Modeling Oral-Esophageal Squamous Cell Carcinoma in 3D Organoids

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Abstract

Esophageal squamous cell carcinoma (ESCC) is prevalent worldwide, accounting for 90% of all esophageal cancer cases each year, and is the deadliest of all human squamous cell carcinomas. Despite recent progress in defining the molecular changes accompanying ESCC initiation and development, patient prognosis remains poor. The functional annotation of these molecular changes is the necessary next step and requires models that both capture the molecular features of ESCC and can be readily and inexpensively manipulated for functional annotation. Mice treated with the tobacco smoke mimetic 4-nitroquinoline 1-oxide (4NQO) predictably form ESCC and esophageal preneoplasia. Of note, 4NQO lesions also arise in the oral cavity, most commonly in the tongue, as well as the forestomach, which all share the stratified squamous epithelium. However, these mice cannot be simply manipulated for functional hypothesis testing, as generating isogenic mouse models is time- and resource-intensive. Herein, we overcome this limitation by generating single cell-derived three-dimensional (3D) organoids from mice treated with 4NQO to characterize murine ESCC or preneoplastic cells *ex vivo*. These organoids capture the salient features of ESCC and esophageal preneoplasia, can be cheaply and quickly leveraged to form isogenic models, and can be utilized for syngeneic transplantation experiments. We demonstrate how to generate 3D organoids from normal, preneoplastic, and SCC murine esophageal tissue and maintain and cryopreserve these organoids. The applications of these versatile organoids are broad and include the utilization of genetically engineered mice and further characterization by flow cytometry or immunohistochemistry, the generation of isogeneic organoid lines using CRISPR technologies, and drug screening or syngeneic transplantation. We

believe that the widespread adoption of the techniques demonstrated in this protocol will accelerate progress in this field to combat the severe burden of ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is the deadliest of human squamous cell carcinomas, owing to its late diagnosis, therapy resistance, and metastasis[1,2]. ESCC arises from the stratified squamous epithelium, which lines the luminal surface of the esophagus. The squamous epithelium is comprised of proliferative basal cells and differentiated cells within the suprabasal cell layer. Under physiologic conditions, basal cells express markers such as p63, Sox2, and cytokeratin K5 and K14, while differentiated cells express K4, K13, and IVL. Basal cells themselves are heterogeneous and include putative stem cells defined by markers such as K15[3] and CD73[4]. In homeostasis, basal cells undergo post-mitotic terminal differentiation within the suprabasal cell layer, whereas differentiated cells migrate and desquamate into the lumen to complete epithelial renewal. Reminiscent of their cells of origin, ESCC displays squamous cell differentiation to varying degrees. ESCC is often accompanied by multifocal histologic precursor lesions, known as intraepithelial neoplasia (IEN) or dysplasia, comprising atypical basaloid cells. In addition to epithelial changes, ESCC displays tissue remodeling within the subepithelial compartment, where the activation of cancer-associated fibroblasts (CAFs) and the recruitment of immune/inflammatory cells take place to foster the tumor-promoting microenvironment.

The pathogenesis of ESCC involves genetic changes and exposure to environmental risk factors. Key genetic lesions include the inactivation of the tumor suppressor genes *TP53* and *CDKN2A* (p16INK4A) and the activation of the

CCND1 (cyclin D1) and *EGFR* oncogenes, which culminate in impaired cell cycle checkpoint function, aberrant proliferation, and survival under genotoxic stress related to exposure to environmental carcinogens. Indeed, genetic changes interact closely with behavioral and environmental risk factors, most commonly tobacco and alcohol use. Tobacco smoke contains human carcinogens such as acetaldehyde, which is also the major metabolite of alcohol. Acetaldehyde induces DNA adducts and interstrand DNA crosslinks, leading to DNA damage and the accumulation of DNA mutations and chromosomal instability. Given excessive mitogenic stimuli and aberrant proliferation from oncogene activation, the malignant transformation of esophageal epithelial cells is facilitated by mechanisms to cope with genotoxic stress, including the activation of antioxidants, autophagy, and epithelial-mesenchymal transition (EMT). Interestingly, these cytoprotective functions are often activated in ESCC cancer stem cells (CSCs) that are characterized by high CD44 (CD44H) expression and have the capabilities of tumor initiation, invasion, metastasis, and therapy resistance[5,6,7].

ESCC has been modeled in cell culture and in rodent models[8,9]. In the last three decades, robust genetically engineered mouse models of ESCC have been developed. These include *CCND1* and *EGFR* transgenic mice[10,11] and *p53* and *p120Ctn* knockout mice[12,13]. However, single genetic changes do not typically result in rapid-onset ESCC. This challenge has been overcome with the use of esophageal carcinogens that recapitulate well the human genetic lesions in ESCC[14]. For example, 4-nitroquinoline-1-

oxide (4NQO) accelerates ESCC development in *CCND1* transgenic mice[15]. In recent years, putative esophageal epithelial stem cells, progenitor cells, and their respective fates have been investigated in cell lineage-traceable mouse models[3,4]. Furthermore, these cell lineage-traceable mice have been utilized to explore the cells of origin of ESCC and how such cells give rise to CD44H CSCs via conventional histology and omics-based molecular characterization[7].

One emerging area related to these mouse models is the novel application of cell culture techniques to analyze live ESCC and precursor cells in a three-dimensional (3D) organoid system in which the architecture of the original tissues is recapitulated *ex vivo*[7,8,9]. These 3D organoids are rapidly grown from a single-cell suspension isolated from murine tissues, including primary and metastatic tumors (e.g., lymph node, lung, and liver lesions). The cells are embedded in basement membrane extract (BME) and fed with a well-defined serum-free cell culture medium. The 3D organoids grow within 7-10 days, and the resulting spherical structures are amenable for subculture, cryopreservation, and assays for analyzing a variety of cellular properties and functions, including CSC markers, EMT, autophagy, proliferation, differentiation, and apoptotic cell death.

These methods can be broadly applied to 3D organoid cultures established from any stratified squamous epithelial tissue, such as the head and neck mucosa (oral cavity, tongue, pharynx, and larynx) and even the forestomach. The head and neck mucosa are contiguous with the esophagus, and the two tissues share similar tissue organization, function, and susceptibility to disease. Both head and neck squamous cell carcinoma (HNSCC) and ESCC share genetic lesions and lifestyle-related environmental risk factors such as tobacco and alcohol exposure. Underscoring this similarity,

mice treated with the tobacco smoke mimetic 4NQO readily develop both HNSCC and ESCC. Given the ease with which the protocols described below can be applied to modeling HNSCC, we include specific instructions for establishing 3D organoid cultures from these lesions.

Herein, we provide detailed protocols for generating murine esophageal 3D organoids (MEOs) representing normal, preneoplastic, and ESCC lesions that develop in mice treated with 4NQO. Various mouse strains can be utilized, including common laboratory strains such as C57BL/6 and cell lineage-traceable and other genetically engineered derivatives. We emphasize the key steps, including the isolation of normal or diseased murine esophageal epithelium, the preparation of single-cell suspensions, the cultivation and monitoring of the growing 3D organoids, subculture, cryopreservation, and the processing for subsequent analyses, including morphology and other applications.

Protocol

The murine experiments were planned and performed in accordance with regulations and under animal protocol #AABB1502, reviewed and approved by Columbia University's Institutional Animal Care and Use Committee. The mice were housed at a proper animal care facility that ensures the humane treatment of mice and provides appropriate veterinary care for the mice and laboratory safety training for the laboratory personnel.

1. Treatment of mice with 4NQO to induce esophageal IEN and ESCC lesions (time consideration: up to 28 weeks)

NOTE: To generate MEOs representing neoplastic esophageal lesions, the mice are subjected to 4NQO-mediated chemical carcinogenesis as previously described

by Tang et al.[14] . Normal/non-neoplastic MEOs are generated from untreated mice.

1. Mice

   1. House four to five mice per cage, and acclimate them to the animal facility for at least 1 week before starting an experiment. To reduce the possibility that age-related disorders result in premature termination of the full 28 week experiment, start with 8 week old to 16 week old mice.
   NOTE: C57BL/6 mice weighing approximately 20-30 g were utilized in this protocol. For shorter experiments, older mice may be used. Male or female mice are acceptable. Control (no treatment, see section 1.3.1) mice must be matched in age and sex.

2. Preparation of drinking water containing 4NQO

   1. Prepare 1 mg/mL 4NQO stock solution in ethylene propylene glycol (**Table of Materials**). Dissolve 100 mg of 4NQO into 100 mL of 99.9% ethylene propylene glycol in a 500 mL glass beaker covered with sealing film. Mix thoroughly at room temperature (RT) using a magnetic stirrer at 800 rpm for 30 min. Store at 4 °C.

   2. Add 900 mL of autoclaved deionized water to 100 mL of 1 mg/mL 4NQO stock solution, and mix by inversion in a 2 L plastic graduated cylinder covered with sealing film. A volume of 1 L of 100 µg/mL 4NQO in 10% ethylene propylene glycol will serve two mouse cages equipped with a 500 mL drinking bottle.
   CAUTION: Of note, 4NQO is a synthetic chemical carcinogen that may cause cancer. Handle with nitrile gloves and a long-sleeved lab coat, and wear

closed-toe shoes. Consider proper eye protection, face protection, and head covering. For waste disposal, 4NQO should be placed in a labeled container in accordance with institutional guidelines for hazardous waste management by environmental health and safety.

3. Treatment with 4NQO and monitoring

   1. Attach the drinking bottle, and administer 4NQO via the drinking water *ad libitum* to the mice for 16 weeks. Use 10% (w/v) propylene glycol as a vehicle (no treatment) control.
   NOTE: Shorter durations of 4NQO treatment may be used to induce IEN.

   2. Refill the water once per week.

   3. Weigh each mouse weekly by placing it in a plastic container on a laboratory balance.

   4. At the end of the 16 week 4NQO treatment period, start giving the mice regular drinking water during the post-4NQO observation period for up to 12 weeks (**Figure 1**).

   5. Monitor the mice daily for signs of distress (e.g., impaired mobility, hunched habitus, and withdrawn behavior), dysphagia, and dehydration. Additionally, evaluate the mice weekly for changes in body weight or food and liquid intake. Should the body weight drop by more than 10% from the initial body weight, feed the mice with a liquid dietary supplement.
   NOTE: A loss of body weight refractory to liquid dietary supplements may be indicative of ESCC, and mice that lose more than 20% of their body weight should be euthanized. Importantly, MEOs can be generated from prematurely euthanized mice. Note that C57BL/6 mice without genetic modifications

do not typically display signs of morbidity or have visible ESCC lesions until the end of the post-4NQO observation period.

4. Animal preparation

   1. Euthanize the mice in a CO_2 chamber filled with CO_2 at a flow rate that displaces 30%-70% of the chamber volume per minute. Confirm death by cervical dislocation.

   2. Pin the limbs and nose of the mouse in the supine position to the dissection platform using 21 G needles.

   3. Disinfect the ventral surface of the mouse with 70% ethanol.

5. Dissection (time consideration: 0.5 h)

   1. Open the skin by pinching the midabdominal fur and skin to ensure it is released from the viscera below. Use the surgical scissors to make a craniocaudal, ventral midline incision from the lower abdomen to the chin.

   2. Starting at the midline incision, use surgical scissors to make radial cuts extending to the limbs on both sides of the mouse. Flay the skin flaps open.

   3. To expose the cervical trachea, use the dissecting scissors to divide the salivary glands at the midline. The trachea lies deep in the glands.

   4. To expose the thoracic trachea, remove the sternum.

      1. Gently pinch and lift the peritoneum with forceps, and use scissors to divide the peritoneum craniocaudally and laterally along the rib cage.

      2. Gently retract the liver from the caudal surface of the diaphragm, and use scissors to make a small incision in the diaphragm at the sternal notch, specifically at the dorsal surface of the xiphoid process. This releases the lung and heart from the visceral pleura.

      3. Separate the ribcage from the thoracic contents. Insert scissors into the incision in the diaphragm, and dissect cranially to the cervical girdle. During this dissection, adhere closely to the dorsal surface of the sternum to avoid damage to the organs below. Ensure that the plane of dissection is anterior to the trachea.

      4. Cut the ribs on either side of the sternum using scissors, and remove the sternum. Ensure that the thoracic contents are exposed.

5. Expose the abdominal esophagus. Gently lift the stomach anteriorly by holding the antrum with forceps. Dissect the spleen, pancreas, and mesentery off the stomach and esophagus with scissors.

6. Expose the thoracic esophagus (**Figure 2**).

   1. Gently lift the trachea immediately caudal to the thyroid cartilage, and dissect the esophagus of the dorsal side of the trachea using iris scissors.

   2. Divide the trachea at the thyroid cartilage with iris scissors.

   3. Peel the trachea off the remainder of the esophagus via careful dissection in the caudal direction.

   4. Remove the lung, heart, and thymus *en masse* with the trachea. Take care to avoid damage to

the esophagus when dissecting and dividing the aorta and vena cava.

7. Divide the stomach at the pylorus with scissors.

8. Separate the esophagus from the vertebra by holding the antrum with forceps and dissecting cranially.

9. Divide the esophagus at the level of the thyroid cartilage, and harvest the esophagus and stomach *en masse* (**Figure 3**).

10. Separate the stomach and esophagus by dividing the esophagus at the cardia (**Figure 4**, top panel, red line).

11. Dissect off any fascia on the outer surface of the esophagus, and split longitudinally with scissors. Place the esophagus in cold PBS on ice.

12. Open the stomach along the greater curvature, and wash with PBS sufficiently. Separate the forestomach, and wash with cold PBS. Place the forestomach in cold PBS on ice.

13. To harvest the tongue, remove the 21 G needle on the nose, and pull out the tongue with tweezers. Cut the tongue as long as possible. Place the tongue in cold PBS on ice.

2. Establishment of murine esophageal organoid (MEO) culture

NOTE: This protocol can also be used to establish a murine tongue organoid culture with the addition of a step in which the tongue tissue is minced before trypsinization. See the note in step 2.2.3.

1. Preparation of reagents

NOTE: A list of reagents may be found in the **Table of Materials**. Prepare and store the stock solutions according to the manufacturer's instructions unless otherwise indicated.

1. Ensure that the single-use aliquots of the basement membrane matrix (BME) utilized in this protocol are stored at −20 °C until the day of use, are subsequently thawed on ice or at 2-8 °C, and are kept on ice at all times when not in use.

2. Dissolve 250 mg of soybean trypsin inhibitor (STI) in 1,000 mL of PBS (250 mg/mL stock concentration), and filter-sterilize (0.22 μm) it. Dispense 50 mL aliquots into conical tubes, and store for up to 6 months at 4 °C.

3. Prepare the mouse organoid medium (MOM): Supplement advanced DMEM/F12 with 1 mM N-acetyl-L-cysteine (NAC), 2% R-Spondin and Noggin conditioned medium (RN CM), 1x N-2 supplement, 1x B-27 supplement, 10 mM HEPES, 1x antibiotic-antimycotic, 1x GlutaMAX supplement, and 100 ng/mL mouse epidermal growth factor (mEGF). Prepare the MOM 500 mL at a time, divide it into 50 mL aliquots, and store at 2-8 °C until ready to use. Add 0.5 μg/mL amphotericin B and 10 μM Y-27632 just prior to use.

4. Prewarm the MOM, 0.25% trypsin, and soybean trypsin inhibitor (STI) to 37 °C in a water or bead bath before use.

2. Isolation of keratinocytes from the dissected mouse tissue (time consideration: 2 h)

1. Transfer the esophageal tissue into 500 μL of dispase in PBS (2.5-5 units total), and incubate in a thermomixer for 10 min at 37 °C and 800 rpm.

2. Transfer the tissue to a culture dish, and carefully remove the muscle layer from the epithelium using forceps (**Figure 4**).

   NOTE: This step can also be performed by an experienced researcherprior to incubation with dispase.

3. Transfer the epithelium to a microcentrifuge tube containing 500 µL of 0.25% trypsin, and incubate in a thermomixer for 10 min at 37 °C and 800 rpm.

   NOTE: If the starting material is tongue tissue, mince the tissue with a sterile scalpel into smaller pieces, approximately 1-2 mm^2 in size, before adding the trypsin.

4. Briefly centrifuge for 5-10 s at 2,000 x *g* to pellet the tissue. Prepare a 50 mL conical tube with a 100 µm cell strainer. Transfer the tissue/cell suspension through the strainer with a wide bore tip using circular motions.

5. Add 3 mL of STI through the strainer, using circular motions to wash.

6. Scrub the strainer with the base of a 1 mL tuberculin syringe plunger to push the cells through.

7. Wash the strainer with 3 mL of PBS 3-5 times, scrubbing the strainer with the base of the syringe between washes.

8. Centrifuge the tube at 300 x *g* for 10 min at 4 °C to pellet the cells.

9. Remove the supernatant, leaving 1 mL of solution in the tube.

10. Resuspend the pellet in the remaining 1 mL, and transfer the cell suspension through a 70 µm cell strainer into a new 50 mL conical tube.

11. Centrifuge the tube at 300 x *g* for 10 min at 4 °C to pellet the cells.

12. Resuspend the pellet in 100 µL of MOM; adjust the volume as needed. Perform an automated cell count by trypan blue exclusion.

3. Seeding of the initial cell suspension (time consideration: <1 h)

   1. Prewarm a 24-well cell culture plate in a 37 °C incubator.

   2. Plate 5,000 viable cells in 75% (v/v) BME/MOM with 50 µL total volume per well. Maximize the number of wells plated and prepare enough cells in BME for one extra well according to the example calculations below.

      NOTE: Cryopreserve any excess cells in the cryopreservation medium (10% DMSO in FBS) at a maximum concentration of 1 x 10^6 cells/mL. Store the cryovials in a freezing container overnight at −80 °C. Transfer them to vapor phase liquid nitrogen for long-term storage.

   3. In a microcentrifuge tube, prepare an appropriate cell dilution in MOM first, and then add BME using a wide bore tip just before plating.

   4. Using a 200 µL wide bore tip, slowly add a 50 µL droplet to the center of the well, avoiding contact between the tip and the bottom or sides of the well (**Figure 5**). Be careful not to use too much force to expel the liquid from the tip, or the dome will flatten.

   5. Allow the BME to solidify for 30 min in a 37 °C, 5% CO_2, 95% relative humidity (RH) incubator.

   6. Carefully add 500 µL of MOM per well supplemented with 0.5 µg/mL amphotericin B and 10 µM Y-27632.

NOTE: Add amphotericin to all the MOM throughout the initial primary culture. Add Y-27632 only on the day of passaging (day 0) for all passages.

7. Change the MOM on days 3-4 and then every 2-3 days after that until ready to passage.

8. On days 7-10, image the organoids, and measure the organoid formation rate (OFR) by dividing the number of organoids formed by the number of cells initially seeded.
   Example calculations:

$$6 \text{ wells} + 1 \text{ extra well} = 7 \text{ wells} \times 5{,}000 \frac{cells}{well} = 35{,}000 \text{ cells total}$$

$$350 \text{ μL total volume (for 7 wells)} \div 4 = 87.5 \text{ μL of cells in BCM} + 262.5 \text{ μL of BME}$$

$$35{,}000 \text{ cells total} \div \text{live cell count in} \frac{cells}{mL} = \text{volume cell suspension needed}$$

$$(100 \text{ organoids formed} \div 5000 \text{ single cells plated}) \times 100 = 2 \text{ (OFR)}$$

4. Passaging and cryopreservation of murine esophageal organoids (MEOs) (time consideration: <1.5 h)

   1. Thaw and keep the BME on ice. Prewarm MOM, 0.05% trypsin, and STI to 37 °C in a water or bead bath before use. Prewarm a 24-well cell culture plate in a 37 °C incubator.

   2. Using a wide bore micropipette tip, collect the organoids in the BME dome along with the supernatant. Disrupt the BME by pipetting up and down.
      NOTE: Combine the wells containing identical samples into a single microcentrifuge tube.

   3. Briefly centrifuge for 10-15 s at 2,000 x g to pellet the organoids. Remove and discard the supernatant.

   4. Gently dislodge the pellet, and resuspend the pellet in 500 μL of 0.05% trypsin.

5. Incubate the tube(s) in a thermomixer at 37 °C and 800 rpm for 10 min.

6. Inactivate the trypsin with 600 μL of STI.

7. Centrifuge the tube at 300 x g for 5 min at 4 °C to pellet the cells.

8. Remove and discard the supernatant. Resuspend the cell pellet in 100 μL of MOM. Perform an automated cell count by trypan blue exclusion.
   NOTE: The volume may be adjusted as needed.

9. Plate 2,000-5,000 viable cells in 75% (v/v) BME/MOM with 50 μL total volume per well. Maximize the number of wells plated and prepare enough cells in BME for one extra well according to the example calculations mentioned previously.
   NOTE: Cryopreserve any excess cells in the cryopreservation medium (10% DMSO in FBS) at a maximum concentration of 1 x 10^6 cells/mL. Store the cryovials in a freezing container overnight at −80 °C. Transfer them to vapor phase liquid nitrogen for long-term storage.

10. In a microcentrifuge tube, prepare an appropriate cell dilution in MOM first, and then add BME using a wide bore tip just before plating.

11. Using a 200 μL wide bore tip, slowly add a 50 μL droplet to the center of the well, avoiding contact between the tip and the bottom or sides of the well. Be careful not to use too much force to expel the liquid from the tip, or the dome will flatten.

12. Incubate the plate for 30 min in a 37 °C, 5% CO_2, 95% RH incubator.

13. Carefully add 500 μL of MOM per well supplemented with 10 μM Y-27632.

NOTE: Add Y-27632 only on the day of passaging (day 0). It is unnecessary to add it during the media changes. Adding amphotericin B is no longer necessary.

14. Change the MOM on days 3-4 and then every 2-3 days after that until ready to passage.

15. On days 7-10, image the organoids, and measure the OFR.

5. Thawing and recovery of the murine esophageal organoids (MEOs) (time consideration: <1 h)

1. Thaw and keep the BME on ice. Prewarm a 24-well cell culture plate in a 37 °C incubator.

2. Prepare 10 mL of cold or RT MOM or PBS in a 15 mL conical tube.

3. Thaw a cryovial in a 37 °C water bath or bead bath for approximately 30 s to 1 min or until a small ice pellet remains.

4. With a pre-wetted pipet tip, slowly transfer the cell suspension to the tube containing MOM or PBS in a dropwise manner.

5. Centrifuge the tube for 300 x g and 4 °C for 5 min to pellet the cells.

6. Remove and discard the supernatant. Resuspend the cell pellet in 100 µL of MOM; adjust the volume as needed. Perform an automated cell count by trypan blue exclusion.

7. Plate 5,000-10,000 viable cells in 75% (v/v) BME/ MOM with 50 µL total volume per well. Maximize the number of wells plated and prepare enough cells in BME for one extra well according to example calculations mentioned previously.

8. Continue with the remaining steps of the protocol for the passaging and cryopreservation of murine esophageal organoids (MEO) (see step 2.4.11).

3. Preparation of organoids for paraffin embedding (time consideration: <1 h [plus 1.5 h for reagent preparation])

1. Using a wide-bore micropipette tip, collect three wells per microcentrifuge tube. Collect the organoids in the BME dome along with the supernatant. Disrupt the BME by pipetting up and down.

2. Briefly centrifuge for 10-15 s at 2,000 x g to pellet the organoids. Remove and discard the supernatant.

3. Gently dislodge the pellet, and resuspend the pellet in 300 µL of 4% paraformaldehyde (PFA).

4. Fix the organoids overnight at 4 °C.

5. Briefly centrifuge for 10-15 s at 2,000 x g to pellet the organoids. Remove and discard as much PFA as possible.

6. Gently dislodge the pellet, and resuspend the pellet in 500 µL of PBS.
   NOTE: Fixed organoids can be stored at 4 °C for up to 2 weeks before proceeding to the next step.

7. Prepare a 50 mL stock of agar gel (2% agar plus 2.5% gelatin).
   NOTE: Prepare the agar gel stock in advance, due to the incubation time, followed by carrying out the autoclave cycle.

   1. Resuspend 1 g of Bacto-agar and 1.25 g of gelatin in 50 mL of water in a 150 mL autoclavable glass beaker.

   2. Swirl the suspension, and let it sit for 30-60 min at RT.

3. Autoclave for 20 min at 121 °C.

4. Cool slightly and dispense 5 mL aliquots in 15 mL conical tubes.

5. Store for up to 6 months at RT.

8. Prepare an embedding surface by inverting a microcentrifuge tube rack and covering the surface with a sheet of sealing film. Label with the corresponding organoid ID(s).

9. Centrifuge the tube at 300 x *g* for 5 min to pellet the organoids. Remove and discard the supernatant.

10. Meanwhile, liquefy the agar gel by placing a 15 mL conical tube containing the agar gel into a 150 mL glass beaker containing 100 mL of water and microwaving on the highest power setting for 1-2 min or until the water starts to boil and the agar gel is in a liquid state.
CAUTION: Loosen the cap of the conical tube containing the agar gel before microwaving.

11. Partially submerge the microcentrifuge tube containing the organoid pellet into the warm water without introducing any water into the microcentrifuge tube.

12. Carefully overlay the organoid pellet by adding 50 μL of agar down the side of the tube.

13. Without disturbing the pellet (do not resuspend, keep the pellet intact), transfer the pellet in the agar gel droplet to the sealing film on the embedding surface.

14. Repeat step 3.12 and step 3.13 with an additional 50 μL of liquid agar gel to collect any remaining organoid pellet, and carefully add to the same gel droplet.

15. Incubate the droplet containing the organoid pellet for 45 min at 4 °C.

16. Using forceps, carefully transfer the droplet containing the organoid pellet to a labeled pathology cassette.

17. Store the cassette in 70% ethanol at 4 °C for up to 1 month.

18. Proceed with paraffin embedding via routine histological processing to prepare paraffin blocks.

Representative Results

This protocol describes the process of generating murine esophageal organoids (MEOs) from normal esophageal tissue or ESCC tumor tissue from 4NQO-treated mice according to a specific treatment regimen consisting of 16 weeks of 4NQO administered in drinking water, followed by a 10 week to 12 week observation period (**Figure 1**). The mice are then euthanized for the dissection of the tongue or esophageal tissue (**Figure 2** and **Figure 3**). We describe a method for the isolation of the epithelial layer from the intact esophagus (**Figure 4**) to be utilized for subsequent single-cell isolation. Esophagus-derived epithelial cells are initially plated at 5,000 cells per well in 50 μL droplets containing cells in a basement membrane matrix and well-characterized serum-free cell culture medium (**Figure 5**) and allowed to form organoids over the course of 7-10 days on average. Murine esophageal, tongue, and forestomach organoids can be further characterized by morphological analysis by phase-contrast/brightfield imaging and histopathology (**Figure 6**). The self-renewal capacity of organoids can be assessed by determining the OFR upon subculture (**Figure 7**). The OFR is largely influenced by the content of the proliferative basaloid cells, as revealed by the growth kinetics analysis coupled with their morphology (**Figure 8**). Finally, these organoids, including normal structures from 4NQO-untreated mice, grow

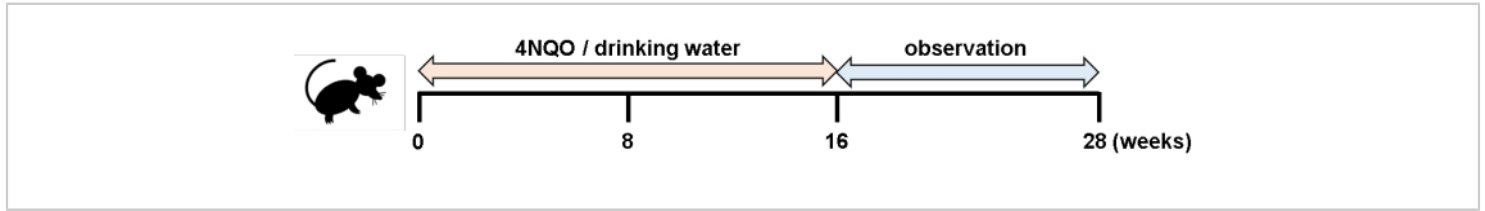continuously and can be maintained for a long time (**Figure 9**).

Figure 1: The 4NQO treatment regimen. The mice are treated with 4NQO in drinking water for 16 weeks, followed by a 10 week to 12 week observation period. Please click here to view a larger version of this figure.

Figure 2: Exposing the thoracic esophagus. The trachea (blue lines) is peeled away from the esophagus (white lines). Please click here to view a larger version of this figure.
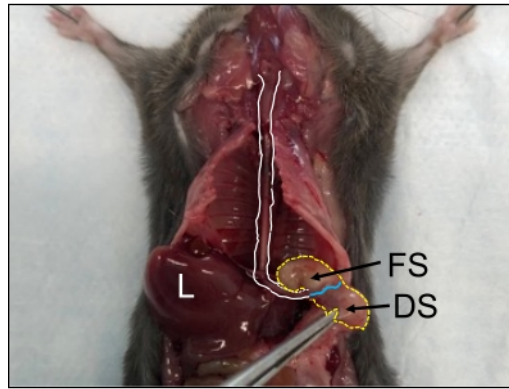
Figure 3: Esophagus (white lines) connected to the stomach (yellow lines) at the squamous columnar junction (blue line). Abbreviations: FS = forestomach, DS = distal stomach, L = liver. Please click here to view a larger version of this figure.
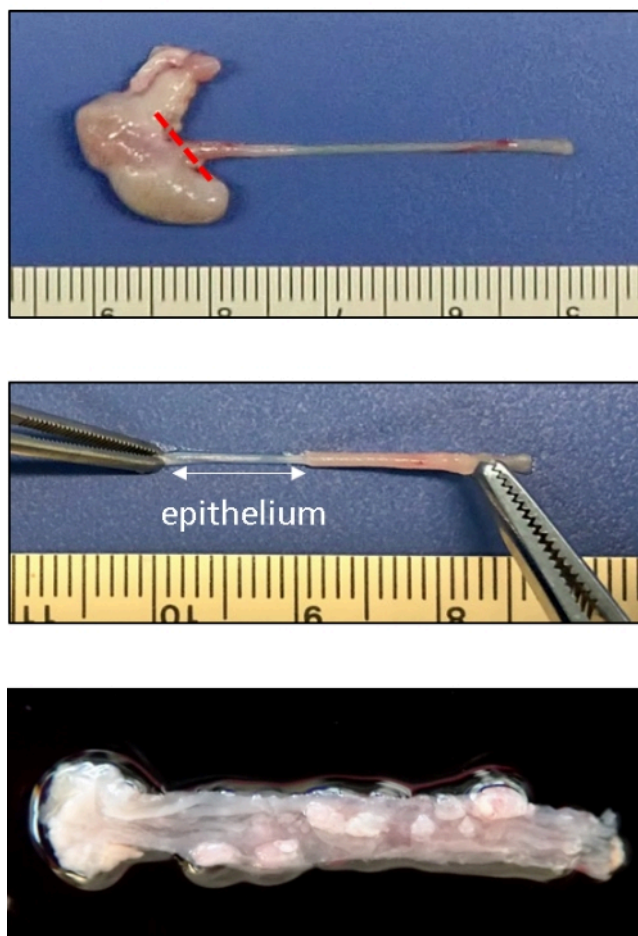
Figure 4: Separating the stomach and esophagus and isolating the epithelium. Upper: The stomach is separated from the esophagus. The red line indicates where the esophagus should be detached during dissection. Middle: The epithelium (white line) is peeled away from the muscle layer. Lower: The esophagus bearing tumors following the treatment schedule described in **Figure 1**. Please click here to view a larger version of this figure.
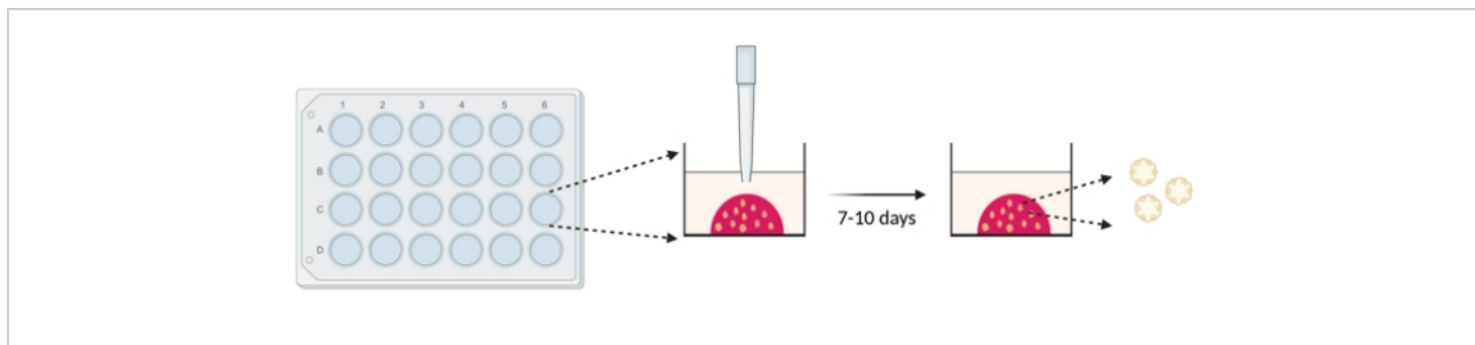
Figure 5: Plating the BME droplet containing single cells using a wide bore tip. The organoids begin to form around days 4-7 and are ready to be passaged after 7-10 days on average. Created with BioRender.com. Please click here to view a larger version of this figure.
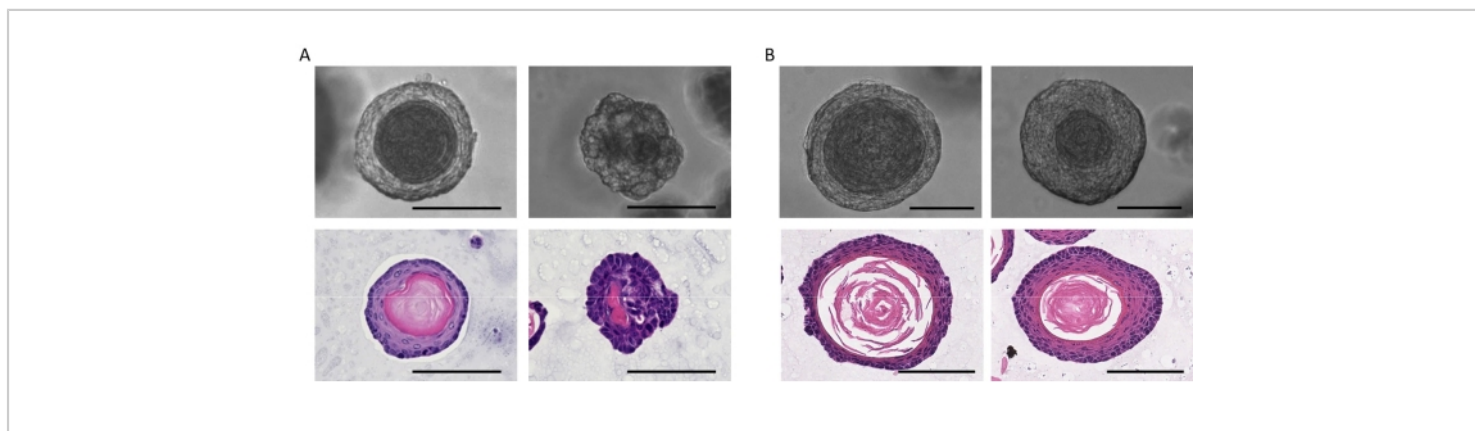
Figure 6: Characterizing the organoids by morphological analysis. (**A**) Representative images of normal and ESCC MEOs. Upper left: Brightfield image of a normal MEO. Lower left: H&E staining of a normal MEO. Upper right: Brightfield image of an ESCC MEO from a 4NQO-treated mouse. Lower right: H&E staining of an ESCC MEO from a 4NQO-treated mouse displaying nuclear atypia and abrupt keratinization, the latter being reminiscent of a keratin pearl, representing a well-differentiated compartment within an SCC tumor. (**B**) Representative images of normal mouse tongue and forestomach organoids (MTO and MFO, respectively). Upper left: Brightfield image of a normal MTO. Lower left: H&E staining of a normal MTO. Upper right: Brightfield image of a normal MFO. Lower right: H&E staining of a normal MFO. All scale bars = 100 μm. Please click here to view a larger version of this figure.
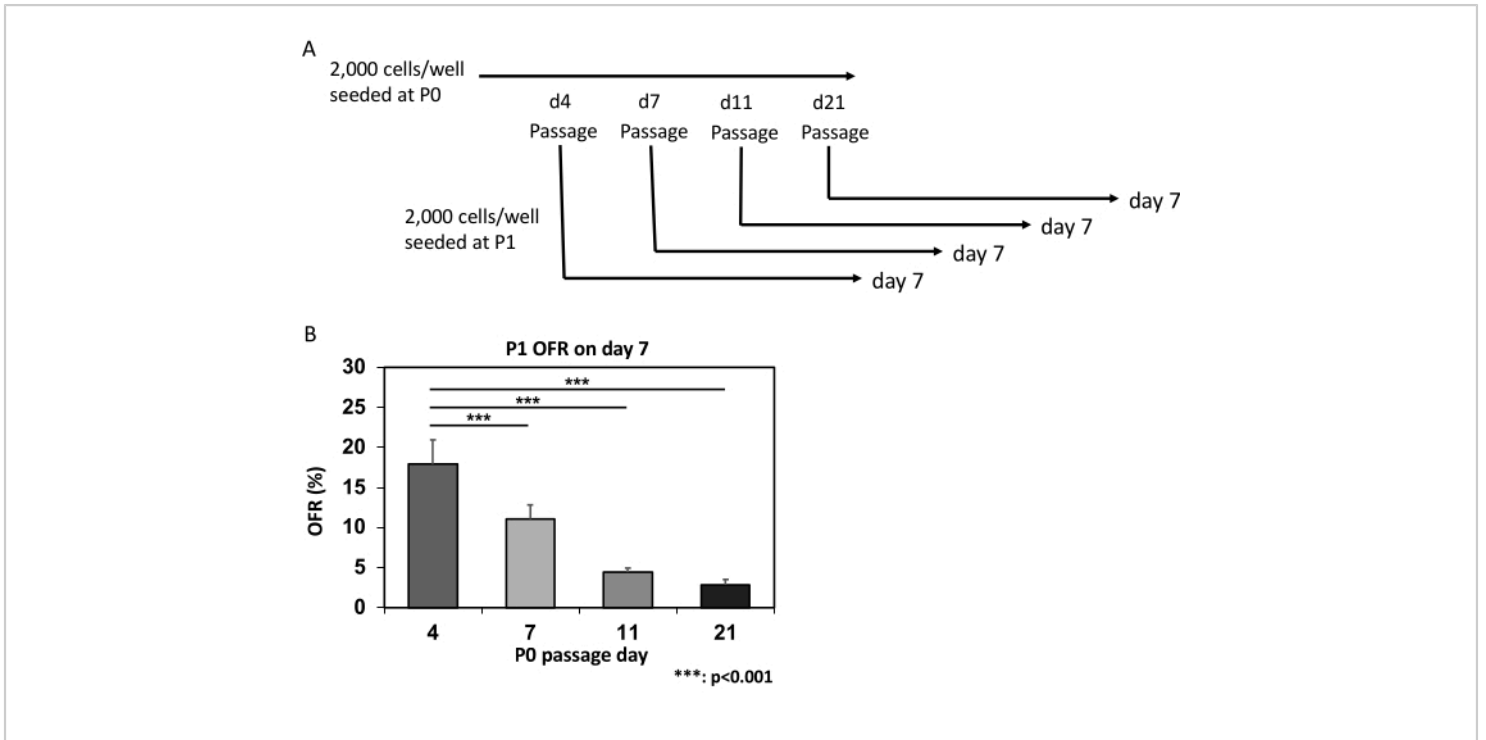
Figure 7: Determining organoid renewal capacity. (**A**) Experimental design to determine the organoid renewal capacity by subculture. The growing primary organoids (P0) are dissociated at various time points and made into single-cell suspensions. These cells are passaged to determine the OFR at day 7 in the subculture (P1). (**B**) Representative OFR data from passaged MEOs generated from 4NQO-untreated mice. Note that the OFR decreases as a function of time, reflecting decreased proliferative basaloid cells in the matured organoids, which contain more cells that have undergone post-mitotic terminal differentiation (see **Figure 8**). *** $p < 0.001$ (n = 6) OFR at day 7, day 11, and day 21 versus OFR at day 4. Please click here to view a larger version of this figure.
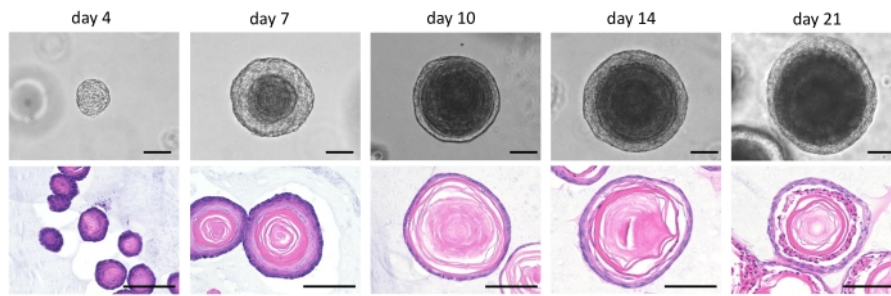
Figure 8: Growth kinetics of organoids as revealed by their morphology at various time points. Representative bright-field and H&E staining images of a normal MEO from 4NQO-untreated mice are shown. Note that the keratinization of the inner core of organoids becomes prominent by day 10. The proliferative basaloid cells remain in the outermost cell layer even at the day 14 and day 21 time points. For immunohistochemistry/immunofluorescence, accumulated keratin may cause non-specific staining, as is the case for original squamous epithelial tissues, warranting a careful optimization of the staining conditions, controls (e.g., secondary antibody only), and data interpretation. All scale bars = 100 μm. Please click here to view a larger version of this figure.
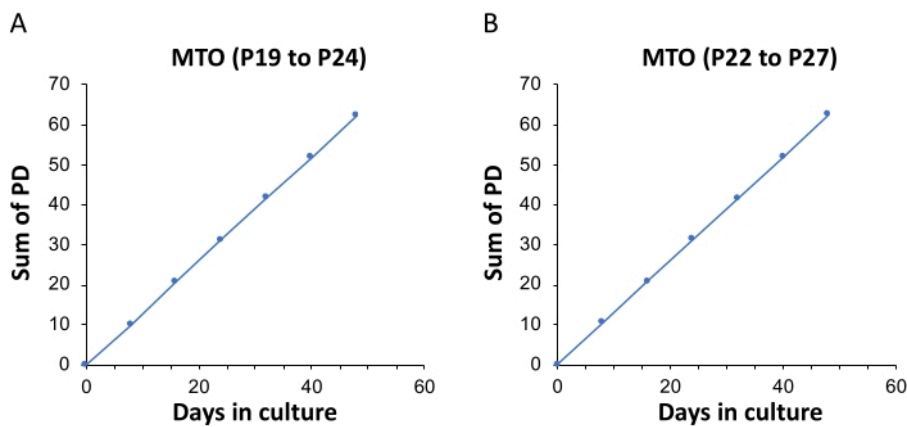

Figure 9: A population doubling curve of a representative normal MTO generated from 4NQO-untreated mice. These organoids were previously generated, cryopreserved, and thawed to demonstrate their steady growth over multiple passages and long-term culture. Please click here to view a larger version of this figure.

Discussion

There are several critical steps and considerations for the generation and analysis of MEOs in the protocols described here. To ensure reproducibility and rigor in MEO experiments, biological and technical replicates are both important. For biological replicates, two to three independent mice bearing

ESCC are generally sufficient per experimental condition. However, the appropriate number of biological replicates may vary depending on the parameters to be tested in individual studies. For example, it is currently unknown how early and how frequently neoplastic organoids may be detected from 4NQO-treated mice without macroscopically visible lesions or histologic IEN and ESCC lesions. Although 4NQO induces esophageal lesions in various mouse strains[14], MEOs have been generated from C57BL/6 mice only. The development and progression of ESCC are accelerated in mice with genetically engineered genetic lesions such as Trp53 loss and cyclin D1 overexpression[7,14,15]. In such mice, neoplastic MEOs may emerge more frequently and earlier than in wild-type C57BL/6 mice during or after 4NQO treatment. If necessary, pilot studies should be performed to estimate the appropriate sample size of mice in planned experiments. With multi-well cell culture plates, technical replicates (n = 3-6) are easily created. However, careful pipetting and practice are warranted to minimize well-to-well variability as the cells are dispensed in a viscous medium containing BME.

A near 100% success rate may be expected for MEO establishment in the protocols described. However, successful MEO culture depends on the viability of the initial isolated esophageal epithelial cells, which is largely related to the quality of the starting material. When the mice cannot be immediately dissected upon euthanasia, the carcasses may be kept at 4 °C overnight to isolate the esophagi the following day. However, the carcasses should not be frozen, as frozen tissue will not yield viable cells. The dissociated esophageal epithelial cells may be cryopreserved as a single-cell suspension in a freezing medium (90% FBS and 10% DMSO) and stored in liquid nitrogen to generate organoids at a later date. The esophagi (epithelial sheets) may be cryopreserved in a similar fashion, albeit less optimally. While MEOs can be initiated with cells purified by fluorescence-activated cell sorting (FACS) and other methods to purify unique subsets of esophageal epithelial cells, the cell viability may be diminished after purification. It should be noted that cell viability assays such as the trypan blue exclusion test can distinguish live cells from dead cells but not dying cells, thus underestimating the cell viability prior to organoid culture initiation. After establishment, MEOs from both 4NQO-treated and untreated mice can be passaged indefinitely (>20 passages), with >80% viability expected at each passage in the medium specified. It is noteworthy that there are organoid medium components whose requirements remains unclear. Zheng et al. recently demonstrated that a supplement comprising Wnt3A, RSpondin-1, and Noggin is required to grow murine esophageal organoids[16]. However, we do not utilize Wnt3A in this protocol as organoids from the tongue, esophagus, and forestomach grow and can be passaged multiple times (>10 passages) without it[7,3,17]. It should be noted that the requirements of these factors have not been tested individually.

Epithelial sheets are utilized to generate MEOs from the normal esophagus or the esophageal mucosa without macroscopically visible lesions such as tumors. The use of epithelial sheets permits the enrichment of epithelial cells as a starting material. However, the isolation of the epithelial sheets from the tumor-bearing esophageal mucosa is difficult due to ESCC invasion into the subepithelial compartments. The whole esophagus can be used to initiate MEO culture, although the presence of non-epithelial cell populations may decrease the organoid formation rate (OFR) in the initial culture. The OFR is expected to rise in the subsequent passages, as the current MEO culture conditions allow epithelial cells to grow within the BME. Of note,

the protocols described here can also be applied to other organs, especially the oral tongue and the forestomach, both of which are susceptible to 4NQO-induced squamous cell carcinogenesis. Of note, we have generated mouse tongue organoids (MTOs) and mouse forestomach organoids (MFOs) from the whole tissues without isolating the epithelial cells. If necessary, these organoids can be made from a subpopulation of epithelial cells purified by fluorescence-activated cell sorting (e.g., cancer stem cells characterized by high CD44 expression, CD73+ putative esophageal stem cells).

Additionally, non-epithelial cells, fibroblasts in particular, may migrate out of the BME to grow in a monolayer on the plastic surface, thereby allowing the concurrent establishment of murine esophageal fibroblasts, including cancer-associated fibroblasts. The selectivity for epithelial cell growth in the 3D BME may limit the co-culturing of MEOs with immune cells and other cell types under the current conditions described here. Optimization for co-culture experiments is underway. Additionally, the tumor invasive front may be better recapitulated in the epithelial-stromal interface modeled by alternative 3D culture methods such as organotypic 3D culture[8,18]. Moreover, MEOs may not be a suitable platform with which to measure the epithelial barrier function, which can be better evaluated by utilizing air-liquid interface culture to assess the transepithelial electrical resistance[19,20,21].

The foremost advantage of MEOs, and indeed MTOs and MFOs, is the rapid growth of single cell-derived structures that recapitulate the original epithelial structures, both benign and malignant. The doubling time (DT) of these organoids is typically estimated to be <20 h. The growth kinetics of normal MTOs from 4NQO-untreated mice illustrate both the capability for long-term culture of normal organoids,

as well as the expected population doubling (PD) level and DT (**Figure 9**). In this example, roughly 10 population doublings occurred during each passage, with a calculated DT of 18.5 h and 18.3 h, on average. After the initial primary culture, an OFR of 15%-25% is typically expected, regardless of seeding density, and MEOs may emerge as early as day 4 (**Figure 8**), though they may be smaller in size. Under phase-contrast microscopy, organoids from the normal esophageal mucosa display spherical structures with smooth surfaces. As a function of time, normal organoids display a concentric structure as the inner cell mass undergoes post-mitotic terminal differentiation, generating a differentiation gradient that mimics the stratified squamous epithelium. Neoplastic organoids show irregular surfaces, reflecting the high proliferative activity of the basaloid cells, which may expand in an outward fashion.The ability of MEOs to display a proliferation-differentiation gradient can be utilized to study how proliferative esophageal basal keratinocytes may propagate and undergo post-mitotic terminal differentiation. By subculturing dissociated MEO cells, one may evaluate epithelial cell self-renewal (**Figure 7**), as well as regeneration or transformation under genotoxic stress induced by 4NQO. Similar studies may also be carried out utilizing the MTO model in the context of oral cancer, broadening the application of the described 4NQO mouse organoid model to head and neck SCC. While epithelial renewal may imply stem cells, one potential weakness of the MEO system is that it may not detect quiescent or rarely dividing stem cells, as MEO formation depends upon cell proliferation. A recent single-cell analysis study on MEOs provided substantial insight into esophageal epithelial cell heterogeneity[22].

Malignant transformation can be assessed by the careful morphological assessment of individual MEO structures and

the nuclear atypia of cells comprising MEOs. The molecular profiling of MEOs by whole-exome sequencing and RNA sequencing may reveal the distinct nature of preneoplastic and ESCC lesions. However, the ultimate test for malignant transformation may require the transplantation of MEOs into either immunodeficient or immunocompetent mice to document the tumorigenicity of the ESCC cells. ESCC MEOs transplanted in syngeneic immunocompetent mice may also provide an excellent platform to investigate the tumor immune microenvironment.

The applications of MEOs are broad. Besides morphology, biochemistry, and multi-omics approaches, flow cytometry can be utilized not only to analyze cellular processes such as DNA synthesis, apoptosis, autophagy, and mitochondrial respiration but also cell surface markers that define unique subsets of cells within MEOs corresponding with genetic and pharmacological modifications *ex vivo*[7,23]. Furthermore, organoids may be used for co-culture experiments to evaluate the interactions between the epithelial cells and the stroma, the immune cells, or even the microbiome[24]. Finally, MEOs can be generated from mice carrying cell lineage-traceable genetic modifications, such as fluorescent reporter protein expressed under a cell type-specific gene regulatory promoter (e.g., Sox2, cytokeratins Krt5, and Krt15)[3,4,7,25].

In summary, MEOs are an invaluable tool for the study of ESCC. The protocols described here aim to show that MEOs represent a versatile model that is relatively easy to generate, maintain, and characterize and has the ability to recapitulate the features of ESCC and esophageal neoplasia.

Disclosures

The authors declare no conflicts of interest.

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