarithmic growth phase.
- Microvascular endothelial cells isolated from the retina of the macaque monkey exhibit a mean doubling time of 44.5 h during the first (3–5) days of culture and 23 h at (6–8) days of culture, forming a confluent monolayer within (12–14) days.

NB: A 75 mm flask with 1 × 10^6 cells takes (2–3) days to reach 90% confluence. An endothelial cell may divide for about 50 passages, following which cells undergo apoptosis.

3.5 Types of Blood Vessels in Humans

Based on the diameter, human blood vessels are divided into the overleaf two general groups:

Macro blood vessels
Micro blood vessels

The following is a brief discussion of these vessels and the endothelial cells present in them:

3.5.1 Macro Blood Vessels

These include the following:

Coronary artery/vein
Pulmonary artery/vein
Aorta and large arteries/veins present in the brain and limbs

3.5.2 Micro Blood Vessels

These are the blood vessels (such as **capillary, arteriole, or venule)** of the micro-circulatory system. In mammals, each organ has its blood vessels and vascular bed.

Arterioles are the typical, small-diameter blood vessels that extend and branch out from an artery, leading to capillaries.

Capillaries: These are perhaps the smallest blood vessels.

Metarterioles: These are the vessels linking arterioles and capillaries.

Venules: The vessels that allow deoxygenated blood to return from the capillary beds to veins.

Thoroughfare channel: A venous vessel receiving blood directly from capillary beds. It is a tributary of venules.

NB: Blood flows away from the heart to arteries, then into arterioles, which further narrow down into capillaries. Capillaries flow into venules and connect to veins, which return blood to the heart.

Endothelial cells are present in the lumen of all blood vessels and serve a very important role in the structural and functional integrity of the vessels.

Type 2 diabetes is more closely associated with "microvascular" complications (retinopathy, nephropathy, and neuropathy) and "macrovascular" complications affecting the heart, brain, and foot.

3.5.3 Some Physiological Distinctions Among the Endothelial Cells Originated from Various Vascular Beds

Endothelial cells differ in their macro- versus microvessel distribution besides the characteristic distinctions between the manifold vascular origins or even within the different portions of the same vascular bed.

Some physiological differences between various endothelial cells are as follows:

- Human microvascular endothelial cells differ from macrovascular endothelial cells in their matrix metalloproteases (MMPs) expression, bradykinin degradation (Jackson and Nguyen 1997; Gräfe et al. 1994), and plasminogen activator inhibitor-1 activity (Gräfe et al. 1994).
- Microvascular endothelial cells have a different prostaglandin and prostanoid secretion profile than their macrovascular counterparts and endocardium (Mebazaa et al. 1995).
- Endothelial cells isolated from the porcine coronary artery release more endothelin 1 (ET-1) than those from the aorta.
- Receptors for adenosine, α-thrombin, histamine, and acetylcholine appear to be much more abundant in microvascular guinea pig coronary artery endothelial cells than those of bovine aorta (Mehrke et al. 1990).
- *The cerebral vascular endothelial cells have distinctive features, which may differ from one other species. For example, humans and dogs have different vascular beds with different endothelial cell characteristics (*Gerhart et al. 1988).
- The fibrinolytic potential of cerebral and umbilical vein endothelial cells is differentially modulated by alpha thrombin (α-thrombin) (Shatos et al. 1995).
- There are structural and functional differences between pulmonary micro- and macrovascular endothelial cells (Stevens 2011).
- There are differences in cell adhesion molecule expression in micro- and macrovascular endothelial cells. Microvessel endothelial cells express a larger contingent of adhesion molecules than macrovessel endothelial cells.
- At a functional level, the homing of leukocytes to specific lymphoid and nonlymphoid tissues occurs exclusively within the microvascular compartments and is mediated by differentiation and sequential expression of specific adhesion molecules in a tissue-specific manner (Ades et al. 1992).

- In 2007, *Arid WC* in two consecutive publications described the endothelium heterogeneity, along with their structures and functions, in parallel, emphasizing the value of viewing endothelium as an integrated system (Arid 2007a, b).

3.6 Isolation and Culture of Vascular Endothelial Cells from Human Umbilical Veins

- The human umbilical cord contains two small-diameter arteries and one large-diameter vein (Gruber et al. 2021). The human umbilical vein (HUV) is the source of the simplest and most easily isolated endothelial cells and is widely used in biomedical research.
- Although HUVECs exhibit all general attributes of endothelial cells, they are of fetal and venous origin.
- One can also isolate umbilical artery endothelial cells. However, this is technically more difficult because of the smaller native diameter.
- Endothelial cells may also be isolated from many other blood vessels, including the aorta or microvessels.
- The isolation procedure was described by Jaffe et al. 1973.

Isolation of endothelial cells from the human umbilical cord vein involves the following steps.

Step 1: Collection of umbilical cord
Step 2: Washing of the umbilical cord vein lumen
Step 3: Enzymatic digestion of the umbilical cord vein lumenal surface
Step 4: Collection of endothelial cells from the digested vein
Step 5: Coating the cell culture vessels (containers) with 0.1% gelatin
Step 6: Plating and culture of endothelial cells in gelatin-coated containers

What follows is a brief discussion of the above steps:

3.6.1 Step 1: Collection of umbilical cord

The followings are the key points about umbilical cord collection:

- Collect fresh (within 1–3 h after baby delivery), (5–20)-cm-long umbilical cords.
- Place harvested cords into sterile containers containing sterile Dulbecco's phosphate buffer saline (DPBS) or Hanks' balanced salt solution (HBSS) media without Ca^{+2} and Mg^{+2}.
- Cords should be inspected and all areas with clamp marks should be excised to lessen the chances of cell contamination (e.g., smooth muscle cells/fibroblasts) from the clamp-damaged regions.
- Discard the cord if one is below 12 cm in length or the clamp marks are less than 12 cm apart.

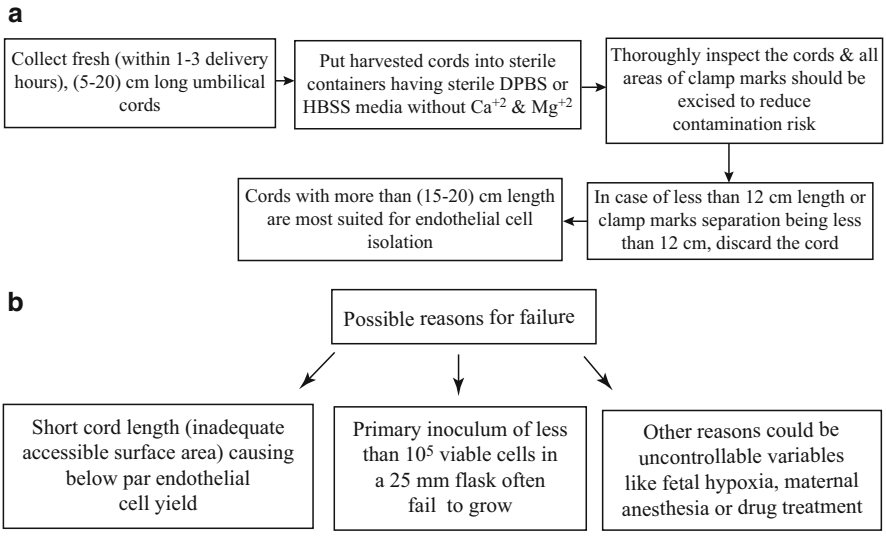

Fig. 2 (**a**) Chronological steps for harvesting the umbilical cord cells. (**b**) Possible reasons for not being able to harvest the umbilical cord cells

- In general, cords with more than (15–20) cm in length are most suitable for endothelial cell isolation.
- Cords must be processed within (1–3) hours of birth and should be discarded if the processing is delayed for more than 3 h.

NB: About one-third of the cords fail to provide viable cultures despite the use of fresh medium and all necessary additives.

- A major factor for failure is the short length of the cord (insufficient intimal surface area) leading to insufficient endothelial cell yield.
- A primary inoculum of less than **1×10^5 viable cells** in a 25 mm flask usually fails to grow.
- Figure 2(a) depicts the functional steps for harvesting the umbilical cord cells, and the possible reasons for its failure due to uncontrollable variables such as fetal hypoxia, maternal anesthesia, or drug treatments are shown in Fig. 2(b).

3.6.2 Step 2: Washing of the umbilical cord vein lumen

The over-leafed points need to be remembered regarding washing of umbilical cord vein:

- Bring the umbilical cord container into the laminar flow hood.
- Rinse the cord exterior with sterile DPBS, without Ca^{+2} and Mg^{+2}.
- **Identify the Umbilical Cord Vein**

Fig. 3 The axial view of an umbilical cord

An umbilical cord has two arteries and one vein. Arteries are smaller and thick-walled because of a thicker smooth muscle coat. The umbilical vein is substantially larger in diameter than the umbilical arteries. The umbilical vein is easily dilated (Fig.3).

- Insert a cannula or tube attached to a 20 cc syringe into one end of the umbilical vein lumen.
- Then, use this 20 cc syringe to flush the vessel with (200–300) ml sterile DPBS or HBSS for removing blood inside the vein lumen.
- Allow the vessel to drain the DPBS or HBSS by gravity from the other end.
- Do not introduce air into the lumen.

3.6.3 Step 3: Enzymatic digestion of the umbilical cord vein lumenal surface

The following details need to be adhered to during luminal digestion of an umbilical cord:

- Tie off or seal one end of the umbilical vein and leave the other end open.
- Fill a sterile syringe with 20 ml sterile prewarmed (37 °C) 0.1% collagenase solution.
- Place a syringe-connected tube into the open end of the umbilical vein before filling the lumen with 0.1% collagenase type 1 from *Clostridium histolyticum* (Gibco-BRL).
- Remove the syringe and clamp the end of the umbilical cord.
- Now both ends of the umbilical cord are sealed.
- Place the cord into a sterile container, close the container, and remove it from the laminar flow hood.
- Incubate the container at 37 °C inside a CO_2 incubator.
- Collagenase will remove the luminal endothelial cell layer of the umbilical vein.
- After (15–20) minutes of incubation in the CO_2 incubator, place the cord into a laminar flow hood. Gently massage the vein and remove the seal/clamp from one end of the vein, keeping the other inside a sterile container.
- Open the other end of the umbilical vein.
- Insert a needle of a syringe at the latter opened end of the umbilical vein before flushing the umbilical vein once with sterile DPBS into the same container that contains collagenase extract.

3.6.4 Step 4: Collection of endothelial cells from the digested vein

- Place the DPBS/collagenase-containing endothelial cells suspension into a 50 ml sterile centrifuge tube.
- Centrifuge the tube at 1000 rpm for 5 min.
- The yield in this procedure should be in the range of (0.5–1.5) $\times$ 10^5 cells.

NB: Any preparation contamination at this stage can usually be detected. A bulky gelatinous pellet indicates the presence of connective tissue matrix and cellular elements from deeper areas of the vessel wall, while the presence of red cells indicates an inadequate luminal blood flushing.

The collected umbilical cord should be used within 3 h, otherwise human umbilical vein endothelial cells (HUVECs) do not grow properly.

After the isolation, the umbilical cord and all other used materials may be treated with a 10–15% solution of bleach for 15 min before putting into biohazard containers.

3.6.5 Step 5: Coating the cell culture containers with 0.1% gelatin

- Endothelial cells are adherent by nature. While there are many mammalian cell adhesive agents available including poly-L-lysine, laminins, and fibronectin, gelatin is the cheapest, most easily available, and most useful.
- For the preparation of gelatin, add 100 mg in 100 ml of deionized water before autoclaving and cooling. This results in 0.1% gelatin. Store this gelatin stock solution in a 4 °C refrigerator.
- In the laminar flow hood, add (2–3) ml of 0.1% gelatin in a 100 mm Petri plate or (4–5) ml in a 75 mm flask. Close the container. Shake to cover the entire cell culture surface of the container. Incubate the cell culture vessel/container at 37 °C for 30 min. Gelatin will attach to the plate surface.
- Following incubation, take the container to a laminar flow hood, remove the gelatin solution, and rinse the containers with DPBS without Ca^{+2} or Mg^{+2}.
- Until use, place DPBS in the container to prevent dryness.

NB: The gelatin-coated plates can be stored up to a month at 4 °C for future use.

3.6.6 Step 6: Plating and culture of endothelial cells in the gelatin-coated containers

- Collect the cell pellet from the centrifuge and resuspend it in a 10 ml complete medium. Place it into a 0.1% gelatin-coated 100 mm Petri plate.
- Alternatively, resuspend the cells in a 15 ml complete medium and place them into a 75 mm flask.
- Incubate the cell culture container overnight in a CO_2 incubator at 37 °C in a 5% CO_2 environment.
- Henceforth, examine for cell growth using an inverted phase-contrast microscope (Fig. 4).
- At the time of plating, 90% of the harvested cells should be viable using Trypan Blue exclusion.

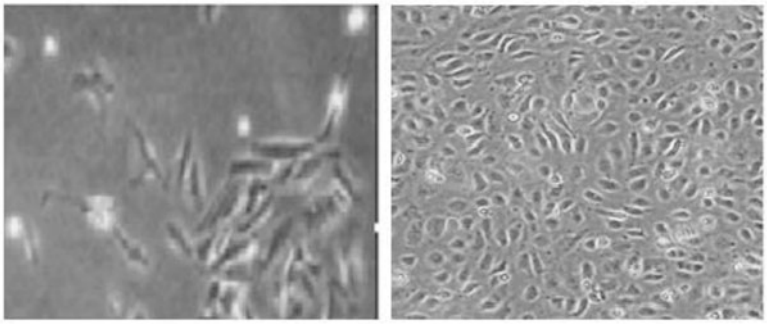

Fig. 4 The culture of endothelial cells from the human umbilical vein as observed through inverted microscope: (a) endothelial cell growth after 24 h of seeding; (b) completely confluent human umbilical vein endothelial 4–5 days after seeding

- On being 12 h in culture, nearly (25–30)% of cells become adherent. Cellular aggregates may attach to the plate as clusters.
- Change the medium to remove dead cells and allow the living cells to further expand.
- Check the growth characteristics of endothelial cells daily. The approximate morphology after 9 days of preserving in culture medium at 100% confluent stage is depicted in Fig.4.
- Harvest, subculture, and freeze HUVECs.

3.7 Limitations of Isolation of Endothelial Cells from Umbilical Cord

- The umbilical cord is of fetal origin.
- The endothelial cells isolated from the umbilical cord generally are of venous origin.

NB: Umbilical cords have one large-diameter vein and two small-diameter arteries.

3.8 Rationale for Isolation of Endothelial Cells from Umbilical Cords

- Umbilical cords are abundantly available.
- In comparison, pulmonary artery, coronary artery, and cerebral artery are available primarily when there is a need for transplantation surgery or in case of accidental death.
- Consent of the donor is essential before collecting any organ.

3.9 Isolation and Culture of Vascular Endothelial Cells from Microvessels

In 1992, ***Ades and colleagues*** generated a human dermal microvascular endothelial cell line (HMEC-1). This cell line is currently the most widely used for microvascular endothelial cell culture for diverse research requirements (Ades et al. 1992).

The following points need to be considered when culturing microvascular endothelial cells:

- During in vitro culture, microvascular endothelial cells undergo morphological differentiation into capillary-like structures much more rapidly and readily than large blood vessels.
- Loss of microenvironment-driven control when culturing endothelial cells is a major hurdle in obtaining an accurate depiction of the molecular heterogeneity of in vivo functioning.
- The isolation and culture procedures for microvascular endothelial cells are generally the same as for HUVECs.
- Several groups have described the isolation and culture of microvascular endothelial cells.

NB: In general, since microvasculature is very small in diameter it is very difficult to isolate the endothelial cells.

- The number of cells isolated from the microvasculature is very small. Moreover, growing such a small number of cells in cell culture containers is difficult due to missing cell-to-cell signaling.
- Microvascular endothelial cells are much more fastidious in growth; the in vitro culture requirements of microvascular cells are more complex than large vessel endothelial cells with a significant extent of biological differentiation and functional distinctions.
- To partially overcome these problems, *Ades and associates* have established an immortal dermal microvascular endothelial (**HMEC-1**) cell line, which is now widely used for experimental purposes (Ades et al. 1992).

3.10 Phenotypic Identification of Endothelial Cells

The following are the phenotypic characteristics of endothelial cells, as observed through an inverted microscope:

Shape of Endothelial Cells in Two-Dimensional Cultures
In general, endothelial cells appear flat having a central polygonal morphology with a cobblestone surface.
Thickness of Endothelial Cells in Two-Dimensional Cultures
Endothelial cells are (1–2) μm in thickness when cultured in two dimensions.

Diameter of Endothelial Cells in Two-Dimensional Cultures

Endothelial cells from various blood vessels (e.g., micro- versus macrovessels) exhibit a significant variation in their diameters, ranging from (3 to 20) μm.

3.11 Marker Proteins Expression-Based Identification of Endothelial Cells

As mentioned, endothelial cells are phenotypically identified by a cobblestone or polygonal morphology. During embryonic development, these cells differentiate from a common precursor called angioblast and acquire organ-specific properties. One of the important determinants of endothelial cell differentiation is the local environment and especially the interaction with surrounding cells. This interaction may occur through the release of soluble cytokines, cell-to-cell adhesion molecules, communication proteins, as well as the synthesized matrix proteins on which the endothelium adheres and grows. The acquisition and maintenance of specialized properties by endothelial cells are highly essential for the functional homeostasis of different organs. For instance, in the brain, alteration of the blood–brain barrier (***BBB***) properties may have important consequences on functional integrity. Von Willebrand factor (***VWF***), together with the Weibel–Palade bodies (***WPB***), angiotensin-converting enzyme (***ACE, CD143***), and the cobblestone morphology specific for monolayer cultures, have all been previously referred to as a few obligate criteria to confirm the authenticity and purity of cultured endothelial cells.

Here is a list of the most common endothelial cell phenotype markers. These molecules and related antibodies may be valuable tools for endothelial cell isolation and characterization (Garlanda and Dejana 1997).

Endothelial cells are screened by the presence of the following molecules:

Weibel–Palade Body

Microscopically, endothelial cell cytoplasm is characterized by the large rod-shaped organelles called Weibel–Palade bodies (WP bodies). These organelles store von Willebrand factor (blood glycoprotein involved in homeostasis) and P-selectin (the cell adhesion molecule on the surface of activated epithelial cells, lining inner surfaces of blood vessels and activated platelets).

Von Willebrand Factor

A large proportion of adhesive glycoprotein is secreted into the blood, facilitating coagulation following hemorrhage. It is also known as Factor VIII-related antigen. The deficiency of this molecule may lead to hemophilia.

CD31

A cluster of glycoproteins is expressed on the surface of endothelial cells. The protein cluster of differentiation 31 (CD31) is also known as platelet endothelial cell adhesion molecule-1 (PECAM-1).

Vascular Endothelial Cadherin

An implicit endothelial cell adhesion molecule is located at junctions between endothelial cells, also known as a CD.

Blood Group Antigens

ABO blood group antigens (proteins) are present on the surface of endothelial cells. They are not specific for endothelial cells as these are also expressed by RBC.

Acetylated Low-Density Lipoprotein

Endothelial cells take up the acetylated low-density lipoproteins (LDLs), which can also be used as endothelial cell markers (Garlanda and Dejana 1997).

3.12 Utility of Endothelial Cells

Endothelial cells are involved in the following aspects of vascular biology:

3.12.1 Barrier Function

- The endothelium acts as a semiselective barrier between the vessel lumen and surrounding tissue, controlling the constituent's passage and the transit of white blood cells in and out of the bloodstream. The passage of various molecules across the endothelium is regulated by four different arrangements of endothelial cells in the endothelium, forming four alternative structures.
- In continuous structure, the single layer of endothelial cells is a continuous arrangement in such a way that it completely encloses the vessel lumen. The only gaps are the sealed cellular locations via junctional complexes. This structural regime is most restrictive toward the diffusion of biomolecules and proteins.
- The second type of endothelium, called fenestrated endothelium, contains small holes (25 nm) that allow the diffusion of molecules and small proteins. Diaphragms across the fenestrae and the basement membrane limit the diffusion of materials. This type of endothelium is located in the intestines and kidneys.
- The third type of endothelial cells comprises a discontinuous arrangement. Larger gaps, lack of diaphragms, and altered basement membrane characterize these endothelial cells. These endothelial cells are found in the liver and spleen.
- The final limiting diffusion of molecules across the endothelium is provided by the junctional complex between endothelial cells (where one endothelial cell meets its neighbor). Junctional complexes prevent paracellular diffusion of materials.
- Figure 5 summarizes the diversity of endothelial tissue, based on their molecular transport abilities and characteristic prevalence within the body. The permeability of the endothelium is determined by its specific regime, the basement membrane, the glycocalyx, and intercellular junctions. Transcytosis is a highly selective process that regulates the movement of proteins across endothelial cells.

NB: Excessive or prolonged increments in the permeability of endothelial cell monolayer, as in the case of chronic inflammation, may lead to tissue edema/swelling.

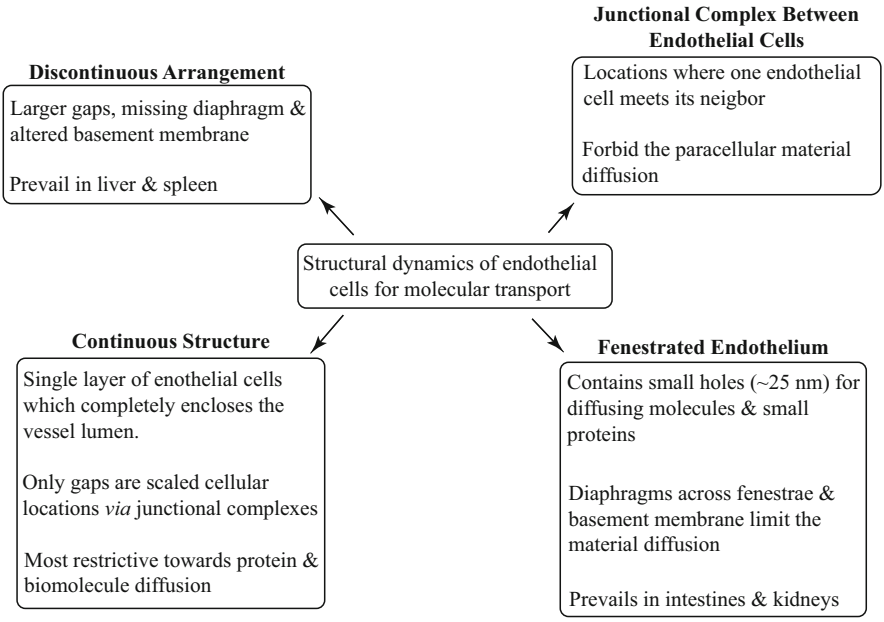

Fig. 5 Diversity of endothelial cells in terms of their molecular transport ability

3.12.2 Prevention of Clotting in the Blood Vessels

The endothelium normally provides a non-thrombogenic surface because it contains, for example, heparan sulfate (HS), a cofactor for activating antithrombin and a protease that inactivates several factors in the coagulation cascade.

3.12.3 Angiogenesis

- Angiogenesis is the typical formation of new blood vessels. Low oxygen in the blood (e.g., during tumor/cancer formation) and wounding of the endothelium (e.g., during atherosclerosis) initiate the growth of new blood vessels.
- One of the initial steps of angiogenesis is the expression of angiopoietin 2 (vascular growth factor promoting cell death through disruption of vascularization) and vascular endothelial growth factor (**VEGF**).
- Angiopoietin-2 interacts with VEGF to activate specialized endothelial cells called tip cells. Tip cells divide and invade/migrate into surrounding tissues by extending filopodia and tube formations. This leads to the formation of new blood vessels.
- Experiments in culture demonstrate that endothelial cells in a culture medium containing suitable growth factors spontaneously form the capillary tubes, even if they are isolated from other cells.
- Since capillary tubes that develop in culture do not contain blood and nothing travels through them, blood flow and pressure are not required for the initiation of a new capillary network.

3.12.4 Vascular Tone and Blood Pressure

- Endothelial cells are the source of various vasoactive agents, including nitric oxide (**NO**), prostacyclin, arterial natriuretic peptide (**ANP**), and endothelin 1 **(ET-1**).
- Other humoral agents, including angiotensin II (**Ang. II)**, aldosterone, and bradykinin, act on the endothelial cells.
- These vasoactive molecules regulate vasomotion (vasoconstriction and vasodilation) and thus control blood pressure.

3.12.5 Fluid Filtration

- Some endothelial cells are involved in fluid filtration. One example of such endothelial cells is the cells in the glomerulus of the kidney (Aird 2007a, b).

3.13 Human Diseases Related to Vascular Endothelium Defects

Since vascular endothelium controls all the biological functions of capillaries, any defect of vascular endothelium leads to multiple diseased conditions. The list of these diseases includes venous thrombosis, peripheral vascular disease, insulin resistance, diabetes, chronic kidney failures, tumors, and cancers.

- Immunological cells in the blood (e.g., macrophages) roll onto endothelial cells and attach to their surface. Attachment of blood cells (e.g., monocytes) requires a cell surface receptor, such as very late antigen 4 (**VLA4**), which binds to endothelial cell surface adhesion molecules [e.g., vascular cell adhesion molecule 1 [**VCAM-1]**).
- Following attachment, monocytes and other leukocytes transmigrate to the subendothelial space, leading to localized inflammation. Subsequently, these subendothelial leukocytes accumulate oxidized lipids and proteins inside them, leading to **fatty streak formation**. Fatty streak formation is an early stage of **atherogenesis**.
- Isolation and culture of endothelial cells serve as a useful tool for the study of atherosclerosis (fatty streak formation), arteriosclerosis (hardening of the artery) hypertension (increase in blood pressure), angiogenesis (new blood vessel formation), cancer therapy, burn therapy, wound healing and regeneration, cell signaling (e.g., **NO signaling**), gene expression profiling, drug/toxic testing and screening, and tissue engineering (Rajendran et al. 2013).

4 Isolation and Primary Culture of Vascular Smooth Muscle Cells

4.1 Vascular and Nonvascular Smooth Muscle Cells

- Smooth muscles are involuntary muscle tissues, confined within the walls of viscera and blood vessels. These are comprised of nonstriated, spindle-shaped cells.
- Based on their location, smooth muscle cells are placed into two groups, vascular and nonvascular.
- Smooth muscle cells present in the tunica media of large blood vessels (macrovessels) are called vascular smooth muscle cells (VSMCs).
- VSMCs maintain the structural integrity of macrovessels and regulate their diameter by contracting and relaxing in response to vasoactive stimuli. Thus, VSMCs help in regulating vascular tone and blood pressure.
- Smooth muscle cells (SMCs) also prevail in the walls of hollow organs, including the stomach, intestines, urinary bladder, uterus, the respiratory, urinary, and reproductive systems, and eye and skin tissue. These cells are referred to as nonvascular smooth muscle cells or simply SMCs.

NB: The intermediate filament and actin composition of VSMCs reflects a differentiation pathway, separate from other nonvascular SMCs present in other organs.

4.2 Distinctions Between Endothelial and Smooth Muscle Cells

- Smooth muscle cells constitute the **middle layer of blood vessels,** whereas endothelial cells constitute the **inner layer of a blood vessel**.
- By transmission electron microscopy (**TEM)**, cultured endothelial cells could be visualized as comprised of cytoplasmic inclusions (**Weibel–Palade bodies**). These inclusions are also found in endothelial cells lining umbilical veins but are not seen in smooth muscle cells or cultured fibroblasts, in situ.
- Cultured endothelial cells contain abundant quantities of smooth muscle actomyosin (actin–myosin).
- However, endothelial cells do not stain as intensely for actin as do the SMCs. This distinction is a preferred approach for determining the SMC's presence in disorders such as atherosclerosis.
- Cultured endothelial cells also contained ABH antigens appropriate to the tissue donor's blood type; these antigens are not detectable in cultured SMCs or fibroblasts.
- Human endothelial cells express FasL (death factor-inducing apoptosis) on the surface to a higher extent than do VSMCs.
- When subjected to variable durations of supercooling and rewarming, SMCs undergo higher apoptosis compared to endothelial cells.

4.3 Prevalence of Vascular Smooth Muscle Cells

VSMCs are located throughout the large blood vessels. However, for isolation and culture of VSMCs, the following three large vessels are primarily used not only because of their large diameter and accessible locations but also because of their involvement in **atherosclerotic plaque formation.**

- Human ascending (thoracic) aorta
- Human descending (abdominal) aorta
- Pulmonary arteries

The most common sources for isolating VSMCs are the following:

- Aorta
- Coronary arteries
- Pulmonary arteries
- Other sources include the placenta, umbilical cord, and mammary blood vessels

4.4 Dissecting Instruments for Isolation of Smooth Muscle Cells

The instruments are described in the isolation and culture of vascular endothelial cells, Sect. 4 of this chapter.

4.5 Materials for Isolation and Culture of Vascular Smooth Muscle Cells

4.5.1 Sodium Pentobarbital

1 gm·ml^{-1} pentobarbital is used most often as the anesthetic for animals.

4.5.2 Phosphate-Buffered Saline

Sterile PBS without Ca^{+2} and Mg^{+2} is used to wash the sample tissue/organs.

4.5.3 70% Ethanol (ETOH)

ETOH is used as a disinfectant for the laminar flow hood working area and other materials.

4.5.4 Cell-Dissociating Enzymes

- Collagenase from *Clostridium histolyticum* stock (10 mg·ml^{-1}, freshly prepared).
- Add 10 mg collagenase in 1 ml HBSS, filter sterilize, and use immediately.
- Do not store an extra amount or reuse the latter. Final concentration needed is 0.5 mg·ml^{-1}.

4.5.5 Fungizone

0.25 mg·ml^{-1} fungizone is used as an antimycotic agent

4.5.6 Containers and Associated Materials

Flasks (25 mm/75 mm), plates (30 mm/60 mm/100 mm), (15 and 50) ml centrifuge tubes, and pipettes of various capacities are needed for cell culture. All these materials must be sterilized before use and should be disposable, of mammalian cell culture grade and polystyrene constituted. Membrane filters having various pore sizes are used for filter sterilization of the cell culture medium.

4.5.7 Cell Culture Medium of Vascular Smooth Muscle Cells

The complete medium is **DMEM** with 10% FBS, 2 mM L-glutamine, penicillin (100 U·ml^{-1}), and streptomycin (100 μg·ml^{-1}).

NB: Instead of using DMEM, many researchers use a medium comprising 1:1 DMEM and F12K.

4.6 Cell Division Capacity and Growth Properties of Vascular Smooth Muscle Cells

The VSMCs in the mature blood vessels rarely proliferate or migrate. However, these cells express various smooth muscle-specific contractile proteins such as α-SMA and calponin. Thus, these VSMCs contract as and when necessary.

On the contrary, whenever an injury happens to blood vessels, there is a significant phenotypic change in VSMCs, converting the contractile phenotype to proliferative and migrative phenotype. The extracellular matrix synthesis is also noticed under this condition.

- In 1993, *Kirschenlohr HL and colleagues* demonstrated that "VSMCs from adult human aortas proliferated in culture in response to FCS supplementation, with a population doubling time of **(70–85) hours** compared to **(35 ± 5) hours** for VSMC derived from adult rat aortas" (Kirschenlohr et al. 1993).
- In 2012, *Proudfoot D and colleagues* described that human aortic VSMCs have a typical doubling time of about **44 h** (Proudfoot and Shanahan 2012).

4.7 Isolation and Culture of Vascular Smooth Muscle Cells from Mouse Aorta

Several groups have established methods for isolating and culturing VSMCs from mouse aorta.

A discussion of some of these methods is as follows:

The method comprises the following steps:

- Use two ~10-week-old mice.
- Euthanize them by intraperitoneal injection of 0.25 ml ($1gm \cdot ml^{-1}$) sodium pentobarbital using a 26-gauge needle.

NB: Alternatively, one can asphyxiate mice by exposing them to CO_2 for 2 min.

- Spray mice with 70% ethanol.
- Place mice under a dissecting lamp with a magnifier.
- Surgically, open mice from the abdomen all along to the thorax one by one, without cutting any blood vessels to avoid bleeding.
- Collect the blood via cardiac puncture using a 1 ml syringe with a 26-gauge needle.
- Remove/discard all the organs except the heart to allow a clear view of the aorta.
- Place the mouse under a microscope.
- Dissect the aorta from its origin at the left ventricle to the iliac bifurcation.
- Leave the aorta attached to the left ventricle.
- Use a 3 ml syringe fitted with a 26-gauge needle to puncture the left ventricle.
- Perfuse with 3 ml sterile PBS, so that the aorta is flushed and all the blood cells are removed from the lumen.
- Remove the aorta, using sterile microdissecting scissors, and place in a 100 mm Petri dish containing a drop or two of fungizone solution.
- Remove fat from the aorta vicinity.
- When the vessel is free of the fibrous material and fat, make two cuts, one below the arch, and another in diaphragm proximity.
- Place the aortas into the PBS in a 15 ml conical flask and keep cool.
- Remove adventitia and endothelial layers.
- Remove aortas from the 15 ml conical flask.
- Rinse the aortas in PBS and put them in a dish afterward.
- Add proteolytic enzymes.
- Incubate the tray at 37 °C for 10 min.
- Put the aorta into a new culture plate and rinse with DMEM:F12 media (1:1) to wash off enzymes.
- Remove the medium and add a fresh enzyme solution and incubate at 37 °C in 5% CO_2 within the incubator for about an hour.
- Check whether the vessels are dissolved and the cells are floating.
- If not, the incubation time may be increased for another couple of minutes.
- Titrate the cells with a Pasteur pipette.
- Suspend and wash the cells with 4 ml of DMEM:F12 media (1:1), followed by centrifugation at 1500 RPM for 5 min.
- Decant the supernatant medium and wash the pellet once again by repeating the above step.
- Remove the supernatant medium.
- Suspend the cells in 1.5 ml DMEM: F12 media (1:1).
- Put the cell suspension in 3 wells (0.5 ml each) of 48-well cell culture dish.

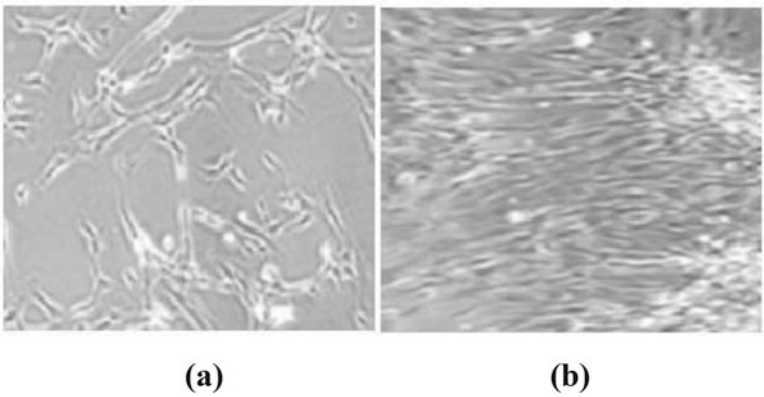

Fig. 6 Growth pattern of VSMCs: (a) hill and (b) valley morphologies

- Grow for 1 week, till 80–90% confluency.
- Subculture the cells and grow for 3–5 passages.

4.7.1 Growth Pattern of the Isolated Cells.

VSMCs' growth characteristics exhibit hill and valley patterns **(**Fig. 6**)**.

4.7.2 Confirmatory Identification of Vascular Smooth Muscle Cells

- VSMCs can be confirmed by positive immunohistological staining for α-smooth muscle actin, using a commercial kit and hematoxylin-assisted counterstaining.
- Besides α-smooth muscle actin, CD3 (lymphocytes), CD68 (macrophages), and CD31 (endothelial cells) are also recognized as negative VSMC screening markers.

4.7.3 Passaging Vascular Smooth Muscle Cells

- Split the cells into 48-well plates as follows:
- 1–3 wells (1^{ST}p) up to 6 wells (2ND p) to 12 well (3RD p) to 24 wells (4TH p) to 60 wells (p).

Do not grow SMCs for more than (10–12) passages because of the following reasons:

- Changes in cell function
- Loss of growth capacity
- Spreading of cells

4.8 Isolation and Culture of Vascular Smooth Muscle Cells from Human Aorta

In 2012, Proudfoot and Shanahan established a detailed method for isolating and culturing VSMCs from human blood vessels (Proudfoot and Shanahan 2012)**.**

A detailed and complete protocol is available from their publication.

4.9 Identification of Vascular Smooth Muscle Cells Based on Phenotype (Contractile vs. Synthetic Phenotype)

4.9.1 Diameter of Vascular Smooth Muscle Cells

The largest SMCs exist in the uterus during pregnancy **(12 × 600) μm.**

The smallest are found in small arterioles **(1 × 10) μm.**

4.9.2 Shape of Vascular Smooth Muscle Cells Based on Their Phenotype

- Smooth muscle consists of spindle-shaped cells. However, unlike some terminally differentiated cells, VSMCs maintain marked phenotypic plasticity.
- VSMCs exhibit two distinct phenotypes, **contractile** and **synthetic**.

A brief discussion of phenotypes is as follows:

4.9.3 Contractile Phenotype of Vascular Smooth Muscle Cells

- Under normal physiological conditions, VSMCs remain in a contractile phenotype. This phenotype is only present in intact blood vessels or very early primary cultures.

The contractile phenotype of VSMCs is characterized by the following:

- High expression of contractile proteins (e.g., α-SMA).
- Low in the rough endoplasmic reticulum (rough ER).
- Small Golgi apparatus.
- Low proliferative index and fusiform morphology (Fig.7).
- This phenotype is also called "differentiated phenotype," but not "terminally differentiated phenotype" (Beamish et al. 2010).

4.9.4 Synthetic Phenotype of Vascular Smooth Muscle Cells

- Within a few days of 2D culture, VSMCs transform from a contractile to synthetic phenotype, also known as ***"de-differentiated phenotype."***

This dedifferentiated VSMCs' phenotype is characterized by the following:

- Gradual loss of myofilament bundles.
- Formation of an extensive rough endoplasmic reticulum (rough ER) (Fig.8).

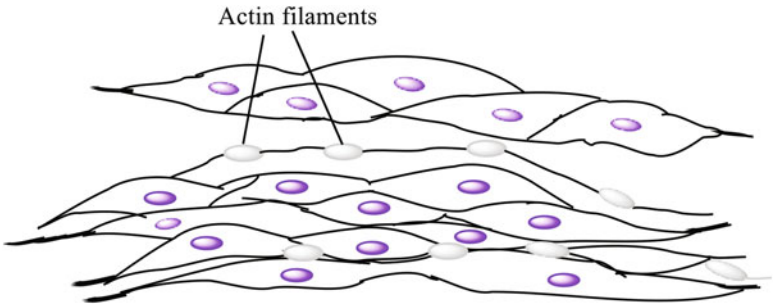

Fig. 7 Contractile phenotype of SMCs has actin expression as their hallmark

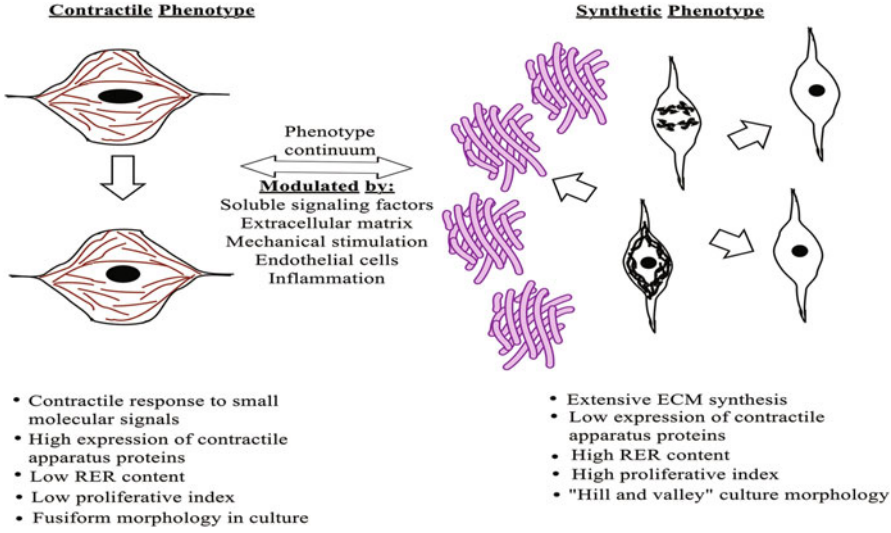

Fig. 8 Distinction of contractile and synthetic VSMC phenotypes

- Large Golgi complex.
- This evolution provides VSMCs with the ability to synthesize DNA and divide when stimulated with serum or purified growth factors (Pahk et al. 2017).

NB: Recent studies indicate that some SMCs exhibit a synthetic phenotype even under* in vivo *conditions.

In a significant 2010 study, ***Beamish JA and colleagues*** described the molecular regulation of contractile smooth muscle cell phenotype and its implications for vascular tissue engineering (Beamish et al. 2010).

4.10 Identification of Vascular Smooth Muscle Cells Based on Marker Proteins Expression

4.10.1 Markers for Contractile Phenotype of Vascular Smooth Muscle Cells

The best markers for the contractile phenotype in VSMCs are tropoelastin, a matrix protein; α-smooth muscle actin, γ-smooth muscle actin, calponin, and phospholamban.

In 1993, *CM Shanahan and colleagues* identified SM22α as VSMCs' contractile protein. They also identified CHIP28, a putative membrane channel protein that is not highly expressed in other SMCs as a new VSMC marker.

The developing bone marker osteopontin and matrix Gla protein (MGP) are strongly expressed in aortic VSMCs, but not in other SMCs. These proteins are responsible for calcification that commonly occurs in vascular lesions.

Recent studies indicate that smooth muscle myosin heavy chain (SM-MHC) and smothelin are specific markers *for a contractile phenotype.*

4.10.2 Marker Proteins for Synthetic Phenotype of Vascular Smooth Muscle Cells

- Identifying features of an SMC synthetic phenotype include elevated collagen type III, cyclophilin A and matrix metallopeptidase-9 in the neointima, elevated FDG uptake into the atherosclerotic carotid artery, and acutely high glucose transporter 1 expression in the neointima.

4.11 Contractile to Synthetic Phenotype Transformation of Vascular Smooth Muscle Cells

1. Vascular smooth muscle cells (VSMCs) are capable of both contractile and synthetic functions, although, in native form, they exist primarily in contractile phenotypes.
2. Three major factors have been identified that enable the transition of the contractile phenotype to the synthetic form. These are an expression of specific biochemical factors, ECM components, and physical factors including stretch and shear stress.
3. Studies reveal a decrease in contractile marker protein expression levels upon culturing SMCs. Slowly, the expression intensity of contractile proteins decreases while that for synthetic proteins increases.
4. Biochemical factors influencing the SMC phenotype include PDGF, transforming growth factor-beta (TGF-β), activin a, retinoid, angiotensin II, and tumor necrosis factor-α (TNFα).
5. For example, the two PDGF isoforms, namely, PDGF-A and PDGF-B, induce a more synthetic phenotype in human adult SMCs. PDGF-B also induces a contractile pig coronary artery to develop a rhomboid synthetic morphology in an elevated proliferation.

6. In contrast to PDGF, the TGF-β isoforms are deemed essential for contractile SMC phenotype formation.
7. Other ECM constituents that foster a contractile SMC phenotype include fibrillar collagen type I, collagen type IV, and laminin, all preferentially favoring the contractile phenotype.
8. While most ECM proteins support a contractile phenotype, fibronectin preferentially supports a synthetic phenotype.
9. Hyaluronan, a major ECM glycosaminoglycan, contributes to atherosclerosis progression and also favors the synthetic phenotype.
10. The specific organization of ECM proteins also affects the VSMCs' phenotype. As one example, 3D cultures enable higher collagen I expression and consequently favor a synthetic phenotype.
11. Thus, both the composition and organization of ECM components influence SMC phenotype (Rensen et al. 2007).

4.12 The Rationale for Culturing Vascular Smooth Muscle Cells

- VSMCs create the tunica media of mammalian arteries and regulate vasomotion/vascular tone by contraction and relaxation.
- VSMCs in culture serve as an important aid to investigate the mechanism by which VSMCs contribute to vessel wall contraction.
- Since VSMCs are one of the important constituents of major blood vessels, they perform a decisive role in vasculogenesis and angiogenesis, restenosis, and thrombosis.
- Like all other cells, VSMCs are also generated from the preexisting VSMCs or progenitor VSMCs by the process of proliferation and pericyte maturation.
- Experimentally, it is observed that alteration of a specific VSMC phenotype may be related to one or more specific pathophysiological conditions.

5 Isolation and Primary Culture of Vascular and Nonvascular Fibroblasts

5.1 Properties of Fibroblasts

- Fibroblasts are a type of connective tissue cells of mesenchymal (mesodermal) origin that produce collagen, elastin, and various other ECM components.
- In the large blood vessels, fibroblasts are present in the tunica adventitia (the outer layer).
- In certain conditions, epithelial cells can give rise to fibroblasts, through epithelial–mesenchymal transition (EMT).
- On the other hand, in the process of development, tissue repair, and tumor/cancer growth, fibroblasts may undergo mesenchymal–epithelial transition (MET).

5.1.1 The Relationship Between Fibroblasts and Fibrocytes

Fibroblasts and fibrocytes are two states of the same cells. The less active state of fibroblasts is called fibrocytes, typically responsible for maintenance and tissue metabolism.

5.2 Locations of Fibroblasts in Humans

5.2.1 Blood Vessels

Tunica adventitia, comprising the outer layer of macrovessels containing fibroblasts.

5.2.2 Heart

The heart contains special fibroblasts, called **myofibroblasts.**

5.2.3 Skin

The dermis layer of the skin contains fibroblasts.

5.2.4 Lung

The airways and interstitium of the lung contain fibroblasts.

5.2.5 Alimentary Canal

The matrix of the digestive tract contains fibroblasts.

5.2.6 Reproductive System

The vas deference and uterus (stroma) contain fibroblasts.

NB: Myofibroblasts are the cells representing an intermediate phenotype between fibroblasts and smooth muscle cells.

5.3 Dissecting Instruments for Isolation of Tissues and Organs Containing Fibroblasts

The required instruments are described in the isolation and culture of vascular endothelial cells, Sect. 4 of this chapter.

5.4 Materials for Isolation and Culture of Fibroblasts

The following materials are needed for fibroblast isolation and culture:

5.4.1 Sodium Pentobarbital

- 1 gm·ml^{-1} of pentobarbital is used as an anesthetic for animals.
- Use directly from the stock without any dilution.

5.4.2 Dulbecco's Phosphate-Buffered Saline

Sterile Dulbecco's phosphate-buffered saline (DPBS) is used to wash the tissue/organ samples.

5.4.3 Hanks' Balanced Salt Solution

Hanks' balanced salt solution (HBSS) is used for washing buffer and digestive solution.

5.4.4 70% Ethanol

70% ethanol is used as a disinfectant for the laminar flow hood working area and other materials.

5.4.5 Cell-Dissociating Enzymes

- Collagenase from *Clostridium histolyticum* stock (10 $mg \cdot ml^{-1}$ freshly prepared).
- Add 10 mg collagenase in 1 ml HBSS, filter sterilize, and use immediately.
- Do not store or reuse it later.
- The final concentration is 0.5 $mg \cdot ml^{-1}$.

5.4.6 Penicillin/Streptomycin

- Use as 1% (v/v) from the stock.
- The final concentrations of penicillin and streptomycin are (100 $U \cdot ml^{-1}$) and (100 $\mu g \cdot ml^{-1}$), respectively.

5.4.7 Fungizone

- 0.25 $mg \cdot ml^{-1}$ fungizone is used as an antimycotic agent.
- Use a final concentration of (0.25–2.5) $\mu g \cdot ml^{-1}$.
- Nystatin dehydrate can be used instead of fungizone.

5.4.8 DNase I from Bovine Pancreas

- Dilute 10 mg DNase I into 10 ml HBSS.
- Filter-sterilize aliquots and store at -20 °C.
- Use 100 $\mu g \cdot ml^{-1}$ as the final concentration.

5.4.9 0.1% Gelatin

Mammalian cell culture containers must be coated with 0.1% sterile gelatin as described in endothelial cell culture, a cell-adhesive agent in the culture containers.

5.4.10 Mammalian Cell Culture Containers and Associated Materials

- Flasks (25 mm/75 mm) or plates (30 mm/60 mm/100 mm), (15 and 50) ml centrifuge tubes, and pipettes of various capacities are needed for cell culture.
- All these materials are sterile, disposable, typically of mammalian cell culture grade, and made up of polystyrene.
- Membrane filters having various pore sizes are used for filter sterilizing the cell culture medium.

5.4.11 Cell Culture Medium for Fibroblasts Culture

- The complete medium contains DMEM with 4.5 g D-glucose, 10% FBS, 1% L-glutamine (2 mM final), 4 ng·ml^{-1} fibroblast growth factor (FGF), penicillin (100 U·ml^{-1}), and streptomycin (100 μg·ml^{-1}).
- If necessary, sodium bicarbonate may be added to adjust the medium pH.

The following are examples of methods for isolating and culturing fibroblasts:

5.5 Isolation and Culture of Fibroblasts from Mouse Lung Using Enzymatic Digestion

- Fibroblasts are fast-growing and can be rapidly expanded from small samples.

Fibroblasts can be isolated by any of the following three procedures:

- Enzymatic digestion (e.g., with collagenase or trypsin) of the tissue pieces.
- Explant culture of the small-sized ((1–3) mm in diameter) tissue pieces.
- A combination of enzymatic digestion and explant culture.

NB: Some protocols recommend that fibroblasts be cultured in a 3% (rather than 20%) oxygen environment.

- Some protocols recommend the use of fetal serum protein fetuin in the cell culture medium for more robust growth.
- Important publications in the field of fibroblast culture are credited to Vangipuram et al. 2013; SeluanovA and Gorbunova 2010; Keira et al. 2004; and Takashima 2001.

5.5.1 Isolation and Culture of Lung Fibroblasts

This protocol is a modified version of Das et al. 2002**.**

- Take three mice, each at least 10 weeks old.
- Euthanize the mice by intraperitoneal, 0.25 ml sodium pentobarbital injection using a 26-gauge needle.
- Alternatively, asphyxiate mice with CO_2 for 2 min.
- Spray the mice with 70% ethanol.
- Place the mice under a dissecting lamp with a magnifier.
- Surgically open all the mice (from the abdomen to the thoracic region) without cutting any blood vessels to avoid bleeding.
- Now expose the lungs and perfuse them with sterile tissue culture grade DPBS, and remove the system lungs–trachea–heart from the mice body.
- Discard the heart and trachea.
- Make 15 ml washing solution by adding penicillin (100 U·ml^{-1}) and streptomycin (100 μg·ml^{-1}) final concentration in HBSS.
- In a Petri dish, wash the lungs with the washing solution and place them in a Falcon tube in the washing solution.

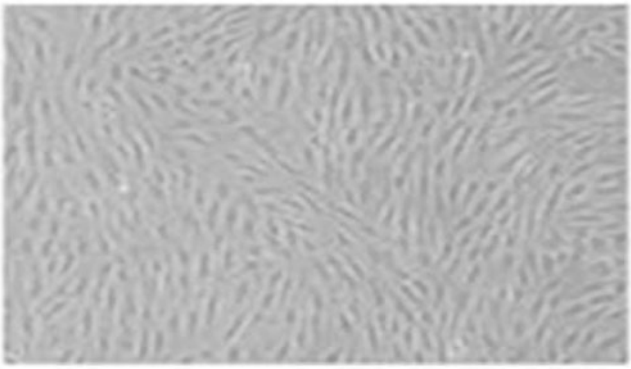

Fig. 9 Typical morphology of mice fibroblasts, segregated from pulmonary artery adventitia using the limited dilution cloning method. As visible, the isolated cells revealed a **rhombus morphology**. The study referred to here also isolated the fibroblasts from adventitial fibroblasts, which revealed a spindle shape (not shown here). Together, the differential origin fibroblasts were characterized by round or elongated morphology. (Das et al. 2002)

- Close Falcon tubes inside a lab hood and transfer the Falcon tubes to the cell culture hood.
- Open the tubes inside the hood.
- In a Petri dish, cut the lungs of the three mice into small pieces (2 mm in diameter) using a previously unused scalpel blade.
- Collect the lung pieces in one big Falcon tube.
- Make 15 ml digestion solution by adding 0.75 ml collagenase stock, 1.5 ml DNase stock, 150 μl penicillin/streptomycin stock, and then making to 15 ml using HBSS.
- Add 1.5 ml digestion solution to the Falcon tube containing the lung pieces and incubate inside a shaking water bath at 37 °C.
- After 10 min, pipette the solution inside the culture hood and dilute to neutralize proteolytic enzymes.
- Repeat the extraction six times (10 min $\times$ 6 = 60 min) to get a sufficient number of cells.
- Combine the extracts and centrifuge at 1200 rpm, 4 °C for 10 min to remove collagenase.
- Resuspend the cells in a 10 ml complete medium and place them in a T-75 flask.
- Place the flask in the CO_2 incubator.
- While live fibroblasts adhere to the plates, the dead cells float.
- Replace the medium the following day.
- If cells seem too sparse, replace the medium after 3 days instead of 1 day.
- While changing the medium, the floating dead cells will be removed.
- Subculture the cells as described for other mammalian cells.
- Figure 9 depicts the typical morphology of mice fibroblasts isolated from pulmonary artery using the limited dilution cloning method, wherein a rhombus morphology is inferred.

NB: Mouse fibroblasts are sensitive to oxidative stress and will senesce within ~ 14 population doubling times when maintained at 20% (atmospheric) oxygen.

Mouse fibroblasts can be maintained for more passages when maintained at 3% O_2.

5.6 Isolation and Culture of Fibroblasts from Rat Coronary Artery

In 2007, Jenkins and associates established a rat coronary artery fibroblast cell culture model.

- They used tunica adventitia of the artery to isolate fibroblasts.
- For detailed protocol, refer to ***Jenkins* et al.** 2007 publication *(*Jenkins et al. 2007*)*.

5.7 Isolation and Culture of Fibroblasts from Human Skin

- Fibroblasts are one of the important constituent cells of dermal (skin) tissues. The culture of skin fibroblasts would help to understand the skin connective tissue production, as well as the characteristic skin pathophysiology.
- In vitro cultured primary skin fibroblasts are widely utilized to understand the process of wound healing.
- Other major experiments that are conducted on in vitro cultured skin fibroblasts include studying the effects of various growth factors, toxicity studies, various drugs, and others.

NB: For culturing skin fibroblasts, the epidermis is either dissected out or enzymatically removed to prevent epidermal cell contamination into the skin fibroblasts culture.

The presented protocol is a modification of the original protocol, standardized by Keira et al. 2004**.**

This is an explant culture method, generally accepted as the best one.

5.7.1 Initial Processing of Human Skin Specimen

- For various reasons, portions of the human subjects (patients), that is, skin are required to be dissected or surgically removed by specialized doctors.
- After receiving written consent from the subject concerned, the skin sample is collected in a 50 ml sterile tube containing DMEM.
- The sample can be processed immediately in a laminar flow hood or stored at 4 °C, if it needs time but must be processed within 4 h of collection.
- It is recommended that the collected skin sample should be cleaned multiple times (exhaustively) with sterile PBS before processing.

Fig. 10 Skin fragments in the cell culture medium for attachment and growth

5.7.2 Primary Culture of Human Dermal Fibroblasts

- As mentioned above, the original protocol has been borrowed from the work of Keira et al. 2004.
- This is an **explant culture** method.
- Experimentally, the epidermis is removed from the dermis after which the isolated dermis is fragmented into 03 mm pieces.
- Now, 60 mm Petri dishes are taken and 0.5 ml cell culture medium is added. Henceforth, the dermis fragments are added to the culture plates.
- The Petri dishes are maintained in a semi-open state in a laminar flow for 40 min to adhere to the dermis specimen along the culture surface **(**Fig.10**)**.
- After 40 min of incubation, 5 ml of DMEM with 20% FBS, penicillin (100 $U \cdot ml^{-1}$), and streptomycin (100 $\mu g \cdot ml^{-1}$) are added to the culture plates. Now the culture plates are incubated at 37 °C in a CO_2 incubator under a 5% CO_2 environment.
- Change the medium at every 2 days interval.
- Approximately in 1-week time, fibroblast proliferation is observed.

5.8 Identification of Fibroblasts Based on Their Phenotype

5.8.1 Diameter of Fibroblasts

(10–15) μm.

5.8.2 Shape of Fibroblasts

Fibroblasts can take a **wide array of shapes in different tissues**.

Typically, fibroblasts acquire either bipolar or multipolar morphology.

5.8.3 Phenotype of Fibroblasts

- Fibroblasts are large, flat, elongated (spindle-shaped), or round-shaped with extensions radiating out from the ends of the cell body.
- The cell nucleus is flat and oval.

5.9 Identification of Fibroblasts Using Marker Proteins Expression

- Fibroblasts are identified based on their spindle shape combined with positive staining for the mesenchymal (mesodermal) marker vimentin.
- By immunohistochemistry (IHC), vimentin and TE-7 identify both dermal and lung fibroblasts.
- The fibroblast surface antigen (**FSA**), a glycoprotein produced by connective tissue cells (mesenchymal cells, fibroblasts, and astroglial cells), can also be used for fibroblasts and fibroblast cell line detection.
- Fibroblasts present in the skin, alveolus, and blood vessels express CD34, a marker of hematopoietic stem cells (HSCs).
- Other markers of fibroblast differentiation in the human dermis are endogenous peroxidases.
- By immunohistochemistry, heat shock protein 47 (HSP47) can also be used as a marker for skin fibroblasts (Kuroda and Tajima 2004).

5.9.1 Significance of Fibroblasts Culturing

- Fibroblasts do not carry mutations in their proto-oncogenes and tumor suppressor genes. Therefore, these types of cells possess a normal cell cycle, rather than defected one, as observed in tumor and cancer cells. These cells are used to understand the normal cell cycle, cell synchronization, and its regulation by various molecules, and finally differences in cell cycle between a normal cell and a tumor or cancer cell.
- Fibroblasts are an important example of cells to understand the normal process of cell proliferation by the process of mitosis, the normal process of programmed cell death or apoptosis, and the process of various kinds of DNA repair in completely normal cells.
- The fibroblasts of the blood vessels produce collagen that provides structural support by anchoring the blood vessels to nearby tissues.
- Fibroblasts also play a critical role in wound healing.
- Abnormal proliferation, activation, or differentiation of fibroblasts to myofibroblasts causes excessive synthesis and accumulation of collagen (a connective tissue material) and other ECM materials, leading to tissue fibrosis.
- As discussed previously, in the vascular wall the fibroblasts are present in the tunica adventitia. During the process of vascular injury, these cells get activated, proliferate, and respond to establish normalcy.
- The adventitial fibroblasts produce cytokines and chemokines during pathological conditions. In the next step, these cytokines and chemokines induce

infiltration of immunological cells into the adventitial layer of a vessel wall. Immune cell infiltration into the adventitia results in adventitial inflammation and can lead to cardiovascular diseases (CVDs).
- Fibroblasts are suitable for functional, biochemical, and genomic studies.
- Collectively, all the above observations indicate that fibroblasts provide an ideal cell model system for studying various normal physiological events and diseased states (An et al. 2015).

5.9.2 Examples of Studies One Can Conduct Using Fibroblasts

The significance of the fibroblast culture section has discussed the importance of fibroblast culture.

Briefly, the fibroblast culture has the following importance:

- Understanding the normal cell cycle, cell proliferation, and differentiation.
- Understanding the differences between normal cells and cancer cells, particularly concerning cell cycle regulation.
- Screening for drugs and toxins.
- Gene delivery to cells.
- Genome editing or CRISPR technology application.
- Understanding the process of wound healing.
- Understanding the physiology and various pathophysiologies of skin.

6 Isolation and Primary Culture of Epithelial Cells

6.1 Properties of Epithelium

The epithelium is the thin tissue layer, either single or multiple, forming the inner or outer surface of organs, glands, the mouth, nostrils, trachea, alveoli, ducts lumen of mammary glands, the lining of the alimentary canal, and urinary bladder.

Salient features of epithelium include the following:

- All surfaces, including **cutaneous, serous, and mucus,** are covered by epithelium.
- **No blood vessels** are present in the epithelium.
- The epithelium is not present in the inner lining of blood or lymph vessels. Endothelial cells replace the epithelial cells/epithelium in these vessels.
- Epithelial cells form the epithelium.

6.2 Differences Between Endothelial and Epithelial Cells

- Endothelial cells are present in the endothelium, which is the innermost layer of the blood vessels or circulatory vessels. These cells are never exposed to exteriors. The epithelial cells are present in the epithelium, which cover the structures

exposed to the exterior surfaces of the body (e.g., skin, intestine, urinary bladder, urethra, and many other organs).

- The endothelium is made up of a single layer of endothelial cells. On the other hand, the number of layers for epithelial cells (in an epithelium cell) varies in different tissue layers.
- Both endothelial and epithelial cells are of epithelial origin. However, while endothelial cells have **vimentin,** epithelial cells have **keratin.**
- Endothelial cells also contain von Willebrand factor or factor 8 antigen. Epithelial cells do not possess the von Willebrand factor.
- Endothelium provides a **non-thrombogenic surface, which is not so for e**pithelium.

6.3 Differences Between Epithelial Cells and Fibroblasts

- Epithelial cells are tightly connected and arranged in monolayers. They have numerous functions, including protection, diffusion, secretion, absorption, excretion, and separation of compartments. In contrast, fibroblasts comprise the structural framework of tissues and synthesize ECM.
- Epithelial cells undergo growth arrest in response to TGF-β, whereas fibroblasts undergo morphological changes and proliferate in response to TGF-β.
- Fibroblasts have much higher 5′-nucleotidase activity than epithelial cells and exhibit a faster 8-azaguanosine-5′-monophosphate degradation to 8-azaguanosine. As a result, epithelial cells (isolated from rat liver) are more sensitive to the toxic effects of the purine analog, 8-azaguanine.
- Fibroblasts can migrate as individual cells while epithelial cells cannot.
- Epithelial cells express the markers, pan-cytokeratin, CK8, and E-cadherin while fibroblasts express a high α-SMA.

6.4 Foremost Locations of Epithelial Cells in Humans

- Striated skin layers.
- The lining of the mouth, taste buds, nose, trachea, alveoli, alimentary canal, including esophagus, stomach, small and large intestine, urinary and gall bladder.
- Organelles in the kidney, pancreas, ear, and eye.
- Ducts and glands such as the bile ducts and salivary gland.
- Male and female primary and secondary reproductive organs include the testes, prostate, ovary, uterus, and mammary glands.

6.5 Classification of Epithelium Based on the Cell Layering

6.5.1 Simple Epithelium

The epithelium is composed of a single layer of cells.

6.5.2 Stratified Epithelium

This epithelium comprises multiple layers of cells.

6.5.3 Pseudo-Stratified

Epithelium of this regime possesses fine hair-like extensions, cilia, unicellular glands, and goblet cells that secrete mucus.

6.6 Additional Classifications of Epithelium

6.6.1 Keratinized Epithelium

- The most exterior or apical layer of dead cells contains keratin as a resistant protein.
- An example of this type of epithelium is found in mammalian skin, conferring water-resistant ability.

6.6.2 Transitional Epithelium

- This type of epithelia is found in tissues such as the urinary bladder, wherein cells change their shape due to stretching.
- Here, columnar epithelium may be converted to the cuboidal epithelium.

6.7 Dissecting Instruments for Isolating Epithelial Cells

The instruments are described in the isolation and culture of vascular endothelial cells, Sect. 4 of this chapter.

6.8 Materials for Isolation and Culture of Epithelial Cells

- This section describes the isolation and culture of epithelial cells from the trachea and alveoli of lung and mammary glands.
- The materials for isolation and culture of these cells are mentioned in their specific section on isolation and culture.

6.9 Isolation and Culture of Pulmonary Tracheal Epithelial Cells

Human pulmonary epithelial cells can be isolated either from the trachea or bronchioles or from the alveolus. In general, the trachea and bronchi are lined by a pseudo-stratified mucociliary epithelium, consisting of three cell types, as follows.

6.9.1 Basal Cells

Around 30% of tracheal epithelial cells are basal cells. These cells serve as a pool of progenitor cells that can repopulate a damaged epithelial cell layer.

6.9.2 Ciliated Cells

These cells propel mucus and clear particles from the respiratory tract.

6.9.3 Secretory Cells

These cells secrete mucus and other factors contained within the mucus layer.

Pulmonary epithelial cells can be cultured in two distinct ways:

Air Interface Culture

With this method, airway epithelial cells can be grown on porous membranes with the medium in the basolateral chamber but not on the apical side. The primary importance of this culture is to mimic the air-exposed environments to have similar in vivo airway conditions.

Liquid Submerged Culture

In this method, cells can be cultured with the medium on both sides, meaning cells are submerged in the medium.

Here, we discuss first the isolation and culture of tracheal and then alveolar, epithelial cells.

6.10 Comments Regarding Isolation and Culture of Pulmonary Tracheal Epithelial Cells

- It is very difficult to get trachea or human lung samples except during lung transplantation or accidental death.
- Additionally, important ethical issues limit the collection of human organs and tissues.
- Therefore, animals such as mice and rats are predominantly utilized to isolate tracheal epithelial cells.
- There are several protocols for isolation and culturing respiratory epithelial cells.

Some landmark studies in epithelial cell culture include the following:

- In 1977, ***Collier*** **et al.** described tracheal ring organ cultures**.**
- In Goldman and Baseman 1980, ***Goldman*** **et al.** described the enzymatic dissociation of epithelial cells followed by an investigation of various culture conditions.
- In 1988, ***Whitcutt*** **et al.** described the air–liquid interface (ALI) for the culture of tracheal epithelial cells.

6.11 Isolation and Culture of Human Tracheal Epithelial Cells

The method described here is a minor modified version of the one demonstrated by Bals **et al.** ***in*** 2004 ***with a minor modification.***

- It involves ALI cultures of human airway epithelial cells.
- There are two well-standardized protocols. The first one involves the culture of human epithelial cells from large airways while the second protocol involves the culture of human epithelial cells from distal airways. In both methods, cells are obtained from human lung pieces collected during surgery. The following sections describe the steps involved in human tracheal epithelial cell isolation and culture.

6.12 Reagent Preparation for Isolation and Culture of Human Tracheal Epithelial Cells

6.12.1 Preparation of Incubation Medium for Isolating Epithelial Cells from Large Airways

Dulbecco's modified eagle medium (DMEM) supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), tobramycin (40 μg/ml), ceftazidime (50 μg/ml), amphotericin B (2.5 μg/ml), imipenem-cilastatin (50 μg/ml), DNase (10 μg/ml), and dithiothreitol (0.5 mg/ml).

NB: All chemicals can be obtained from Sigma.

6.12.2 Preparation of Incubation Medium for Isolating Epithelial Cells from Distal Airways

DMEM/Ham's F12 medium supplemented with penicillin (100 U/ml)/streptomycin (100 μg/ml) gentamicin (0.5 mg/ml), and amphotericin B (10 μg/ml). For cystic fibrosis lung specimens, add ceftazidime (500 μg/ml) and ticarcilline (500 μg/ml).

6.12.3 Digestion Medium for Isolating Epithelial Cells from Large Airways

Incubation medium +0.1% protease 14 (Sigma).

6.12.4 Digestion Medium for Isolating Epithelial Cells from Distal Airways

MEM/Ham's F12 medium supplemented with 0.1% protease and 0.1% DNase.

6.12.5 Coating the Cell Culture Containers for Large Airways with Adhesive Agents

- Transwells are coated with collagen type 1.
- To prepare collagen, add 1 mg collagen to 1 ml 0.1 M acetic acid. Stir at room temperature for (1–3) hours or until collagen dissolves.
- Dilute ten-fold in distilled water to a resulting 0.1% working solution, store at 4 °C, and use within 4 weeks.
- To coat 1.2 cm inserts, add 100 μl collagen, whereas for 3 cm inserts, add 1 ml collagen.
- Incubate the collagen-filled culture vessels overnight at 37 °C.

- The next day, remove excess fluid from the coated culture containers and dry the culture containers for 30–60 min.
- The coated flasks are sterilized via exposure to UV light for 2–3 h in a laminar flow hood.

6.13 Coating the Cell Culture Containers for Distal Airways with Adhesive Agents

For distal airways, use a homemade collagen membrane affixed to Plexiglas support.

6.13.1 Preparation of Medium for Air–Liquid Interface Cell Culture from Large Airways

As discussed in the previous section, this protocol is adapted from the research work of Bals et al. 2004 ***with minor modifications.***

- Ultroser G2 is supplemented to the DMEM/Ham's F12.
- Mostly, the large airways, trachea, or main bronchus are dissected.
- Start the proximal dissection and proceed distally.
- Airways: DMEM/Ham's F12 medium supplemented with insulin (5 μg/ml), transferrin (7.5 μg/ml), hydrocortisone (10^6 M), endothelial cell growth supplement (2 μg/ml), EGF (25 ng/ml), triiodothyronine (3×10^8 M), L-glutamine (1 mM), penicillin/streptomycin (100 μg/ml), gentamicin (50 μg/ml), and amphotericin B (5 μg/ml).
- For cystic fibrosis cultures, add ceftazidime (125 μg/ml) and tobramycin (100 μg/ml).
- Chemicals should be procured from Sigma/other standard companies.

6.14 Isolation and Culture of Epithelial Cells from Large Airways

As discussed in the previous section, this protocol is adapted from the research work of Bals et al. 2004 ***with minor modifications.***

- Retrieve resected lung tissue prepared on a clean working surface and store in sterile PBS without Ca^{+2} and Mg^{+2} for up to 2 h (for laboratory transport of a sample).
- Before processing, aseptically clean the large airways multiple times in sterile PBS.
- The attached soft tissue or lung parenchyma is dissected.
- Open the airways longitudinally by cutting.
- Incubate in an incubation medium for 6–24 h at 4 °C.
- Remove medium and replace it with the digestive medium before storing at 4 °C for 2 h.
- Now, transfer the digestion solution and the airway into plastic dishes.

- Hold the airway with forceps and use a scalpel to scrape the luminal surface of the airway 4–5 times.
- The luminal epithelial cells detach and gradually come into the solution.
- The rest of the airway material is discarded and put into a biohazard disposal container.
- Spin down the cell suspension at 170 × *g* for 10 min.
- Remove the supernatant and resuspend the cell pellet in the **bronchial epithelial cell growth medium (BEGM).**
- To remove mucus and other materials present in the cells, spin the solution at 40 × *g* for 30 s.
- The epithelial cells containing supernatant are transferred into a culture vessel (size depends on the number of cells) and cultivate at 37 °C in a humidified atmosphere of 5% CO_2 air for 24 h.
- Following incubation, spin down the cells using low-speed centrifugation.
- Now that the cells are ready for being seeded into the transwells coated with collagen (collagen type I, Sigma C9791), wet the membrane using a 10-min immersion in the medium before emptying the wells.
- Fill the lower reservoir first with culture medium (1 ml for large 3 cm inserts, 0.2 ml for smaller 1.2 cm inserts).
- Fill the upper reservoir with a culture medium containing the appropriate number of cells to obtain a density of approximately 1×10^6 cells/cm^2 (0.5 million cells for small inserts and 4×10^6 cells for large inserts).

NB: The lower reservoir should be guarded for possible contamination with the cell-containing solution.

6.15 Isolation and Culture of Epithelial Cells from Distal Airways

As discussed in the previous section, this protocol is adapted from the research work of Bals et al. 2004 ***with minor modifications.***

- Collect the lung pieces.
- Wash with the incubation medium.
- Place in a clean location for further processing.
- Identify bronchioles by the devoid wall cartilage and ~ 1 mm outer diameter.
- Now remove the larger structures such as bronchi and vessels.
- Remove lung parenchyma from bronchioles using sharp curved scissors and binoculars.
- Cut the bronchioles into small segments (3–10 mm).
- Put the bronchiolar segments into a dish containing the incubation medium at 4 °C.
- For cystic fibrosis (CF) bronchioles, put the segments for at least 4 h in the medium to purge the contaminated bacteria (if any).

- Cut and open the bronchioles longitudinally to expose the epithelium to the digestion medium.
- Put the containers at 4 °C for incubating the bronchioles in the digestive medium.
- The epithelial cells would be gradually detached, coming into the digestive medium.
- Following incubation, neutralize the enzymes by adding 10% FBS to the digestion medium.
- Now remove the undigested bronchioles.
- Perform low-speed centrifugation of the epithelial cells.
- Resuspend the pellet into the culture medium.
- Observe the cells in a microscope and count their number.
- Fill the lower reservoir with the culture medium.
- Seed the cells on the collagen membrane (**Invitrogen**) at a **4×10^4 cells/mm^2** loading rate.

6.16 Culture After Seeding

- Following seeding, incubate the culture containers in a CO_2 incubator at 37 °C in a 5% CO_2, 95% moisture, environment.
- Cells should be settled and may be confluent if checked within 24 h of seeding.
- For **bronchiolar** but not bronchial cultures, no medium is added to the cell surface after seeding.
- For bronchial cultures, remove the apical medium when cells are completely confluent, (usually after 3 days).
- Subsequently, PBS is used to wash the apical side of the epithelial layer for 2–4 days.
- Every 24 h, the basolateral culture medium would be changed.

NB: Avoid contaminating the upper reservoir with the basolateral culture medium.

- At this juncture, the cell differentiation could be monitored using microscopical inspection of beating **cilia** and using an **ohmmeter** to measure the transepithelial electrical resistance.

6.17 Isolation and Culture of Porcine Tracheal Epithelial Cells

***This method was established by Yu W,* et al *with a little modification*. *Here are the step-by-step procedures established by the scientists*.**

The authors claimed that every material used in this procedure, including the small instruments, should be sterile besides doing all the isolation work inside the laminar flow hood.

- Remove the skin with the help of clean surgical scissors and scalpel.
- Cut along the sternum to open the upper abdomen.
- Now remove the rib cage.
- Cut the trachea and put it into a 50 ml conical tube containing 30 ml Ham's F12 medium supplemented with antibiotics, on ice. The medium will wash the trachea.
- In the next step, transfer the trachea into a 100 mm Petri plate containing 10 ml Ham's F12 medium supplemented with antibiotics.
- With the help of sterile forceps and surgical scissors, gently dissect away the connective tissue.
- To expose the lumen, cut the trachea along the vertical axis.
- In the next digestion step, the trachea is treated with 0.75% protease in M199 medium at 4 °C for 14 h.
- Now is the step of harvesting the ciliated cells. The harvesting is done by shaking the mucosa in an M199 medium enriched with 10% FCS and 1% penicillin-streptomycin.
- To minimize fibroblast contamination, allow the cells to settle for 1 h in a plastic Petri dish.
- At this point, while the fibroblasts remain adhered to the bottom of the Petri dish the resulting ciliated cells may remain clustered and float in the media.
- Collect resulting clusters of ciliated cells and wash them after 5 min of centrifugation at 220 × *g*, in an M199 medium.
- Resuspend the cells in an M199 medium.
- From each of the trachea(s), (5–10) ciliated cell culture samples may be obtained.

6.18 Isolation and Culture of Mouse Tracheal Epithelial Cells

This method was established by Lam et al. 2011 ***with a minor modification.***

6.18.1 Reagent Preparation for Isolation and Culture of Mouse Tracheal Epithelial Cells

Before commencing the isolation, prepare the following solutions:

- **Ham's F12 Medium Preparation**

  In 250 ml of Ham's F12 basal medium, add penicillin (100 $U \cdot ml^{-1}$), streptomycin (100 $\mu g \cdot ml^{-1}$), and fungizone (3 $\mu g \cdot ml^{-1}$). Store at 4 °C for up to 4 weeks.
- **0.5% Pronase Solution Preparation**

  In 10 ml Ham's F12 medium, add 15 mg pronase, antibiotics, and antimycotics. Make the solution freshly and keep on ice until use.
- **Collagen 1 Solution Preparation**

  Prepare collagen 1 solution at a concentration of 50 $\mu g \cdot ml^{-1}$ in 0.02 N acetic acid.

  Add 1.0 ml of this collagen solution into each well of a 12-well transwell plate (Corning).

Now, cover the wells with paraffin and incubate overnight at room temperature.

- **DNase 1 Solution Preparation**
  To 18 ml antibiotic containing Ham's F12 medium, add 2 ml, 10 $mg \cdot ml^{-1}$ bovine serum albumin (BSA) stock solution, and 10 mg crude pancreatic DNase I. Make 1 ml aliquots and store them at -20 °C until use.
- **Preparation of Ham's F12 Medium Containing Antibiotics with 20% Fetal Bovine Serum**
  For a 200 ml Ham's F12 basal medium, add 40 ml FBS, 100 $U \cdot ml^{-1}$ penicillin, 100 $\mu g \cdot ml^{-1}$ streptomycins, and 3 $\mu g \cdot ml^{-1}$ fungizone.

6.18.2 Prepare Mouse Tracheobronchial Epithelial Cell Basic Medium Containing Antibiotics

To 475 ml DMEM/F12 basic medium, add 7.5 ml (1 M) HEPES solution, 10 ml (200 mM) glutamine solution, 2 ml (7.5%) $NaHCO_3$ solution, 100 $U \cdot ml^{-1}$ penicillin, 100 $\mu g \cdot ml^{-1}$ streptomycin, and 3 $\mu g \cdot ml^{-1}$ fungizone.

6.18.3 Prepare Mouse Tracheal Epithelial Cell Medium with 10% FBS

- Take 45 ml mouse tracheal epithelial cell basic medium containing antibiotics.
- In this medium, add 5 ml heat-inactivated FBS to make it a 10% serum-containing medium.
- Like all other cell isolation procedures, a clean work surface area, including a laminar flow hood, is necessary to isolate mouse tracheal epithelial cells.
- Standardized small instruments as required for other cell isolation instruments, such as laminar flow hood, humidified CO_2 incubators, inverted microscope, disposable plastic pipette, culture ware, table-top cell centrifuges, and cold room, are necessary.

NB: To add the exact concentration of antibiotics/other chemicals for cell culture, one may need to check the company booklet from where the chemicals are sourced.

6.18.4 Protocol for Isolation of Mouse Tracheal Epithelial Cells

As discussed above, this method was established by Lam et al. 2011 ***with a minor modification.***

- Commercially available mouse strains such as C57Bl/6, male 6–8 weeks old were used by the authors.
- For the culture of a 12-well transwell plate, around six mice are needed that will yield 1.5–2.0) $\times$ 10^5cells/mouse.
- The mice were euthanized using a standard procedure such as CO_2–-induced necrosis or by injecting pentobarbital.
- Remove the skin with the help of clean surgical scissors and scalpel around the tracheal area and expose the trachea.

- The upper abdomen needs to be opened before cutting the sternum and removing the rib cage.
- Continue removing the tissue until the end of the trachea is exposed.
- Now, place the trachea into a 50 ml conical tube containing 30 ml Ham's F12 medium with antibiotics on ice. This will cleanse the trachea and minimize the contamination risk.
- In the next step, transfer the tracheal tissue to a sterile 100 mm Petri dish containing 10 ml Ham's F12 medium supplemented with antibiotics.
- With the help of sterile forceps and surgical scissors, gently dissect the connective tissue.
- Expose the lumen-cut trachea along the vertical axis.
- Take a 50 ml tube, add 10 ml (0.15%) pronase solution and now transfer the trachea into it and incubate overnight at 4 °C.
- On the next day (after ~24 h), gently rock the tube 10–12 times and then let it stand for (30–60) minutes at 4 °C.
- Add 10 ml Ham's F12 medium containing 20% FBS and antibiotics to the tube and rock 12 times.
- Get the 3, 15 ml conical tubes prepared to contain 10 ml Ham's F12 medium having antibiotics and 20% FBS.
- The trachea is removed from the pronase solution before being placed in an ice solution.
- In the next step, the trachea is transferred to the first conical tube containing Ham's F12 and then inverted 12 times. Repeat this process twice.
- Henceforth, take one 50 ml tube and merge the pronase solution with three supernatants.
- Discard the remaining tissue.
- Centrifuge the collected supernatant at 1400 rpm (390 × *g*) for 10 min.
- Discard the supernatant.
- Resuspend the pellet in a 1 ml DNase solution ((100–200) µl/trachea) and incubate for 5 min on ice.
- Again centrifuge at 1400 rpm (390 × *g*) for 5 min at 4 °C.
- Discard the supernatant.
- Finally, resuspend the cell pellet in an 8 ml MTEC medium containing 10% FBS.
- Transfer the cell suspension on Primaria plates (Falcon).
- Incubate the Primaria plates at 37 °C in an atmosphere of 95% air and 5% CO_2 for 5 h.

NB: The following steps are used for the negative selection of fibroblasts.

- Take the plates to retrieve the cell suspension.
- Rinse the plates with 4 ml MTEC containing 10% FBS.
- Take a 50 ml conical tube and pool the cell suspension into it.
- **NB:** Put 1 ml separately for cytospin and cell counting.
- In a table-top centrifuge, spin at 5000 rpm for 5 min.
- Remove 500 µl and resuspend the pellet in the remaining supernatant.

- For cell counting by Trypan Blue vital staining method, use 100 μl cell suspension.
- Conserve four aliquots of 100 μl each for cytospin analysis.
- Spin the remaining 15 ml cell suspension at 1400 rpm (390 × *g*) at 4 °C for 10 min.

6.18.5 Mouse Tracheal Epithelial Cells at Air–Liquid Interface: Propagation and Differentiation

Retinoic Acid Stock Solution Preparation

- Make a 5 mM stock solution of retinoic acid (mol. wt. 300.44 g μg/mol) in 95% ethanol.
- Store in a foil-wrapped tube at −80 °C.

NB: It is a light-sensitive molecule. So, preparation must be done in dark. It is herewith referred to as stock solution A.

Stock Solution B Preparation

- For the preparation of 50 μM stock solution B, add 50 μl stock A, 500 μl BSA solution (100 mg/ml), and 49.5 ml **HBSS**.
- Store in a foil-wrapped tube at −80 °C for up to 4 weeks.

6.18.6 Mouse Tracheal Epithelial Cells Proliferation Medium with Retinoic Acid Preparation

To prepare mouse tracheal epithelial cells basal medium (**MTEC**), add the following:

47.5 ml MTEC with antibiotics
2.5 ml FBS (heat inactivated)
1 ml retinoic acid stock B.
250 μl insulin solution (2 mg/ml insulin in 4 mM HCl).
250 μl epidermal growth factor solution (5 μg/ml EGF in HBS containing 1 mg/ml BSA) 200 μl bovine pituitary extract (15 mg/ml in HBS containing 1 mg/ml BSA).
50 μl transferrin solution (5 mg/ml transferrin in HBS containing 1 mg/ml BSA.
50 μl cholera toxin solution (100 mg/ml in HBS containing 1 mg/ml BSA).
Filter-sterilize the medium and use it within 2 days.
Remove the collagen solution from the transwell plates.
Wash with sterile PBS twice.
Centrifuge the content.
Resuspend the cell pellet in ~500 μl.
Plate 7.5×10^4 -1.0×10^5 cells per well.
In the basal compartment of the transwell plate, add a 1.5 ml proliferation medium.
The submerged MTEC cultures should be incubated at 37 °C in a humidified incubator containing 95% air and 5% CO_2 for (7–10) days.

Change the medium after 3 days.
Thereafter, change the medium every 24 h.
Monitor cultures by visual inspection and measurement of transepithelial cell resistance (EVOM Ohm voltmeter, World Precision Instruments, Sarasota, FL).
When cells appear confluent and epithelial resistance reaches 1000 Ω/cm^2, they are ready to differentiate.

6.18.7 Mouse Tracheal Epithelial Cells Basal Medium Preparation

Take a sufficient quantity of MTEC and add 2% NuSerum.

- Add retinoic acid stock B to a final concentration of $\mathbf{1 \times 10^{-7}\ M}$ before using.
- Prepare fresh and use within 2 days.
- Allow cells to differentiate for (10–14) days by removing the apical medium and replacing the basal medium with a 750 μl MTEC basal medium containing 2% NuSerum and retinoic acid.
- Change the basal medium and wash the apical side with MTEC containing 2% NuSerum every 24 h.

6.19 Isolation and Culture of Pulmonary Alveolar Epithelial Cells

- The human pulmonary alveolus contains two kinds of epithelial cells. They are alveolar type 1 epithelial **(ATI)** cells that cover around (90–97)% alveolar surface area and alveolar type II epithelial **(ATII)** cells that cover around (3–10)% alveolar surface area.
- AT-II cells become AT I cells when AT I cells are injured or die.
- Therefore, **AT-II cells act as progenitors for AT I cells**.

6.19.1 Identification and Characterization of Alveolar Type 1 Epithelial Cells

AT I cells cover (90–97)% alveolar wall.

6.19.2 Morphology and Structure of Pulmonary Alveolar Type I Cells

AT I cells can be identified based on their morphology, while being observed using an electron microscope with the following characteristics:

Large squamous epithelial cells
Flattened nuclei
Few mitochondrion
Few cellular inclusions
No lamellar bodies

6.19.3 Metabolic Activity and Cell Division of Pulmonary Alveolar Type I Cells

Metabolically inactive, rarely divide or may not divide at all.

6.19.4 Functions of Alveolar Type 1 Epithelial Cells

AT1 cells provide a barrier function to alveoli and help in gas exchange.

NB: Isolation and culture of ATI cells are difficult for the following reasons:

- They have very thin cytoplasmic extensions.
- They form very tight intercellular junctions.
- They are metabolically inactive.
- They rarely divide or do not divide at all.

6.19.5 Identification and Characterization of Alveolar Type 1I Epithelial Cells

AT-II cells cover (3–10)% of the alveolar wall.

6.19.6 Morphology and Structure of Alveolar Type 1I Epithelial Cells

Small cuboidal epithelial cells.

A large number of lamellar bodies with a histology can be confirmed using electron microscopy [via modified papanicolaou or nile red (a fluorescent dye) staining].

Abundant mitochondrial presence.

6.19.7 Metabolic Activity and Cell Division

- Highly metabolically active and actively dividing cells.
- AT-II cells are readily activated by TNFα and produce various cytokines, SP-C, and intercellular adhesion molecule 1 (ICAM-1).

6.19.8 Functions of Alveolar Type II Epithelial Cells

Produces surfactants and pulmonary host defense proteins.

6.19.9 Culture of Alveolar Type II Epithelial Cells

- The isolation of rat alveolar type II (AT-II) epithelial cells was first published in 1974.
- Since then, a large number of studies isolated and cultured AT-II epithelial cells from various mammalian species including humans.
- Some important methods in this field were established by Dobbs 1990; Corti et al. 1996; Wang et al. 2007; and Ballard et al. 2010.
- In 1996, *Rannels and colleagues* described the culturing of primary rat AT-II epithelial cells at various stages of differentiation.

The following is a protocol for isolation and culture of human primary alveolar type II epithelial cells:

6.19.10 Specimens Collection and Isolation of Alveolar Type II Epithelial Cells

The protocol presented here for isolation and culture of human primary AT-II epithelial cells was published by Mao* et al.** 2015. ***This method of isolation was

established by the adaption and modification of several previous protocols. Here is the step-by-step protocol:

- Obtain the specimen of distal normal lung tissue (6–10) g from patients undergoing lung resection under sterile conditions.
- The lung specimen should be cut into a 1 cm^3 cube.
- Extensively wash the pieces with Hanks' balanced salt solution (**HBSS**).
- With the help of scissors, mince the lung pieces (0.5 mm^3) and transfer them to a sterilized beaker containing 50 ml BSS.
- Mix gently.
- Filter it by passing through a mesh (150 μm) cell strainer (BD Falcon, San Jose, CA).
- Incubate the lung tissue for 45 min in a mixture of proteolytic enzymes containing 3 ml trypsin (10,000 U/ml) and 300 µl elastase (5.1 U/ml) in a shaking water bath at 37 °C. Add DNase I at 2 ml (10,000 U/ml) for 15 min.
- Stop the proteolytic digestion using 40 ml inhibition solution (30 ml DMEM: F-12, 1:1, 10 ml FBS and 1 ml DNase I 10,000 U/ml).
- Further, dilute the digested suspension by adding 400 ml HBSS and thorough whiffling for 10 min using a Pasteur pipette.
- Now, filter the suspension using the cell strainers at the size of (150, 75, and 40) μM in tandem to collect the crude cell extract.
- In the next step, centrifuge the suspension at 400 × g for 10 min at room temperature.
- Resuspend the cell pellet in 100 mm Petri dishes containing 10 ml of each adhesion medium for macrophages and fibroblasts.
- The medium constitutes the following: 1:1 mixture (22.5 ml each) of DMEM-F-12 and small airway epithelial cell growth medium, SAGM; 5 ml FBS and 1 ml DNase I 10,000 U/ml.
- Incubate the cells at 37 °C for 150 min.
- Early attachment of macrophages and fibroblasts with the cell culture containers was noticed.
- Epithelial cells attach late. This characteristic distinction helps in separating the epithelial cells from macrophages and fibroblasts.
- Centrifuge and resuspend the cell pellets in 3 ml DMEM-F12 and layer the crude cell suspension onto a (1.040–1.089) g/ml discontinuous Percoll gradient.
- A solution of 40 ml light gradient (1.040 g/ml) containing 4 ml PBS, (10 × 12.55) ml Percoll solution (MP Biomedical, Solon, OH), and 23.45 ml distilled water, is preserved.
- A solution of 40 ml heavy gradient (1.089 g/ml) containing 4 ml PBS (10 × 25.96) ml Percoll solution (MP Biomedical), 10.04 ml distilled water, and one drop of phenol red is obtained.
- Take 15 ml centrifuge tubes in which 3 ml from each solution is gradually added to 5 ml FBS. Subsequently, conduct the centrifugation at 300 × g for 20 min at 4 °C, with the aid of a swing-out rotor. This results in the formation of a dense

layer of AT-II cells along with the interface between the two Percoll gradient layers.
- Conduct deceleration via turning off the power as the designated centrifugation duration is neared.
- Relocate the intensified cell pellets (of AT-II cells) in a 15 ml centrifuge tube already having 13 ml BSS. Perform centrifugation twice at 300 × *g* till 10 min at room temperature.
- Resuspend the pellets in a 2 ml HBSS and attempt a magnetic bead-assisted separation to get rid of any kind of macrophage contamination (Anti-CD^{14} MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany).
- Subject the isolated AT-II cells at 300 × *g* centrifugation for 10 min at room temperature. Thereafter, resuspend the cells in a 2 ml SAGM, carrying 1% FBS. Thereafter, incubate the cell mixture in 60 mm culture dishes at 37 °C for 60 h, changing the medium every 24 h.
- Subject the cells to subculturing, either in SAGM or DMEM having either 1% or 10% FBS till 21 days. The 10% FBS, in general, exhibits a greater growth.

6.19.11 Limitations of the Culture of Pulmonary Alveolar Type II Epithelial Cells

- Several investigators, including Campbell et al. 1999 ***and*** Meleady et al. 1998**,** have suggested an important feature of isolated AT-II epithelial cells is their loss of specific features within days of in vitro culture and the acquisition of AT I epithelial cells characteristics.
- This process is highly dependent on cultural conditions and is termed **trans-differentiation**.
- Of note, in vitro trans-differentiation is at least partially reversible, but it is not known whether the reversibility of AT-II epithelial cell differentiation into AT I epithelial cells is a potential regulatory mechanism in vivo.

6.20 Isolation and Culture of Mammalian Mammary Epithelial Cells.

6.20.1 Basic Structure of Mammary Gland

Some basic aspects of the mammary glands are as follows:

In mammals, mammary glands (breast) produce milk and also act as secondary sexual organs.

- The human breast comprises a specialized branching duct network that culminates in clusters of smaller ductules. Together, these comprise the terminal ductal lobular units (TDLUs).
- Of note, TDLU may comprise (30–50) acinar cells grouped as a lobule along with their associated ducts.
- Interestingly though, acinar cells are the smallest functional structures in a breast.

- A terminal duct, in general, arises from each acinar cell. The branching ducts besides acini prevail at the terminus of each ductal system.
- The primary function of the acini is to secrete milk.
- Milk secreted by the acinar cells passes through the lobule and duct to reach the nipple.
- The cellular structures predominating the breast are epithelial cells. Significantly, the mammary epithelium is quite distinct contrary to several other human body tissues as it persists to develop right after birth. Major events in course of this development comprise vigorous remodeling with branching cycles, acini generation, and dissolution of epithelial structures amidst puberty, pregnancy, lactation, and involution.

The branching ducts and acini are composed of three types of cells, which are as follows:

Mammary stem cells
Myoepithelial cells
Luminal epithelial cells

The followings are a brief discussion of the three types of human mammary epithelial cells.

6.20.2 Mammary Stem Cells

- Mammary stem cells (**MaSCs**) are small progenitor cells located at the basal or outer layer of the duct/ductules/acini.
- MaSCs are located at the top of the epithelial hierarchy and have unique traits, including self-renewal and multidirectional differentiation.
- These cells proliferate and differentiate into myoepithelial and luminal progenitor cells that further differentiate into myoepithelial cells or luminal epithelial cells.

6.20.3 Myoepithelial Cells

- The outer layer of the ductal/lobular/acini structures of the mammalian breast is located at the surface of the basement membrane and is composed of myoepithelial cells.
- These cells are flattened and elongated in their morphology.
- These cells contain numerous myofibrils (the contractile units), the absence of rough-surfaced endoplasmic reticulum (rough ER), and lipid droplets.
- Milk flow into the ducts is driven by the contractions of the myoepithelial cells (Fig.11).

6.20.4 Luminal Epithelial Cells

- The inner layer of the ductal/lobular/acini structure of the mammalian breast is composed of luminal epithelial cells, with apical microvilli.

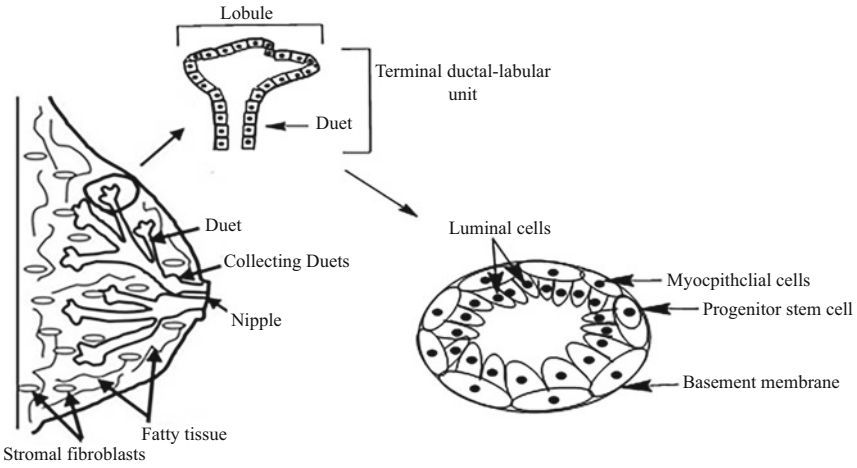

Fig. 11 Schematic depiction of the location of various types of epithelial cells in the human breast. (Dimri et al. 2005; Breast Cancer Research] with modifications)

- The luminal lineage can be further subdivided into ductal and alveolar luminal cells that line the ducts and constitute the alveolar units arising during pregnancy (Fig.11).
- These cells are arranged radially with tight junctions and narrow widths. These cells secrete milk.

6.20.5 Isolation, Purification, and Characterization of Mammary Epithelial Cells

- A large number of studies have established the isolation and culture methods of epithelial cells from mammalian breast tissues.
- Notable examples in this field include publications by Shipitsin et al. 2007; Labarge et al. 2013; and Raouf and Sun 2013.
- In 2015, *ZubeldiaPlazaola* et al compared the different isolation and culture methods of mammalian breast luminal and myoepithelial cells.
- The cell yield and viability of the mammary epithelial cells isolated by various methods depend on the following factors:

  Mechanical Handling

  Whether or not to discard adipose tissue and the specific size of a piece of tissue.

  Proteolytic Absorption

  This method critically depends on digestion time. Type and concentration of digestive enzymes, viz., collagenase/hyaluronidase or a stoichiometric blend of the two.

  Separation of Cell Portion

  This is accomplished using sequential filtering or differential centrifugation.

  Final Cell Isolation

  This is done either using immunogenetic beads or via sorting.

- Primary cultures suffer from certain restrictions such as the episodes of senescence, once (10–40) population doublings have happened. Thereby, these cultures are often not useful for long-term requirements.
- Studies by Garbe et al. (2009) established that the medium configuration and the inclusion of Rho-associated protein kinase (ROCK) hinder or prolong the senescence happening after digestion. Such changes or hallmarks result in improved cell proliferation by preventing anoikis (perishing of cells due to loss of attachment).
- Technical snags demonstrated to date have a significant impact on cell viability and yield, and impede the primary cells culture, suggesting a requirement to streamline the technology for apt segregation of epithelial and myoepithelial cells.

6.20.6 Materials for Isolation and Culture of Mammalian Mammary Epithelial Cells

Materials needed for isolation and culture of mammary (breast) epithelial cells are the same as for isolation and culture of other mammalian cells. Materials specifically needed are as follows:

- DMEM: F12 in 1:1 ratio with antibiotics and antimycotics
- Bovine serum albumin (BSA)
- Collagenase type IV
- Hyaluronidase
- ROCK inhibitor or Y27632
- Epithelial and organoid culture medium: M87A formula previously described (Garbe et al. 2009)

6.21 Isolation and Culture of Human Mammary Epithelial Cells

This protocol is developed from the publication by Jin et al. 2018 ***with minor modifications. The following steps describe the protocol:***

- All the tasks must be completed aseptically in a laminar flow hood.
- Collect normal human breast tissues from surgical specimens with written informed consent from the patient and store them at 4 °C before processing. The breast tissue must be processed within 3 h of collection and discarded if it is not processed more than 3 h after collection.
- The tissue is minced or cuts into 1 mm^2 pieces.
- Now the breast tissue is treated with collagenase and hyaluronidase mixture overnight at 37 °C.
- Following overnight treatment with enzymes, the mixture is filtered using a nylon strainer.
- Collect the flow-through fractions and centrifuge the fractions at 1200 rpm for 10 min to obtain cell pellets.

- Use Accumax to further dissociate the cells and obtain single cells.
- Resuspend the cells in F-medium. F-medium contains Dulbecco's Modification of Eagle's Medium: Ham's F-12 (1:1) with 2 mM L-glutamine, 5% FBS, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 8.4 ng/ml cholera toxin, 10 ng/ml epidermal growth factor, and 24 μg/ml adenine with 10 μM Y-27632.
- Now, seed the cells on a layer of 50% confluent irradiated or mitomycin-treated 3 T3-J2 feeder cells for expansion. Maintain all cultures at 37 °C and 5% CO_2.
- To reach 80–90% confluence in 6-well plates, the initial colony expansion may take 1–2 weeks.
- Passage cells with 1:10 splitting.
- Now the cells take lesser time (within 4–6 days) for (80–90)% confluence.
- These cells can be passaged with accutase (Innovative Cell Technologies) to avoid the negative impact of enzymatic conditions.

6.22 Isolation and Primary Culture of Human Mammary Epithelial Cells

The presented protocol is the one proposed by Kothari et al. 2003 ***with minor modifications and consists of the following steps:***

- Collect the breast tissue following surgery.
- Mince or cut it into small pieces.
- Mince the breast tissue and digest overnight at 37 °C using type 1A collagenase (1 mg/ml) in RPMI-1640 plus 5% FCS and 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 U/ml polymixin B, and 2.5 mg/ml amphotericin B.
- Now decant off the fat tissue.
- Use the medium to wash the remaining organoids and cells three times.
- Wait for 20 min so that the organoid settles down.
- Remove the supernatants and resuspend in RPMI-1640 containing 1% FCS.
- Complete another two rounds of sedimentation.
- Now, digest the pellet with trypsin/EDTA (0.05/0.02% in PBS) plus 0.4 mg/ml of DNase I for (15–30) minutes at 37 °C.
- Terminate the reaction by adding cold RPMI plus 10% FCS.

6.22.1 Purification of Epithelial Cells

- The isolated epithelial cells are purified via immunoaffinity using super-paramagnetic, polystyrene beads (Dynal Ltd., New Ferry, Wirral, UK) coated with a mouse IgG1 monoclonal antibody (MAbBer-EP4) specific for two (34 kDa and 39 kDa) glycopolypeptide membrane antigens as described by Latza et al. 1990.
- Culture the purified cells in BCM (DMEM: F-12 (1:1), supplemented with 15 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 U/ml polymixin B, 2.5 mg/ml amphotericin B, 5 mg/ml insulin, 10 mg/ml

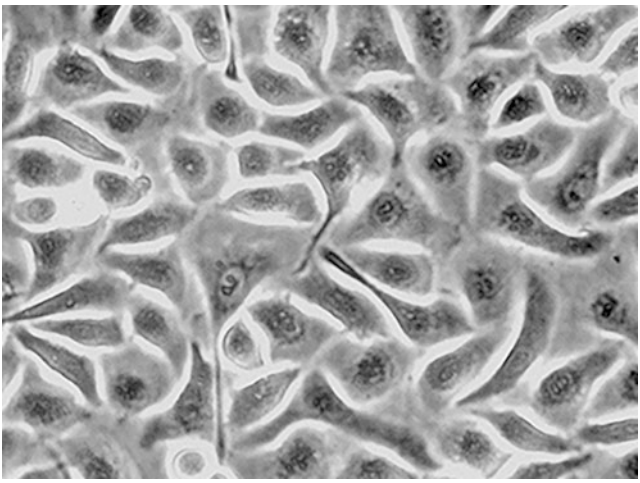

Fig. 12 Phenotypic growth pattern of cultured mammary epithelial cells (100% confluent) as observed through an inverted microscope

apotransferrin, 100 mM ethanolamine, 1 mg/ml hydrocortisone, 10 ng/ml EGF, and 10% FCS as described by Gomm et al. 1995.

- Figure 12 depicts the phenotype morphology of cultured mammary epithelial cells in a 100% confluent state.

6.23 Isolation and Culture of Human Mammary Epithelial Cells

The presented protocol follows the method of Stampfer and Yaswen 1993 ***with minor modifications. A brief description of the protocol is as follows:***

6.23.1 Dissociation Buffer

Tissue mix medium: Mammary epithelial cell growth medium (MEGM) without additives from Cambrex or Ham's F12 + insulin (10 μg/ml**),** penicillin (100 U/ml), streptomycin (100 μg/ml), polymixin B (50 U/ml), and fungizone (3 μg/ml). Store at 4 °C.

6.23.2 Preparation of Enzyme Solution

- For preparing collagenase solution, dissolve 1500 U/ml collagenase in the appropriate amount of tissue mix medium at 37 °C.
- Filter the collagenase solution through a 500 ml bottle top filter.
- For preparing hyaluronidase solution, dissolve 1000 U/ml hyaluronidase in the appropriate amount of tissue mix medium at 37 °C.
- Filter-sterilize just like in the case of collagenase solution preparation.
- Aliquot 30 ml into 50 ml conical tubes, and store at -70 °C for up to 1 year. (This is a 5X solution).

6.23.3 Digestion and Isolation of Mammary Epithelial Cells

As discussed in the above section, this method was established by Stampfer and Yaswen 1993***, with minor modifications.***

- Obtain human mammary tissue as discarded material from surgical procedures.
- Place material in sterile containers containing sterile buffer or tissue mix medium with 10% FBS for up to 72 h.
- Separate the epithelium from the stromal matrix in sterile 150 mm Petri dishes using a combination of a sterile scalpel, forceps, and scissors.
- The epithelium appears as white strands embedded in the stromal matrix. Dissect these areas, scraping away the grossly fatty material.
- Prepare a 50 ml conical tube for the tissue by adding 5 ml FBS and an 18.3 ml tissue mix medium.
- Transfer the minced epithelium-containing tissue into a conical centrifuge tube.
- Fill the tube to full volume with enzyme solution (10 ml), leaving only a small air space to allow gentle mixing during rotation.
- Place tubes on a tube rotator and rotate overnight at 37 °C.
- Centrifuge tubes at 600 × g for 5 min.
- Discard the supernatant fat and medium.
- Dilute a small aliquot of the pellet in a medium to microscopically examine the degree of digestion.
- Digestion is complete when microscopic examination shows clumps of cells (organoids) with ductal, alveolar, or ductal–alveolar structures free from the attached stroma.
- If the tissue is not fully digested, resuspend the pellet in fresh tissue digestion medium at approximately the same pellet to medium ratio.
- Reincubate with rotation at 37 °C for additional (4–12) hours.
- Recentrifuge the tubes and recheck the pellet.
- If digestion is still not complete, add fresh digestion medium and incubate again till overnight.
- The concentration of enzymes in the digestion medium can vary according to the needed extent of digestion.
- Washing and freezing buffers: CPMI: Add 15 ml FBS and 10 ml DMSO to 75 ml, 1:1 DMEM and F12 mixture; shake gently and store indefinitely at −20 °C.
- Wash the remaining organoids and single cells with medium at least thrice.
- To enrich the preparation for ductal and lobular elements and to eliminate free blood cells, fibroblasts, and endothelial cells, complete three (30–60) minutes of sedimentation at 1000 × *g* (on the bench-top centrifuge).
- Remove the supernatant containing stromal cells.
- A pellet of (5–10) ml organoids should be obtained from each preparation.
- Pellet the organoids by centrifugation at 600 × *g* for 5 min and remove the supernatant.
- Add 1 ml CPMI for every 0.1 ml pellet.
- Seed a Petri dish for each tube by placing 0.1 ml resuspended material into 35 mm dishes drop by drop to fill and cover the dish surface.

- Disperse the organoids in the dish by gently rocking the dish to spread out the medium.
- Let it sit for 1 min and then add 1 ml growth medium to the dish.
- Incubate at 37 °C and check for attachment and sterility the following day.
- Store fractions in liquid nitrogen until use.

6.23.4 Identification of Epithelial Cells Based on Phenotypes

Shape of the Epithelial Cells

The epithelium is classified into various types based on the shape of the superficial cells and the number of cell layers.

Squamous Epithelium

Flattened plate-like cells**.**

Cuboidal Epithelium

Approximately same height and width.

Columnar Epithelium

Cells have a greater height than width.

Diameter of the Epithelial Cells

Epithelial cells show a range of diameters, from (8 to 21) microns with 97% of measurements lying in the (9–17) micron range. The mean cell size is 12.7 microns.

6.23.5 Identification of Various Epithelial Cells Based on Marker Proteins Expression

Although epithelial cells are located throughout the body, in this section we describe the isolation and primary culture of epithelial cells only from the trachea, alveoli, and mammary glands.

Epithelial marker proteins of these organs are described as follows.

Tracheal Epithelial Cells Marker Proteins

Both human and mouse tracheal epithelial cells are characterized by the expression of the following molecules:

- Cytokeratins include cytokeratin 5 (Krt 5) and cytokeratin 14 (Krt 14).
- Mucin proteins include MUC16 and MUC5AC.
- Transcription factors include Trp63.

Alveolar Type I Epithelial Cells Marker Proteins

AT1 cell markers include T1α protein, aquaporin 5 (AQP-5), and caveolin-1.

Alveolar Type II Epithelial Cells Marker Proteins

Aquaporin 3, surfactant proteins (SP-A, SP-B, SP-C, and SP-D), CK-8, KL-6, α ENaCRTII 70, and LB180.

Mammary Stem Cells Marker Proteins

- In 2007, ***Shipitsin*** **et al.** identified CD44, CD29, CD49F or integrinα-6, EpCAM, and CD24 as mammary stem cell markers in the epithelium.
- In 2007, ***Ginestier*** **et al** reported that normal and malignant human mammary stem cells express aldehyde dehydrogenase (ALDH).
- In 2007, ***Chen*** **et al** identified Lin^{-} $Procr^{+}$ $CD24^{+/med}$ $CD29^{hi}$ $CD49f^{hi}$ $Sca1^{low/-}$ and $Lin^{-}CD49F^{+}EpCAM^{neg-low}$ or $CD10^{+}$ as markers of mouse and human breast stem cells, respectively.

Mammary Myoepithelial Cells Marker Proteins

- Myoepithelial cells are characterized by the expression of cytokeratin 14 (CK 14), alpha-smooth muscle actin (α-SMA), and vimentin.
- Another important marker protein present in myoepithelial cells is CD10 (Fu et al. 2014; Batistatou et al. 2003).

Mammary Luminal Epithelial Cells Marker Proteins

- Several proteins are recognized and well-characterized as markers of luminal epithelial cells.
- The general luminal epithelial cell markers are cytokeratin 8 (CK8), cytokeratin 18 (CK-18), cytokeratin 19 (CK-19), Mucin 1 (Muc 1), and the epithelial cell adhesion molecule (EpCAM).
- The K8 and K18 are reported as a pair of keratin filaments, restricted to luminal cells in mouse and human mammary glands.
- In 2014, *Fu* et al. identified CD227, EpCAM, CD44, and CD24 in luminal epithelial cells.

Differences in Keratin Expression Between Luminal Epithelial Cells and Myoepithelial Cells

- As discussed in the previous section, a protective structural protein is expressed by various types of epithelial cells. Several keratin proteins are expressed by epithelial cells and hair. While in luminal epithelial cells, keratin 8 and keratin 18 are expressed, in the mammary epithelial cells, keratin 19 appears to be associated with the most mature, well-differentiated cells.
- In addition, luminal epithelial cells also express **mucin** and **PEM,** which can be detected by their respective specific antibodies.
- For basal epithelial cells, the marker proteins include **vimentin, keratins, smooth muscle actin,** and acute lymphoblastic leukemia antigen (**CALLA**).
- Myoepithelial cell identification in a basal cell population requires evidence for the presence of contractile myofibrils or oxytocin receptors.

- p16 expression is one of the important post-selection characteristics. The cells maintain certain expressions of keratins 5, 7, and 14 while acquiring increasing keratins 8, 18, and PEM expressions.

6.23.6 Utility of Epithelial Cells

- Present at the boundary and lining of all cavities, organs, and exterior–interior surfaces, epithelial cells protect organs and help in maintaining their structural and functional integrity.
- Epithelial cells are involved in the transportation, absorption, secretion, lubrication, and movement of organs.
- To understand the structure–function relationships of epithelial cells in specific organs, it is necessary to culture them.
- While epithelial cells can be isolated and cultured from any of the abovementioned organs or tissues, the source of the two most widely studied epithelial cells is the respiratory tract and the female breast.
- Approximately 80% of mammalian cancers are of epithelial origin.

7 Conclusions

This chapter describes the isolation and culture of endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells. Depending upon the type, these cells are present in both the vascular and nonvascular regions of a mammalian body. While endothelial cells are present only in the vascular bed (micro- and macrovascular) of the body, smooth muscle and cells and fibroblasts are present both in the vascular and nonvascular regions of the body and epithelial cells are present in both outer and inner body surfaces without any blood supply (vascular bed). Each of these cells has a specific distinct morphology and phenotype through which these can be identified and confirmed. For example, endothelial cells have **cobblestone** morphology, smooth muscle cells are **spindle-shaped,** fibroblasts are **large, flat, elongated (spindle-shaped) or round cells with extensions radiating out from the ends of the cell body,** and finally, epithelial cells have three distinct morphologies such as **flattened plate-like** (squamous epithelium), **approximately same height and width as cubes** (cuboidal epithelium) and the cells having **greater height than width as column** (columnar epithelium**)**. These cells can also be separated and distinguished from each other based on their specific size, marker protein expression, required culture medium, and doubling time. The isolation and culture procedure of these four cell types described in this chapter is based on the personal research experience of the authors as well as modification of the various well-established techniques by other eminent researchers. Based on the numerous physiological and pathophysiological roles played by these four types of cells, learning about the isolation and culture of endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells is undoubtedly essential.

8 Cross-References

- Culture of Continuous Cell Lines
- Culture of Neuron and Glia Cells
- Primary Culture of Immunological Cells
- Stem Cell Culture and Its Applications

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