Protocol

Three-Dimensional Organotypic Culture of Stratified Epithelia

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One of the limitations of conventional tissue culture on flat two-dimensional surfaces is the loss of complex interactions between the epithelium and stroma. We have devised a culture system that recreates the salient features of the stratified epithelium using primary cell cultures from mouse models. The protocol described here is applicable to the esophageal epithelium, but stratified epithelial cells from other organs (e.g., skin) can be grown. Once established, the system can be used to interrogate the effect of various pharmacologic and genetic manipulations on epithelial homeostasis and invasion.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS)
Epithelial cells (1×10^7 cells/mL)

Prepare this cell suspension from stratified epithelia after collagenase and trypsin digestion as described in Harada et al. (2003).

Ethanol (70%)
Formalin (10%)
Phosphate-buffered saline (PBS) <R>
Solution A for organotypic culture <R>
Solution B for organotypic culture <R>
Solution C for organotypic culture <R>
Solution D for organotypic culture <R>
Solution E for organotypic culture <R>

Equipment

Cellulose chromatography paper (Fisher 05-714-4)

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Histology cassette pads (Fisher 22038221)
 Histosette II tissue cassettes (Fisher 15182701C)
 Pasteur pipettes (glass, sterile)
 Scalpel
 Tissue-culture dishes (100 mm, sterile)
 Tissue-culture incubator (37°C, 5% CO₂)
 Transwell (24 mm) with 3.0-μm membrane insert (Corning 3414)
 Transwell carrier (six well; Organogenesis TS01001)
 Tubes (50 mL)

METHOD

1. Place transwell inserts into their wells.
2. Prepare Solution A in an ice cold 50-mL tube.
3. Distribute 1 mL of Solution A onto each transwell filter. Incubate at room temperature for 15 min until the solution solidifies.
4. Prepare Solution B in an ice cold 50-mL tube.
5. Add 3 mL of Solution B on top of the solidified collagen layer, making sure no air bubbles are trapped between the layers.
6. Incubate the transwell plates at 37°C for 45 min, until both layers have solidified.
Make sure not to agitate the plate.
7. Add 10 mL and 2 mL of 10% FBS/DMEM to the bottom and top wells, respectively. Incubate overnight at 37°C in a 5% CO₂ incubator.
8. Dislodge the collagen matrix from the walls of the transwell insert by gently running a sterile Pasteur pipette (held vertically) around the edge without puncturing the transwell membrane or breaking the pipette tip.
9. Add 2 mL of 10% FCS/DMEM to the transwell and return it to incubator. Over the next 5 d, confirm that the collagen matrix contracts, creating an indentation in the middle of the basal layer. The medium does not need to be changed during this time.
See Troubleshooting.
10. Prepare Solution C.
11. Gently aspirate media from both compartments of each transwell without disturbing the basal matrix layer.
12. Add 10 mL and 2 mL of Solution C to the bottom and top wells, respectively. Place the plate in the incubator for 1 h.
13. Remove Solution C and gently add 50 μL of epithelial cells (1×10^7 cells/mL; 5×10^5 total cells per well) into the indentation at the center of the matrix.
14. Return the plate to the incubator for 2 h.
15. Prepare Solution D.
16. Add 10 mL and 2 mL of Solution D to the bottom and top wells, respectively, and return the plate to the incubator. Store the remaining media at 4°C.
17. After 48 h, replace media from both wells with 10 mL and 2 mL of Solution D in the bottom and top compartments, respectively. Return the plate to the incubator.
18. After 48 h, remove media from both compartments. Add 7.5 mL of Solution E to the bottom compartment only. Return the plate to the incubator.

19. After 48 h, replace media in bottom compartment with 7.5 mL of Solution E.
20. After 48 h, carefully replace media with 10 mL and 2 mL of 10% formalin in the bottom and transwell compartments, respectively. Incubate at 4°C for 1 h.
21. Transfer each transwell to a sterile tissue-culture dish. Remove the transwell membrane from the carrier by gently cutting the membrane using a scalpel.
22. Transfer each membrane into a tissue cassette lined with a precut sheet of 100% chromatography paper. Gently close the cassette.
23. Wash the cassettes in PBS in a beaker for 10 min at room temperature. Repeat two more times.
24. Transfer the cassettes to 70% ethanol and store at 4°C.
25. Process the tissue for paraffin embedding using standard histologic protocols. Cut sections vertically for staining.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 9): The collagen matrix did not contract.

Solution: Healthy fibroblasts are crucial for matrix contraction. If there is inadequate contraction, consider using another source of fibroblasts (preferably low passage). Also, ensure that the fibroblasts are evenly mixed when applying to the transwell. Contraction may also be inhibited by an acidic pH. pH may vary with each batch of collagen from the supplier.

Problem (Step 9): There is asymmetric collagen contraction.

Solution: If there is asymmetric contraction of the collagen layer, then it is likely still affixed to the walls of the transwell. Gently detach the layer using a sterile Pasteur pipette.

Problem (Step 25): The stratified epithelial layer is poorly organized.

Solution: Healthy epithelial cells are essential for the creation of a stratified epithelial layer. Ensure that input epithelial cells are viable by minimizing the time from trypsinization to transfer to transwell. Use low passage, nonconfluent cells.

DISCUSSION

This protocol, adapted from Kalabis et al. (2012), describes conditions for recreating stratified epithelium from primary or immortalized epithelial cells *ex vivo*. The resultant organotypic cultures contain a basal layer containing stroma and fibroblasts layered on a basement membrane.

These cultures can be analyzed in a number of downstream assays, such as immunohistologic analysis (Kalabis et al. 2008). We have previously shown using conventional H&E analysis that wild-type esophageal cells yield a stable stratified layer in this system. However, the use of TE12 human esophageal cancer cells results in an abnormal stratified epithelium featuring isolated areas of invasion into the basal compartment (Harada et al. 2003). Laser microdissection of invading cells led to the identification of an invasion-associated transcriptional signature (Okawa et al. 2007). Potential genes involved in invasion were then confirmed functionally by genetically manipulating TE12 cells with shRNA and assessing the affect on invasion by histology.

Similarly, stromal–epithelial interactions can be dissected easily in organotypic culture (Okawa et al. 2007). Genetically manipulated fibroblasts can also be used. Further, soluble factors and cytokines can be studied in this system either by the exogenous addition of neutralization or by specific immunoglobulins.

RECIPES

Phosphate-Buffered Saline (PBS)

Reagent	Amount to add (for 1× solution)	Final concentration (1×)	Amount to add (for 10× stock)	Final concentration (10×)
NaCl	8 g	137 mM	80 g	1.37 M
KCl	0.2 g	2.7 mM	2 g	27 mM
Na ₂ HPO ₄	1.44 g	10 mM	14.4 g	100 mM
KH ₂ PO ₄	0.24 g	1.8 mM	2.4 g	18 mM

If necessary, PBS may be supplemented with the following:

CaCl ₂ •2H ₂ O	0.133 g	1 mM	1.33 g	10 mM
MgCl ₂ •6H ₂ O	0.10 g	0.5 mM	1.0 g	5 mM

PBS can be made as a 1× solution or as a 10× stock. To prepare 1 L of either 1× or 10× PBS, dissolve the reagents listed above in 800 mL of H₂O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H₂O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle or by filter sterilization. Store PBS at room temperature.

Solution A for Organotypic Culture

Reagent	Volume
Eagle's minimal essential medium (EMEM; 10×, Lonza 12-684F)	690 μL
Fetal bovine serum	700 μL
L-Glutamine (200 mM; Cellgro 25-005CI)	60 μL
Sodium bicarbonate (NaHCO ₃ ; Lonza 17-613E)	140 μL
Bovine collagen type I (Organogenesis 200-055)	5.6 mL

Combine the reagents in the order listed. The final volume is sufficient for six inserts.

Solution B for Organotypic Culture

Reagent	Volume
Eagle's minimal essential medium (EMEM; 10×, Lonza 12-684F)	1.8 mL
Fetal bovine serum	2 mL
L-Glutamine (200 mM; Cellgro 25-005CI)	160 μL
Sodium bicarbonate (NaHCO ₃ ; Lonza 17-613E)	380 μL
Bovine collagen type I (Organogenesis 200-055)	11.4 mL
Matrigel (BD Biosciences 354234)	3.8 mL
Fibroblasts (6 × 10 ⁵ cells/mL)	1.6 mL

Combine the reagents in the order listed. The final volume is sufficient for six inserts.

Solution C for Organotypic Culture

Reagent	Volume
Dulbecco's modified Eagle's medium (DMEM)	60 mL
Ham's F12 (Life Technologies 11765-054)	20 mL

The final volume is sufficient for six inserts.

Solution D for Organotypic Culture

Reagent	Volume
Dulbecco's modified Eagle's medium (DMEM)	218 mL
Ham's F12 (Life Technologies 11765-054)	72 mL
L-Glutamine (200 mM; Cellgro 25-005CI)	6 mL
Hydrocortisone (Sigma-Aldrich H0888)	600 μ L
ITES supplement (500 $\times$; Lonza 17839Z)	600 μ L
O-Phosphorylethanolamine (Sigma-Aldrich P0503)	600 μ L
Adenine (Sigma-Aldrich A9795)	600 μ L
Progesterone (Sigma-Aldrich P8783)	600 μ L
Triiodothyronine (Sigma-Aldrich T5516)	600 μ L
Newborn calf serum (NBCS) (HyClone SH3011802)	300 μ L
Gentamicin sulfate (50 mg/mL; Cellgro 30-005-CR)	300 μ L

The final volume is sufficient for 12 inserts.

Solution E for Organotypic Culture

Reagent	Volume
Dulbecco's modified Eagle's medium (DMEM) (Cellgro 10-013-CV)	95 mL
Ham's F12 (Life Technologies 11765-054)	95 mL
L-Glutamine (200 mM; Cellgro 25-005CI)	4 mL
Hydrocortisone (Sigma-Aldrich H0888)	400 μ L
ITES supplement (500 $\times$; Lonza 17839Z)	400 μ L
O-Phosphorylethanolamine (Sigma-Aldrich P0503)	400 μ L
Adenine (Sigma-Aldrich A9795)	400 μ L
Triiodothyronine (Sigma-Aldrich T5516)	400 μ L
Newborn calf serum (NBCS) (HyClone SH3011802)	4 mL
Gentamicin sulfate (50 mg/mL; Cellgro 30-005-CR)	200 μ L

The final volume is sufficient for 12 inserts.

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REFERENCES

- Harada H, Nakagawa H, Oyama K, Takaoka M, Andl CD, Jacobmeier B, von Werder A, Enders GH, Opitz OG, Rustgi AK. 2003. Telomerase induces immortalization of human esophageal keratinocytes without p16INK4a inactivation. *Mol Cancer Res* 1: 729–738.
- Kalabis J, Oyama K, Okawa T, Nakagawa H, Michaylira CZ, Stairs DB, Figueiredo JL, Mahmood U, Diehl JA, Herlyn M, et al. 2008. A subpopulation of mouse esophageal basal cells has properties of stem cells with the capacity for self-renewal and lineage specification. *J Clin Invest* 118: 3860–3869.
- Kalabis J, Wong GS, Vega ME, Natsuzaka M, Robertson ES, Herlyn M, Nakagawa H, Rustgi AK. 2012. Isolation and characterization of mouse and human esophageal epithelial cells in 3D organotypic culture. *Nat Protoc* 7: 235–246.
- Okawa T, Michaylira CZ, Kalabis J, Stairs DB, Nakagawa H, Andl CD, Johnstone CN, Klein-Szanto AJ, El-Deiry WS, Cukierman E, et al. 2007. The functional interplay between EGFR overexpression, hTERT activation, and p53 mutation in esophageal epithelial cells with activation of stromal fibroblasts induces tumor development, invasion, and differentiation. *Genes Dev* 21: 2788–2803.

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