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Original Article

Organoid culture of human prostate cancer cell lines LNCaP and C4-2B

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Abstract: Organoids mimic the architecture and functions of a small organ. Organoid culture technique has been rapidly accepted by all research communities during the past decade to study stem cells, organ development and function, and patient-specific diseases. A protocol for organoid culture of human and mouse prostate epithelial and cancer tissues has been reported. However, organoid culture of the commonly used human prostate cancer cell lines has yet to be established. We followed the published protocol and performed organoid culture of LNCaP and C4-2B cells in Matrigel™ and organoid culture medium for 14 days. We found that both LNCaP and C4-2B cell lines formed organoids that presented glandular structures. The cells within the organoids were androgen receptor-positive adenocarcinoma cells, but not p63-positive basal cells. The cells in the organoids responded to interleukin-17A treatment differently from the cells in the monolayer culture. The present study suggests that LNCaP and C4-2B cells are able to form organoids under the defined organoid culture conditions.

Keywords: Organoid, organoid culture, monolayer culture, prostate cancer, interleukin-17A

Introduction

Prostate cancer is one of the most prevalent cancers throughout the world. According to the American Cancer Society, in 2017 there will be 161,360 new cases of prostate cancer diagnosed in the US and about 26,730 deaths caused by prostate cancer [1]. Prostate cancer is the most common malignancy and the third common cause of cancer-related deaths among American men [1]. The etiology has not been clearly known. Many *in vitro* cell lines and *in vivo* animal models have been developed to investigate the initiation and progression of prostate cancer [2]. It has been known that there is a reciprocal relationship between the cancer cells and the cells in the microenvironment, in which the cells in the microenvironment (i.e., the stroma) can promote tumorigenesis, and in turn, the cancer cells can promote further pro-tumor changes in the microenvironment [3]. In the tumor microenvironment, the presence of persistent immune infiltrates contributes to the recruitment and reprogramming of other non-immune stromal cells including cancer-associated fibroblasts to support continued cancer growth, invasion, and metastasis [3]. The interactions between the cancer cells and stroma have been investigated through a variety of methods. Traditionally, the interactions are studied using tumor specimens and immunohistochemical staining to demonstrate the static relationship between tumor cells and their surrounding environmental cells [4-7]. The functional assays are carried out using co-culture of tumor cells and environmental cells in dishes, either as monolayer culture or separated in Boyden Chambers [8]. These assays do not completely mimic the real interactions of cancer cells and their microenvironment in

human or animal bodies. However, when tumor cells and their environmental cells are co-injected into animals for *in vivo* studies, the technical challenge is that it is not easy to observe the dynamic interactions between the cancer cells and their environmental cells in real-time. The development of intravital multi-photon microscopy has made a significant progress in observing the *in vivo* interactions of cancer cells and their microenvironment [9]. However, this method is limited in studying the organs in the deep body cavities such as the prostate. Thus, new methods are needed.

Organoid culture is an *in vitro* culture of stem cells in the extracellular matrix such as Matrigel™ (BD Biosciences, San Jose, CA), which is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma. Matrigel™ contains 9-12 mg/mL proteins including laminin (a major component), collagen IV, heparin sulfate proteoglycans, entactin/nidogen, and a number of growth factors. The stem cells differentiate and proliferate to self-organize into multi-cellular architecture resembling an organ (hence the name "organoid"). Because the organoids mimic the architecture and functions of a small organ, this technique has been rapidly accepted by all research communities during the past decade to study stem cells, organ development and function, and patient-specific diseases [10-12]. In particular, a standard protocol for organoid culture of human and mouse prostate epithelial and cancer tissues has been published [11]. Based on this published protocol, we performed organoid culture of human prostate cancer cell lines LNCaP and C4-2B. Here we report our findings.

Materials and methods

Reagents

LNCaP cell line (the American Type Culture Collection, Manassas, VA, USA; catalog #CRL-1740) was first isolated from a human metastatic prostate adenocarcinoma found in a lymph node [13]. LNCaP cell line is androgen responsive with mRNA/protein expression of androgen receptor (AR) and prostate-specific antigen (PSA) [2].

C4-2B cell line was isolated from a mouse vertebral metastasis in 1994 as a subline of LNCaP xenografts [14], which was a gift from Dr. Leland Chung, Cedars-Sinai Medical Center, Los Angeles, CA, USA. C4-2B cells express AR and PSA mRNA/protein and grow tumors in either intact or castrated mice [2].

293T-HA-RspoI-Fc cell line was purchased from Trevigen, Inc., Gaithersburg, MD. This cell line was originally developed by Dr. Calvin Kuo at Stanford University to produce R-spondin 1-conditioned medium [15].

Dulbecco's Modified Eagle's medium (DMEM) (Life Technologies, catalog #31966).

Roswell Park Memorial Institute (RPMI)-1640 (Fisher Scientific, catalog #SH3002701).

0.25% Trypsin-ethylenediaminetetraacetic acid (EDTA) (Genesee Scientific, Catalog #25-510).

Advanced DMEM/F12 (Fisher Scientific, catalog #SH30126.01) that became adDMEM/F12 +/+/+ when penicillin/streptomycin (100 U/ml), 10 mM HEPES, and 2 mM GlutaMax were added.

GlutaMAX, 100 × (Gibco™, catalog #350500-61).

Penicillin-streptomycin (Life Technologies, catalog #15140-122).

HEPES (Life Technologies, catalog #15630-080).

Zeocin (Life Technologies, catalog #R250-01).

Phosphate-buffered saline (PBS), 1X, without Ca, Mg, Phenol Red (Genesee Scientific, catalog # 25-507).

Matrigel™, growth factor reduced, Phenol Red-free (Corning™, catalog #356238). The original bottle of Matrigel™ was thawed overnight at 4°C on ice, mixed well by pipetting, and divided into 1-ml aliquots in 1-ml cryovials. Aliquots can be stored at -20°C until the expiration date.

B27 supplement, 50 × (Fisher Scientific, catalog #17504044). Store at -20°C until the expiration date.

Nicotinamide (Sigma-Aldrich, catalog #N0636), dissolved 1.2 g in 10 ml of PBS to prepare a 100 × 1 M stock solution. Aliquots were stored at -20°C until the expiration date.

N-acetyl-L-cysteine (Sigma-Aldrich, catalog #A9165), dissolved 81.5 mg/ml in H_2O to prepare a 400 × 500 mM stock solution for storage up to 1 month.

A83-01 (Tocris Bioscience, catalog #2939), dissolved 10 mg in 950 µl of dimethyl sulfoxide (DMSO) to obtain a 25 mM 50,000 × stock solution for storage up to 1 month.

Y-27632 dihydrochloride (Abmole Bioscience, catalog #M1817), dissolved 50 mg in 1.5 ml of H_2O to prepare a 10,000 × 100 mM stock solution for storage up to 1 month.

Human fibroblast growth factor (FGF)10 (PeproTech, catalog #100-26), dissolved 500 µg in 5 ml of PBS + 0.1% (weight/volume) bovine serum albumin (BSA) to prepare a 10,000 × 0.1 mg/ml stock solution for storage up to 1 month.

Human FGF2 (PeproTech, catalog #100-18B), dissolved 50 µg in 100 µl of 5 mM Tris, pH 7.6 (0.5 mg/ml), and then diluted to a 10,000 × 50 µg/ml stock solution by adding 900 µl of PBS + 0.1% (weight/volume) BSA to 100 µl of 0.5 mg/ml solution for storage up to 1 month.

Human epidermal growth factor (EGF) (PeproTech, catalog #AF-100-15), dissolved 1 mg in 2 ml of PBS + 0.1% (weight/volume) BSA to prepare a 10,000 × 0.5 mg/ml stock solution for storage up to 1 month.

Recombinant human Noggin (PeproTech, catalog #120-10C), dissolved 100 µg in 1 ml of PBS + 0.1% (weight/volume) BSA to prepare a 1,000 × stock solution for storage up to 1 month.

R-spondin 1-conditioned medium (see preparation method below) or recombinant R-spondin 1 protein (R&D Systems, catalog #4645RS025).

Prostaglandin E2 (PGE2) (Tocris Bioscience, catalog #2296), dissolved 10 mg in 2.84 ml of DMSO to prepare a 10,000 × 10 mM stock solution for storage up to 1 month.

SB202190 (Sigma-Aldrich, catalog #S7076), dissolved 25 mg in 2.75 ml of DMSO to prepare a 30 mM 3,000 × stock solution for storage up to 1 month.

(DiHydro) testosterone (5α-Androstan-17β-ol-3-one) (DHT) (Sigma-Aldrich, catalog #A8380), dissolved 1 mg in 3.44 ml of 100% ethanol to obtain a 1 mM solution, and then diluted to 1,000 × in 100% ethanol to prepare a 1,000 × 1 µM stock solution that was stored at -20°C until the expiration date.

Fetal bovine serum (FBS) (GEMIN Bio-Products, catalog #900-208).

Recombinant human interleukin-17A (IL-17A) (R&D Systems, catalog #317-ILB-050).

NucleoSpin® RNA kit (Takara Bio USA, catalog # 740955.250).

PrimeScript™ reverse transcription kit (Takara Bio USA, catalog #RR037B).

Human C-X-C ligand (CXCL) 1, C-C ligand (CCL) 20, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were ordered from Eurofins MWG Operon (Huntsville, AL, USA). CXCL1 primer sequences: Forward 5'-CACCCAAACCGAAGTCATAG-3', Reverse 5'-AAGCCAGCGTTCACCAGA-3'); CCL20 primer sequences: Forward 5'-AACTGGGTGAAAAGGGCTGT-3', Reverse 5'-GTCCAATTCCATCCCAAAAA-3'); GAPDH primer sequence: Forward 5'-CCACATCGCTCAGACACCAT-3', Reverse 5'-TAAAAGCAGCCCTGGTGACC-3'.

iQ SYBR Green Supermix (Bio-Rad Laboratories, catalog #1708884).

Mouse anti-p63 monoclonal antibody (Biocare Medical, catalog #CM163A).

Rabbit anti-androgen receptor polyclonal antibody (Santa Cruz Biotechnology, catalog #sc-816).

Preparation of R-spondin 1-conditioned medium

293T-HA-RspoI-Fc cell line (less than 15 passages) was cultured in DMEM with 10% FBS, penicillin/streptomycin (100 U/ml), and zeocin (300 µg/ml) in 100 × 20 mm culture dishes until confluency in a humidified incubator with 5% CO_2 at 37°C. The cells were split in 1 to 6 ratio and cultured in DMEM with 10% FBS and penicillin/streptomycin (100 U/ml), but without zeocin for 3-4 days until confluency. The medium was replaced with adDMEM/F12 +/+/+. After 1 week, the medium was collected into a 50-ml tube and centrifuged at 450 × g for 5

min at 4°C. The supernatant (i.e., R-spondin 1-conditioned medium) was filtered through a 0.22-μm filter and stored at -20°C for up to 6 months. The R-spondin 1-conditioned medium was split into aliquots to avoid repeated freeze-thaw cycles.

Monolayer culture

LNCaP and C4-2B cell lines were cultured in RPMI-1640 medium with 10% FBS and penicillin/streptomycin (100 U/ml) in 100 × 20 mm cell culture dishes in a humidified incubator with 5% CO_2 at 37°C. As a control to the organoid culture, 10,000 cells per well were plated in 6-well tissue culture plates and cultured with the organoid culture medium before IL-17A treatment (see below).

Organoid culture procedures

Pre-warm 24-well tissue culture plates overnight at 37°C.

Culture LNCaP and C4-2B cells in RPMI-1640 + 10% FBS + penicillin/streptomycin (100 U/ml) in 100 × 20 mm cell culture dishes until 80% confluency.

Wash the cells 3 times with 10 ml PBS.

Digest the cells with 5 ml of 0.25% trypsin-EDTA for 3-5 min at 37°C.

Harvest the cells into 50-ml tubes.

Centrifuge the tubes at 150 × g for 5 min at 4°C.

Aspirate the supernatant and wash the cells once with 10 ml adDMEM/F12 +/+/+, and then centrifuge the tubes at 150 × g for 5 min at 4°C.

Aspirate the supernatant and resuspend the cells in 10 ml adDMEM/F12 +/+/+.

Count the cells using a hemocytometer and dilute the cells to 250,000 cells/ml in adDMEM/F12 +/+/+.

Take 1 ml cell suspension into 50-ml tubes and centrifuge the tubes at 150 × g for 5 min at 4°C.

Aspirate the supernatant and add 1 ml thawed Matrigel™.

CRITICAL STEP: Stir the mixture gently and avoid pipetting up and down to make any bubbles. Suck the mixture using a 10-ml pipette. Before plating the cells, must deplete the air in the tip of the pipette.

Let Matrigel™ drip from the pipette into the middle of each well of 24-well tissue plates; each drop contains approximately 10,000 cells in 40-μl Matrigel™.

CRITICAL STEP: the 10-ml pipette must be pre-cooled and the plating procedure must be performed on ice; work quickly to ensure that Matrigel™ does not solidify before plating.

Place the tissue culture plates upside down in the incubator with 5% CO_2 at 37°C for 15 min to allow the Matrigel™ to solidify into a drop, rather than spreading and adhering onto the plate bottom.

Pre-warm the organoid culture medium in a 37°C water bath for 10 min before adding it to the organoid culture. The organoid culture medium for human prostate cancer cells contains 50 × diluted B27, 1.25 mM N-acetyl-L-cysteine, 5 ng/ml EGF, 100 ng/ml Noggin, 500 ng/ml recombinant R-spondin 1 (or 10% of the R-spondin 1-conditioned medium), 500 nM A83-01, 10 ng/ml FGF10, 5 ng/ml FGF2, 1 μM PGE2, 10 mM nicotinamide, 10 μM SB202190, 1 nM DHT, and 10 μM Y-27632 dihydrochloride [11].

Gently pipette 500 μl of pre-warmed (37°C) organoid culture medium with 10 μM Y-27632 dihydrochloride into each well, and then place the plates in the incubator with 5% CO_2 at 37°C.

Refresh the organoid culture medium with 10 μM Y-27632 dihydrochloride every 2-3 days. From 7 days after initial plating, an organoid culture medium without Y-27632 dihydrochloride should be used. The organoid culture was ended on day 14.

Immunohistochemical (IHC) staining

At the end of 2-week organoid culture, organoids embedded in Matrigel™ were fixed in the 24-well plates with 4% paraformaldehyde (PFA) in PBS for 60 min at room temperature.

Organoids from 6 wells were pooled into 15-ml tubes and centrifuged at 150 × g for 5 min.

CRITICAL STEP: The fixation process does not lead to degradation of the Matrigel™. Use a 10

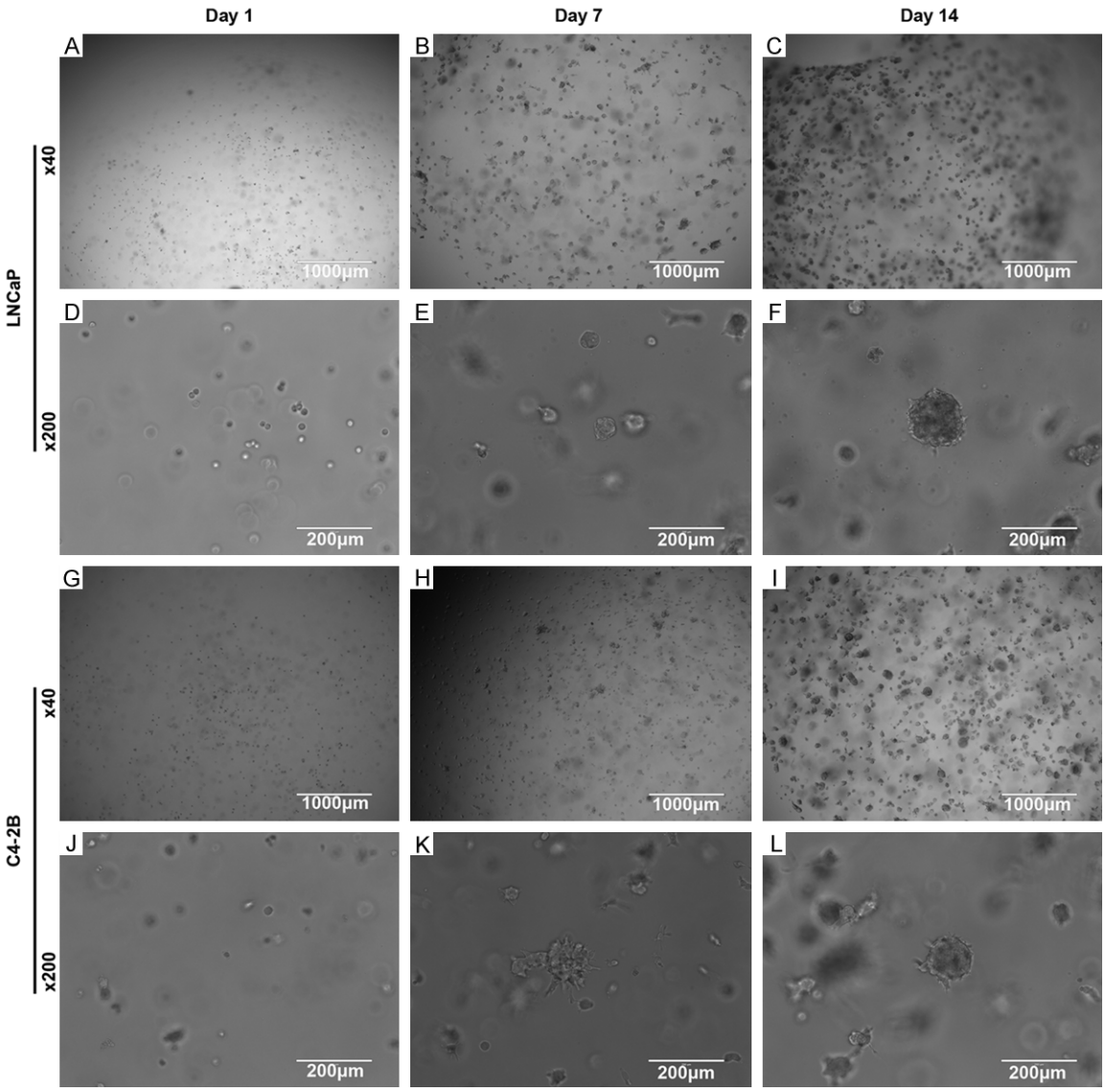

Figure 1. Representative photomicrographs of organoid culture of LNCaP and C4-2B cells at different time points.

ml pipette to collect the organoids from 24-well plates into 15-ml tubes.

PFA was aspirated.

10 ml water was added into the tubes.

The tubes were centrifuged again and PFA was removed.

Pre-staining of the organoids was performed using trypan blue, in order to identify the organoids during paraffin embedding and sectioning.

Organoids were dehydrated with 95% ethanol at 4°C overnight, followed with 95% ethanol for 15 min, 100% ethanol for 15 min, and 100% ethanol for 15 min.

Organoids were cleared in xylene twice and embedded in paraffin.

Paraffin-embedded organoids were cut into 4-μm thick sections.

Organoid sections were stained with hematoxylin (Biocare Medical, catalog #CATHE-MM) & eosin (Biocare Medical, catalog #HTE-MM) solutions according to the manufacturer's instructions.

Organoids sections were stained with mouse anti-p63 antibody (1:500 dilution) and rabbit

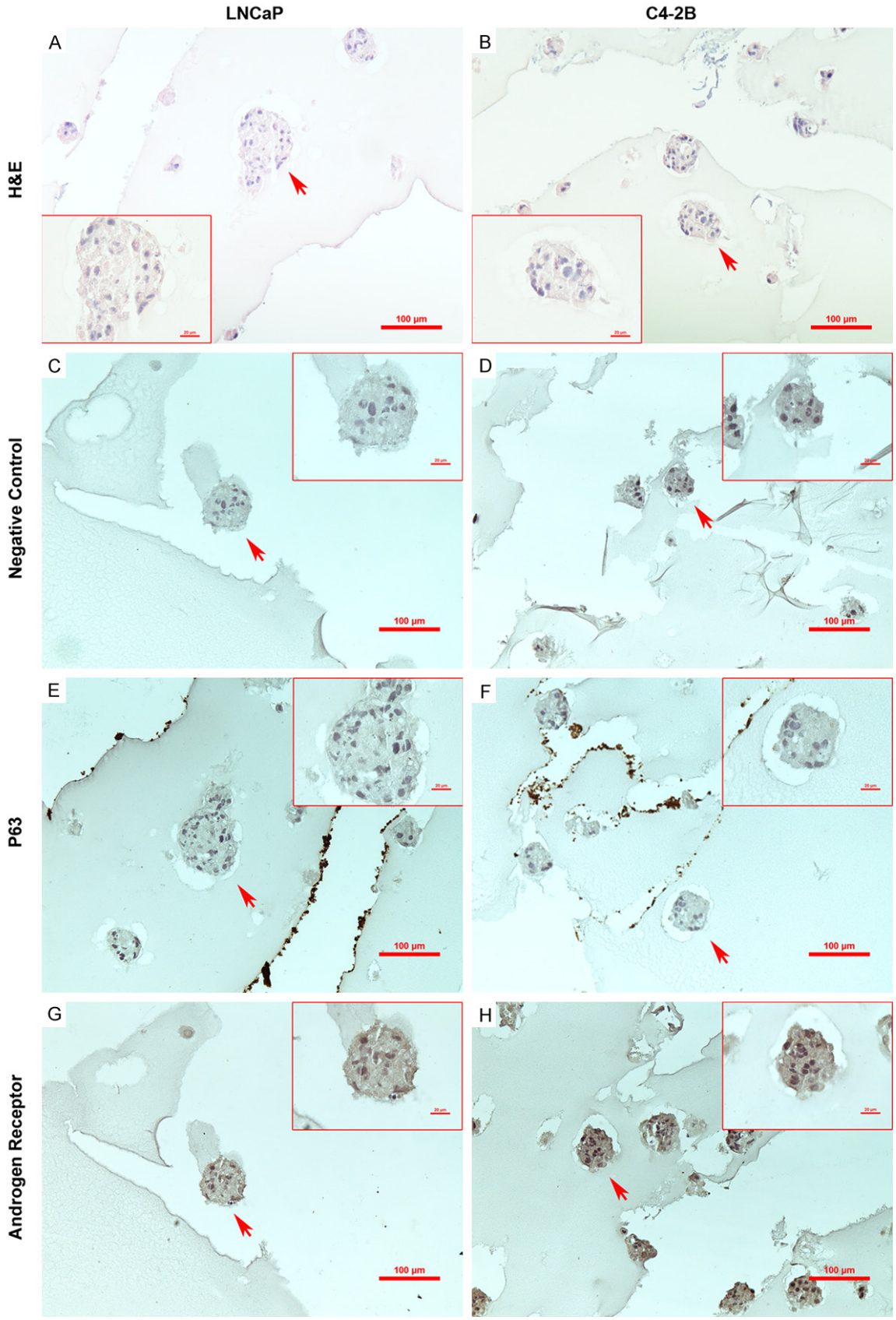
LNCaP
C4-2B
H&E
Negative Control
P63
Androgen Receptor
A
B
C
D
E
F
G
H
100 μm

Figure 2. Representative photomicrographs of stained LNCaP and C4-2B organoid sections. A, B. Hematoxylin and eosin (H&E) staining. C, D. Negative control of immunohistochemical staining using PBS to replace the primary antibody. E, F. Immunohistochemical staining of p63. G, H. Immunohistochemical staining of androgen receptor. Arrows indicate the organoids. Magnification: × 100 for the background images and × 400 for the images in the red frames.

anti-androgen receptor antibody (1:200 dilution), together with Vectastain® ABC kits (Vector Laboratories) as described previously [16]. For negative control, PBS was used to replace the primary antibody.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Organoids (2 wells per group) and monolayer-cultured cells (1 well of 6-well plates per group) were treated without (as control group) or with 20 ng/ml recombinant human IL-17A for 3 hours.

Organoids (2 wells of organoids were pooled together into one sample) and monolayer-cultured cells were harvested for total RNA extraction using a NucleoSpin® RNA kit according to the manufacturer's instructions.

cDNA was synthesized from total RNA using a PrimeScript™ reverse transcription kit according to the manufacturer's instructions.

qRT-PCR was performed in triplicates with the iQ SYBR Green Supermix and an iQ5 iCycler PCR machine (Bio-Rad Laboratories) following the manufacturer's protocols.

Results were normalized to GAPDH levels using the formula Δ cycle threshold (Ct) = Ct of target gene-Ct of GAPDH. The mRNA level of the control group (not treated with IL-17A) was used as the baseline; therefore, ΔΔCt was calculated using the formula ΔΔCt = ΔCt of target gene - ΔCt of the baseline. The fold change of mRNA level was calculated as fold = $2^{-\Delta\Delta Ct}$.

Statistical analysis

All experiments were replicated three times. Data were represented with mean ± standard error of the mean (SEM) of 3 independent experiments (n=3). Student's *t* test (2-sided) was used to analyze the data. A $P < 0.05$ is considered statistically significant.

Results

At the beginning of organoid culture (day 1), LNCaP cells (**Figure 1A**, **1D**) and C4-2B cells (**Figure 1G**, **1J**) mostly presented as single cells suspended in Matrigel™. A few cells presented as an aggregate of two cells or three cells. At day 7, LNCaP cells (**Figure 1B**, **1E**) and C4-2B cells (**Figure 1H**, **1K**) started to present aggregates of multiple cells. At day 14, organoids were formed as aggregates of LNCaP cells (**Figure 1C**, **1F**) and C4-2B cells (**Figure 1I**, **1L**). It was technically difficult to count the number of cells per organoid, but we measured the size of representative organoids. The average diameter of LNCaP organoids was 120.7 μm (standard deviation: 30.6 μm; range: 73.9-180.9 μm; n=15). The average diameter of C4-2B organoids was 90.9 μm (standard deviation: 32.5 μm; range: 48.0-160.6 μm; n=15).

To check the internal structure of the organoids, we stained the organoid sections with hematoxylin and eosin (H&E). We found that both LNCaP organoids and C4-2B organoids presented as glandular structures (**Figure 2A**, **2B**). We also stained the organoid sections for p63 (a marker of prostatic basal cells) and AR (a marker of prostatic luminal cells). We found that all cells in the organoids were negative for p63 staining (**Figure 2C-F**). On the other hand, all cells in the organoids were positive for AR staining (**Figure 2G**, **2H**).

To assess if the cells in the organoids respond to external stimuli differently from the cells in the monolayer culture, we treated the organoids and monolayer-cultured cells with recombinant IL-17A for 3 hours and analyzed expression of IL-17A-downstream genes CXCL1 and CCL20. We found that IL-17A induced higher levels of CXCL1 and CCL20 expression in monolayer-cultured LNCaP cells than LNCaP organoids (**Figure 3A**). In contrast, IL-17A induced higher levels of CXCL1 and CCL20 expression in C4-2B organoids than monolayer-cultured C4-2B cells (**Figure 3B**).

Discussion

Organoid culture has been rapidly accepted by all research communities during the past decade to study stem cells, organ development and function, and patient-specific diseases [10-

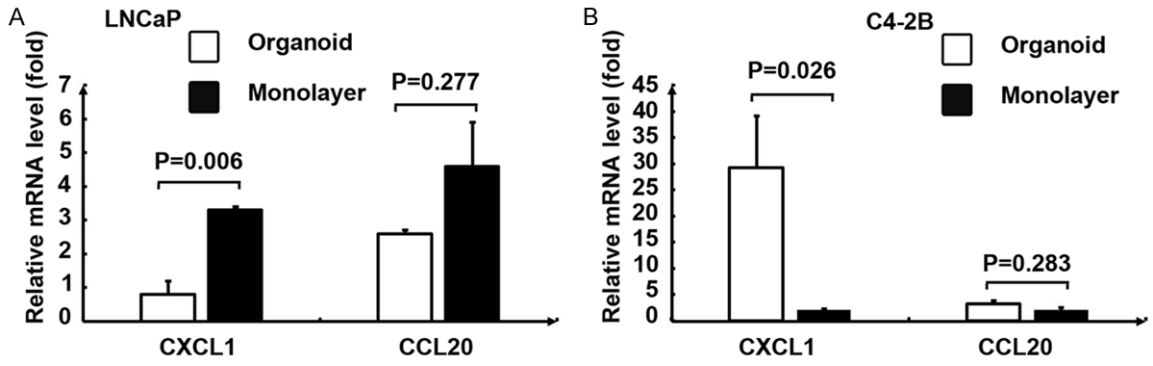

Figure 3. Expression of CXCL1 and CCL20 in the organoids and monolayer-cultured cells. The organoids and monolayer-cultured LNCaP and C4-2B cells were treated without or with IL-17A for 3 hours. The mRNA levels were analyzed by qRT-PCR. Data represent mean ± SEM (error bars) of 3 independent experiments (n=3).

12]. A standard protocol for organoid culture of human and mouse prostate epithelial and cancer tissues has been published previously [11]. However, organoid culture of the commonly used human prostate cancer cell lines LNCaP and C4-2B has not been reported. In the present study, we followed the reported protocol [11] to set up organoid culture of LNCaP and C4-2B cells. We found that LNCaP and C4-2B organoids were formed after 14-days culture in Matrigel™ and the organoid culture medium that was previously defined [11]. The organoids contained luminal adenocarcinoma cells as evidenced by AR expression, but not basal cells that express p63. We also found that the cells in the organoids responded to IL-17A treatment differently from the monolayer-cultured cells. The exact mechanisms that caused the differences are not clear, but hypothetically it reflects the differences between the 3-dimensional organoid culture and 2-dimensional monolayer culture. We learned a few things through establishing the organoid culture of LNCaP and C4-2B cells. First, 293T-HA-RspoI-Fc cell line is needed for large-scale organoid culture because the recombinant R-spondin 1 protein is very expensive to purchase, yet it is essential for organoid culture. This cell line can be purchased from Trevigen, Inc. (Gaithersburg, MD) with a simple Material Transfer Agreement. The original developer Dr. Calvin Kuo does not provide this cell line anymore. Second, a few critical steps are noticed. The most critical step is how to make the drops of Matrigel™ containing the cells. The published protocol did not describe how to make the 40-μl drop containing 20,000 cells [11]. We found that a drop of Matrigel™ was approximately 40 μl if dripped from a 10-ml pipette, and each drop contained approximately 10,000 cells if 1 ml Matrigel™ was mixed gently with 250,000 cells. We think that the detailed procedures presented in this manuscript will be very helpful to prostate cancer researchers who want to perform organoid culture of LNCaP and C4-2B cell lines as well as other prostate cancer cell lines.

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Disclosure of conflict of interest

None.

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