Video Article

In vitro Organoid Culture of Primary Mouse Colon Tumors

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Abstract

Several human and murine colon cancer cell lines have been established, physiologic integrity of colon tumors such as multiple cell layers, basal-apical polarity, ability to differentiate, and anoikis are not maintained in colon cancer derived cell lines. The present study demonstrates a method for culturing primary mouse colon tumor organoids adapted from Sato T *et al.* [1], which retains important physiologic features of colon tumors. This method consists of mouse colon tumor tissue collection, adjacent normal colon epithelium dissociation, colon tumor cells digestion into single cells, embedding colon tumor cells into matrigel, and selective culture based on the principle that tumor cells maintain growth on limiting nutrient conditions compared to normal epithelial cells.

The primary tumor organoids if isolated from genetically modified mice provide a very useful system to assess tumor autonomous function of specific genes. Moreover, the tumor organoids are amenable to genetic manipulation by virus meditated gene delivery; therefore signaling pathways involved in the colon tumorigenesis could also be extensively investigated by overexpression or knockdown. Primary tumor organoids culture provides a physiologic relevant and feasible means to study the mechanisms and therapeutic modalities for colon tumorigenesis.

Video Link

The video component of this article can be found at https://www.jove.com/video/50210/

Introduction

The intestinal epithelial cells proliferate and turn over at an extraordinary rate, outpacing all other tissues in the vertebrate body [2,3]. The dividing cells including intestinal stem cells (ISC) and transit-amplifying cells differentiate into either secretory (goblet, Paneth and enteroendocrine) cells or enterocytes [3]. The ISC is located at the base of the crypt. Paneth cells move down to the bottom of crypts and are long-lived, whereas other lineages migrate upwards to the villi [3,4]. Here the cells are exposed to the gut contents including microbiota and are shed from the villus tips through an anoikis-induced apoptotic mechanism. Although the colon lacks villi and Paneth cells, the mechanism for maintaining homeostasis is similar [4].

The Wnt signaling pathway has been implicated in playing a crucial role in intestinal proliferation and ISC maintenance [4]. Deletion of the transcription factor TCF4, a downstream effector of Wnt signaling, leads to loss of intestinal stem cells and subsequent breakdown of the tissue [5]. Similarly, transgenic expression of the Wnt inhibitor DKK1 reduces the epithelial proliferation and depletes secretory cell lineages [6]. Conversely, overexpression of the Wnt agonist R-spondin-1 induces potent and rapid proliferation of intestinal crypt cells [7].

Given the importance of Wnt signaling for intestinal homeostasis, Wnt pathway mutations are frequently observed in colon cancer [8]. Colon cancer is the third leading cause of death from cancer in United States [9]. Excess dietary intake with red meat and alcohol, reduced physical activity, and inherited and somatic mutations are considered to be risk factors of colon cancer [10,11]. The adenomatous polyposis coli (Apc) gene, a key Wnt signaling factor, is mutated in a majority of patients with familial, sporadic, and colitis-associated colon cancer [12,13]. Mutations of other factors involved in Wnt signaling pathway including Axin2 and β-catenin are also observed in colon cancer [14,15]. However, the precise mechanism and effective therapy for colon cancer are still lacking. To facilitate the investigation of the molecular mechanisms for colon cancer, human colon cancer cell lines representing different stages of cancer progression from a benign to an aggressive cell type have been established [16-18]. Mouse colon carcinoma cell lines with different metastatic properties are also available [19,20]. Nevertheless, primary cells or organoid cultures are preferred over transformed cell lines because they closely mimic the *in vivo* state and generate more physiologically relevant data [21]. Most colon-cancer derived cell lines grow as monolayer attached to the plate or as cell suspensions, lacking of apical-basolateral orientation and tight junctions between cells. Also, normal and tumor intestinal epithelial cells *in vivo* undergo a spontaneous form of apoptosis termed anoikis as the differentiated cells reach the villus tips and are shed [22]. These features are difficult to recapitulate in cell lines but are important in the developmental process of colon cancer [23]. These features are maintained in primary organoids. In addition, the tumor organoid cultures provide an efficient system to assess tumor autonomous functions of genes compared to *in vivo* studies. Genetic manipulation *in vivo* of the intestine is a time-consuming process mainly through creating transgenic and/or knockout mice using intestine-specific drivers. However, the tumor organoids are readily amenable to viral mediated genetic manipulations and thus a great tool for assessing

precise molecular mechanisms. Primary intestinal tumor organoid cultures have been demonstrated to be a feasible and powerful technique. Primary intestinal cell culture can establish functional intestinal organoids with crypt-villi structure *in vitro* from a single adult Lgr5+ stem cell [24]. These organoids can be transplanted and engrafted into damaged colon tissue for regeneration [25]. Further adaption of the culture conditions had made similar epithelial organoids from mouse colon and human small intestine and colon feasible [1]. For primary normal colon epithelium culture, basal culture medium as well as growth factors including EGF, Noggin, R-spondin and Wnt3a are essential, whereas basal culture medium and EGF is sufficient for growing primary mouse colon tumor organoids [1]. Here we describe a detailed protocol to isolate, culture, and generate colon tumor organoids.

Protocol

1. Colon Tumor Isolation and Cell Dissociation

1. Intestinal tumors can be isolated from any sporadic or treatment-induced colon cancer model. The mice should be euthanized with CO_2. Colons are then collected, flushed with cold phosphate-buffered saline (PBS) and opened longitudinally. Identify regions containing tumors using a stereomicroscope, dissect out with a pair of scissors, and wash with cold PBS.
2. Incubate intestinal fragments containing tumors in EDTA chelation buffer (2 mM EDTA, 5.6 mmol/L Na_2HPO_4, 8.0 mmol/L KH_2PO_4, 96.2 mmol/L NaCl, 1.6 mmol/L KCl, 43.4 mmol/L sucrose, 54.9 mmol/L D-sorbitol, 0.5 mmol/L DL-dithiothreitol in distilled water) for 60 min on ice.
3. After chelation, most of the normal intestinal epithelial cells will be detached, while tumor cells will remain attached to the mesenchyme. Aspirate off the chelation buffer containing normal epithelial cells and wash the remnant tumor fragments one more time with 5 ml cold chelation buffer.
4. Aspirate off the chelation buffer, wash tumor fragments with 5 ml cold 1x PBS.
5. Aspirate off the 1x PBS, incubate tumor fragments in digestion buffer (2.5% fetal bovine serum, 1 unit/ml of penicillin, 1 µg/ml of streptomycin, and 2.5 ng/ml of amphotericin B, 200 U/ml type IV collagenase, 125 µg/ml type II dispase in Dulbecco's Modified Eagle Medium) for 2 hr at 37 °C.
6. Allow the tumor fragment to settle under normal gravity for 1 min, and collect the supernatant in a 15 ml falcon tube. Pellet the single cell tumor suspension supernatant by centrifuging at 200 x g for 3 min and wash once with 5 ml PBS by centrifuging at 200 x g for 3 min.

2. Culture of Intestinal Tumor

1. Resuspend the tumor cell pellet with 500 µl PBS, count isolated single tumor cells using a hemocytometer.
2. Pellet tumor cells by centrifuging at 200 x g for 3 min, and resuspend them in 5 mg/ml Matrigel on ice and plate in 24-well plates at 15,000 cells per 50 µl of Matrigel per well.
3. Let the Matrigel polymerize for 15 min at 37 °C, and add 500 µl/well basal culture medium (1 unit/ml of penicillin, 1 µg/ml of streptomycin, and 2.5 ng/ml of amphotericin B, 10 mmol/L HEPES, 2mM Glutamax, 1x N2 supplement, 1x B27 supplement, 1 mmol/L N-acetylcysteine in Advanced Dulbecco's Modified Eagle Medium/F12) containing 50 ng/ml murine EGF.

3. Maintenance of Established Organoids

1. Change basal culture medium containing EGF every 2 days and passage organoids 1:5 once a week.
2. For passaging, replace the culture medium with fresh basal culture medium. Mechanically disrupt organoids and Matrigel using a P1000 pipette with tips cut off and transfer into a 15 ml falcon tube. Further mechanical dissociation is achieved using a fire polished Pasteur pipette.
3. Wash dissociated organoids with 5 ml of basal culture medium and centrifuge at 200 x g for 2 min.
4. Discard the supernatant, resuspend the pellet with Matrigel and add culture medium as described above.

4. Storage and Recovery of Established Organoids

1. For long-term storage, freeze organoids in liquid N_2 which are stable for at least 2 years. For freezing organoids, disrupt using a P1000 pipette with tips cut off and transfer into a 15 ml falcon tube.
2. Wash dissociated organoids with 5 ml of basal culture medium and centrifuge at 200 x g for 2 min.
3. Discard the supernatant, resuspend the pellet with Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO).
4. Transfer cells into 1.5 ml cryotubes, then put the tubes in a Nalgene Mr. Frosty freezing container and store in an -80 °C freezer to achieve a cooling rate of -1 °C/min. After overnight incubation, transfer tubes into liquid N_2.
5. For recovery, the frozen organoids are rapidly thawed in a 37 °C water-bath, wash with basal culture medium, spin down, resuspend in Matrigel and culture with conditions described above.

5. RNA Extraction, Protein Extraction and Immunohistochemistry

1. RNA Extraction: Cells are collected as described above for passage. RNA is isolated with PicoPure™ RNA Isolation Kit according to manufacturer's instructions.
2. Protein Extraction: Cell pellets are collected as described above and lysed in 100 µl radioimmunoprecipitation assay buffer (RIPA; 50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% NP-40, 0.1% SDS) with 1x protease inhibitor.
3. Immunohistochemistry: Aspirate off cell culture medium, transfer tumor organoids with P1000 pipette with tips cut off into Cryo-mold and freeze immediately in tissue freezing medium (O.C.T.) with dry ice. Frozen sections were cut at 7 µm and stained as described previously [26].

Representative Results

The time course of a colon tumor organoid formation from a three-month-old *Apc*$^{min/+}$ mouse is shown in **Figure 1**. At day 0, single cells could be observed several hours following plating (**Figure 1A**). At day 1, survived colon tumor epithelial cells with refractory nuclei could be observed. At day 3, the size of cells doubled. At day 6, the size of organoid expanded more than ten-fold and showed signs of apoptosis in the middle. At day 14, the orgnoids would grow into irregular compartments (**Figure 1B**). Here we had 39.33 ± 22.05 organoids/well formed under the digestion condition of 200 U/ml collagenase for 2 hr, compared to no organoids formed under the digestion condition of 75 U/ml collagenase for 30 min in this study (**Figure 1C**).

To show this organoid culture model could be used for functional study, immunofluorescent staining of β-catenin, an integral component for Wnt signaling pathway is shown in **Figure 2**. The positive stained cells for β-catenin are located in the out layers of the organoid, which confirmed growth pattern with a central lumen proposed by Dr. Clevers' group [24].

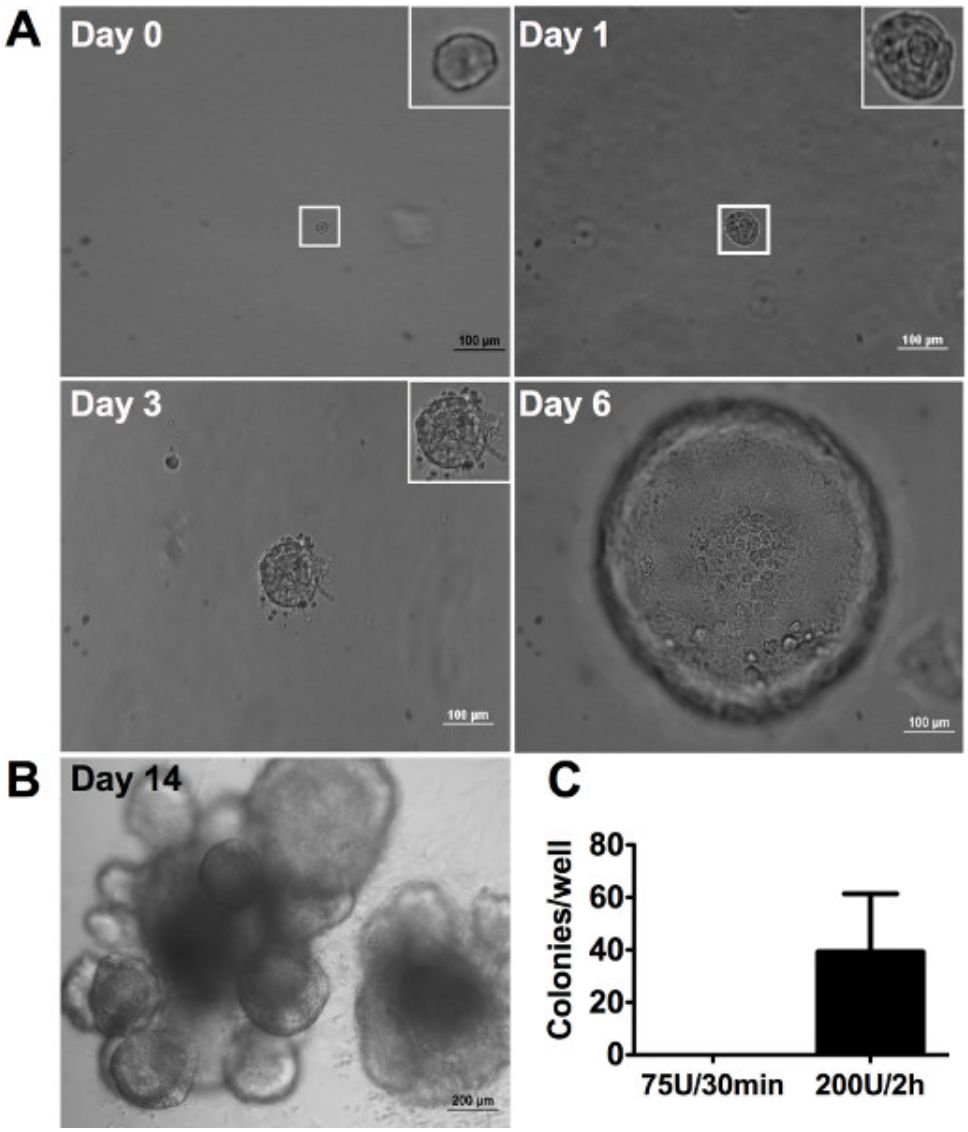

Figure 1. A representative organoid formation derived from colon tumor taken from a three-month-old *Apc*$^{min/+}$ mouse was cultured for (A) 0, 1, 3, 6 and (B)14 days is shown. (C) The successful rate of organoid formation under two different collagenase digestion conditions.

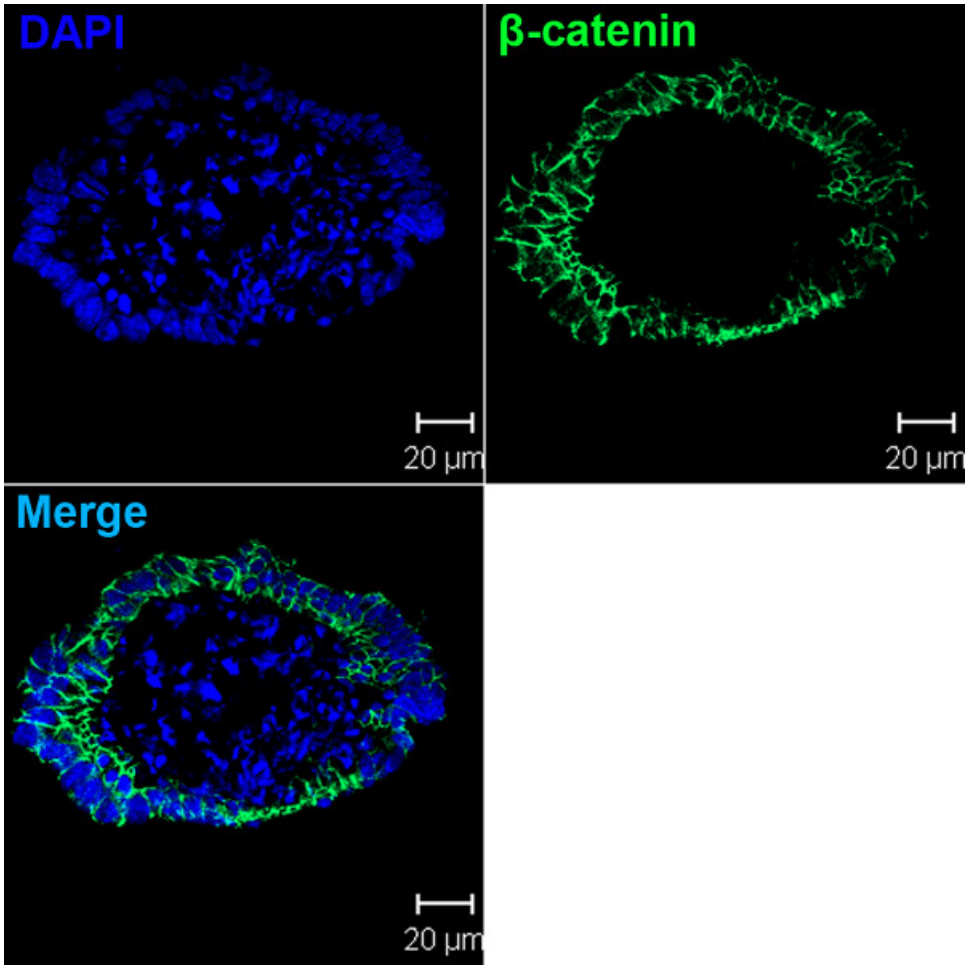

Figure 2. Immunofluorescent staining of β-catenin for organoids derived from a colon tumor taken from a three-month-old *Apc*$^{min/+}$ mouse is shown. 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) was used to stain nuclei.

Discussion

The experimental procedures described in this protocol will allow for isolation and culture of primary murine colon tumors. The protocol is adapted from seminal work done by Dr. Clevers group [1,24,27]. We optimized the digestion time and collagenase concentration to get a better yield of tumor organoids. The critical steps include tumor cell digestion into single cells, Matrigel resuspension, and selective culture. For tumor cell digestion, in order to obtain efficient dissociation of colon tumors and maintain their viability, the digestion and concentration of collagenase should be empirically derived and optimized. For example when using collagenase at 75 U/ml and incubation time of 30 min, we did not obtain single tumor cell suspensions and no organoids were formed under this condition in our hands. Maintaining cell-cell contact is reported to be critical for culturing cancer cells [28], here when using collagenase at 300 U/ml and incubation time of 3 hr, stromal cells contamination is also observed. Similarly, Matrigel resuspension, the concentration, and solidification time should also be determined empirically. The organoids allow precise assessment of the tumor epithelium. The organoids established from intestinal tumors are capable of long-term culture. We have readily used established organoids for several months without appreciable differences in proliferation and differentiation. The established organoid can be genetically manipulated for knockdown and overexpression. Moreover, the tumor organoids can be generated from human tumor biopsy samples; therefore the culturing technique can be used to study basic mechanism in a patient specific manner. The Wnt pathway is critical for intestinal embryonic development. In the adult intestine Wnts are critical for the normal proliferation of transit-amplifying cells and dysregulation of the Wnt pathway is critical for colon tumorigenesis [29]. Selective culture with EGF is sufficient for maintaining colon tumor organoid growth. However, when supplemented with Wnt3a, the organoids grow more efficiently. Therefore, the cells are responsive to the growth promoting effects of Wnt ligands, unlike several colon-derived cell lines.

The organoids more closely mimic the intestinal epithelium than established colon cancer derived lines, and we believe will provide more accurate and physiologically relevant data. As described above, this technique is useful for investigating the initiation and progression mechanisms for colon cancer. Also the tumor organoids will provide more accurate data when assessing the therapeutic efficacy of colon cancer treatments.

Disclosures

We have nothing to disclose.

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