Organoid culture systems for prostate epithelial and cancer tissue

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This protocol describes a strategy for the generation of 3D prostate organoid cultures from healthy mouse and human prostate cells (either bulk or FACS-sorted single luminal and basal cells), metastatic prostate cancer lesions and circulating tumor cells. Organoids derived from healthy material contain the differentiated luminal and basal cell types, whereas organoids derived from prostate cancer tissue mimic the histology of the tumor. We explain how to establish these cultures in the fully defined serum-free conditioned medium that is required to sustain organoid growth. Starting with the plating of digested tissue material, full-grown organoids can usually be obtained in ~2 weeks. The culture protocol we describe here is currently the only one that allows the growth of both the luminal and basal prostatic epithelial lineages, as well as the growth of advanced prostate cancers. Organoids established using this protocol can be used to study many different aspects of prostate biology, including homeostasis, tumorigenesis and drug discovery.

INTRODUCTION

The prostate is a gland of the male reproductive system that produces seminal fluid. The prostate consists of luminal, basal and rare neuroendocrine cells. Androgen receptor (AR) signaling is essential for prostate development and homeostasis, as well as for prostate cancer initiation and progression. As generally acknowledged1–2, prostate cancer research has been hampered by the lack of suitable in vitro model systems. Although powerful in vivo models are available, these are often expensive, time-consuming and technically challenging. Most in vitro research is performed using cell lines derived from neoplastic lesions, and most of these do not have an intact AR signaling pathway, making them poor representatives of healthy prostate and prostate cancer tissue. We have developed a testosterone-responsive prostate organoid culture system derived from primary prostate tissue and advanced prostate cancer tissue3–4, which opens new opportunities to study prostate homeostasis and cancer. In this protocol, we provide further details of how to set up and evaluate these culture systems.

Comparison with other methods

The culture system we describe here was developed by adapting and optimizing the culture conditions that were previously used to establish mouse and human small intestine and colon organoid cultures5,6. We added different compounds and growth factors to generic organoid medium (containing epidermal growth factor (EGF), Noggin and R-spondin 1 (Box 1)5), enabling us to establish culture conditions that support the long-term growth of mouse and human prostate tissue and advanced prostate cancers. The composition of the medium we use is summarized in Table 1. By using this culture system, we have shown that: (i) both the luminal and basal lineages harbor multipotent progenitor cells and can be propagated for long-term growth, (ii) organoids functionally recapitulate AR signaling, (iii) organoids derived from prostate cancer mouse models recapitulate mouse phenotypes and (iv) human prostate cancer–derived organoids genetically and phenotypically mimic the tumor from which they were derived3,4.

Several groups have demonstrated the in vitro growth of primary prostatic tissue. However, in contrast to our prostate organoid cultures, most of these models only support short-term growth, mainly support growth of basal cells and do not allow for full luminal differentiation (limiting androgen responsiveness)7–10. Moreover, these methods do not allow efficient growth of prostate cancer tissue. A protocol developed by Liu et al.11 enables the indefinite growth of reprogrammed prostatic epithelial cells. Interestingly, the Rho-associated kinase (ROCK) inhibitor Y-27632 and the presence of feeder cells—producing factors that may also be present in our defined prostate culture medium—are essential in this system. However, cells cultured under these conditions do not closely resemble the in vivo prostate, and, additionally, androgen responsiveness is limited in this system. Finally, Chua et al.12 recently demonstrated a culture system that exclusively allows the growth of organoids from single luminal cells, albeit at a lower plating efficiency than reported with our method (0.2–0.3% described by Chua et al.12 versus 1–2% when using our method3). Under these conditions, organoids that resemble the in vivo prostate are formed. However, basal cell–derived organoids cannot be propagated for a prolonged time. In addition, in contrast to our method, their medium is not fully defined. It is possible that the medium’s undefined additions (e.g., fetal calf serum) contain growth factors that are present in our defined medium. No studies to date have investigated whether prostate cancer tissue can be propagated under these conditions.

Limitations of the method

With an average split ratio of 1:2 every 2 weeks, the proliferation rate of sorted human luminal cells is not very high. Organoid lines derived from advanced prostate cancers may have a similarly
low average proliferation rate, although this rate can vary greatly between cultures derived from different donors. Moreover, with our culture protocol, we have not yet succeeded in growing organoids derived from primary prostate cancers. This is probably because tumor cells do not have a selective advantage over normal cells in our prostate culture medium. On the contrary, the normal prostate cells that are present within each sample seem to overgrow the tumor cells.

**Experimental design**

**Overview of the procedure.** The following step-by-step procedure describes how to obtain mouse and human prostate organoids from healthy prostate tissue and advanced prostate cancers. The main procedure (Steps 1–3) describes the dissection and digestion of tissue, as well as the subsequent cell plating and organoid passaging. Organoids are plated in Matrigel and cultured in defined prostate culture medium, which is refreshed every 2–3 d. Box 2 describes how to culture organoids derived specifically from luminal and basal lineages. Separation of luminal and basal lineages requires antibody staining and FACS separation. We also describe protocols for cryopreservation of the organoids once they have been established (Step 4A), RNA isolation and cDNA production (Step 4B), and genomic DNA isolation (Step 4C). The organoids are genetically and phenotypically stable, and they can be genetically modified using many different genome editing systems\(^3,4,13–16\), making them a very suitable tool for studying tissue homeostasis and cancer.

**Culture medium.** In this protocol, mouse and human prostate organoids are grown in defined prostate culture medium

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**Box 1 | Preparation of R-spondin 1-conditioned medium**

**TIMING 10–11 d**

| Additional materials required |
|-------------------------------|
| 293T-HA-RspoI-Fc cell line\(^19\) (can be obtained from the Calvin Kuo laboratory Stanford University) |

**CAUTION** The cell lines used should be regularly checked to ensure that they are authentic and that they are not infected with *Mycoplasma*.

**Procedure**

1. Cultivate the 293T-HA-RspoI-Fc cell line\(^19\) (low-passage cells (up to 15 passages) must be used) in DMEM + 10% (vol/vol) FBS + penicillin/streptomycin + zeocin (300 µg/ml) in 175 cm\(^2\) flasks until confluency.
2. Passage confluent flask (split ratio ~1:6) and grow the cells in DMEM + 10% (vol/vol) FBS + penicillin/streptomycin, without zeocin.
3. When the cells reach confluency (after 3–4 d), replace the DMEM + 10% (vol/vol) FBS + penicillin/streptomycin medium with adDMEM/F12 +/-/+.
4. After 1 week, collect the medium in a 50-ml Falcon tube.
5. Centrifuge the tube at 450g for 5 min at 4 °C.
6. Pass the medium through a Stericup-GP with a 0.22-µm filter.

**CRITICAL STEP** It is important that all 293T cells are removed from the R-spondin 1–conditioned medium.

7. Store the R-spondin 1–conditioned medium at −20 °C for up to 6 months. Avoid repeated freeze-thaw cycles.

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**Box 2 | Overview of culture medium components for mouse and human prostate organoids.**

| Factor              | Mouse organoids                                                                 | Human organoids                                                                 |
|---------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| B27                 | 50× diluted                                                                     | 50× diluted                                                                     |
| N-acetylcysteine    | 1.25 mM                                                                         | 1.25 mM                                                                         |
| EGF                 | 50 ng/ml                                                                        | 5 ng/ml                                                                        |
| Noggin              | 100 ng/ml                                                                       | 100 ng/ml                                                                       |
| R-spondin 1         | 500 ng/ml or 10% conditioned medium                                             | 500 ng/ml or 10% conditioned medium                                             |
| A83-01              | 200 nM                                                                          | 500 nM                                                                          |
| FGF10               | —                                                                               | 10 ng/ml                                                                        |
| FGF2                | —                                                                               | 5 ng/ml                                                                         |
| Prostaglandin E2    | —                                                                               | 1 µM                                                                            |
| Nicotinamide        | —                                                                               | 10 nM                                                                           |
| SB202190            | —                                                                               | 10 µM                                                                           |
| DHT                 | 1 nM                                                                            | 1 nM                                                                            |
| Y-27632 dihydrochloride\(^a\) | 10 µM                                                                            | 10 µM                                                                            |

\(^a\)Y-27632 dihydrochloride is only added to the medium during establishment of the culture and after passaging the organoids using TrypLE.
Box 2 | Establishment of luminal- and basal-derived organoid cultures

**TIMING** 14 d

**Antibodies required**
- CD26–FITC–conjugated antibody (anti-human 1:200, M-A261, eBioscience)
- CD49f–Alexa Fluor 647–conjugated antibody (anti-human/mouse 1:200, GoH3, BD Biosciences)
- CD49f–PE–conjugated antibody (anti-human/mouse 1:200, GoH3, BD Biosciences)
- CD24–Alexa Fluor 647–conjugated antibody (anti-mouse 1:200, 30-F1, eBioscience)

**Procedure**
1. Prewarm the tissue culture plates overnight at 37 °C.
2. Enzymatically digest mouse (Fig. 3a) or human (Fig. 3b) prostate tissue as described in Step 2A(i–xii) and Step 2B(i–v) of the main PROCEDURE, respectively.
3. Wash the digested single cells in 10 ml of blocking solution in a 15-ml Falcon tube.
4. Centrifuge the tube at 200g for 5 min at 4 °C.
5. Aspirate the supernatant.
6. Resuspend the cells in 1 ml of blocking solution.
7. Pass the resuspended cells through a cell strainer of a 5-ml polystyrene round-bottom tube.
8. Incubate the cells on ice for 15 min.
9. Centrifuge the cells at 200g for 5 min at 4 °C and aspirate the supernatant. Set aside some cells (about one-tenth of the total) as an unstained negative control. Use the remaining cells for antibody staining (as described in step 10 below).
10. Incubate the cells with 500 μl of staining solution for 60 min on ice and in the dark with CD26-FITC-conjugated antibody (M-A261, 1:200) and CD49f–Alexa Fluor 647–conjugated antibody (GoH3, 1:200) for human organoids, or CD24–Alexa Fluor 647–conjugated antibody (30-F1, 1:200) and CD49f–PE–conjugated antibody (GoH3, 1:200) for mouse organoids.
11. Wash all aliquots of cells from steps 9 and 10 with 10 ml of adDMEM/F12 +/-/+ plus Y-27632 dihydrochloride (10 μM) plus dihydrotestosterone (1 nM).
12. Centrifuge the cells at 200g for 5 min at 4 °C.
13. Repeat steps 11 and 12.
14. Resuspend the cells in staining solution (with no antibody added).
15. Add DAPI to each cell sample (to give 1.0 μg/ml final concentration).
16. Add DNaseI to each cell sample (to give 0.5–1.0 U/μl final concentration).
17. Isolate the cells using FACS (Fig. 3). On average, 200 mg of prostate tissue should contain ~150,000 luminal cells and 600,000 basal cells.

**Troubleshooting**
18. Plate luminal and basal cells as described in Step 2A(xiv–xvii) (for mouse cells) and Step 2B(viii–x) (for human cells) of the main PROCEDURE (Fig. 3).
19. Refresh the medium with Y-27632 dihydrochloride every 2–3 d and check organoid growth using a light microscope. After 2–3 d, organoids should appear from basal cells, whereas small organoids should appear from luminal cells after 5–7 d. For culture beyond 7 d, do not include Y-27632 dihydrochloride in the medium. From 7 d onward, medium without Y-27632 dihydrochloride should be used.
20. After ~14 d, passage organoids as described in PROCEDURE Step 3A (mouse) and Step 3B (human).

**Controls.** The separation of luminal and basal cells from mouse and human prostate tissue (Box 2) is based on double antibody staining for marker gene expression followed by FACS. To specifically stain luminal cells, we use conjugated antibodies against CD26 and CD24 (human and mouse, respectively). For specific staining of basal cells, we use a conjugated CD49f-specific antibody for both human and mouse. As a negative control, an isotype-matched control antibody generated from the same species as the target antibody can be used. In addition, single-color staining controls (staining for either the luminal or basal marker) can also be included.

To verify that the organoids are indeed derived from prostate tissue, (q-) RT-PCR can be performed for the expression of prostate-specific genes (see ANTICIPATED RESULTS and Karthaus et al.3). In addition, to confirm the growth of prostate tumor organoids, whole-genome sequencing can be performed to analyze mutation spectra. In this protocol, we also describe the procedures to isolate RNA, produce cDNA and isolate genomic DNA from organoids.

containing R-spondin 1. Either R-spondin 1–conditioned medium (for preparation, see Box 1) or recombinant R-spondin 1 (R&D Systems) at a final concentration of 500 ng/ml can be used. We have not observed any differences in organoid establishment, maintenance or morphology when using prostate culture medium containing either R-spondin 1–conditioned medium or recombinant R-spondin 1. To achieve high efficiency for culture establishment and successful maintenance, it is essential to use fresh medium (stored at 4 °C for not more than 1 week) and to use effective and appropriately stored growth factors and chemical compounds.

**Mouse age.** To establish mouse prostate organoids, prostates should be isolated from mice that are at least 8 weeks old. The maximum age of the mice for efficient organoid establishment has not been carefully addressed. However, we have successfully established organoids from mice with an age of up to 2 years.
**MATERIALS**

**EQUIPMENT**
- Falcon tubes, 15 ml
- Falcon tubes, 50 ml
- SepMate-50 (StemCell Technologies, cat. no. 15450)
- Polyethylene terephthalate bottom tube with cell-strainer caps, 5 ml (Falcon)
- Microcentrifuge tubes, 1.5 ml
- Shaking platform, 37 °C
- Plates, 6 well (Greiner Bio-One, cat. no. 657 160)
- Plates, 12 well (Greiner Bio-One, cat. no. 665 180)
- Plates, 24 well (Greiner Bio-One, cat. no. 662 160)
- Plates, 48 well (Greiner Bio-One, cat. no. 677 180)
- Cell culture dishes 100 × 20 mm (Greiner Bio-One, cat. no. 664 160)
- Glass Pasteur pipette (Kowa International, cat. no. 87144E)
- Glass Pasteur pipettes (VWR, cat. no. 612-1701)
- Light microscope (Nikon, Eclipse TS100)
- Dissection microscope (Leica, MZ75)
- Dissection tools (Neolab)
- FACs machine (DaKo MoFlo)
- Disposable scalpels (Swann-Morton, code 0501)
- Centrifuge for 15-and 50-ml Falcon tubes (Eppendorf, cat. no. 5810R)
- Microcentrifuge (Eppendorf, cat. no. 5424)
- CO2 incubator (5% CO2, 37 °C)
- Biosafety cabinet
- CoolCell freezing container (BioCision)
- Stericup-GP, 0.22 μm, polyethersulfone, 500 ml, radio-sterilized (Millipore, cat. no. SCG5UP05RE)

**REAGENTS**
- Recombinant RNasin RNase inhibitor (Promega, cat. no. N2511)
- Oligo(dT)
- Reliaprep gDNA tissue miniprep system (Promega, cat. no. A2052)
- Recovery cell culture freezing medium (Life Technologies, cat. no. 12648-010)
- Plates, 48 well (Greiner Bio-One, cat. no. 677 180)
- Plates, 12 well (Greiner Bio-One, cat. no. 665 180)
- Plates, 24 well (Greiner Bio-One, cat. no. 662 160)
- Plates, 6 well (Greiner Bio-One, cat. no. 657 160)
- Plates, 6 well (Greiner Bio-One, cat. no. 677 180)
- Glass Pasteur pipette (Kowa International, cat. no. 87144E)
- Glass Pasteur pipettes (VWR, cat. no. 612-1701)
- Light microscope (Nikon, Eclipse TS100)
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- Microcentrifuge (Eppendorf, cat. no. 5424)
- CO2 incubator (5% CO2, 37 °C)
- Biosafety cabinet
- CoolCell freezing container (BioCision)
- Stericup-GP, 0.22 μm, polyethersulfone, 500 ml, radio-sterilized (Millipore, cat. no. SCG5UP05RE)

**PROTOCOL**

**REAGENT SETUP**

**Mouse and human material** The procedure can be applied to whole mouse prostate, pieces of human prostate tissue (minimum size 1 mm³), pieces of human metastasis biopsy (minimum size 1 mm³) or blood samples from patients with advanced prostate cancer (8 ml of blood is required). We have successfully grown organoids from all these starting materials. Although fresh material should preferably be used, we have been able to establish organoids from both mouse and human tissue stored overnight at 4 °C in adDMEM/F12 +/- (+/+, i.e., adDMEM/F12 containing penicillin-streptomycin, 10 mM HEPES and 2 mM Glutamax). CAUTION All mouse experiments must be in compliance with local animal welfare laws and guidelines. CAUTION Informed consent must be obtained from all subjects. Studies must conform to all relevant institutional and governmental regulations.

**Mouse prostate culture medium** The final medium composition is summarized in Table 1. To make up the medium, combine 1.0 ml of B27, 125.0 µl of N-acetyl-L-cysteine (500 mM in PBS), 5.0 µl of EGF (0.5 mg/ml in PBS + 0.1% (wt/vol) BSA), 2.0 µl of A83-01 (5 mM in DMSO), 50.0 µl of Noggin (100 µg/ml in PBS + 0.1% (wt/vol) BSA), 50.0 µl of R-spondin 1 (500 µg/ml in PBS + 0.1% (wt/vol) BSA or 10% conditioned medium) and 50.0 µl of dihydrotestosterone (1 mM in ethanol). Top it up to 50 ml with adDMEM/F12 +/- (+/+, +). CRITICAL As detailed in the PROCEDURE, after cell passaging, Y-27632 dihydrochloride (ROCK inhibitor) should be included in the culture medium to a final concentration of 10 µM. To make up this medium, add 5.0 µl of 100 mM Y-27632 dihydrochloride to 50 ml of mouse prostate culture medium. CRITICAL The culture medium should not be stored for longer than 1 week at 4 °C.

**Human prostate culture medium** The final medium composition is summarized in Table 1. To make up the medium, combine 1.0 ml of B27, 500 µl of nicotinamide (1.0 M in PBS), 125.0 µl of N-acetyl-L-cysteine (500 mM in PBS), 0.5 µl of EGF (0.5 mg/ml in PBS + 0.1% BSA), 5.0 µl of A83-01 (5 mM in DMSO), 50.0 µl of Noggin (100 µg/ml in PBS + 0.1% (wt/vol) BSA), 50.0 µl of R-spondin 1 (500 µg/ml in PBS + 0.1% (wt/vol) BSA or 10% conditioned medium), 50.0 µl of dihydrotestosterone (1.0 µM in ethanol), 5.0 µl of FGF2 (50 µg/ml in PBS + 0.1% (wt/vol) BSA), 5.0 µl of FGF10 (0.1 mg/ml in PBS + 0.1% (wt/vol) BSA), 5.0 µl of prostaglandin E2 (10 mM in DMSO) and 16.7 µl of SB202190 (30 mM in DMSO). Top it up to 50 ml with adDMEM/F12 +/- (+/+, +). CRITICAL As detailed in the PROCEDURE, after cell passaging, Y-27632 dihydrochloride should be included in the culture medium to a final concentration of 10 µM. To make up this medium, add 5.0 µl of 100 mM Y-27632 dihydrochloride to 50 ml of human prostate culture medium. CRITICAL The culture medium should not be stored for longer than 1 week at 4 °C.

**Reverse transcription mix** For each reaction, combine 4.0 µl of GoScript reverse transcription mix (Promega, cat. no. A5003) and 1.0 µl of GoScript reverse transcriptase (Promega, cat. no. A5003) to prepare a 10,000× 1M stock solution for up to 1 month.

**Human EGF** Dissolve 500 µg in 5 ml of PBS + 0.1% (wt/vol) BSA to prepare a 10,000× 0.1 mg/ml stock solution for up to 1 month.

**N-acetyl-L-cysteine** Dissolve 81.5 mg/ml H₂O to prepare a 400× 500 mM stock solution for up to 1 month.

**R-spondin 1** Dissolve 500 µg in 5 ml of PBS + 0.1% (wt/vol) BSA to prepare a 10,000× 0.1 mg/ml stock solution for up to 1 month.

**Noggin** Dissolve 1.2 mg in 10 ml of PBS to prepare a 100× 1 mg/ml stock solution. Store aliquots at −20 °C until the expiration date.

**R-spondin 1 medium** Make up the medium as described in Box 1. N-acetyl-L-cysteine Dissolve 81.5 mg/ml H₂O to prepare a 400× 500 mM stock solution for up to 1 month.

**FGF-10** Dissolve 500 µg in 5 ml of PBS + 0.1% (wt/vol) BSA to prepare a 10,000× 0.1 mg/ml stock solution for up to 1 month.

**N-cysteine** Dissolve 1.2 mg in 10 ml of PBS to prepare a 100× 1 mg/ml stock solution. Store aliquots at −20 °C until the expiration date.

**Human EGF** Dissolve 1 mg in 2 ml of PBS + 0.1% (wt/vol) BSA to prepare a 10,000× 0.5 mg/ml stock solution for up to 1 month.

**N-EGF** Dissolve 100 µg in 1 ml of PBS + 0.1% (wt/vol) BSA to prepare a 1,000× 100 mM stock solution for up to 1 month.

**Y-27632 dihydrochloride** Dissolve 50 mg in 1.5 ml of H₂O to prepare a 10,000× 100 mM stock solution for up to 1 month.

**TRIRETinine** Dissolve 100 µg in 1 ml of PBS + 0.1% (wt/vol) BSA to prepare a 1,000× 100 mM stock solution for up to 1 month.
**SB202190** Dissolve 25 mg in 2.75 ml of DMSO to prepare a 30 mM 3,000× stock solution for up to 1 month.

**A83-01** Dissolve 10 mg in 950 µl of DMSO to obtain a 25 mM 50,000× stock solution for up to 1 month.

**FGF2** Dissolve 50 µg in 100 µl of 5 mM Tris, pH 7.6 (0.5 mg/ml). Dilute it to a 10,000× 50 µg/ml stock solution by adding 900 µl of PBS + 0.1% (wt/vol) BSA to 100 µl of 0.5 mg/ml solution for up to 1 month.

**PGK2** Dissolve 10 mg in 2.84 ml of DMSO to prepare a 10,000× 10 mM stock solution for up to 1 month.

**DHT** Dissolve 1 mg in 3.44 ml of 100% ethanol to obtain a 1000× 1 mM stock solution for up to 1 month.

**PGE2** Dissolve 50 µg in 2.75 ml of DMSO to prepare a 30,000× 50 µg/ml stock solution by adding 900 µl of 0.5 mg/ml solution for up to 1 month.

**A83-01** Dissolve 10 mg in 950 µl of DMSO to obtain a 25 mM 50,000× stock solution for up to 1 month.

**Collagenase type II** Dissolve 5 mg of collagenase type II in 1 ml of adDMEM/F12 +/+/+ to make a 5 mg/ml solution. Add Y-27632 dihydrochloride to a final concentration of 10 µM and dihydrotestosterone at a final concentration of 1 nM. Collagenase 5 mg/ml solution should be freshly prepared.

**Blocking solution** Add 2.5 ml of FBS to 47.5 ml of adDMEM/F12 +/+/+ to get a 5% (vol/vol) blocking solution. Add Y-27632 dihydrochloride to a final concentration of 10 µM and dihydrotestosterone at a final concentration of 1 nM. Blocking solution should be freshly prepared.

**Staining solution** Add 25.0 µl of FBS to 49.975 ml of adDMEM/F12 +/+/+ to get a 0.05% (vol/vol) blocking solution. Add Y-27632 dihydrochloride to a final concentration of 10 µM and dihydrotestosterone at a final concentration of 1 nM. Staining solution should be freshly prepared.

**PROCEDURE**

**Establishing and culturing prostate organoid cultures**

1. Prewarm tissue culture plates overnight at 37 °C.
2. If you are establishing mouse prostate organoid cultures, follow option A. For human prostate organoid cultures from human prostate tissue, follow option B. For organoids from prostate cancer metastasis biopsies, follow option C. For organoids from circulating prostate tumor cells, follow option D.

**(A) Establishing mouse prostate organoid cultures**

- (i) Euthanize a male mouse at <8 weeks of age, and then isolate the urogenital system (Fig. 1a).
- (ii) Remove the seminal vesicles by breaking or cutting the blood vessels and connective tissue and making an incision at the base of the urethra (Fig. 1b,c; for a detailed isolation protocol of the mouse prostate, see ref. 17).
- (iii) Remove the vas deferens by cutting it near the prostate (Fig. 1d).
- (iv) Remove the bladder by cutting it near the base of the urethra (Fig. 1e).
- (v) Remove the remaining vesicles and fat tissue by gently cutting (Fig. 1f).
- (vi) Remove the urethra; carefully pull the prostate lobes so that they are no longer attached to the urethra (Fig. 1g).

▲ **CRITICAL STEP** Do not isolate the ampullary gland, which is located between the two lobes of the anterior prostate (Fig. 1i,j) but is not considered to be part of the prostate.

- (vii) Isolate each lobe individually (anterior prostate, ventral prostate and dorsolateral prostate); alternatively, continue with the whole prostate (Fig. 1g,h,k).

- (viii) Use a scalpel to mince the whole prostate or prostate lobes into small pieces (~1 mm³) in a 10-cm culture dish.

- (ix) Digest the prostate in 5 mg/ml collagenase type II with 10 µM Y-27632 dihydrochloride in a 15-ml Falcon tube for 1–1.5 h at 37 °C on a shaking platform. Use 1 ml of 5 mg/ml collagenase type II per ~50 mg of minced tissue.

**Figure 1** Dissection of the mouse prostate. Overview of the procedure for isolating the prostate from the mouse urogenital system. Further information on how to isolate the mouse prostate can be found in the earlier protocol by Lukacs et al. (11). (a–e) Briefly, the seminal vesicles are removed (a–c), followed by removal of the vas deferens (d) and the bladder (e). (f–h) The prostate is cleaned (f) and the lobes are isolated and separated (g,h) before being treated with collagenase and TrypLE. (f,j) The ampullary gland (f and j; inset of i) should not be isolated, as it is not considered to be part of the prostate. (k) A schematic representation of the anatomy of the mouse prostate. AP, anterior prostate; DLP, dorsolateral prostate; VP, ventral prostate.
(x) Wash once by topping up to 10 ml with adDMEM/F12 +/-/+-.
(xi) Centrifuge the tube at 150g for 5 min at 4 °C.
(xii) Aspirate the supernatant and resuspend the pellet in 1 ml of TrypLE with 10 µM Y-27632 dihydrochloride, and then digest it for ~15 min at 37 °C.
   ▲ CRITICAL STEP Pipette up and down with a P1000 pipette every 5 min to ensure efficient digestion.
   ? TROUBLESHOOTING
(xiii) Wash once by topping up to 10 ml with adDMEM/F12 +/-/+-, and then centrifuge the tube at 150g for 5 min at 4 °C.
(xiv) Count the cells using a hemocytometer and plate 20,000 cells in a 40-µl drop in the middle of one well of a 24-well tissue culture plate (Fig. 2a and Table 2). On average, one whole prostate will yield 25 drops.
   ▲ CRITICAL STEP Work quickly to ensure that the Matrigel does not solidify before plating.
   ▲ CRITICAL STEP To ensure efficient plating, do not dilute the Matrigel too much. The average final percentage of Matrigel should be ~75%.
(xv) Place the tissue culture plate upside down in the CO₂ incubator (5% CO₂, 37 °C) for 15 min to allow the Matrigel to solidify.
   ▲ CRITICAL STEP The plate should be placed upside down in the incubator to prevent adherence to the plate bottom.
(xvi) Prewarm the mouse prostate culture medium in a 37 °C water bath for ~10 min before adding it to the organoids.
(xvii) Gently pipette 500 µl of prewarmed (37 °C) mouse prostate culture medium plus 10 µM Y-27632 dihydrochloride into each well, and then place the plate into the CO₂ incubator (5% CO₂, 37 °C).
(xviii) Refresh the medium with 10 µM Y-27632 dihydrochloride every 2–3 d. From 7 d after initial plating, medium without Y-27632 dihydrochloride should be used. Examples of the typical morphology of prostate organoids seen after 1 and 7 d of culture are shown in Figure 2b.

(B) Establishing human prostate organoid cultures ● TIMING up to 14 d
(i) Use a scalpel to mince human prostate tissue into small pieces (~1–5 mm³, Fig. 3) in a 10-cm culture dish.
(ii) Digest the tissue overnight in 5 mg/ml collagenase type II with 10 µM Y-27632 dihydrochloride in a 15-ml Falcon tube at 37 °C on a shaking platform. Use 1 ml of 5 mg/ml collagenase type II per ~50 mg of minced tissue.
(iii) Wash once by topping up to 10 ml with adDMEM/F12 +/-/+-.
(iv) Centrifuge the tube at 200g for 5 min at 4 °C.
(v) Resuspend the pellet in 1 ml of TrypLE with 10 µM Y-27632 dihydrochloride, and then digest it for ~15 min at 37 °C.
   ▲ CRITICAL STEP Pipette up and down every 5 min to ensure efficient digestion (P1000 pipette).
   ? TROUBLESHOOTING
(vi) Wash once by topping up to 10 ml with adDMEM/F12 +/-/+-.
(vii) Centrifuge the tube at 200g for 5 min at 4 °C.
(viii) Count the cells using a hemocytometer, and plate ~20,000 cells in a 40-µl drop in the middle of one well of a 24-well plate (Fig. 2a and Table 2).
   ▲ CRITICAL STEP Work quickly to ensure that the Matrigel does not solidify before plating.
   ▲ CRITICAL STEP To ensure efficient plating, do not dilute the Matrigel too much. The average final percentage of Matrigel should be ~75%.

Figure 2 | Establishment of mouse prostate organoid cultures. (a) Photo of a Matrigel disc in the correct position in a well of a 24-well tissue culture plate. (b) Representative pictures of organoids grown from mouse prostate tissue 1 and 7 d after plating. Mouse organoid procedures were performed in compliance with the institutional animal review committees at the Hubrecht Institute and Memorial Sloan Kettering Cancer Center (MSKCC). Scale bars, 100 µm.

| Plate     | Matrigel volume (µl) | Matrigel discs | Medium (µl) |
|-----------|----------------------|----------------|-------------|
| 96-well   | 10                   | 1              | 100         |
| 48-well   | 20                   | 1              | 250         |
| 24-well   | 40                   | 1              | 500         |
| 12-well   | 40                   | 3–5            | 1,000       |
| 6-well    | 40                   | 10–15          | 2,000       |
(ix) Place the plate into the CO₂ incubator (5% CO₂, 37 °C) for 15 min to allow the Matrigel to solidify.

▲ CRITICAL STEP Place the plate upside down in the incubator to prevent adherence to the plate bottom.

(x) Gently pipette 500 µl of prewarmed (~10 min in a 37 °C water bath) human prostate culture medium plus 10 µM Y-27632 dihydrochloride into each well, and place the plate into a CO₂ incubator (5% CO₂, 37 °C).

(xi) Replace the medium with medium containing 10 µM Y-27632 dihydrochloride every 2–3 d and continue to culture for 7 d.

(xii) At the next culture change after the initial 7 d of culture, replace the medium with medium that does not contain Y-27632 dihydrochloride. Proceed to the next step (organoid passage) 1–2 weeks after starting the culture.

▲ CRITICAL STEP Human organoids should be split at a 1:2 ratio every 1–2 weeks. The exact timing is dependent on the density and whether the organoids are derived from luminal or basal lineages. For instance, the day 7 basal-derived and the day 14 luminal-derived organoids depicted in Fig. 3 are of the size and density to be passaged.

(C) Establishing organoids from prostate cancer metastasis biopsies ● TIMING up to 14 d

(i) Use a scalpel to mince human advanced prostate cancer biopsy tissue (minimum size ~1 mm³) into small pieces (~1–5 mm³, Fig. 3b).

(ii) Digest the tissue in 5 mg/ml collagenase type II with 10 µM Y-27632 dihydrochloride in a 15-ml Falcon tube for 1 h at 37 °C. Use 1 ml of 5 mg/ml collagenase type II per ~50 mg of minced tissue.

(iii) Wash once with 10 ml of adDMEM/F12 +/+/+.

(iv) Centrifuge the tube at 200g for 5 min at 4 °C.

(v) Resuspend the pellet in 1 ml of TrypLE with 10 µM Y-27632 dihydrochloride, and then digest it for ~10 min at 37 °C.

▲ CRITICAL STEP Pipette up and down every 5 min to ensure digestion (P1000 pipette).

(vi) Wash once with 10 ml of adDMEM/F12 +/+/+.

(vii) Centrifuge the tube at 200g for 5 min at 4 °C.

(viii) Repeat the wash (Step 2C(vi,vii)).
(ix) Count the cells using a hemocytometer, and then plate ~50,000 cells in a 40-µl drop into the middle of one well of a 24-well plate (Fig. 2a and Table 2).

**A CRITICAL STEP** Work quickly to ensure that the Matrigel does not solidify before plating.

**A CRITICAL STEP** To ensure efficient plating, do not dilute the Matrigel too much. The average final percentage of Matrigel should be ~75%.

**A CRITICAL STEP** Seed the prostate cancer cells at high density (~50,000 cells in 40 µl of Matrigel).

(x) Place the dish into the CO₂ incubator (5% CO₂, 37 °C) for 15 min to allow the Matrigel to solidify.

**A CRITICAL STEP** Place the plate upside down in the incubator to prevent adherence to the plate bottom.

(xi) Gently pipette 500 µl of prewarmed (~10 min at 37 °C) human prostate culture medium plus 10 µM Y-27632 dihydrochloride into each well, and then place the plate into a CO₂ incubator (5% CO₂, 37 °C).

(xii) Replace the medium with medium containing Y-27632 dihydrochloride every 2–3 d, and check organoid growth using a light microscope. From 7 d after initial plating, medium without Y-27632 dihydrochloride should be used. After 5–7 d, small organoids should appear.

(xiii) Proceed to passaging organoids (Step 3) after ~14 d.

(D) Establishing organoids from circulating prostate tumor cells (CTCs) ● TIMING up to 14 d

(i) Collect 8 ml of blood from a patient with advanced prostate cancer.

**A CRITICAL STEP** The minimal total CTC number should be ~50 in 8 ml of blood. A CTC count can be performed using the Cellsearch circulating tumor cell kit (http://www.cellsearchctc.com).

(ii) Incubate the blood sample with 400 µl of RosetteSep human CD45 depletion cocktail for 20 min at room temperature (~20 °C).

(iii) Pipette 15 ml of Ficoll into a SepMate 50-ml tube.

(iv) Carefully add the blood to the Ficoll in the SepMate tube so that it forms a layer on top of the Ficoll.

(v) Centrifuge the tube at 1,200 g for 20 min at room temperature.

**A CRITICAL STEP** To keep layers intact, centrifuge without brake.

(vi) Collect the interphase with the desired cells into a 15-ml Falcon tube.

(vii) Aspirate and discard the supernatant.

(viii) Add 10 ml of PBS + 2% (vol/vol) FBS and mix by inverting the tube.

(ix) Pipette 15 ml of Ficoll into a SepMate 50-ml tube.

(x) Carefully add the blood to the Ficoll in the SepMate tube so that it forms a layer on top of the Ficoll.

(xi) Centrifuge the tube at 1,200 g for 20 min at room temperature.

**A CRITICAL STEP** To keep layers intact, centrifuge without brake.

(xii) Collect the interphase with the desired cells into a 15-ml Falcon tube.

(xiii) Add 10 ml of PBS + 2% (vol/vol) FBS and mix by inverting the tube.

(xiv) Centrifuge the tube at 200 g for 5 min at 4 °C.

(xv) Repeat the wash as described in Step 2D(vii,viii).

(xvi) Wash once by topping up to 10 ml with adDMEM/F12 +/+/+

**CRITICAL STEP** Work quickly to ensure that Matrigel does not solidify before plating.

**CRITICAL STEP** To ensure efficient plating, do not dilute the Matrigel too much. The average final percentage of Matrigel should be ~75%.

(xvii) Gently pipette 500 µl of ice-cold Matrigel and pipette them up and down 5–10 times to mix.

**CRITICAL STEP** Work quickly to ensure that Matrigel does not solidify before plating.

**CRITICAL STEP** To keep layers intact, centrifuge without brake.

(xviii) Collect 8 ml of blood from a patient with advanced prostate cancer.

(xix) Proceed to passaging organoids (Step 3) after ~14 d.

3 | If you are culturing mouse tissue, follow option A; if you are culturing human tissue, follow option B.

(A) Passaging of organoids derived from mouse tissue ● TIMING variable

(i) Collect organoids (~7 d after starting the culture; Fig. 2b) in the culture medium in the well containing the organoids, and transfer organoids from the same sample into one 15-ml tube.

**TROUBLESHOOTING**

(ii) Dissociate the organoids by trituration with a fire-polished glass pipette. The glass pipette should have an opening of ~0.5–1 mm in diameter after polishing.

**CRITICAL STEP** Trituration with a fire-polished glass pipette breaks down the organoids into small clumps of cells.

(iii) Pipette up and down 15–20 times.

(iv) Add 5 ml of ice-cold adDMEM/F12 +/+/+ to dissolve any residual Matrigel.

(v) Centrifuge the tube at 150g for 5 min at 4 °C.

(vi) Aspirate and discard the supernatant.

(vii) Resuspend the pellet in 160 µl of Matrigel and place a drop of 40 µl of Matrigel into the middle of one well of a 24-well plate (Table 2 and Fig. 2a), thus splitting each organoid culture at a 1:4 ratio.
(viii) Place the plate into the CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37 °C) for 15 min to allow the Matrigel to solidify.

▲ CRITICAL STEP Place the plate upside down in the incubator to prevent adherence to the plate bottom.

(ix) Gently pipette 500 µl of prewarmed (37 °C) mouse prostate culture medium into each well and place into a CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37 °C).

(x) Replace the medium every 2–3 d.

▲ CRITICAL STEP It is not necessary to add Y-27632 dihydrochloride to the medium at this stage of the culture. Y-27632 dihydrochloride is only required in the culture medium when organoids have been recently passaged using TrypLE. TrypLE treatment gives a high percentage of single cells, and Y-27632 dihydrochloride enhances outgrowth of single cells after plating.

(B) Passaging of organoids derived from human tissue ● TIMING variable

(i) Collect organoids in the culture medium in the well containing the organoids, and transfer organoids from the same sample into one 15-ml tube.

? TROUBLESHOOTING

(ii) Dissociate the organoids enzymatically using 0.5 ml of TrypLE containing 10 µM Y-27632 dihydrochloride on a shaking platform for 5 min at 37 °C.

▲ CRITICAL STEP The best method for splitting human organoids is with TrypLE. However, if the organoids are small, but the density is high, we do not recommend splitting with TrypLE, but instead we recommend using a fire-polished pipette as described in Step 3A for mouse organoids.

(iii) Inactivate the TrypLE by adding ~5 ml of adDMEM/F12 +/-+ containing 5% (vol/vol) FBS.

(iv) Centrifuge the tube at 200g for 5 min at 4 °C.

(v) Aspirate and discard the supernatant.

(vi) Resuspend the pellet in 80 µl of Matrigel. Plate 40-µl drops into the middle of each well of a 24-well plate, thus splitting each organoid culture at a 1:2 ratio (total two wells).

(vii) Place the dish into the CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37 °C) for 15 min to allow the Matrigel to solidify.

▲ CRITICAL STEP Place the plate upside down in the incubator during this incubation to prevent adherence to the plate bottom.

(viii) Gently pipette 500 µl of prewarmed (37 °C) human prostate culture medium plus 10 µM Y-27632 dihydrochloride into each well, and place the plate into a CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37 °C).

(ix) Replace the medium every 2–3 d. For the first 7 d, replace the medium with medium containing Y-27632 dihydrochloride. From 7 d onward, medium without Y-27632 dihydrochloride should be used.

Downstream assays

4) At appropriate time points during culture, cultures can be cryopreserved (option A), RNA can be isolated and cDNA produced (option B), or genomic DNA can be isolated (option C).

(A) Cryopreservation of prostate organoids ● TIMING 30 min

(i) Aspirate the medium from the Matrigel disc.

(ii) Use a P1000 pipette to resuspend the Matrigel disc in 1 ml of TrypLE plus 10 µM Y-27632 dihydrochloride.

(iii) Transfer the suspension to a 15-ml Falcon tube.

(iv) Incubate the tube for 5–10 min at 37 °C. If needed, pipette up and down to break up the organoid structures after incubation (using a P1000 pipette).

(v) Add 10 ml of ice-cold adDMEM/F12 +/-+.

(vi) Centrifuge the tube at 300g for 5 min at 4 °C.

(vii) Resuspend organoids from one well of a 24-well plate in 500 µl of Recovery cell culture freezing medium and transfer it into a 1.5-ml cryovial. Freeze the cells using a CoolCell freezing container (BioCision) or comparable freezing container at −80 °C.

■ PAUSE POINT Cells can be stored for up to 1 month at −80 °C. For long-term storage (>1 month), transfer the cryovial(s) to liquid nitrogen.

(viii) When you wish to defrost cells, thaw the cells in the cryovial in a 37 °C water bath.

(ix) Transfer the thawed cells into 15-ml Falcon tube and add 10 ml of adDMEM/F12 +/-+ dropwise to wash away freezing medium.

(x) Centrifuge the tube at 200g for 5 min at 4 °C.

(xi) Aspirate the supernatant and take up the cell pellet in the desired volume of Matrigel.

(xii) Plate the organoids as described in Step 3 above.

(B) RNA isolation and cDNA production from prostate organoids ● TIMING 2 h

(i) Aspirate the prostate culture medium from the Matrigel disc.
(ii) Collect the organoids (at least 50 µl of Matrigel containing organoids is required to obtain sufficient RNA for most applications) directly in 350 µl of RLT buffer (Qiagen) (addition of β-mercaptoethanol is preferable, but not essential).

(iii) Incubate the tube at room temperature for 15 min on a shaking platform.
(iv) Add 350 µl of 70% (vol/vol) ethanol and mix it by pipetting.
(v) Transfer the mixture to an RNeasy column (Qiagen).
(vi) Centrifuge for 30 s at 8,000g and discard the flow-through.
(vii) Add 700 µl of buffer RW1 (Qiagen).
(viii) Centrifuge for 30 s at 8,000g and discard the flow-through.
(ix) Add 500 µl of buffer RPE (Qiagen).
(x) Centrifuge for 30 s at 8,000g and discard the flow-through.
(xi) Add 500 µl of buffer RPE.
(xii) Centrifuge for 2 min at 8,000g and discard the flow-through.
(xiii) Place the column in a clean collection tube and centrifuge it for 1 min at full speed.
(xiv) Elute the RNA with 30 µl of RNase-free H₂O.

? TROUBLESHOOTING

(xv) Pipette 100–500 ng of RNA in an RNase-free 1.5-ml microcentrifuge tube.
(xvi) Add 1 µl of oligo(dT)₁₅, and bring the mixture to a total volume of 5 µl with RNase-free H₂O.
(xvii) Incubate the tube for 5 min at 70 °C and place it on ice.
(xviii) Add 15.0 µl of reverse transcription mix (see Reagent Setup) to the RNA/oligo(dT)₁₅ mix.
(xix) Perform reverse transcription using the following incubations:

| Step | Temperature | Time  |
|------|-------------|-------|
| 1    | 25 °C       | 5 min |
| 2    | 42 °C       | 60 min|
| 3    | 70 °C       | 15 min|

(xx) Use the cDNA product for specific (q-) RT-PCR analyses of interest.

(C) Genomic DNA isolation from organoids ● TIMING 90 min

(i) Collect the organoids (at least 50 µl of Matrigel containing organoids is required to obtain sufficient DNA for most applications) in the culture medium covering the organoids, and transfer them into a 1.5-ml microcentrifuge tube.

(ii) Centrifuge the tube for 5 min at 750g at 4 °C.
(iii) Aspirate the supernatant.
(iv) Resuspend the pellet in 160 µl of PBS.
(v) Add 20 µl of proteinase K solution (Promega).
(vi) Add 200 ml of cell lysis buffer (CLB; Promega) to the tube.
(vii) Mix by vortexing thoroughly.
(viii) Incubate the tube at 56 °C for 1 h; vortex every 15 min.
(ix) Add 20 µl of RNaseA solution (Promega).
(x) Mix by vortexing thoroughly.
(xi) Incubate the tube at 56 °C for 10 min.
(xii) Add 250 µl of binding buffer (BBA; Promega) and mix by vortexing.
(xiii) Transfer the solution to a ReliaPrep binding column (Promega).
(xiv) Centrifuge for 1 min at maximum speed, and discard the flow-through.
(xv) Add 500 µl of column wash solution (Promega) to the column.
(xvi) Centrifuge for 2 min at maximum speed, and discard the flow-through.
(xvii) Repeat Step 4C(xv,xvi) twice (steps performed three times in total).
(xviii) Place the column in a clean 1.5-ml microcentrifuge tube.
(xix) Add 50 µl of nuclease-free H₂O to elute the genomic DNA from the column.
(xx) Centrifuge for 1 min at 20,000g at room temperature. The flow-through contains genomic DNA, which can be stored at −20 °C.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.
This protocol describes an efficient method for establishing organoid cultures from mouse and human prostate tissue. The efficiency of establishing these cultures is >95%, although the efficiency of organoid establishment from advanced prostate cancers is substantially lower (~15–20%), mainly because of the small amount of input material. After sorting, basal cells have an organoid-forming capacity of ~70% (of which >95% are solid), whereas ~1–2% of sorted luminal cells give rise to organoids (>95% are cystic). To reach these efficiencies and to maintain growth, it is essential to use fresh medium (stored at 4 °C for no longer than 1 week) and to use effective and appropriately stored growth factors and chemical compounds. Prostate organoids can be genetically modified, and they can thus be used to study the involvement of genes in prostate homeostasis and cancer. We have not been successful in growing organoids derived from primary prostate cancers, most probably because of overgrowth by normal prostate epithelium present within each sample.

For mouse and human organoid culture, small organoids can usually be detected within 2–3 d after plating (Figs. 2b and 3a,b). Mouse organoids are generally cystic (Figs. 2b and 3a), whereas unsorted newly established human organoid cultures mainly consist of solid basal cell–derived organoids during the initial passages. After 5–7 d, small cystic organoids can be observed from sorted luminal cells (Fig. 3b). The morphology of organoids derived from advanced prostate cancers can vary greatly between patients (e.g., cystic and solid structures) and—because of tumor heterogeneity—even within cultures derived from the same patient.4

To confirm that the organoids are indeed derived from prostate tissue, expression of prostate-specific genes such as that encoding prostate-specific antigen (PSA) can be determined. Expression analysis of luminal-specific markers (e.g., the AR cytokeratin 8, cytokeratin 18, probasin, PSA) and basal-specific markers (e.g., p63, cytokeratin 5) should be used to confirm the presence of both lineages in the established cultures.

For samples derived from advanced prostate cancers, growth speed and morphology are highly variable (ranging from weekly 1:2–1:3 split ratios to monthly 1:2–1:3 split ratios). Confirmation of the cancerous origin of these organoids can be achieved by genomic analysis either by whole-genome sequencing or by comparative genomic hybridization4.

### TABLE 3 | Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|------|---------|----------------|----------|
| 2A(xii), 2B(v) | Large tissue pieces remain after digestion | Inefficient digestion | Increase the digestion time. The duration of the enzymatic digestion is variable and dependent on the initial tissue size. Pipette up and down with a fire-polished glass pipette after digestion. |
| 3A(i), 3B(i) | No, few or small organoids appear | Inadequate growth factor activity in the culture medium | Change the medium every 2–3 d. Make fresh medium to ensure that the growth factors in the culture medium are active. |
| 4B(xiv) | No or low yield from RNA isolation | The amount of organoids used as input was too low | Use more organoids for the isolation. |
| Box 2, step 16 | Cell suspension remains viscous after DNaseI addition | High concentration of genomic DNA in the suspension | Add more DNaseI to the suspension. |
| Box 2, step 17 | High percentage of contaminating (non-prostate) cells (e.g., immune cells) in the sorted population | The tissue was not properly dissected | Identify epithelial cells by co-staining for an epithelial marker (e.g., Epcam). Check the culture conditions used (non-epithelial cells should not grow under the described prostate culture conditions). |

**TIMING**

Steps 1–3, establishing and culturing prostate organoid cultures: variable; up to ~2 weeks
Step 4, downstream assays: variable
Box 1, preparation of R-spondin 1–conditioned medium: 10–11 d
Box 2, establishment of luminal cell– and basal cell–derived organoid cultures: 14 d

**ANTICIPATED RESULTS**

This protocol describes an efficient method for establishing organoid cultures from mouse and human prostate tissue. The efficiency of establishing these cultures is >95%, although the efficiency of organoid establishment from advanced prostate cancers is substantially lower (~15–20%), mainly because of the small amount of input material. After sorting, basal cells have an organoid-forming capacity of ~70% (of which >95% are solid), whereas ~1–2% of sorted luminal cells give rise to organoids (>95% are cystic). To reach these efficiencies and to maintain growth, it is essential to use fresh medium (stored at 4 °C for no longer than 1 week) and to use effective and appropriately stored growth factors and chemical compounds. Prostate organoids can be genetically modified, and they can thus be used to study the involvement of genes in prostate homeostasis and cancer. We have not been successful in growing organoids derived from primary prostate cancers, most probably because of overgrowth by normal prostate epithelium present within each sample.

For mouse and human organoid culture, small organoids can usually be detected within 2–3 d after plating (Figs. 2b and 3a,b). Mouse organoids are generally cystic (Figs. 2b and 3a), whereas unsorted newly established human organoid cultures mainly consist of solid basal cell–derived organoids during the initial passages. After 5–7 d, small cystic organoids can be observed from sorted luminal cells (Fig. 3b). The morphology of organoids derived from advanced prostate cancers can vary greatly between patients (e.g., cystic and solid structures) and—because of tumor heterogeneity—even within cultures derived from the same patient.

To confirm that the organoids are indeed derived from prostate tissue, expression of prostate-specific genes such as that encoding prostate-specific antigen (PSA) can be determined. Expression analysis of luminal-specific markers (e.g., the AR cytokeratin 8, cytokeratin 18, probasin, PSA) and basal-specific markers (e.g., p63, cytokeratin 5) should be used to confirm the presence of both lineages in the established cultures.

For samples derived from advanced prostate cancers, growth speed and morphology are highly variable (ranging from weekly 1:2–1:3 split ratios to monthly 1:2–1:3 split ratios). Confirmation of the cancerous origin of these organoids can be achieved by genomic analysis either by whole-genome sequencing or by comparative genomic hybridization.
Moreover, urogenital sinus mesenchyme (UGSM) recombination assays (described in detail in Xin et al.\(^{18}\)), whereby single prostate organoid cells are mixed with mesenchymal cells derived from the urogenital sinus of mouse embryos and placed under the kidney capsule, can be used to confirm that healthy or tumor organoids produce prostate glands or neoplastic growth in vivo, respectively.

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