Published in final edited form as:
Nat Protoc. ; 7(2): 235–246. doi:10.1038/nprot.2011.437.

Isolation and characterization of mouse and human esophageal epithelial cells in 3D organotypic culture

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Abstract

This protocol describes the isolation and characterization of mouse and human esophageal epithelial cells and the application of 3D organotypic culture (OTC), a form of tissue engineering. This model system permits the interrogation of mechanisms underlying epithelial-stromal interactions. We provide guidelines for isolating and cultivating several sources of epithelial cells and fibroblasts, as well as genetic manipulation of these cell types, as a prelude to their integration into OTC. The protocol includes a number of important applications, including histology, immunohistochemistry/immunofluorescence, genetic modification of epithelial cells and fibroblasts with retroviral and lentiviral vectors for overexpression of genes or RNA interference strategies, confocal imaging, laser capture microdissection, RNA microarrays of individual cellular compartments and protein-based assays. The OTC (3D) culture protocol takes 15 d to perform.

INTRODUCTION

The esophagus is a hollow tubular organ whose wall comprises a mucosa or epithelium (with sparse lamina propria), submucosa (containing mucus-secreting glands and papillae), muscle (the proximal esophagus has striated muscle and the distal esophagus has smooth muscle) and adventitia. The epithelium is squamous and stratified, similar to the skin, oropharynx, trachea and anogenital tract. The stratified squamous esophageal epithelium protects against mechanical and chemical insults, and, in this context, has a barrier function. Infectious organisms, such as viruses (cytomegalovirus virus, Epstein-Barr virus, herpes

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AUTHOR CONTRIBUTIONS J.K., G.S.W., M.E.V. and M.N. contributed to the experimental results; E.S.R., M.H., H.N. and A.K.R. contributed to the experimental design; all authors contributed to the manuscript preparation and writing.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

simplex virus, human papillomavirus and human immunodeficiency virus), bacteria and fungi (*Candida albicans*) may cause esophagitis. Acid reflux from the stomach and bile reflux from gastroduodenal contents may cause erosive esophagitis, often in the distal esophagus. It is known that acid and bile reflux can cause either an incomplete intestinal metaplasia at the esophagogastric junction or Barrett's esophagus, which is a precursor to esophageal adenocarcinoma. The other major subtype of esophageal malignancy is esophageal squamous cell carcinoma (ESCC).

The esophageal epithelium has several compartments: (i) a proliferative basal cell compartment in which cells reside on the basement membrane; (ii) a differentiating suprabasal cell compartment; and (iii) a superficial squamous cell compartment. Basal cells migrate in an outward direction to the lumen, undergoing a transition from proliferation to terminal differentiation. Eventually, cells desquamate at the luminal surface. This process is renewed on a continuous basis[1]. There are distinct keratins expressed in stratified squamous epithelia[2]. As with the skin[3,4], the esophageal basal cells express cytokeratins 5 and 14 (CK5, CK14)[5]. Whereas the skin suprabasal and superficial layers express CK1 and CK10 (ref. 4), the esophageal suprabasal layer express CK4 (ref. 6) and CK13 (ref. 7). The superficial layer of the esophagus expresses involucrin and filaggrin[8,9]. The mouse esophageal epithelium has a keratinized layer on its surface; the human esophageal epithelium lacks this feature.

Human esophageal epithelial cells can be cultured in 2D, and may or may not use a 3T3 fibroblast layer as a support[10,11]. Apart from the human esophagus, there have been reports of cultivating esophageal epithelial cells from different species, such as rabbit[12]. In human esophageal epithelial cells, SV40 T-antigen was introduced to induce immortalization[13]. We have successfully isolated primary human esophageal cells (EPCs) that undergo replicative senescence after 42–45 population doublings. Retroviral transduction with human telomerase reverse transcriptase (hTERT) overcomes replicative senescence and these cells are immortalized[14]. EPC-hTERT cells can be placed into 3D OTC with recapitulation of the normal stratified squamous epithelium[14]. The organotypic 3D culture has been used to provide insights into mechanisms underlying normal esophageal epithelial biology and esophageal cancer biology[14–18].

Applications of esophageal organotypic (3D) culture

There are intrinsic advantages in evaluating EPCs in 3D culture versus 2D culture. The 3D OTC system for esophageal epithelial cells is unique. Its advantages relate to the normal polarization and differentiation patterns of cells, the expression of genes involved in the adherens junctions and tight junctions and the gene signatures that resemble *in vivo* tissues. Esophageal epithelial cells immortalized with hTERT can constitute a complete stratified squamous epithelium after exposure to a liquid-air interface[14]. Perturbations in the esophageal epithelium can be observed with epidermal growth factor receptor (EGFR) overexpression[15], resulting in epithelial hyperplasia. The expression of inducible AKT in esophageal epithelial cells results in an expansion of the proliferating basal cells and impaired (delayed) differentiation[16]. Epithelial cells may be transformed by the introduction of a combination of oncogenes and/or inactivated tumor suppressor genes (e.g., EGFR, cyclin D1 and mutant p53), and the resulting transformed epithelial cells invade into the underlying matrix, thereby providing a platform to investigate properties of tumor cells and the cross talk between invading tumor cells and activated stromal fibroblasts[17–19]. Genes that mediate tumor invasion can be identified using laser capture microdissected cells from OTC[17,20]. Gene expression can be modified using retrovirally or lentivirally mediated shRNA in esophageal epithelial cells and fibroblasts[16,19,21]. In addition, gene expression can be modulated in the 3D context using inducible systems[22]. Cell signaling pathways in the reconstituted epithelia can be interrogated by pharmacological inhibitors, although such

agents may influence both epithelial and fibroblast functions[15,22]. In addition, the self-renewal capacity of mouse esophageal stem cells can be demonstrated using OTC[23].

Experimental design

The steps involved in the development of OTC are outlined in Figure 1. The process involves the casting of an acellular collagen matrix on the bottom of an insert, followed by the casting of a layer of esophageal fibroblasts mixed with collagen type I and Matrigel. Matrigel is not required for the formation of stratified squamous epithelium, but it facilitates invasion of transformed epithelial cells or ESCC cells. These two layers serve as a substitute for the esophageal 'mesenchyme' and are cultured initially for up to 7 d, thereby allowing for fibroblast-mediated constriction of the collagen matrix. Sources of epithelial cells for OTC are the following: (i) primary mouse esophageal keratinocytes, (ii) primary mouse esophageal stem cells using fluorescence-activated cell sorting (FACS), (iii) primary human esophageal keratinocytes (EPCs), (iv) immortalized human esophageal keratinocytes (EPC-hTERT cells) and (v) esophageal cancer cell lines. On day 5, the epithelial cells are seeded on the surface of the constricted matrix. The medium of the OTC is changed every 2 d and the epithelium is exposed to air to create a liquid-air interface, thereby promoting epithelial stratification and differentiation. Finally, on day 15, the resulting OTC may be processed for histology, and subsequent immunohistochemistry or immunofluorescence. In addition, the epithelium may be peeled away from the matrix and processed for RNA or protein isolation. Specific cell populations (e.g., epithelial cells, regions of fibroblasts in the matrix) may be isolated using laser capture microdissection (LCM) for RNA isolation and subsequent *in vitro* RNA amplification and microarray analysis or quantitative reverse-transcription PCR (Fig. 2). The conditioned medium from OTC has been used for the detection of proteins by western blot analysis, ELISA or proteomics.

MATERIALS

REAGENTS

- Tissues: use esophagi from wild-type or genetic (transgenic/knockout) mice as a source for fibroblasts and epithelial cells

  ! CAUTION Procedures must be approved by the local Institutional Animal Care and Use Committee.

- Cells: passaged human esophageal fibroblasts (FEF3 and others)[18], freshly isolated primary mouse esophageal fibroblasts and epithelial cells[23], immortalized and transformed human esophageal epithelial cells (EPC-hTERT and derivatives)[14,19] and ESCC cell lines (i.e., TE series, T.T, HCE4 and HCE7)[24]. See Supplementary Tables 1 and 2 for comprehensive lists of fibroblast cell lines and epithelial cell lines validated in 3D OTC.

- Keratinocyte–serum-free medium (KSFM) without calcium chloride ($CaCl_2$), supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF; Invitrogen, cat. no. 10725-018)

- $CaCl_2$ (300 mM; Lonza, cat. no. CC-4202)

- KSFM with $CaCl_2$, supplemented with BPE and EGF (Invitrogen, cat. no. 10724-011)

- Dulbecco's modified Eagle's medium (DMEM; Cellgro, cat. no. 10-013-CV)

- Fetal bovine serum (FBS; HyClone, cat. no. SH30071.03)

- Penicillin-streptomycin (10,000 U ml $^{-1}$ of penicillin G sodium and 10,000 μg ml $^{-1}$ of streptomycin sulfate in 0.85% (wt/vol) saline; Invitrogen, cat. no. 15140-122)
- Dulbecco's phosphate-buffered saline (PBS; Invitrogen, cat. no. 14190-136)
- Hanks' balanced salt solution (HBSS; Invitrogen, cat. no. 14175-079)
- Nystatin suspension (10,000 U ml $^{-1}$; Sigma-Aldrich, cat. no. N1638)
- Gentamicin sulfate, liquid (50 mg ml $^{-1}$; Cellgro, cat. no. 30-005-CR)
- Dispase I (Roche, cat. no. 210455)
- Propidium iodide (PI; Calbiochem, cat. no. 537059)
- Collagenase, lyophilized (Invitrogen, cat. no. 17018-029)
- Hoechst 33342 (Sigma-Aldrich, cat. no. 14533)
- Verapamil hydrochloride (Sigma-Aldrich, cat. no. V4629)
- Trypsin-EDTA (0.05% (wt/vol) trypsin, 0.53 mM EDTA·4Na in Hank's balanced salt solution without $CaCl_2$, $MgCl_2 \cdot 6H_2O$ and $MgSO_4 \cdot 7H_2O$; Invitrogen, cat. no. 25300-054)
- Trypsin inhibitor from *Glycine max* (soybean; Sigma-Aldrich, cat. no. T9128)
- Dimethyl sulfoxide (DMSO; Fisher Scientific, cat. no. BP231-1)
- Ham's F12 (Invitrogen, cat. no. 11765-054 or Mediatech 10-080-CV)
- Eagle's minimum essential medium (EMEM, 10×; Lonza, cat. no. 12-684F)
- Sodium bicarbonate ($NaHCO_3$, 7.5% (wt/vol); Lonza, cat. no. 17-613E)
- L-Glutamine (200 mM solution; Cellgro, cat. no. 25-005CI)
- Bovine collagen type I (1.0–1.2 mg ml $^{-1}$; Organogenesis, cat. no. 200-055)
- Matrigel basement membrane matrix (BD Biosciences, cat. no. 354234; a solubilized basement membrane matrix extracted from the Engelbreth-Holm-Swarm (EHS) mouse tumor, containing laminin, collagen I, entactin, heparan sulfate proteoglycan, growth factors, matrix metalloproteinases and other proteinases[25–27])
- Hydrocortisone (Sigma-Aldrich, cat. no. H0888)
- Ethanol (100% (200 proof); Pharmco, cat. no. 111ACS200)
- Insulin, transferrin, ethanolamine and selenium (ITES; 500×; Lonza, cat. no. 17839Z)
- *O*-Phosphorylethanolamine (Sigma-Aldrich, cat. no. P0503)
- Adenine (Sigma-Aldrich, cat. no. A9795)
- Progesterone (Sigma-Aldrich, cat. no. P8783)
- Triiodothyronine (Sigma-Aldrich, cat. no. T5516)
- Sodium hydroxide solution (NaOH, 10 N; Fisher Scientific, cat. no. SS255-1)
- Newborn calf serum (Hyclone, cat. no. SH 3011802)
- Formalin, phosphate buffered (10% (wt/vol); Fisher Scientific, cat. no. SF100-4)

- Optimum cutting temperature (OCT) compound (Sakura Finetek Tissue-Tek, cat. no. 4583)
- Chelex 100 (Sigma-Aldrich, cat. no. C7901)
- Diethylpyrocarbonate (DEPC)
- Xylene

EQUIPMENT

- Sterilizable sharp-pointed dissecting scissors (Fisher Scientific, cat. no. 08-951-25)
- Sterilizable dissecting tissue forceps (Fisher Scientific, cat. no. 1381236)
- Cell strainer (40 μm; BD Falcon, cat. no. 352340)
- Cell strainer (70 μm; BD Falcon, cat. no. 352350)
- Round-bottom polystyrene tube (5.0 ml; BD Falcon, cat. no. 352058)
- Six-well multiwell plate (BD Falcon, cat. no. 353046)
- Cell culture dish (100 mm × 20 mm; BD Primaria, cat. no. 353803)
- Cell culture flask (75 cm^2; Corning, cat. no. 430641)
- Cell culture flask (150 cm^2; BD Falcon, cat. no. 355001)
- Syringe filters (diameter 25 mm and pore size 0.2 μm; Whatman, cat. no. 6750-2502)
- Filter units (pore size, 0.2 μm; 115-ml capacity, 250-ml capacity, 1,000-ml capacity; Nalgene, cat. nos. 120-0020, 568-0020, 567-0020, respectively)
- Six-well Transwell carrier (Organogenesis, cat. no. TS01001)
- Transwell (24 mm) with 3.0 μm pore polycarbonate membrane insert (Corning, cat. no. 3414)
- Sterile disposable scalpel no. 21 (Feather, cat. no. 2975)
- Histosette II tissue cassettes (Fisher Scientific, cat. no. 15182701C)
- Histology cassettes pads, cellular polyester foam (rectangular; 32 mm × 25 mm × 2 mm; Fisher Scientific, cat. no. 22038221)
- Pure cellulose chromatography paper (thickness: 0.35 mm; Fisher Scientific, cat. no. 05-714-4)
- Single-edge razor blades (0.009-in thickness; Daniel Smith, cat. no. 479118002)
- Nikon Eclipse TE2000-5 inverted microscope with UV laser (Molecular Machines & Industries CellCut System)
- FACSVantage with Diva option (BD Biosciences)
- Isolation cap tubes (Molecular Machines & Industries, cat. no. 50210)
- Arcturus PicoPure RNA isolation kit (Applied Biosystems, cat. no. KIT0204)
- Amicon Ultra-15 centrifugation tubes (Millipore, cat. no. UFC901008)
- Incubator
- Glass-bottom dish no. 1 (35 mm; MatTek Corporation)

- Inverted laser scanning confocal microscope (LSM 410 equipped Axiovert 135; Carl Zeiss)
- LSM version 3.98 software (Carl Zeiss)
- LSM Image Examiner version 2.8 software (Carl Zeiss)

REAGENT SETUP

Growth medium for mouse esophageal keratinocytes (m-KSFM)—Use to the KSFM 500 ml of KSFM without $CaCl_2$. Add 30 μl of 300 mM $CaCl_2$ (final concentration 0.018 mM). Add EGF to a final concentration of 1 ng ml $^{-1}$, 25 mg of BPE (the whole content of the vial supplied) and 5 ml of penicillin-streptomycin. For primary culture, add gentamicin sulfate (5 μg ml $^{-1}$) and nystatin (100 U ml $^{-1}$) for the first 2 weeks. Store at 4 °C for up to 1 month. ▲ **CRITICAL** Warm medium to 37 °C before use for primary culture to avoid cell stress.

Growth medium for primary, immortalized or transformed human esophageal keratinocytes (i.e., EPC, EPC-hTERT and derivatives; h-KSFM)—Use 500 ml of KSFM with 0.09 mM $CaCl_2$. Add EGF (final concentration 1 ng ml $^{-1}$), 25 mg of BPE and 5 ml of penicillin-streptomycin as in m-KSFM. For primary culture, add gentamicin sulfate (5 μg ml $^{-1}$) and nystatin (100 U ml $^{-1}$) for the first 2 weeks. Store at 4 °C for up to 1 month. ▲ **CRITICAL** Warm medium to 37 °C before use.

Growth medium for fibroblasts and esophageal cancer cell lines (DMEM–10% (vol/vol) FBS)—Use 500 ml of DMEM supplemented with 50 ml of 100% FBS and 5 ml penicillin-streptomycin. Store at 4 °C for up to 1 month.

Soybean trypsin inhibitor—Dissolve 250 mg of soybean trypsin inhibitor in 1 liter of Dulbecco's PBS. Filter-sterilize with a filter unit (1,000-ml capacity) and dispense into 50-ml tubes and store at 4 °C for up to 6 months. ▲ **CRITICAL** Trypsin inhibition is essential before human and mouse esophageal keratinocytes are seeded in KSFM-base medium as it contains a low level of protein (i.e., EGF and BPE).

Cell culture and genetic modifications—Grow cells at 37 °C in a 5% CO_2 incubator. If required, perform retrovirus/lentivirus-mediated stable gene transduction in human and mouse esophageal fibroblasts, keratinocytes (i.e., EPC and EPC-hTERT derivatives) and esophageal cancer cell lines with the vectors listed in Table 1 and used for 3D OTC.

HBSS(+) for cell isolation in primary culture—Add penicillin-streptomycin (1% (vol/vol)), gentamicin sulfate (5 μg ml $^{-1}$) and nystatin (100 U ml $^{-1}$) to 1 liter of HBSS and store the mixture at 4 °C for up to 6 months.

Dispase I (1 U ml^{-1})—Dispase I is shipped as a vial containing ~2 mg (≥20 U per vial) lyophilized enzyme; dissolve in HBSS to 10 mg ml $^{-1}$. Sterilize through a 0.2-μm filter membrane, aliquot and store at −20 °C for up to 1 year. Dilute further in HBSS to 1 U ml $^{-1}$ as a working solution.

Collagenase (1 mg ml^{-1})—Reconstitute (≥125 U mg^{-1}) collagenase in HBSS to 1 mg ml^{-1} (≥125 U ml^{-1}). Sterilize through a 0.2-μm filter membrane, aliquot and store at − 20 °C for up to 1 year.

OTC medium components—Store 10× EMEM and 7.5% (wt/vol) $NaHCO_3$ at room temperature (25 °C) for up to 2 years. Avoid repeating freezing and thawing cycles more

than four times. Prepare aliquots of the reagent and store at − 20 °C after filter sterilization through a 0.2-μm membrane. When reconstituted in a 100-ml scale, the authors' laboratory makes 20–50 small aliquots in 1.7-ml tubes (~1,400 μl per tube). The rest is stored at − 20 °C as a large aliquot (12–30 ml per tube) in 15-ml/50-ml tubes. Thaw aliquots of FBS, newborn calf serum, L-glutamine, hydrocortisone, ITES, O-phosphorylethanolamine, adenine, progesterone and triiodothyronine in a water bath at 37 °C. Transfer them onto ice immediately. Matrigel is stored at − 20 °C. However, it should be thawed at 4 °C overnight; Matrigel thawed at 37 °C solidifies rapidly and can no longer be used. ▲ **CRITICAL** All OTC reagents should be chilled on ice before use.

Bovine collagen type I—Prepare aliquots of ~40 ml and store them at 4 °C for up to 6 months. Chill on ice before use. Approximately 35 ml of collagen will be needed to prepare two six-well Transwell carriers (12 wells).

Hydrocortisone—Dissolve 0.0269 g (molecular weight (MW) = 362.46) in 2.5 ml of absolute ethanol. Dilute with 97.5 ml of DMEM to obtain a 74.2 μM stock solution. Filter (0.2 μm), label aliquots 'H' and store at − 20 °C for up to 1 year.

***O*-Phosphorylethanolamine—**Dissolve 0.705 g (MW = 141.06) in 100 ml of DMEM to obtain a 5 mM stock solution. Filter (0.2 μm), label aliquots 'O' and store at − 20 °C for up to 1 year.

Adenine—Dissolve 1.55 g (MW = 171.59) in 100 ml of double-distilled H_2O to obtain a 90 mM stock solution. Filter (0.2 μm), label aliquots 'A' and store at − 20 °C for up to 1 year.

Progesterone—Dissolve 1 mg (MW = 314.46) in 1 ml of absolute ethanol. Add 14.7 ml of double-distilled H_2O. Dilute 1 ml of this in 100 ml of DMEM (final concentration of 2 μM) as a stock solution. Filter (0.2 μm), label aliquots 'P' and store at − 20 °C for up to 1 year.

Triiodothyronine—Dissolve 1 mg (MW = 672.96) in 1 ml of 1 N NaOH. Add 19 ml of DMEM. Further dilute 4 μl of this solution in 31 ml of DMEM to obtain a 10 nM stock solution. Filter (0.2 μm), label aliquots 'T' and store at − 20 °C for up to 1 year.

PROCEDURE

Preparation and use of different cell lines for 3D OTCs ● TIMING 1–1.5 weeks

1| For organotypic 3D culture, freshly isolated esophageal keratinocytes and fibroblasts can be used; set these up as described in option A, in which functions of putative esophageal stem can be tested as well. Alternatively, immortalized or transformed human EPCs and ESCC cell lines can be used along with passaged human esophageal fibroblasts, as described in option B. Primary cells are prone to phenotypic changes (e.g., senescence). By contrast, immortalized or transformed human EPCs and ESCC cells are more stable and easier to manipulate genetically or pharmacologically. See also Supplementary Information for cultivating and passaging the cell lines. If you are using human or mouse fibroblasts, use a 150-cm^2 cell culture flask at 60–80% confluence for two Transwell carriers (i.e., 12-well per inserts) and start growing these cells a week before Step 2. If you are using human or mouse esophageal keratinocytes (human or mouse) and ESCC cells, a 75-cm^2 cell culture flask at 80% confluency yields around 4×10^6 cells, and 3×10^6 cells are used per Transwell

carrier (i.e., six-well per insert). Start growing these cells 1.5 weeks before Step 2.

A. Isolation of primary mouse esophageal keratinocytes, putative stem cells and primary mouse esophageal fibroblasts for OTC

i. Isolation of primary mouse esophageal keratinocytes: euthanize five to ten mice (C57/BL6) at 4–8 weeks of age. Use sterile technique to remove esophagi from mice.

ii. Rinse removed esophagi with sterile HBSS(+) and collect them in a 100-mm cell culture dish containing 10 ml of sterile HBSS(+) until all esophagi are harvested.

iii. Cut each esophagus open longitudinally to expose the mucosal surface. Place open esophagi in new sterile HBSS(+).

iv. Place the esophagi in 1 ml of 1 U ml^{-1} of dispase I (volume for ten esophagi) and incubate them at 37 °C for 10 min.

v. Peel the epithelia from the submucosa and place in 1 ml of HBSS(+), pooling the epithelia together. Retain the submucosa for Step 1A(xxiv).

vi. Place the pooled epithelia in 1 ml of trypsin-EDTA and incubate them at 37 °C for ~10 min.

vii. Vortex for 10 s, then remove trypsin-EDTA with floating cells and pipette into 8 ml of 250 mg per liter soybean trypsin inhibitor to stop trypsinization of cells.

viii. Add 1 ml of trypsin-EDTA to the remaining esophagi and incubate at 37 °C for an additional 5 min.

ix. Vortex for 10 s, then remove trypsin-EDTA with floating cells and pipette into a tube containing soybean trypsin inhibitor and pool with cells from Step 1A(vii).

x. Filter the isolated cell suspension through a 40-μm cell strainer.

xi. Centrifuge at 188*g* for 5 min at 4 °C.

xii. Remove the supernatant from Step 1A(xi).

xiii. Resuspend pelleted cells in 4 ml of m-KSFM medium with gentamicin sulfate and nystatin. Note that the reproducible yield should be 2.5–5.0 × 10^5 esophageal epithelial cells per mouse.

xiv. Plate cells into two wells (2 ml per well) of a six-well plate. Grow cells at 37 °C in a 5% CO_2 incubator for 48 h to allow cells to adhere to the bottom of the culture plate.

xv. Change the medium every 48 h and replace with fresh m-KSFM containing gentamicin sulfate and nystatin. ▲ **CRITICAL STEP** Change the medium every other day (mandatory) regardless of cell density.

xvi. Isolation of a mouse esophageal stem cell fraction (side population): alongside performing Steps 1A(xiii) and 1A(xiv), isolate primary mouse esophageal keratinocytes as described in Step 1A(i–xii) and resuspend the cells in prewarmed (37 °C) m-KSFM (10^6 cells per ml).

xvii. Divide the cells for eventual use in the FACSVantage SE with Diva option. An ascribed property of stem cells is their ability to efflux Hoechst 33342, a fluorescent dye. The Hoechst-negative cells are isolated by fluorescence-activated cell sorting as a so-called side population (SP), whereas the Hoechst-positive or enriched population is the nonside population (NSP). It is believed that the ABCG2 transporter is important as a Hoechst efflux pump. Verapamil is used because of its known ability to block efflux of DNA-binding fluorophores such as Hoechst 33342. Place 2.5×10^5 cells into four tubes, (1.5 ml each). Place the remaining cells (typically 4.5×10^6) in a 15-ml polypropylene tube and add Hoechst 33342 dye to a final concentration of 5 μg ml^{-1}. Add Hoechst 33342 dye and verapamil to the 1.5-ml microcentrifuge tubes such that the final concentration of verapamil is 50 μM. Incubate the tubes in a water bath at 37 °C for 90 min (this is the optimal incubation time in our experience). Periodically agitate (every 15 min) the cells by inverting the tubes twice. ▲ **CRITICAL STEP** Make sure that the water bath temperature is maintained precisely at 37 °C to ensure cell viability.

xviii. Take 10^5 Hoechst 33342 dye-stained cells from the 15 ml tube (50 μl for 10^6 cells per ml) and place in a 1.5 ml tube.

xix. Centrifuge each tube at 480*g* for 4 min at room temperature.

xx. Remove the supernatant and resuspend each cell pellet with ice-cold 0.5 ml of m-KSFM per microcentrifuge tube and 1.5 ml of m-KSFM for the 15-ml tube. Place samples on wet ice for the subsequent steps.

xxi. Transfer the cells into 5.0-ml round-bottom polystyrene tubes. Store tubes on wet ice. Set up the following samples: 10^5 Hoechst unstained cells (label tube H^-P^-); 10^5 Hoechst unstained cells: add 2 μg per ml of PI to obtain a final concentration of 1.5 μg ml^{-1} (label tube H^-P^+); 15-ml tube of Hoechst-stained cells: add 2 μg ml^{-1} of PI to obtain a final concentration of 1.5 μg ml^{-1} (label tube H^+P^+); 10^5 Hoechst-stained cells (label tube H^+P^-); 10^5 Hoechst-stained cells and verapamil hydrochloride–treated cells: add 2 μg ml^{-1} of PI to obtain a final concentration of 1.5 μg ml^{-1} (label tube $H^+P^+V^+$).

xxii. Use negative cells to set up the forward scatter (FSC), side scatter (SSC) and PI-negative fractions of cells.

Confirm live/dead gates with PI-only stained cells (excitation 150 mW, 488 nM, collection 630/22 (log scale)). Adjust the voltages on Hoechst-stained cells to optimize the SP (excitation 100 mW multiline UV, Blue fluorescence 450/20 (linear scale), red fluorescence 675LP (linear scale)), which is split by a 610SP (dichroic). Confirm the loss of SP in the target gate with verapamil-treated cells (Fig. 3).

xxiii. Set gates for the following: esophageal epithelial cells (usually 200–400 FSC-A and 100–400 SSC-A); single cells; live cells (dead cells above PI $10^{1.3}$); SP cells ('tail' of cells with Hoechst red below 300 and Hoechst blue below 400); and NSP cells (Hoechst red above 300, Hoechst blue over 400).

xxiv. Primary mouse esophageal fibroblasts: place the submucosa (remaining after removal of epithelia in Step 1A(v)) into a 50-ml tube containing 1 U ml^{-1} dispase I and 100 U ml^{-1} collagenase and incubate at 37 °C in a water bath for 2 h, vortexing briefly every 20 min.

xxv. Quench the reaction by adding three volumes of DMEM–10% (vol/vol) FBS. Mix gently and place on ice.

xxvi. Filter the stromal cell suspension through a 70-μm cell strainer into a 50-ml tube. Use one strainer per 20 ml of cell suspension.

xxvii. Centrifuge at 368*g* for 5 min at 4 °C.

xxviii. Remove the supernatant and disperse the pellet by flicking the tube. Resuspend stromal cells in 10 ml of DMEM–10% (vol/vol) FBS and count the cells.

xxix. Plate the stromal cells at 2×10^6 cells per 75 cm^2 cell culture flask in DMEM–10% (vol/vol) FBS. Grow cells at 37 °C in a 5% CO_2 incubator for 48–72 h.

xxx. Use exponentially growing (60–80% confluent) fibroblasts at passages 2–9 for casting the cellular layer in OTCs. For future experiments, prepare frozen stocks (30–50 cryogenic vials containing 0.8–1 million cells) and grow fibroblasts at passages 4–6 in two 150-cm^2 cell culture flasks until they become 100% confluent to undergo contact inhibition.

B. Passage of primary, immortalized and transformed human esophageal keratinocytes, as well as ESCC cells, for organotypic 3D culture supported by primary human esophageal fibroblasts

i. Start growing primary human esophageal fibroblasts 1 week before preparing the acellular and cellular collagen matrices on day 1 of OTC. Use a 150-cm^2 cell culture flask to obtain subconfluent fibroblasts enough to make two Transwell carriers (i.e., 12 wells in Transwell inserts). Several independent primary human esophageal

fibroblasts are available[18]. FEF3 (human fetal esophageal fibroblasts) perform well.

ii. For EPCs, the noninvasive, nontransformed EPC2-hTERT cells serve as a good control in OTC. To assess invasion, use EPC2-hTERT-EGFR-p53^{R175H} or TE12 (ESCC) cells. Start growing epithelial cells about 1 week before seeding epithelial cells in Step 18. Seed 5×10^5 epithelial cells per insert per well. Prepare 3×10^6 cells for one Transwell carrier (six wells). Approximately 80% confluent EPC2-hTERT cells and their derivatives grown in a 75-cm^2 cell culture flask yield about 4×10^6 cells. Grow cells at 37 °C in a 5% CO_2 incubator.

Preparation of collagen matrices for OTC (OTC day 1) ● TIMING 1.5–2 h

2| Label six-well Transwell carriers at a corner or two of both the lid and the bottom. This avoids loss of orientation.

3| Place the Transwell inserts into each well of the six-well Transwell carriers with sterile forceps inside the tissue culture hood.

4| Chill empty 50-ml tubes on ice to prepare cellular (label ‘C’) and acellular (label ‘A’) matrices while thawing matrix components (see the REAGENT SETUP section).

5| Mix the acellular matrix components in tube ‘A’ on ice in the order listed below.

	Transwell carriers	
50-ml tube ‘A’ acellular layer components	**× 1 (6 Inserts)**	**× 2 (12 Inserts)**
10× EMEM	690 μl	1,380 μl
FBS	700 μl	1,400 μl
L-Glutamine	60 μl	120 μl
Sodium bicarbonate	140 μl	280 μl
Bovine collagen type I	5.6 ml	11.2 ml

▲ **CRITICAL STEP** Bovine collagen type I is viscous. Use a 25-ml serological pipette to mix carefully but thoroughly until the color of the matrix becomes homogeneous. Avoid air bubbles. Once formed they may stay in the matrix.

6| Use a 5-ml serological pipette to add 1 ml each of acellular matrix into the Transwell inserts.

7| Incubate at room temperature in a tissue culture hood for >10 min until the matrix solidifies.

8| Trypsinize and count the fibroblasts from either Step 1A(xxx) or Step 1B(i), and adjust the cell concentration to 6×10^5 per ml.

9| Mix the cellular matrix components on ice in the order listed below in a chilled 50-ml tube ‘C’ from Step 4.

	Transwell carriers	
50-ml tube 'C' cellular layer components	**× 1 (6 Inserts)**	**× 2 (12 Inserts)**
10× EMEM	1.8 ml	3.6 ml
FBS	2 ml	4 ml
L-Glutamine	160 μl	320 μl
Sodium bicarbonate	380 μl	760 μl
Bovine collagen	11.4 ml	22.8 ml
Matrigel	3.8 ml	7.6 ml
6×10^5 per ml fibroblasts	1.6 ml	3.2 ml

▲ **CRITICAL STEP** Bovine collagen type I and Matrigel are viscous. Use a 25-ml serological pipette to mix carefully but thoroughly until the color of the matrix becomes homogeneous. Avoid air bubbles. Once formed they may stay in the matrix.

10| Use a 10-ml serologic pipette to dispense 3 ml each of the matrix into the Transwell inserts. Ensure that there are no remaining large air bubbles. If present, they can be removed easily by poking with a pipette tip.

11| Carefully carry the filled Transwell carriers with the inserts from tissue culture hood to the incubator. Incubate at 37 °C for 30–45 min to allow the matrix to solidify.

12| Add DMEM–10% (wt/vol) FBS: 10 ml to the bottom of the wells in the Transwell carrier and 2 ml to the Transwell inserts.

Dislodging the matrix (OTC day 2) ● TIMING 10 min

13| Dislodge the edges of the matrix from the side of the Transwell insert. Hold a sterile glass Pasteur pipette vertically to go around (~2 times) the matrix along the inner wall of the insert. Feel the membrane at the bottom as you move the pipette tip, but do not pierce it. Ensure that the tip is not broken. If you feel resistance in the matrix, stop and go the other way around.

14| Add 2 ml of DMEM–10% (vol/vol) FBS into the Transwell inserts. Incubate matrices at 37 °C in a 5% CO_2 incubator. Observe the matrix contract over the next few days forming a crater-like top where epithelial cells are to be seeded. Wait until OTC day 7 before seeding epithelial cells. There is no need to change medium until Step 16.

? TROUBLESHOOTING

Seeding epithelial cells (OTC day 7) ● TIMING 4 h

15| Prepare presaturation medium by mixing DMEM and Ham's F12 in a 3:1 ratio in a sterile vessel as described below.

	Transwell carriers	
Presaturation medium	**× 1 (6 Inserts)**	**× 2 (12 Inserts)**
DMEM	60 ml	120 ml

	Transwell carriers	
Presaturation medium	**× 1 (6 Inserts)**	**× 2 (12 Inserts)**
Ham's F12	20 ml	40 ml

16| Remove the old medium (from Step 14) by aspiration from the Transwell carriers and inserts. Do not disturb the matrix formed within the inserts. Add DMEM + Ham's F12 (3:1) from Step 15: 10 ml to the bottom and 2 ml to the top of the Transwell carriers.

17| Incubate at 37 °C in a 5% CO_2 incubator for 1 h.

18| Trypsinize and count the epithelial cells and adjust the cell concentration to 1×10^7 per ml in the medium used for the epithelial cell growth (e.g., h-KSFM for EPC-hTERT).

19| Remove presaturation media and add 50 μl (5×10^5 per Transwell-insert per well) of the epithelial cells to the center of the surface of contracted matrix. Do not disturb the matrix formed within the inserts.

20| Incubate without medium in the tissue culture incubator (37 °C, 5% CO_2) for 2 h.

21| Prepare epidermalization media (EPM1 and EPM2) by mixing the medium components in the order listed below.

For two Transwell carriers (12 wells; two medium changes)	**EPM1 (300 ml), store at 4 °C**	**EPM2 (200 ml), store at 4 °C**
DMEM	218 ml	95 ml
Ham's F12	72 ml	95 ml
L-Glutamine	6 ml	4 ml
Hydrocortisone (H)	600 μl	400 μl
ITES	600 μl	400 μl
O-Phosphorylethanolamine (O)	600 μl	400 μl
Adenine (A)	600 μl	400 μl
Progesterone (P)	600 μl	—
Triiodothyronine (T)	600 μl	400 μl
NBCS	300 μl	4 ml
Gentamicin sulfate	300 μl	200 μl

22| Add EPM1: 10 ml to the bottom (Transwell carrier) and 2 ml to the top (insert). Store the remaining EPM1 at 4 °C. This will be used also for day 7 in Step 23. Incubate the cells at 37 °C in a 5% CO_2 incubator.

Medium change (OTC day 9) ● TIMING 10 min

23| Aspirate the old medium to add EPM1: 10 ml to the bottom well (Transwell carrier) and 2 ml to the top (insert). Incubate the cells at 37 °C in a 5% CO_2 incubator.

Medium change (OTC day 11) to create liquid-air interface ● TIMING 10 min

24| Aspirate the old medium to add EPM2: 7.5 ml to the bottom well (Transwell carrier) only. Incubate the cells at 37 °C in a 5% CO_2 incubator.

Medium change (OTC day 13) ● TIMING 10 min

25| Aspirate the old medium to add EPM2: 7.5 ml to the bottom well (Transwell carrier) only. Incubate the cells at 37 °C in a 5% CO_2 incubator.

Harvesting OTCs (day 15) ● TIMING 1.5–2 h (plus processing time)

26| Harvest the cultures. If you wish to proceed with paraffin embedding and histological processing, proceed with option A. For LCM follow option B. For RNA and protein isolation follow option C. To process for conditioned medium follow option D. To image cells follow option E.

A. Fixation for paraffin embedding and histological processing ● TIMING 3 d

i. Remove the medium and add 10% (wt/vol) formalin: 10 ml to the bottom well (Transwell carrier) and 2 ml to the top (insert).

ii. Incubate at 4 °C for 1 h.

! CAUTION Fixation can be done overnight. However, excessive fixation may affect the antigenicity of certain molecules (e.g., E-cadherin) for detection by immunohistochemistry.

? TROUBLESHOOTING

iii. Remove the inserts from the Transwell carriers and place them on a clean plastic surface and cut the membrane (containing the OTC matrix) off the insert using a scalpel.

iv. Place the membrane into a labeled Histosette II tissue cassette with a sheet of pure cellulose chromatography paper placed on top of a histology cassettes pad.

v. Wash cassettes twice in PBS at room temperature for 10 min per wash.

vi. Place the cassettes in 70% (vol/vol) ethanol and store at 4 °C for up to 7 d.

vii. Continue with routine histological processing to prepare paraffin blocks for H&E staining, as well as for immunohistochemistry/immunofluorescence (some of the antibodies validated in OTCs are listed in Table 2).

? TROUBLESHOOTING

B. Alternative processing for LCM ● TIMING 6–8 h

i. Aspirate the medium and place the Transwell insert on a clean and hard plastic surface.

ii. Cut the membrane off the insert using a scalpel.

iii. Cut the OTCs into halves using a single-edge razor blade.

iv. By using sterile forceps, carefully embed each half of the OTC in OCT compound and snap-freeze using liquid nitrogen.

v. Section the frozen culture halves into 8-μm sections.

vi. Thaw the frozen sections for no longer than 30 s and fix them in 75% (vol/vol) ethanol for 30 s.

vii. Wash with DEPC H_2O for 30 s.

viii. Stain the sections with hematoxylin for 10 s. Wash sections two times with DEPC H_2O for 15 s.

ix. Dehydrate the sections with two washes of 95% (vol/vol) ethanol for 30 s and one wash of 100% ethanol for 30 s.

x. Place the sections in a glass slide container with xylene for 2 min.

xi. Rinse the sections with DEPC H_2O and air-dry on an aluminum foil for 5 min.

xii. Place the stained sections on an inverted microscope and microdissect epithelial cells using UV laser. Carefully transfer microdissected cells from the microscope slide to isolation cap tubes for RNA isolation using the Arcturus PicoPure RNA isolation kit according to the manufacturer's instructions. Isolated RNA can be used for microarray analysis or quantitative reverse-transcription PCR.

C. Alternative processing for RNA and protein isolation ● TIMING 1 h

i. Peel the epithelial sheet off with forceps from the matrix compartment from 26B(iii). This works well for noninvasive epithelial cells.

ii. Homogenize the peeled epithelial sheet in lysis buffers for RNA and protein.

■ **PAUSE POINT** Alternatively, the tissue can be stored in − 80 °C for up to 1 year or until use.

D. Alternative processing for analyzing conditioned medium ● TIMING 10 min

i. At desired time, harvest medium from the bottom wells or inserts containing OTCs. Conditioned medium can be subjected to ELISA, western blotting or proteomics. If necessary, conditioned medium can be concentrated using a centrifugal filter unit.

E. Alternative use of OTCs for imaging of cells ● TIMING 3 h

i. Aspirate medium from inserts of OTCs.

ii. Add 5 ml of 4% (wt/vol) paraformaldehyde to the wells of OTC for 2 h.

iii. Wash twice with PBS for 5 min and add 2 mg ml^{-1} of DAPI after the second wash.

iv. Remove OTCs from the insert using a scalpel and place it on a 35-mm glass-bottom dish no. 1 with epithelium facing the bottom of the glass plate.

v. Immerse individual samples in PBS and place cover slips on the samples. Use a small weight on top of the cover slips to hold each sample as close to the bottom of the plate as possible without damaging the sample. A small glass object (e.g., tissue insert) would be suitable.

vi. Observe the samples and acquire image stacks of roughly 1–40 μm through the *z* dimension, at 1.0 mm apart, for each fluorescent channel using a Zeiss C-Apo 40× W 1.2 DIC lens, mounted on a inverted laser-scanning confocal microscope (LSM 410 equipped Axiovert 135). Note that 15–30 mm is the usual thickness of the epithelial layer.

vii. Use the following settings for emission (DAPI): 488-nm laser line of an Omnichrome argon/krypton ion laser for excitation and a 515- to 565-nm band-pass filter for the emission (FITC), as well as the 351- and 364-nm laser lines of a Coherent Enterprise argon ion laser for excitation and a 410- to 505-nm band-pass filter.

viii. Generate maximum intensity ‘through-focus’ projections of the image stacks by postprocessing, and apply contrast stretching uniformly across images from each channel.

ix. For acquisition, use the Zeiss LSM version 3.98 software, and for postacquisition 3D projections, use the Zeiss LSM Image Examiner version 2.8 software.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.

● **TIMING**

Step 1 Preparation and use of different cell lines for 3D OTCs: 1–1.5 weeks

Steps 2–12 Preparation of collagen matrices for OTC (OTC—day 1): 1.5–2 h

Steps 13 and 14 Dislodging the matrix (OTC—day 2): 10 min

Steps 15–22 Seeding epithelial cells from Step 1A or 1B (OTC—day 7): 4 h

Step 23 Medium change (OTC—day 9): 10 min

Step 24 Medium change (OTC—day 11) to create liquid-air interface: 10 min

Step 25 Medium change (OTC—day 13): 10 min

Step 26 Harvesting OTCs (day 15): 1.5–2 h (plus processing time)

ANTICIPATED RESULTS

Depending on the quality of the fibroblasts used, contraction of the matrix should be detected between days 2 and 4. The contracted matrix forms a concave surface with a depressed center and slightly rising walls that allow for seeding of keratinocytes on the surface of matrix. The matrix may contract further during the remaining cultivation. Examination of the H&E stained section should reveal a mature stratified epithelium on the

surface of the matrix with embedded fibroblasts (Fig. 4). The CK14-positive basal layer contains Ki-67–positive cells (Fig. 4c), on top of which should be progressively more flattening epithelial layers and a nuclear keratinized surface, positive for CK4 and CK13 (Fig. 4e,f). Mouse cell cultures have fewer layers than human cell-derived cultures (compare Fig. 4a and Fig. 5a). Epithelial maturation is impaired by DNMAML1, a genetic pan-Notch inhibitor (Fig. 5a), which affects epithelial formation and expression of differentiation markers such as involucrin (Fig. 5b). Transformed epithelial cells and ESCC cells form dysplastic epithelia and show invasive growth into the underlying collagen matrix compartment (Fig. 2 and Supplementary Fig. 1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge support from the US National Institutes of Health (NIH)/National Cancer Institute (NCI) P01-CA098101, NIH/National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) P30-DK050306 and NIH/NCI U01-CA143056. The Molecular Pathology and Imaging, Molecular Biology/Gene Expression, Cell Culture, Transgenic and Chimeric Mouse Core Facilities of the P30-DK050306 NIH/NIDDK Center for Molecular Studies in Digestive and Liver Diseases at the University of Pennsylvania support this work. We also thank the Penn Flow Cytometry and Microarray Array Core Facilities for supporting this work. Additional sources of support include the American Cancer Society Research Professorship (A.K.R.), NIH/NIDDK R01DK077005 (H.N.), NIH/NCI T32-CA115299 (G.W.) and NIH T32-CA009140-37 (M.E.V.). We thank S. Naganuma for the photomicrographs given in Supplementary Figure 1.

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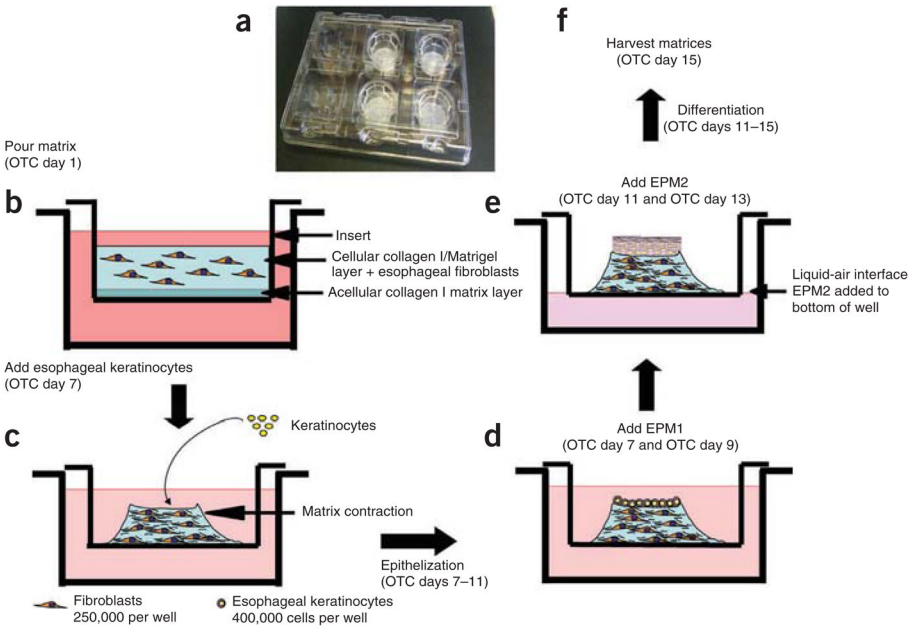

Figure 1.

Establishment of OTC on inserts placed on plates (organogenesis). (**a**) Inserts are placed on plates. (**b**) Initially, there is placement of an acellular collagen matrix on the bottom of an insert, followed by the addition of a layer of esophageal fibroblasts embedded in bovine collagen type I. These two layers are cultured initially for 7 d, thereby allowing for fibroblast-mediated constriction of the collagen matrix. (**c**) On day 7, the epithelial cells are seeded on the surface of the contracted matrix. (**d,e**) The medium of the OTC is changed every 2 d (**d**) and the epithelium is exposed to air to create a liquid-air interface (**e**), thereby promoting epithelial stratification and differentiation. (**f**) Finally, on day 15, the resulting OTC is harvested for histology and other applications such as RNA/protein analyses.

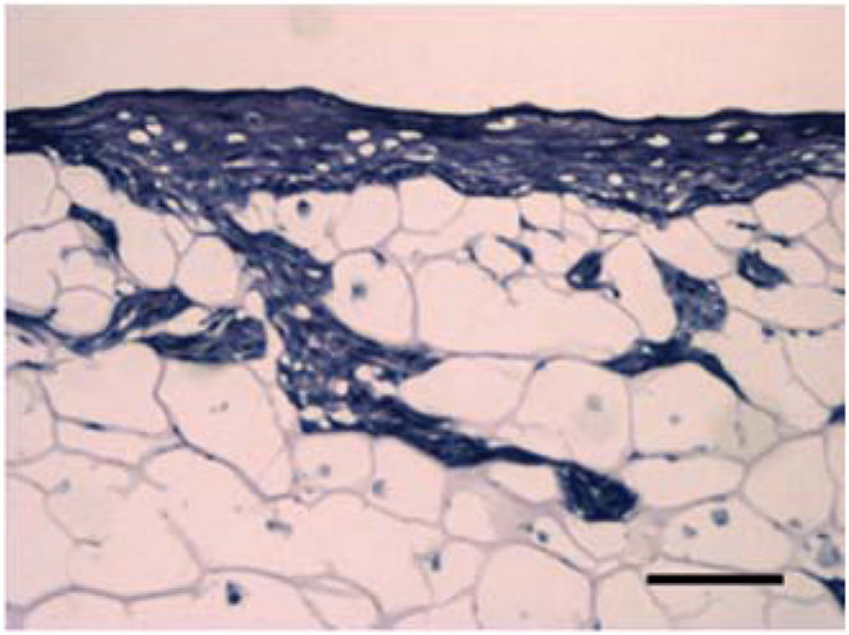

Figure 2.
The use of OTC for LCM. Specific cell populations (e.g., highlighted invading epithelial cells or regions of fibroblasts in the matrix) may be isolated using LCM from frozen sections. Scale bar, 50 μm.

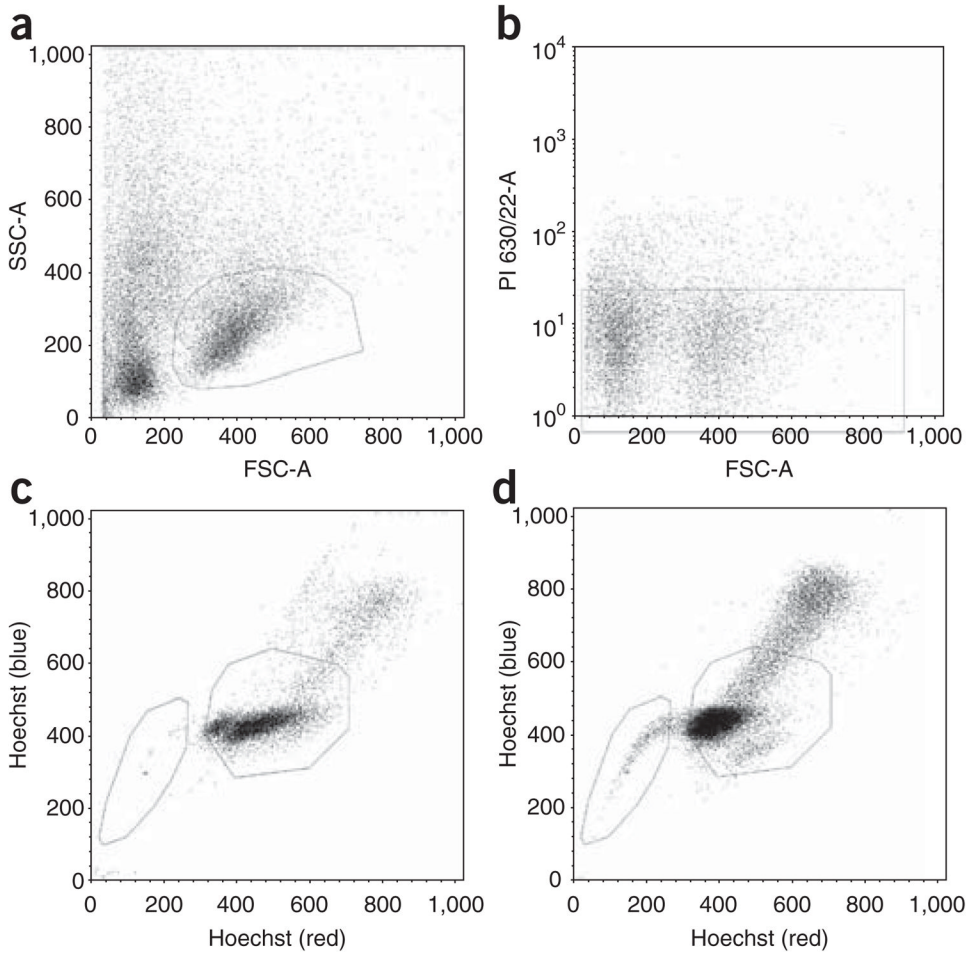

Figure 3.

Detection of side population of esophageal epithelial cells. (**a**) Forward (FSC-A) and side (SSC-A) scatter of primary esophageal epithelial cells. Single cells are gated (usually 200–400 FCS-A and 100–400 SSC-A). (**b**) Live cells gated as PI intensity below $10^{1.3}$ (PI was excited at 488 nm and its emission was measured in a logarithmic scale through a band-pass filter of 630/22A). (**c**) Verapamil-treated cells show diminished side population fraction. (**d**) Side population cells (tail of cells with Hoechst red below 300 and Hoechst blue below 400) and side population–negative cells (Hoechst red above 300, Hoechst blue over 400). Modified from ref. 23 with permission.

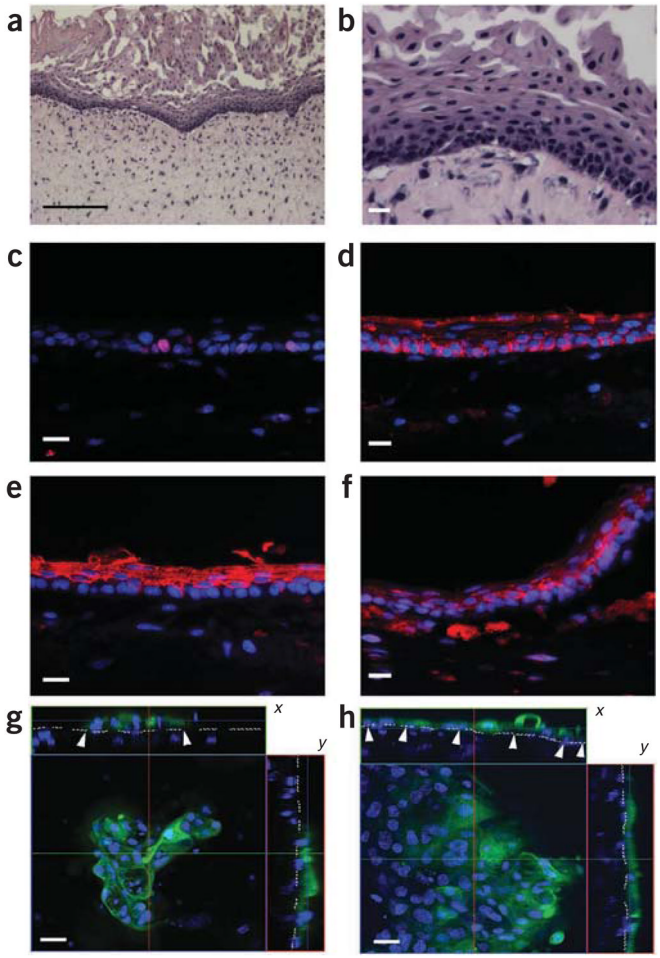

Figure 4.

Morphology of esophageal OTC derived from mouse cells and immunohistological detection of proliferation and differentiation markers. (**a**) SP cells (10^3) from GFP^+ mice mixed with 3×10^4 unsorted GFP^- cells form a stratified epithelium. (**b**) A ×400 magnification of the same figure as in **a**. (**c**–**f**) Immunohistochemical staining for Ki-67 (**c**), CK14 (**d**), CK4 (**e**) and CK13 (**f**). Original magnification, ×400. (**g**) Two-photon confocal microscopy on day 12 after seeding 10^3 NSP or transit-amplifying cells isolated from GFP^+ mice (green) with $DAPI^+$ nuclei (blue); top view, *z*-stack, 53.8 μm in 0.78-μm increments. (**h**) Two-photon confocal microscopy of the complete stratified epithelium on day 12 after seeding 10^3 SP cells isolated from GFP^+ mice (green) with $DAPI^+$ nuclei (blue); top view, *z*-stack, 60 μm in 1-μm increments. Arrowheads indicate basement membrane. Modified from ref. 23 with permission. Scale bars, 25 μm.

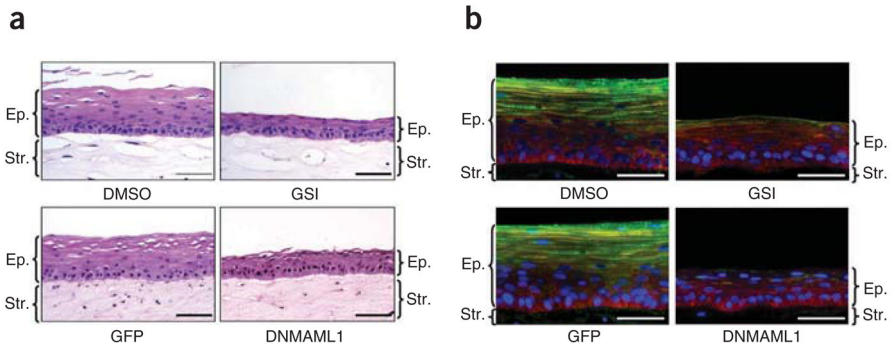

Figure 5.

Morphology of OTC derived from immortalized human keratinocytes (EPC2-hTERT). (**a,b**) Complete stratified epithelium with proliferative basal and differentiated suprabasal cells. Notch inhibition impairs squamous differentiation of esophageal epithelia reconstituted in 3D OTC. EPC2-hTERT cells were grown in the presence or absence of 1 μmol per liter compound E, a γ-secretase inhibitor (GSI) or retroviral DNMAML1 and subjected to histology. (**b**) Immunofluorescence for CK14 (red) and involucrin (green). Ep., epithelium; Str., stroma. Modified from ref. 22 with permission. Scale bars, 50 μm.

TABLE 1

Retroviral and lentiviral vectors validated for use in OTC.

Vector	Selection markers	Transgene expression	Reference
pBABE-puro	Puromycin	Constitutive	28
pBABE-hygro	Hygromycin B	Constitutive	
pBABE-zeo	Zeocin	Constitutive	28
pBABE-bla	Blasticidin S	Constitutive	28
PFBneo	G418	Constitutive	
pBSTR1	Puromycin	Inducible (tet-off)	28
MIGR1	GFP	Constitutive	29
pTRIPZ	Puromycin	Inducible shRNA (tet-on)	
pGIPZ	Puromycin	Constitutive shRNA	

All vectors listed are retroviral except pTRIPZ and pGIPZ, which are lentiviral vectors (Open Biosystems). pFBneo is commercially available (Stratagene). Tet-off/tet-on, tetracycline inducible. Virus production, infection and selection have been described previously[14,16,19,20,22,30].

TABLE 2

Antibodies validated in immunostaining of sections from OTC.

Protein	Primary antibody	Titer and other conditions	Company (cat. no.)	Recognizes	Isotype	Secondary antibody
CK14	Rabbit anti-CK14	(1:3,000) Microwave at pH 6.0	Covance (MK14 PRB155p)	Human, mouse	IgG	IHC, IF[a]
CK13	Mouse anti-CK13 clone 2D7	(1:250) Microwave at pH 6.0	Abcam (ab90096)	Human, mouse, rat	IgG2b	IHC, IF[b]
Ki-67	Rabbit anti-Ki67	(1:1,000) Microwave at pH 6.0	Novocastra/Leica (NCL-Ki67p)	Human	IgG	IHC, IF[a]
Involucrin	Rabbit anti-involucrin	(1:100) Microwave at pH 6.0	Covance (PRB 140C)	Mouse	IgG	IHC, IF[a]
Involucrin	Mouse anti-involucrin clone SY5	(1:750) Microwave at pH 6.0	Sigma-Aldrich (I9018)	Human	IgG1	IHC, IF[b]

IF, immunofluorescence; IHC, immunohistochemistry.

[a]IHC: biotinylated anti-rabbit IgG (H + L), Vector BA-1000, 1:200; IF–cy3 donkey anti-rabbit IgG (H + L), Jackson ImmunoResearch Lab. no. 711–165–152, 1:600.

[b]IHC: biotinylated anti-mouse IgG (H + L), rat absorbed, Vector BA-1000, 1:200; IF–cy3 donkey anti-mouse IgG (H + L) Jackson ImmunoResearch Lab. no. 711–165–150, 1:600.

TABLE 3

Troubleshooting table.

Step	Problem	Possible reason	Solution
14	Poor collagen matrix contraction	Fibroblast number	Optimize fibroblast numbers
		Fibroblast conditions in monolayer culture	Split fibroblasts 2 d before matrix preparation
		Contact inhibition	Use early passage primary fibroblasts
		Senescence	Use fibroblasts growing exponentially
		Viability	Confirm cell viability by trypan blue exclusion test
		Mycoplasma and other contaminations of fibroblasts	Redo fibroblast isolation
		Medium has too-high pH because of acidity in collagen solution	The acellular and cellular matrices should not be too yellowish. Add sodium bicarbonate until the solution turns orange or light pink
14	Inconsistent matrix contraction	Attachment of matrix to the insert	Release the side of matrix with a Pasteur pipette tip (see Step 13). Repeat Step 13 before seeding epithelial cells
		Inconsistent mixture of fibroblasts in the matrix	Mix thoroughly the matrix and fibroblasts before casting in the insert (but avoid air bubbles)
		Premature solidification of gel	Keep collagen, Matrigel and all other matrix components on ice. Minimize exposure to ambient temperature before casting the Transwell inserts
26A(ii)	Poor staining in immunohistochemistry	Fixation in Step 26A	Fixation for 1 h is sufficient for standard morphological analyses. Overnight fixation will not affect regular H&E staining. However, excessive fixation may affect the antigenicity of certain molecules for detection by immunohistochemistry based upon our experiences comparing short fixation (i.e., 1–2 h) versus overnight. Apparently, certain epitopes may be better detected with different fixatives and need to be optimized
26A(vii)	Inconsistent epithelial layer/poor epithelial stratification	Epithelial cell conditions in monolayer culture Overly confluent culture (>90–100%)	Use early passage primary keratinocytes Avoid high cell density as it induces terminal differentiation
		Senescence	Use cells growing exponentially and at earlier passages for primary keratinocytes
		Viability	Confirm cell viability by trypan blue exclusion test
		Terminal differentiation by high Ca^{2+} (>0.6 mM) concentrations	Regular DMEM in EPM1/2 contains 1.8 mM Ca^{2+}
			Use DMEM containing low Ca^{2+} DMEM
			Chelate Ca^{2+} in NBCS used in EMP1. Mix 10 g of Chelex 100 with 100 ml of NBCS, stir at 4 °C for 3 h, filter-sterilize, aliquot and store at –20 °C
	Poor invasion	Fibroblasts	Use FEF3 as a control
		Epithelial cells	Use TE12 as a control