Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

HHS Public Access
Author manuscript
*Cold Spring Harb Protoc*. Author manuscript; available in PMC 2016 December 16.

Published in final edited form as:
*Cold Spring Harb Protoc*. ; 2015(4): 349–353. doi:10.1101/pdb.prot078311.

# Three-dimensional organotypic culture of stratified epithelia

**Andrew D. Rhim** and **Anil K. Rustgi**
Gastroenterology Division, Department of Medicine, Abramson Cancer Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

## Abstract

One of the limitations of conventional tissue culture on flat 2D 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. Stratified epithelial cells from various organs (e.g., skin and esophagus) 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. Below is applicable to the esophageal epithelium.

## Materials

### A. Reagents

Keratinocyte–serum-free medium (KSFM) with and without $CaCl_2$, with bovine pituitary extract and epidermal growth factor (Invitrogen 10724-011 and 10725-018, respectively)

Penicillin-streptomycin (Invitrogen 15140-122).

Dulbecco's phosphate-buffered saline (PBS; Invitrogen 14190-136).

Hanks' balanced salt solution (HBSS; Invitrogen 14175-079).

Nystatin (10,000 U/ml; Sigma-Aldrich N1638).

Gentamicin sulfate (50 mg/ml; Cellgro 30-005-CR).

Dispase I (Roche 210455)

Collagenase (Invitrogen 17018-029).

Hoechst 33342 (Sigma-Aldrich 14533).

Trypsin-EDTA (Invitrogen 25300-054).

Ham's F12 (Invitrogen 11765-054).

Eagle's minimum essential medium (EMEM, 10×; Lonza 12-684F).

Corresponding Author: Anil K. Rustgi, Phone: 215-898-0154, Fax: 215-573-2024, anil2@mail.med.upenn.edu.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Sodium bicarbonate ($NaHCO_3$, Lonza 17-613E).

L-Glutamine (200 mM; Cellgro 25-005CI).

Bovine collagen type I (Organogenesis 200-055).

Matrigel (BD Biosciences 354234).

Hydrocortisone (Sigma-Aldrich H0888).

Insulin, transferrin, ethanolamine and selenium (ITES; 500×; Lonza 17839Z).

*O*-Phosphorylethanolamine (Sigma-Aldrich P0503).

Adenine (Sigma-Aldrich A9795).

Progesterone (Sigma-Aldrich P8783).

Triiodothyronine (Sigma-Aldrich T5516).

Sodium hydroxide (NaOH, Fisher SS255-1).

## B. Equipment

Dissecting scissors (Fisher 08-951-25).

Dissecting tissue forceps (Fisher 1381236).

Round-bottom polystyrene tube (5.0 ml; BD Falcon 352058).

Six-well multi-well plate (BD Falcon 353046).

Cell culture dish (100 mm; BD Primaria 353803).

Six-well Transwell carrier (Organogenesis TS01001).

Transwell (24 mm) with 3.0 μm membrane insert (Corning 3414).

Scalpel no. 21 (Feather 2975).

Histosette II tissue cassettes (Fisher 15182701C).

Histology cassette pads (Fisher 22038221).

Cellulose chromatography paper (Fisher 05-714-4).

## C. Method (Adapted from (Kalabis et al. 2012))

1. Place transwell inserts into wells
2. Make “A” solution in an ice cold 50ml tube
3. Distribute 1ml of A solution onto transwell filters

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

4. Incubate at RT for 15 min until solution solidifies
5. Make B solution in an ice cold 50ml tube.
6. Add 3ml of B solution on top of solidified collagen layer, making sure no air bubbles are trapped between the layers.
7. Incubate at 37°C for 45 minutes, until both layers have solidified. Make sure not to agitate the plate during transfer.
8. Add 10ml and 2ml 10% FBS/DMEM to the bottom and top wells, respectively. Incubate overnight at 37°C in a 5% $CO_2$ incubator.
9. Dislodge the collagen matrix from the walls of the transwell insert by gently running a sterile glass Pasteur pipette (held vertically) around the edge without puncturing the transwell membrane or breaking the pipette tip.
10. Add 2ml 10% FCS/DMEM to the transwell and return to incubator. Over the next five days, confirm that the collagen matrix contracts, creating an indentation in the middle of the basal layer. The media does not need to be changed during this time.

    See troubleshooting
11. Prepare solution C.
12. Gently aspirate media from both compartments, without disturbing the basal matrix layer.
13. Add 10ml and 2ml Solution C to the bottom and top wells, respectively.
14. Place in incubator for 1 h.
15. Remove Solution C and gently add 50μl epithelial cells ($1\times10^7$/ml; $5\times10^5$ total cells per well) into the indentation at the center of the matrix. This cell suspension from stratified epithelia is prepared after collagenase and trypsin digestion as described previously (Harada et al., 2003)
16. Return plate to the incubator for 2h.
17. Prepare Solution D.
18. Add 10ml and 2ml Solution D to the bottom and top wells, respectively and return to incubator. Store remaining media at 4°C.
19. After 48 h, replace media from both wells with 10ml and 2ml Solution D in bottom and top compartments, respectively.
20. After 48 h, remove media from both compartments. Add 7.5ml Solution E to bottom compartment only. Return to incubator
21. After 48 h, replace media in bottom compartment with 7.5ml Solution E.
22. After 48h, gently replace media with 10ml and 2ml 10% formalin in bottom and transwell compartments, respectively.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

23. Incubate at 4°C for 1 h.
24. Transfer transwell to a sterile tissue culture dish. Remove the transwell membrane from the carrier by gently cutting the membrane using a scalpel.
25. Transfer membrane into tissue cassette lined with precut sheet of 100% chromatography paper. Gently close the cassette.
26. Wash cassettes in PBS in a beaker at RT for 10 min. Repeat twice.
27. Transfer cassettes to 70% ethanol and store at 4°C.
28. Process tissue for paraffin embedding using standard histologic protocols. Cut sections vertically for staining.

See troubleshooting

### D. Troubleshooting

#### Problem (Step 10): 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 10): 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 Pasteur pipette.

#### Problem (Step 28): Poor organization of stratified epithelial layer

**Solution:** As in the first troubleshooting step, 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, non-confluent cells.

## Discussion

Here we describe 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. Here, we describe analysis of this system using 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, use of TE12 human esophageal cancer cells result 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

Author Manuscript



identification of an invasion-associated transcriptional signature (Okawa et al. 2007). Potential genes involved in invasion were then were then confirmed functionally by genetically manipulating TE12 cells with shRNA and assessing effect 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 utilized. Further, soluble factors and cytokines can be studied in this system either by the exogenous addition or neutralization by specific immunoglobulins.

## Acknkowledgements

This work is supported by the NIH (K08-DK088945 to ADR; P01-CA098101 and U01-CA 143056 to AKR)

## References

Harada H, Nakagawa H, Oyama K, Takaoka M, Andl CD, Jacobmeier B, von Werder A, Enders GH, Opitz OG, Rustgi AK. Telomerase induces immortalization of human esophageal keratinocytes without p16INK4a inactivation. Mol Cancer Res. 2003; 1(10):729–738. [PubMed: 12939398]

Kalabis J, Oyama K, Okawa T, Nakagawa H, Michaylira CZ, Stairs DB, Figueiredo JL, Mahmood U, Diehl JA, Herlyn M, et al. A subpopulation of mouse esophageal basal cells has properties of stem cells with the capacity for self-renewal and lineage specification. J Clin Invest. 2008; 118(12):3860–3869. [PubMed: 19033657]

Kalabis J, Wong GS, Vega ME, Natsuizaka M, Robertson ES, Herlyn M, Nakagawa H, Rustgi AK. Isolation and characterization of mouse and human esophageal epithelial cells in 3D organotypic culture. Nat Protoc. 2012; 7(2):235–246. [PubMed: 22240585]

Okawa T, Michaylira CZ, Kalabis J, Stairs DB, Nakagawa H, Andl CD, Johnstone CN, Klein-Szanto AJ, El-Deiry WS, Cukierman E, et al. 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. 2007; 21(21): 2788–2803. [PubMed: 17974918]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

| **Recipes** | | |
| --- | --- | --- |
| Solution A (for 6 inserts, combine in order) | | |
| 10x EMEM | 690µl | |
| Fetal bovine serum | 700µl | |
| L-glutamine | 60µl | |
| Sodium bicarbonate | 140µl | |
| Bovine collagen type I5.6ml | | |
| Solution B (for 6 inserts, combine in order) | | |
| 10x EMEM | 1.8ml | |
| Fetal bovine serum | 2ml | |
| L-glutamine | 160µl | |
| Sodium bicarbonate | 380µl | |
| Bovine collagen type I | 11.4ml | |
| Matrigel | 3.8ml | |
| $6x10^5$/ml fibroblasts | 1.6ml | |
| Solution C (for 6 inserts) | | |
| DMEM | 60ml | |
| Ham’s F12 | 20ml | |
| Solution D and E (for 12 inserts) | | |
| | Solution D | Solution E |
| DMEM | 218ml | 95ml |
| Ham’s F12 | 72ml | 95ml |
| L-glutamine | 6ml | 4ml |
| Hydrocortisone | 600µl | 400µl |
| ITES | 600µl | 400µl |
| O-phosphorylethanolamine | 600µl | 400µl |
| Adenine | 600µl | 400µl |
| Progesterone | 600µl | none |
| Triiodothyronine | 600µl | 400µl |
| NBCS | 300µl | 4ml |
| Gentamicin sulfate | 300µl | 200µl |