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Explant Culture of Mouse Embryonic Whole Lung, Isolated Epithelium, or Mesenchyme Under Chemically Defined Conditions as a System to Evaluate the Molecular Mechanism of Branching Morphogenesis and Cellular Differentiation

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Abstract

Lung primordial specification as well as branching morphogenesis, and the formation of various pulmonary cell lineages, requires a specific interaction of the lung endoderm with its surrounding mesenchyme and mesothelium. Lung mesenchyme has been shown to be the source of inductive signals for lung branching morphogenesis. Epithelial–mesenchymal–mesothelial interactions are also critical to embryonic lung morphogenesis. Early embryonic lung organ culture is a very useful system to study epithelial–mesenchymal interactions. Both epithelial and mesenchymal morphogenesis proceed under specific conditions that can be readily manipulated in this system (in the absence of maternal influence and blood flow). More importantly this technique can be readily done in a serumless, chemically defined culture media. Gain and loss of function can be achieved using expressed proteins, recombinant viral vectors, and/or analysis of transgenic mouse strains, antisense RNA, as well as RNA interference gene knockdown. Additionally, to further study epithelial–mesenchymal interactions, the relative roles of epithelium versus mesenchyme signaling can also be determined using tissue recombination (e.g., epithelial and mesenchymal separation) and microbead studies.

Keywords

Organ culture; lung; morphogenesis; branching; epithelium; mesenchyme; epithelial–mesenchymal separation; gain of function; loss of function

1. Introduction

Embryonic lung development begins by evagination of the laryngotracheal groove from the foregut endoderm at embryonic day 9.5. Bronchial branches arise initially as two distal buds. Each bud consists of three different layers: the epithelium, the surrounding mesenchyme, and the mesothelium. It was appreciated early on that the mesenchyme can induce ectopic branching of the trachea, giving rise to extraneous lungs (1). Various growth factors, including those from the epidermal growth factor (EGF), fibroblast growth factor (FGF), sonic hedgehog (SHH), bone morphogenetic protein (BMP), and wingless (Wnt) signaling families, are required to mediate the specific mesenchymal–epithelial–mesothelial interactions that are required and regulate respiratory organogenesis. Many of these growth factors have been extensively studied and affect epithelial branching,

proliferation, and differentiation (2, 3). In order to determine the key molecules involved in the regulation of these epithelial–mesenchymal interactions, many of these studies have used early embryonic lung organ culture experiments as well as epithelial–mesenchymal separation and recombination.

Briefly, both techniques consist in removing embryos from pregnant females at gestational stages of early branching (e.g., embryonic days E11.5–E13.5) and isolating the lungs from these embryos. Early embryonic lung organ culture experiments then consist in placing the isolated lungs on filters and growing them under serum-free, chemically defined conditions, typically for periods from 12 to 96 h (4, 5). This method allows characterization of the overall effect of a particular growth factor on both epithelial and mesenchymal morphogeneses.

In order to further dissect and characterize the molecular mechanism involved and/or inductive role in branching, in the respective epithelial or mesenchymal response to a specific growth factor, the method of choice would be epithelial–mesenchymal separation and recombination. This technique consists in the enzymatic (e.g., dispase) and mechanical separation of the epithelium from the mesenchyme of E12.5–E13.5 lungs. The isolated explants are then placed in Matrigel™ and grown for 24–48 h (6, 7) or mesenchyme can be replaced in apposition to the epithelium (8).

2. Materials

2.1. Embryonic Whole Lung, Epithelium, and Mesenchyme Isolation

1. Timed-pregnant (C57BL6 wild-type or transgenic) mice to be sacrificed on postcoitum day E11.5–13.5, where E0.5 is the day of detection of a copulation plug.
2. Hanks' balanced salt solution (HBSS) (1X), liquid, without calcium chloride, magnesium chloride, or magnesium sulfate (Invitrogen, Carlsbad, CA) supplemented with 50 units/ml of penicillin–streptomycin (Invitrogen, Carlsbad, CA). Store the HBSS at room temperature and at 4°C after opening. Aliquot and store the penicillin–streptomycin at –20°C.
3. Stereoscopic dissecting microscope (Leica, Wetzlar, Germany).
4. Dissection tools including Dumont® forceps, Fine iris scissors (straight), Noyes spring scissors, and Moria® perforated spoon (Fine Science Tools, Foster City, CA).
5. Insect pin holders and Etched tungsten micro-needles (Fine Science Tools, Foster City, CA).

2.2. Embryonic Whole Lung Culture

1. Dulbecco's Modified Eagle Medium: nutrient mix F-12 (D-MEM/F-12) (1X), liquid, 1:1 (v/v). Contains L-glutamine, but no HEPES buffer (Invitrogen, Carlsbad, CA) supplemented with 50 units/ml of penicillin–streptomycin (Invitrogen, Carlsbad, CA). Keep the DMEM/F-12 bottle away from the light (e.g., covered by aluminum foil) and store at 4°C (*see Note 1*). Aliquot and store the penicillin–streptomycin at –20°C.

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¹BGJb media is no longer used. It has a high osmolarity and salt concentration. Also vitamin C supplementation is required and can interact with calcium signaling pathways.

2. Nuclepore polycarbonate track-etch membrane, 8.0 μm , 13 mm (Whatman, Florham Park, NJ).
3. Nunclon™ Δ polystyrene dishes with lids, 4 wells (Rochester, NY).
4. Disposable transfer pipets, sterile (Fisher Scientific, Pittsburgh, PA).

2.3. Isolated Epithelium and Mesenchyme Culture

1. Dulbecco's Modified Eagle Medium: nutrient mix F-12 (D-MEM/F-12) (1X), liquid, 1:1 (v/v). Contains L-glutamine, but no HEPES buffer (Invitrogen, Carlsbad, CA) supplemented with 50 units/ml of penicillin–streptomycin (Invitrogen, Carlsbad, CA). Keep the DMEM/F-12 bottle away from the light (e.g., covered by aluminum foil) and store at 4°C. Aliquot and store the penicillin–streptomycin at –20°C.
2. Dispase (BD biosciences, San Jose, CA). Aliquot the undiluted dispase on ice and store at –20°C.
3. Fetal bovine serum (FBS), certified, heat inactivated (Invitrogen, Carlsbad, CA). Aliquot and store at –20°C.
4. Phosphate-buffered saline (PBS) 7.4 (1X), liquid (Invitrogen, Carlsbad, CA).
5. Nunclon™ Δ polystyrene dishes with lids, 4 wells (Rochester, NY).
6. BD Falcon 60 $\times$ 15 mm Petri dishes (BD biosciences, San Jose, CA).
7. Matrigel™ (BD biosciences, San Jose, CA). Aliquot on ice and store at –20°C.
8. Glass Pasteur pipets, 5–3/4 in. (Fisher Scientific, Pittsburgh, PA) (*see Note 2*).
9. Rubber bulbs for small pipets (Fisher Scientific, Pittsburgh, PA).
10. Diamond scribe (Fisher Scientific, Pittsburgh, PA).
11. Calibrated micropipets, 1–5 μl , with aspirator tube (Drummond, Broomall, PA).

3. Methods

Organ culture and also epithelial or mesenchymal explant cultures are very useful and versatile techniques to study gene and protein expression. These *ex vivo* culture methods can be used as preliminary or in complement to *in vivo* studies.

3.1. Embryonic Lung Isolation

1. Timed-pregnant mice are sacrificed on postcoitum day E11.5–12.5 by CO₂ narcosis according to the NIH and OLAW guidelines (*see Note 3*). The animal is placed in a chamber and 100% CO₂ is introduced. After the animal is unconscious the CO₂ flow is increased. Clinical death of the animal must be ensured.
2. All the following steps need to be completed under sterile conditions in a laminar flow hood.
3. To remove the uterus, the pregnant females are placed on their back and sprayed with 70% ethanol. An incision is made in the abdomen, and the skin is removed by

²Once the lungs have been digested in dispase, they become very sticky, and it is important to use a glass Pasteur pipet and not a plastic transfer pipet to transfer them to another well or to the Petri dish before epithelial–mesenchymal separation. It is also important to make a larger end to the Pasteur pipet using a diamond scribe to allow a cleaner cut of the glass. Then add at the other end a rubber bulb and use this as transfer tool.

³Experiments involving animals must be conducted in accord with the prevailing local and national regulations.

pulling the skin upward while holding the animal's hind legs. The peritoneal cavity is opened and the uterus is dissected free.

4. The blood is rinsed off by placing the uterus in a 50-ml conical tube filled with cold HBSS, and the tube is gently rocked for 2 min.
5. The uterus is then placed in a Petri dish under the stereoscopic dissecting microscope and the embryos released from the uterus by incising the uterine wall using spring scissors. The embryos are harvested using a perforated spoon and placed on ice in a new Petri dish containing HBSS.
6. Under the stereoscopic dissecting microscope, embryonic lungs are dissected one by one in a Petri dish containing cold HBSS (**Fig. 5.1**). For dissection, each embryo is placed on its left flank. The right forelimb is removed. The forceps held by the left hand is placed with one prong in the head and the other in the hindlimb of the embryo, thus holding it steady in the dish. The forceps held by the right hand is used to open the right flank of the embryo from the abdomen to the neck area. The skin above the heart and the heart itself are then removed. The lung should be now observable, lying posterior to the heart and anterior to the spine. The neck area should then gently be opened to remove the tissue surrounding the trachea. The pharynx is divided and gently pulled using the forceps. This will allow the removal of the lung with an intact trachea. If any surrounding tissue (e.g., esophagus or other surrounding organs) is present, it should be gently removed from the isolated lung.
7. The isolated embryonic lung is placed on ice in a Petri dish containing HBSS using a sterile transfer pipet.

3.2. Embryonic Lung Culture

1. 500 μ l–1 ml per well of D-MEM/F-12 supplemented with 50 units/ml of penicillin–streptomycin is added in a Nunclon™ Δ polystyrene dish.
2. A nuclepore polycarbonate track-etch membrane is placed on top of the media (*see Note 4*).
3. A dissected embryonic lung is placed on top of the nuclepore polycarbonate track-etch membrane using a sterile transfer pipet.
4. The embryonic lung position is adjusted using forceps (*see Note 5*).
5. The Nunclon™ Δ polystyrene dish is transferred for the desired culture time in a cell incubator. An example of the results produced is shown in **Fig. 5.2**.

3.3. Isolated Epithelium and Mesenchyme Culture

1. 500 μ l of undiluted dispase is placed in a well of a Nunclon™ Δ polystyrene dish.
2. E12.5–E13.5 dissected lungs are transferred into the dispase using a transfer pipette and incubated on ice for 25–30 min.
3. Dispase activity is stopped by transferring the samples to pure FBS on ice for 15 min.

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⁴When the nuclepore polycarbonate track-etch membrane is placed on top of the media, make sure that the shiny side is against the media and rough side is facing upward.

⁵Embryonic lung placed on the filter should have an intact trachea and be placed with their trachea having a straight position, thus allowing all lungs to have the same internal pressure.

4. Samples are transferred into a well containing D-MEM/F-12 media containing 10% FBS, on ice.
5. One embryonic lung is transferred to a 60 mm Petri dish containing DMEM/F12 media supplemented with 10% FBS. Epithelium and mesenchyme are separated under the dissecting microscope using tungsten needles.
6. Isolated epithelium and mesenchyme are transferred using a calibrated micropipet to respective wells (containing DMEM/F12 media supplemented with 20% FBS). The Nunclon™Δ polystyrene dish is placed on ice.
7. 10–15 µl of Matrigel™ is added to make a dome (*see Note 6*).
8. The epithelial or mesenchymal explants are immediately and as quickly as possible added to the Matrigel™ dome using a calibrated micropipet.
9. Once all of the explants are positioned, the Nunclon™Δ polystyrene dish is placed in the incubator for about 30 min (until it hardens) (*see Note 7*).
10. DMEM/F12 media supplemented with 0.5% FBS is added to each well, and the Nunclon™Δ polystyrene dish is put back in the incubator for 24–48 h.
11. Pictures are taken at time = 0 and every 12 h until the end of the experiment. An example of the results produced is shown in **Fig. 5.3**.

Acknowledgments

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⁶Before making a Matrigel™ dome to culture epithelial or mesenchymal explants, a small flat base of Matrigel™ can be made by adding 2–4 µl of Matrigel™ at the bottom of the well of the Nunclon™Δ polystyrene dish and by letting it harden for 1 min (no longer). This will prevent the spreading of the epithelial or mesenchymal explant by adhesion to the bottom of the dish.

⁷Epithelial or mesenchymal explants grow better when positioned in the middle part of the Matrigel™ dome.

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Fig. 5.1.

Dissection of E12 embryonic lungs. (a) E12.5 whole embryo viewed from the *right side*. (b) Right forelimb has been removed from the embryo. (c) Forceps held by the left hand holding the embryo steady in the dish. *Arrows* indicate plan of dissection. (d) Embryonic lung lies posterior to the heart (removed) and anterior to the spine. This figure shows the lungs after skin and heart removal. (e) Pharynx removal allows separation of the intact lung from the embryo. (f) Extraneous pharyngeal tissue and esophagus have been trimmed away. Dissected E12.5 embryonic lung with intact trachea and larynx is shown. (Cr) Cranial lobe, (Med) Medial lobe, (Ca) Caudal lobe, (Acc) Accessory lobe, (Le) Left lobe.

Fig. 5.2.

FGF9 induces expansion of the mesenchyme and the dilation of the epithelium in lung grown in vitro (6). (a–c) E12.5 lung grown for 48 h in the absence of FGF9. Note the increase in branching over time. (d–f) E12.5 lung grown for 48 h in the presence of FGF9. Note the dilation of the epithelium and mesenchyme as early as after 24 h of culture (e). After 48 h (f), the effect on the epithelium is even more pronounced. Scale bar: (a–f) 400 μm .

Fig. 5.3.

Effect of FGF9 on isolated distal lung epithelium and mesenchyme (6). **(a, b)** Isolated distal mesenchyme grown for 48 h in the absence of FGF9 undergoes necrosis **(b)**. **(c, d)** In the presence of FGF9, the mesenchymal explant grows and many mesenchymal cells invading the Matrigel are observed in the periphery. **(e, f)** Isolated distal epithelium grown for 48 h in the absence of FGF9 undergoes necrosis **(f)**. **(g, h)** In the presence of FGF9, the epithelial explant grows considerably to form a cyst-like structure. Scale bar: **(a–h)** 80 μm .