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Organoid culture systems for prostate epithelial tissue and prostate cancer tissue

Jarno Drost^{1,2,#,*}, Wouter R. Karthaus^{3,*}, Dong Gao³, Else Driehuis^{1,2}, Charles L. Sawyers³, Yu Chen^{3,4,5}, and Hans Clevers^{1,2,#}

¹Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences (KNAW) and UMC Utrecht, 3584CT Utrecht, the Netherlands ²Cancer Genomics Netherlands, UMC Utrecht, 3584CG Utrecht, the Netherlands ³Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Centre, New York, NY 10065, USA ⁴Department of Medicine, Weill Cornell Medical College and New York-Presbyterian Hospital, New York, NY 10065, USA ⁵Department of Medicine, Memorial Sloan Kettering Cancer Centre, New York, NY 10065, USA

Summary

This protocol describes a recently developed strategy to generate 3D prostate organoid cultures from healthy mouse and human prostate (either bulk or FAC-sorted single luminal and basal cells), metastatic prostate cancer lesions and circulating tumour 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 tumour. The stepwise establishment of these cultures and the fully defined serum-free conditioned medium that is required to sustain organoid growth are outlined. Organoids established using this protocol can be used to study many different aspects of prostate biology, including homeostasis, tumorigenesis and drug discovery.

INTRODUCTION

The organoid technology holds great promise to study tissue homeostasis and cancer, but also for regenerative and personalized medicine¹. Since the establishment of culture conditions for mouse small intestine², similar culture protocols have been described for human small intestine and mouse and human colon, stomach, pancreas and liver^{3–9}. These organoids can grow “indefinitely”, remain phenotypically and genetically stable and can be genetically modified using multiple genome editing systems^{10–13}.

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#Correspondence to ; Email: j.drost@hubrecht.eu, ; Email: h.clevers@hubrecht.eu

*These authors contributed equally to this work.

Author contributions

J.D., W.R.K., Y.C., C.S. and H.C. conceived the study. J.D., W.R.K., D.G. and E.D. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Development of the protocol to culture mouse and human prostate organoids

The prostate is a gland of the male reproductive system that produces seminal fluid. The pseudostratified epithelium present in the prostate consists out of luminal, basal and rare neuroendocrine cells. Androgen receptor (AR) signalling is essential for prostate development and homeostasis, as well as for prostate cancer initiation and progression. As generally acknowledged^{14,15}, prostate (cancer) research has been hampered by the lack of suitable *in vitro* model systems. Although powerful *in vivo* models are available for prostate research, 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 signalling pathway, making them poor representatives of healthy prostate and prostate cancer tissue. The recent development of a testosterone-responsive prostate organoid culture system derived from primary prostate and advanced prostate cancer tissue^{16,17} opens new opportunities to study prostate homeostasis and cancer. This model was established by adapting and optimizing the culture conditions previously used to establish mouse and human small intestine and colon organoid cultures^{2,3}. Based on literature, we added different compounds and growth factors to the “generic” organoid medium (containing epidermal growth factor (EGF), Noggin and R-spondin 1; ref. 2) enabling us to establish culture conditions supporting long-term growth of mouse and human prostate tissue and advanced prostate cancers. Using this culture system, we have shown that 1) both the luminal and basal lineages harbour multipotent progenitor cells and can be propagated for long-term, 2) organoids functionally recapitulate AR signalling, 3) organoids derived from prostate cancer mouse models recapitulate mouse phenotypes, 4) human prostate cancer-derived organoids genetically and phenotypically mimic the tumour where they were derived from^{16,17}.

Comparison with other methods

Several other 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 (making androgen responsiveness limited in these cultures)^{18,21}. Moreover, these methods do not allow efficient growth of prostate cancer tissue. A protocol developed by Liu and colleagues enables indefinite growth of reprogrammed prostatic epithelial cells²². Interestingly, the Rho kinase inhibitor Y-27632 and the presence of feeder cells are essential in this system, possibly providing factors that are present in our defined prostate culture medium. Cells cultured under these conditions do not closely resemble the *in vivo* prostate, and androgen responsiveness is limited in this system. Finally, Chua et al. recently demonstrated a culture system that exclusively allows the growth of organoids from single luminal cells²³, albeit at lower plating efficiency than reported with our method (0.2 – 0.3% described by Chua et al. versus 1 – 2% when using our method¹⁶). Under these conditions, organoids that resemble the *in vivo* prostate are formed. However, basal cell-derived organoids cannot be propagated for a prolonged time. Additionally, in contrast to our method, their medium is not fully defined. Possibly, the medium’s undefined additions (e.g. fetal calf serum) contain growth factors present in our defined medium. It has not yet been explored whether prostate cancer can be propagated under these conditions.

MATERIALS

Reagents

Collagenase Type II (Life Technologies, cat. no. 17101-015)

TrypLE Express (Life Technologies, cat. no. 12605-010)

Dulbeccos Modified Eagle Medium (DMEM; Life Technologies, cat. no. 31966)

Advanced DMEM/F12 (adDMEM/F12; Life Technologies, cat. no. 12634-034)

GlutaMAX 100× (Life Technologies, cat. no. 35050-068)

Penicillin-streptomycin (Life Technologies, cat. no. 15140-122)

Hepes (Life Technologies, cat. no. 15630-056)

Zeocin (Life Technologies, cat. no. R250-01)

Phosphate buffered saline

Matrigel, Growth Factor Reduced (GFR), Phenol Red-free (BD, cat. no. 356231)

B27 supplement 50× (Life Technologies, cat. no. 17504-044)

Nicotinamide (Sigma-Aldrich, cat. no. N0636)

N-acetylcysteine (Sigma-Aldrich, cat. no. A9165)

A83-01 (Tocris Bioscience, cat. no. 2939)

Y-27632 (Abmole Bioscience, cat. no. M1817)

Human FGF-10 (PeproTech, cat. no. 100-26)

Human FGF-2 (PeproTech, cat. no. 100-18B)

Human EGF (PeproTech, cat. no. AF-100-15)

Recombinant human Noggin (Peprotech, cat. no. 120-10C)

R-spondin 1-conditioned medium; home made from the 293T-HA-RspoI-Fc cell line²⁴
(derived from Calvin Kuo lab), or recombinant R-spondin 1 protein (R&D Systems, cat. no. 4645-RS-025)

Prostaglandin E2 (Tocris Bioscience, cat. no. 2296)

SB202190 (Sigma-Aldrich, cat. no. S7076)

(DiHydro)Testosterone (5 α -Androstan-17 β -ol-3-one) (Sigma-Aldrich, cat. no. A8380)

Fetal bovine serum (Sigma-Aldrich, cat. no. F7524)

Deoxyribonuclease I (DNase I) from bovine pancreas (Sigma-Aldrich, cat. no. D5025)

DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride; Life Technologies, cat. no. D1306)

RosetteSep® Human CD45 Depletion Cocktail (Stem Cell Technologies, cat. no. 15122)

Ficoll-Paque™ PLUS (GE Healthcare Life Sciences, cat. no. 17-1440-02)

Recovery Cell Culture Freezing medium (Life Technologies, cat. no. 12648-010)

RNeasy mini kit (Qiagen, cat. no. 74104)

Reliaprep gDNA tissue miniprep system (Promega, cat. no. A2052)

GoScript Reverse Transcriptase (Promega, cat. no. A5003)

Oligo(dT) 15 Primer (Promega, cat. no. C1101)

Rec. RNasin RNase Inhibitor (Promega, cat. no. N2511)

Mouse and human material

Whole mouse prostate

Human prostate tissue piece (minimum size 1 mm³)

Human metastasis biopsy (minimum size 1 mm³)

Blood sample from patient with advanced prostate cancer (8 ml)

CRITICAL STEP Although it is preferred to use fresh material, we have been able to establish organoids from tissue that was stored overnight at 4°C in addMEM/F12 (containing penicillin/streptomycin, 10 mM Hepes and GlutaMAX 100× diluted).

Antibodies

CD26-FITC conjugated antibody (anti-human 1:200, M-A261, eBioscience)

CD49f-alexa 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 647 conjugated antibody (anti-mouse 1:200, 30-F1, eBioscience)

EQUIPMENT

Falcon tubes 15 ml

Falcon tubes 50 ml

5 ml polystyrene round-bottom tube with cell-strainer caps (Falcon)

Microcentrifuge tubes, 1.5 ml

37°C shaking platform

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)

Glasstic Slide with hemocytometer counting grid (Kova 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 (DaKo MoFlo)

Disposable scalpels (Swann-Morton, code 0501)

Centrifuge (Eppendorf, 5810R)

Centrifuge (Eppendorf, 5424)

CO₂ incubator

Biosafety cabinet

CoolCell (BioCision)

Stericup-GP, 0.22 µm, polyethersulfone, 500 mL, radio-sterilized (Millipore, cat. no. SCGPU05RE)

REAGENT SETUP

Mouse prostate culture medium

Add 1.0 ml B27, 125.0 µl N-acetylcysteine (500 mM in PBS), 5.0 µl of EGF (0.5 mg/ml in PBS + 0.1% BSA), 2.0 µl A83-01 (5 mM in DMSO), 50.0 µl Noggin (100 µg/ml in PBS + 0.1% BSA), 50.0 µl R-spondin 1 (500 µg/ml in PBS + 0.1% BSA or 10% conditioned medium), 50.0 µl dihydrotestosterone (1 µM in ethanol) and top up to 50 ml with adDMEM/F12 (containing penicillin/streptomycin, 10 mM HEPES and GlutaMAX 100×

diluted). After passaging, Y-27632 is added to the culture medium (e.g. add 5.0 μ l of 100 mM to 50 ml mouse prostate culture medium).

Human prostate culture medium

Add 1.0 ml B27, 500 μ l nicotinamide (1 M in PBS), 125.0 μ l N-acetylcysteine (500 mM in PBS), 0.5 μ l of EGF (0.5 mg/ml in PBS + 0.1% BSA), 5.0 μ l A83-01 (5 mM in DMSO), 50.0 μ l Noggin (100 μ g/ml in PBS + 0.1% BSA), 50.0 μ l R-spondin 1 (500 μ g/ml in PBS + 0.1% BSA or 10% conditioned medium), 50.0 μ l dihydrotestosterone (1 μ M in ethanol), 5.0 μ l FGF2 (50 μ g/ml in PBS + 0.1% BSA), 5.0 μ l FGF10 (0.1 mg/ml in PBS + 0.1% BSA), 5.0 μ l prostaglandin E2 (10 mM in DMSO), 16.7 μ l SB202190 (30 mM in DMSO) and top up to 50 ml with adDMEM/F12 (containing penicillin/streptomycin, 10 mM Hepes and GlutaMAX 100 \times diluted). After passaging, Y-27632 is added to the culture medium (e.g. add 5.0 μ l of 100 mM to 50 ml human prostate culture medium).

CRITICAL STEP The culture media should not be stored for longer than 2 weeks at 4°C.

CRITICAL STEP We have never seen any difference in organoid establishment, maintenance and morphology when using prostate culture medium containing either R-spondin 1-conditioned medium or recombinant R-spondin 1.

Preparation and storage of growth factor stocks

R-spondin 1 medium	See Box 1
N-Acetylcysteine	Dissolve 81.5 mg per ml H ₂ O to prepare a 400 \times 500mM stock solution. Store at -20°C.
FGF-10	Dissolve 500 μ g in 5 ml PBS + 0.1% BSA to prepare a 10000 \times 0.1 mg/ml stock solution. Store at -20°C.
Nicotinamide	Dissolve 1.2 g in 10 ml PBS to prepare a 100 \times 1M stock solution. Store at -20°C.
Human EGF	Dissolve 1 mg in 2 ml PBS + 0.1% BSA to prepare a 10.000 \times 0.5mg/ml stock solution. Store at -20°C.
Rec human Noggin	Dissolve 100 μ g in 1 ml of adDMEM/F12 (containing penicillin/streptomycin, 10 mM Hepes and GlutaMAX (adDMEM/F12 +/+)) to prepare a 1000 \times stock solution. Store at 4°C.
Y-27632	Dissolve 50 mg in 1.5 ml H ₂ O to prepare a 10.000 \times 100mM stock solution. Store at -20°C.
SB202190	Dissolve 25 mg in 2.75 ml DMSO to prepare a 30mM 3000 \times stock solution. Store at -20°C.
A83-01	Dissolve 10 mg in 950 μ l DMSO to get a 25mM 50000 \times stock solution. Store at -20°C.
B27	Provided as 50 \times stock solution. Store at -20°C.
FGF2	Dissolve 50 μ g in 100 μ l 5 mM Tris pH 7.6 (0.5 mg/ml). Dilute to a 10000 \times 50 μ g/ml stock solution by adding 900 μ l of PBS + 0.1% BSA to the 100 μ l 0.5 mg/ml solution. Store at -20°C.
PGE2	Dissolve 10 mg in 2.84 ml DMSO to prepare a 10000 \times 10mM stock solution. Store at -20°C.
DHT	Dissolve 1 mg in 3.44 ml 100% ethanol to obtain a 1 mM solution. Dilute 1000 \times in 100% ethanol to prepare a 1000 \times 1 μ M stock solution.

Store at -20°C .

Collagenase Type II

Dissolve 5 mg Collagenase Type II in 1 ml of adDMEM/F12 +/+/+ to make a 5 mg/ml solution. Add Y-27632 to a final concentration of 10 μM and dihydrotestosterone at a final concentration of 1 nM.

CRITICAL STEP Collagenase 5 mg/ml solution is freshly prepared.

Blocking solution

Add 2.5 ml FBS to 47.5 ml of adDMEM/F12 +/+/+ to get a 5% blocking solution. Add Y-27632 to a final concentration of 10 μM and dihydrotestosterone at a final concentration of 1 nM.

Staining solution

Add 25.0 μl FBS to 49.975 ml of adDMEM/F12 +/+/+ to get a 0.05% blocking solution. Add Y-27632 to a final concentration of 10 μM and dihydrotestosterone at a final concentration of 1 nM.

PROCEDURE

Establishment of mouse prostate organoid cultures (timing 2.5 h)

- 1 Sacrifice male mouse at minimally 8 weeks of age (maximum tested 2 years).
- 2 Isolate the urogenital system (Fig. 1a I).
- 3 Remove seminal vesicles by breaking/cutting blood vessels and connective tissue and making an incision at the base of the urethra (Fig. 1a II, III; for a detailed isolation protocol of the murine prostate see²⁵).
- 4 Remove the vas deferens by cutting it near the prostate (Fig. 1a IV).
- 5 Remove the bladder by cutting it near the base of the urethra (Fig. 1a V).
- 6 Remove remaining vesicles and fat tissue by gentle cutting (Fig. 1a VI).
- 7 Remove urethra; carefully pull the prostate lobes, so they are no longer attached to the urethra (Fig. 1a VII).

CRITICAL STEP The ampullary gland is not considered part of the prostate. The gross anatomy is very similar to prostate. The ampullary gland is located between the two lobes of the anterior prostate (Fig. 1a IX, b). Do not isolate this part.

- 8 Isolate each lobe individually (anterior prostate (AP), ventral prostate (VP), dorsolateral prostate (DLP)), or continue with the whole prostate (Fig. 1a VII, VIII, 1b).
- 9 Mince the prostate (lobes) in small pieces (~ 1 mm³) in a 10 cm culture dish using a scalpel.
- 10 Digest the prostate in 5 mg/ml Collagenase II with 10 μM Y-27632 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 II per ~ 50 mg minced tissue.
- 11 Wash once by topping up to 10 ml with adDMEM/F12 +/+/+.
- 12 Centrifuge at 150 g for 5 min at 4°C.
- 13 Aspirate supernatant and resuspend pellet in 1 ml TrypLE with 10 μM Y-27632 and digest for approximately 15 min at 37°C.
- CRITICAL STEP** Pipet up and down with a P1000 pipet every 5 min to ensure efficient digestion.
- TROUBLESHOOTING**
- 14 Wash once by topping up to 10 ml with adDMEM/F12 +/+/+ and centrifuge at 150 g for 5 min at 4°C.
- 15 Aspirate supernatant and place digested tissue in ice-cold Matrigel (Matrigel protein concentration ~75%). Pipette up and down 5 – 10 times to mix.
- CRITICAL STEP** Work quickly to ensure that Matrigel does not solidify before plating.
- CRITICAL STEP** Do not dilute the Matrigel too much to ensure efficient plating.
- 16 Count cells using hemocytometer and plate 20,000 cells in a 40 μl drop in the middle of one well of a 24-well dish (Fig. 1c, Table 2). On average one prostate will yield 25 drops.
- CRITICAL STEP** Tissue culture plates should be pre-warmed (overnight at 37°C).
- 17 Place the dish into the 37°C incubator 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.
- 18 Gently pipette 500 μl of pre-warmed (37 °C) mouse prostate culture medium plus 10 μM Y-27632 into each well.
- 19 Refresh medium every 2 – 3 days.
- 20 After 7 days Y-27632 can be removed from the medium.

Passaging mouse prostate organoid cultures (timing 30 min)

- 21 After approximately 7 days (Fig. 1d), harvest organoids (e.g. 1 well 24-well dish) in the remaining culture medium and transfer to a 15 ml Falcon tube.

TROUBLESHOOTING

- 22 Dissociate organoids by trituration with a fire-polished glass pipette. The glass pipette should have an opening of about 0.5 – 1 mm after polishing.

- 23 Pipet up and down 15 – 20 times.

- 24 Add 5 ml ice-cold adDMEM/F12 +/+ to dissolve residual Matrigel.

- 25 Centrifuge at 150 g for 5 min at 4°C.

- 26 Aspirate supernatant.

- 27 Resuspend pellet in 160 µl Matrigel (split ratio 1:4) and plate a drop of 40 µl Matrigel into the middle of one well of a 24-well dish (Table 2).

- 28 Place the dish into the 37°C incubator 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.

- 29 Gently pipette 500 µl of pre-warmed (37 °C) mouse prostate culture medium into each well. Trituration with a fire-polished glass pipette breaks down organoids into clumps of cells (TrypLE treatment gives high percentage of single cells). Y-27632 enhances outgrowth of single cells after plating. Therefore, addition of Y-27632 to the culture medium is only required when organoids are passaged using TrypLE.

- 30 Refresh medium every 2 – 3 days.

Establishment of human prostate organoid cultures (timing 20 h)

- 31 Mince human prostate tissue in small pieces (~ 1 – 5 mm³, Fig. 2b) in a 10 cm culture using a scalpel.

- 32 Digest the tissue overnight in 5 mg/ml Collagenase II with 10 µM Y-27632 in a 15 ml Falcon tube at 37°C on a shaking platform. Use 1 ml of 5 mg/ml Collagenase II per ~ 50 mg minced tissue.

- 33 Wash once by topping up to 10 ml with adDMEM/F12 +/+.

- 34 Centrifuge at 200 g for 5 min at 4°C.

- 35 Resuspend pellet in 1 ml TrypLE with 10 μ M Y-27632 and digest for approximately 15 min at 37°C.
CRITICAL STEP Pipet up and down every 5 min to ensure efficient digestion (P1000 pipet).
- 36 Wash once by topping up to 10 ml with adDMEM/F12 +/-/+.
- 37 Centrifuge at 200 g for 5 min at 4°C.
- 38 Aspirate supernatant and place digested tissue in ice-cold Matrigel and pipette up and down 5 – 10 times to mix.
CRITICAL STEP Work quickly to ensure that Matrigel does not solidify before plating.
CRITICAL STEP Do not dilute the Matrigel too much to ensure efficient plating.
- 39 Count cells using hemocytometer and plate approximately 20,000 cells in a 40 μ l drop in the middle of one well of a 24-well dish.
CRITICAL STEP Tissue culture plates should be pre-warmed (overnight at 37°C).
- 40 Place the dish into the 37°C incubator 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.
- 41 Gently pipette 500 μ l of pre-warmed (37 °C) human prostate culture medium plus 10 μ M Y-27632 into each well.
- 42 Refresh medium every 2 – 3 days.
- 43 After 7 days, remove Y-27632 from the medium.
CRITICAL STEP Human organoids are split 1:2 every 1 – 2 weeks. This is dependent on density and whether the organoids are luminal- or basal-derived. For instance, the day 7 basal-derived and the day 14 luminal-derived organoids depicted in Fig. 2b are of the size and density to be passaged. Preferred method of splitting for human organoids is with TrypLE. If organoids are small, but the density is high, do not split with TrypLE, but instead use a fire-polished pipette as described in step 22, 23.

Passaging human prostate organoid cultures (timing 30 min)

- 44 After approximately 14 days, harvest organoids (e.g. 1 well 24-well dish) in the remaining culture medium and transfer to a 15 ml Falcon tube.

TROUBLESHOOTING

- 45 Dissociate organoids enzymatically using TrypLE with 10 μ M Y-27632 on a shaking platform for 5 min at 37 °C.
- 46 Inactivate TrypLE by adding adDMEM/F12 +/+/+ containing 5% FBS.
- 47 Centrifuge at 200 g for 5 min at 4°C.
- 48 Aspirate supernatant.
- 49 Resuspend pellet in 80 μ l Matrigel (split ratio 1:2) and plate 40 μ l drops into the middle of one well of a 24-well dish (total 4 wells).
- 50 Place the dish into the 37°C incubator 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.
- 51 Gently pipette 500 μ l of pre-warmed (37 °C) human prostate culture medium plus 10 μ M Y-27632 into each well.
- 52 Refresh medium every 2 – 3 days. After 7 days, remove Y-27632 from the medium.

Establishment of luminal and basal derived cultures (timing 4 h)

- 53 Enzymatically digest mouse (Fig. 2a) or human (Fig. 2b) prostate tissue as described in step 1 – 13 and step 31 – 35, respectively.
- 54 Wash digested single cells in 10 ml blocking solution in a 15 ml Falcon tube.
- 55 Centrifuge at 200 g for 5 min at 4°C.
- 56 Aspirate supernatant.
- 57 Resuspend cells in 1 ml blocking solution.
- 58 Pass resuspended cells through cell-strainer of a polystyrene round-bottom tube.
- 59 Block cells in blocking solution on ice for 15 min.
- 60 Centrifuge at 200 g for 5 min at 4°C and aspirate supernatant.
- CRITICAL STEP** Keep cells aside (about one tenth of total) for unstained negative control and single color staining controls for FACS. Use the rest for the staining protocol as described from step 61.
- 61 Stain in 500 μ l staining solution for 60 min on ice and in the dark with the following antibodies:
- a. Human
 - i. CD26-FITC conjugated antibody (M-A261, 1:200)

ii. CD49f-alexa 647 conjugated antibody (GoH3, 1:200)

b. Mouse

i. CD24-alexa 647 conjugated antibody (30-F1, 1:200)

ii. CD49f-PE conjugated antibody (GoH3, 1:200)

62 Wash twice with 10 ml addMEM/F12 +/-/+ plus Y-27632 (10 μ M) plus dihydrotestosterone (1 nM).

63 Centrifuge at 200 g for 5 min at 4°C.

64 Resuspend cells in staining solution (no antibody added)

65 Add DAPI (1.0 μ g/ml final concentration).

66 Add DNaseI (0.5 – 1.0 Units/ μ l final concentration).

TROUBLESHOOTING

67 Isolate cells using FACS (Fig. 2). On average, 200 mg of prostate tissue gives approximately 150,000 luminal cells and 600,000 basal cells.

68 Plate cells as described in step 16 – 18 and step 39 – 41 (Fig. 2).

TROUBLESHOOTING

Establishing organoids from prostate cancer metastasis biopsies (timing 2h)

69 Mince human advanced prostate cancer biopsy tissue (minimum size ~ 1 mm³) in small pieces (~ 1 – 5 mm³, Fig. 2b) using a scalpel.

70 Digest the tissue in 5 mg/ml Collagenase II with 10 μ M Y-27632 in a 15ml Falcon tube for 1 h at 37°C. Use 1 ml of 5 mg/ml Collagenase II per ~ 50 mg minced tissue.

71 Wash once with 10 ml addMEM/F12 +/-/+.

72 Centrifuge at 200 g for 5 min at 4°C.

73 Resuspend pellet in 1 ml TrypLE with 10 μ M Y-27632 and digest for approximately 10 min at 37°C.

CRITICAL STEP Pipet up and down every 5 min to ensure digestion (P1000 pipet).

74 Wash once with 10 ml addMEM/F12 +/-/+.

75 Centrifuge at 200 g for 5 min at 4°C.

76 Repeat wash (step 74–75).

77 Place digested tissue in ice-cold Matrigel and pipette up and down 5 – 10 times to mix.

- CRITICAL STEP** Work quickly to ensure that Matrigel does not solidify before plating.
- CRITICAL STEP** Do not dilute the Matrigel too much to ensure efficient plating.
- 78** Count cells using hemocytometer and plate approximately 50,000 cells in a 40 µl drop into the middle of one well of a 24-well dish (Table 2).
- CRITICAL STEP** Tissue culture plates should be pre-warmed (overnight at 37°C).
- CRITICAL STEP** Seed the prostate cancer cells at high density.
- 79** Place the dish into the 37°C incubator 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.
- 80** Gently pipette 500 µl of pre-warmed (37 °C) human prostate culture medium plus 10 µM Y-27632 into each well.
- 81** Refresh medium every 2 – 3 days and check organoid growth using a light microscope.
- CRITICAL STEP** Keep 10 µM Y-27632 in the medium.
- 82** Keep Y-27632 in the medium until the first passage of the prostate cancer organoids.

Establishing and maintenance of circulating prostate tumour cells (CTC)

- 83** Collect 8 ml blood from patient with advanced prostate cancer.
- CRITICAL STEP** Total CTC number should be ~ 50 in 8ml blood (CTC count performed in clinic using cell search circulating tumor cell kit (www.cellsearchctc.com)).
- 84** Incubate blood with 400 µl RosetteSep® Human CD45 Depletion Cocktail for 20 min at room temperature.
- 85** Deplete red and white blood cells using Ficoll-Paque.
- 86** Wash once with adMEM/F12 +/-/+.
- 87** Place cells in 30 µl ice-cold Matrigel and pipette up and down 5 – 10 times to mix.
- CRITICAL STEP** Work quickly to ensure that Matrigel does not solidify before plating.
- CRITICAL STEP** Do not dilute the Matrigel too much to ensure efficient plating.

- 88** Plate circulating tumor cells in a 30 μ l drop into the middle of one well of a 24-well dish.
- CRITICAL STEP** Tissue culture plates should be pre-warmed (overnight at 37°C).
- 89** Place the dish into the 37°C incubator 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.
- 90** Gently pipette 500 μ l of pre-warmed (37 °C) human prostate culture medium plus 10 μ M Y-27632 into each well.
- 91** Refresh medium every 2 – 3 days and check organoids grow every 2 days.
- CRITICAL STEP** Keep 10 μ M Y-27632 in the medium.
- 92** Keep Y-27632 in the medium, until the first passage of the prostate cancer organoids.

Cryopreservation of mouse and human prostate organoids (timing 30 min)

- 93** Aspirate medium from Matrigel disc.
- 94** Resuspend the Matrigel disc in 1 ml of TrypLE using P1000 pipette.
- 95** Transfer suspension to a 15 ml Falcon tube.
- 96** Incubate 5–10 min at 37 °C. If needed pipet up and down to break up organoid structures after incubation.
- 97** Add 10 ml of ice-cold addMEM/F12 +/+/+.
- 98** Centrifuge at 300 g for 5 min at 4 °C.
- 99** Resuspend one 24-well of organoids in 500 μ l of Recovery Cell Culture Freezing medium. Freeze cells using a CoolCell (BioCision) or comparable method.

To verify that the organoids are indeed derived from prostate tissue, (Q-) RT-PCR can be performed for expression of prostate-specific genes (see Anticipated Results and ref. ¹⁶). Additionally, to confirm the growth of prostate tumor organoids, WGS can be performed to analyze mutation spectra ¹⁷. Below we describe the procedures to isolate RNA, produce cDNA and isolate genomic DNA from organoids.

RNA isolation and cDNA production from prostate organoids

- 100** Aspirate prostate culture medium from Matrigel disc.
- 101** Harvest organoids (at least 50 μ l of Matrigel) directly in 350 μ l RLT buffer (addition of β -mercaptoethanol is not essential).
- 102** Incubate at room temperature for 15 min on a shaking platform.

- 103** Add 350 μ l 70% ethanol and mix by pipetting.
- 104** Transfer mixture to a RNeasy column (Qiagen).
- 105** Centrifuge for 30 s at 8000 g and discard flow-through.
- 106** Add 700 μ l Buffer RW1.
- 107** Centrifuge for 30 s at 8000 g and discard flow-through.
- 108** Add 500 μ l Buffer RPE.
- 109** Centrifuge for 30 s at 8000 g and discard flow-through.
- 110** Add 500 μ l Buffer RPE.
- 111** Centrifuge for 2 min at 8000 g and discard flow-through.
- 112** Place column in clean collection tube and centrifuge for 1 min at full speed.
- 113** Elute RNA with 30 μ l RNase-free H₂O.

TROUBLESHOOTING

Components	Amount (μ l)
RNA from step 113 (100 – 500 ng)	×
Oligo(dT) ₁₅	1.0
RNase-free H ₂ O	to 5.0

- 114** Incubate for 5 min at 70°C and place tube on ice

Components	Amount (μ l)
GoScript 5 \times Reaction Buffer	4.0
25mM MgCl ₂	4.0
10mM PCR Nucleotide Mix	1.0
Rec RNasin Ribonuclease Inhibitor	0.5
GoScript Reverse Transcriptase	1.0
RNase-free H ₂ O	to 15.0

- 115** Add 15.0 μ l reverse transcription mix to 5.0 μ l RNA/oligo(dT) mix
- 116** Perform reverse transcription using following incubations:

Step	Temperature	Time
1	25°C	5 min
2	42°C	60 min

Step	Temperature	Time
3	70°C	15 min

- 117 Produced cDNA can be used for subsequent (Q-) RT-PCR using standard protocols.

Genomic DNA isolation from organoids

- 118 Harvest organoids (at least 50 µl of Matrigel) in culture medium and transfer into 1.5 ml microcentrifuge tube.
- 119 Centrifuge 5 min at 3000 rpm.
- 120 Aspirate supernatant.
- 121 Resuspend pellet in 160 µl PBS.
- 122 Add 20 µl Proteinase K (PK) solution.
- 123 Mix by vortexing thoroughly.
- 124 Incubate 56°C for 1 h; vortex every 15 min.
- 125 Add 20 µl RNaseA solution.
- 126 Mix by vortexing thoroughly.
- 127 Incubate 56°C for 10 min.
- 128 Add 250 µl Binding Buffer (BBA) and mix by vortexing.
- 129 Transfer solution to a ReliaPrep Binding Column.
- 130 Centrifuge for 1 min at maximum speed and discard flow-through.
- 131 Add 500 µl of Column Wash Solution (CWD) to the column.
- 132 Centrifuge 2 min at maximum speed and discard flow-through.
- 133 Repeat step 131 and 132 for a total of 3 times.
- 134 Place the column in a clean 1.5 ml microcentrifuge tube
- 135 Add 50 µl of nuclease-free H₂O to elute genomic DNA from the column
- 136 Centrifuge for 1 min at maximum speed. Flow-through contains genomic DNA, which should be stored at -20°C (long-term).

TROUBLESHOOTING

Step	Problem	Possible reasons	Solution
13	Big tissue pieces remaining after digestion	Inefficient digestion	Increase digestion time. The duration of the enzymatic digestion is variable and dependent on the initial tissue size. Pipet up and down with a fire-

Step	Problem	Possible reasons	Solution
			polished glass pipette after digestion.
21 + 44	No/few/small organoids appearing	Inactive/less active growth factors in culture medium. No ROCK inhibitor (Y-27632) added to medium.	Change medium every 2 – 3 days. Make fresh medium to ensure that the growth factors in the culture medium are active. Absence of the ROCK inhibitor (Y-27632) will greatly decrease the efficiency of organoid outgrowth.
66	Cell suspension remains viscous after DNaseI addition	Presence of high concentration of genomic DNA in the suspension.	Add more DNaseI to the suspension.
68	High percentage of contaminating (non-prostate) cells (e.g. immune cells) in sorted population	The tissue was not properly dissected.	Sort out epithelial cells by co-staining for an epithelial marker (e.g. Epcam). Moreover, non-epithelial cells do not grow under the described prostate culture conditions.
113	No/low yield from RNA isolation	Amount of organoids used as input was too low. Organoids were not efficiently lysed.	Use more organoids for the isolation. Snap-freeze samples in liquid nitrogen after step 102 for more efficient lysis

ANTICIPATED RESULTS

The protocol describes an efficient method for establishing organoid cultures from mouse and human prostate tissue. The efficiency of establishing these cultures is > 95%. The efficiency of organoid establishment from advanced prostate cancers is significantly lower (~15 – 20%) mainly because of the small amount of input material. After sorting, basal cells have an organoid-forming capacity of approximately 70% (of which >95% are solid), whereas approximately 1 – 2% of sorted luminal cells give rise to organoids (>95% are cystic). To reach these efficiencies and to be able to maintain growth “indefinitely” it is essential to use medium that is not stored for more than two weeks and to use well-tested and stored growth factors and chemical compounds. Prostate organoids can be genetically modified^{10,16} and 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 due to overgrowth by normal prostate epithelium present within each sample.

For mouse and human organoid culture protocol, small organoids can be detected within 2 – 3 days after plating. Mouse organoids are generally cystic, whereas unsorted newly established human organoid cultures will mainly consist out of solid basal cell-derived organoids during the initial passages. After 5 – 7 days, small cystic organoids can be observed from sorted luminal cells. The morphology of organoids derived from advanced prostate cancer patients can vary greatly between patients and – due to tumour heterogeneity – even within cultures derived from the same patient¹⁷.

To confirm that the organoids are indeed derived from prostate tissue, expression of prostate-specific genes like prostate specific antigen (PSA) can be determined. Luminal-specific

marker (androgen receptor, cytokeratin 8, cytokeratin 18, Probasin, PSA) and basal-specific marker (p63, cytokeratin 5) expression analysis will confirm the presence of both lineages in the established cultures.

For samples derived from advanced prostate cancer, growth speed and morphology are highly variable. Confirmation of the cancerous origin of the organoids can be achieved by genomic analysis either by whole genome sequencing or comparative genomic hybridization. Moreover, urogenital sinus mesenchyme (UGSM) recombination assays (described in detail in ref. ²⁶), where single prostate organoid cells can be mixed with mesenchymal cells derived from the urogenital sinus of mouse embryos and placed under the kidney capsule, can be performed to confirm that healthy or tumour organoids can produce prostate glands or neoplastic growth *in vivo*, respectively.

Human material for organoid cultures

Approval for this study was obtained by the ethics committee of the University Medical Centre Utrecht and Memorial Sloan-Kettering Cancer Center Institutional Review Board. All patients provided informed consent.

Mouse material for organoid cultures

All procedures were performed in compliance with local animal welfare laws and guidelines.

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BOX 1**Preparation of R-spondin 1-conditioned medium**

1. Culture 293T-HA-RspoI-Fc cell line²⁴ in DMEM + 10% FBS + penicillin/streptomycin + zeocin (300 µg/ml) in 175 cm² flasks until confluency.
2. Passage confluent flask (split ratio ~ 1:6) and grow cells in DMEM + 10% FBS + penicillin/streptomycin, without zeocin.
3. When cells reach confluency (after 3 – 4 days), replace DMEM + 10% FBS + penicillin/streptomycin medium with addMEM/F12 +/-/+.
4. After one week, harvest the medium in 50 ml Falcon tubes.
5. Centrifuge at 1500 rpm for 5 min.
6. Pass the medium through a Stericup-GP, 0.22 µm filter.

CRITICAL STEP Step 5 and 6 are included to remove any 293T cells from the R-spondin 1-conditioned medium.

7. Store the R-spondin 1-conditioned medium at –20 °C.

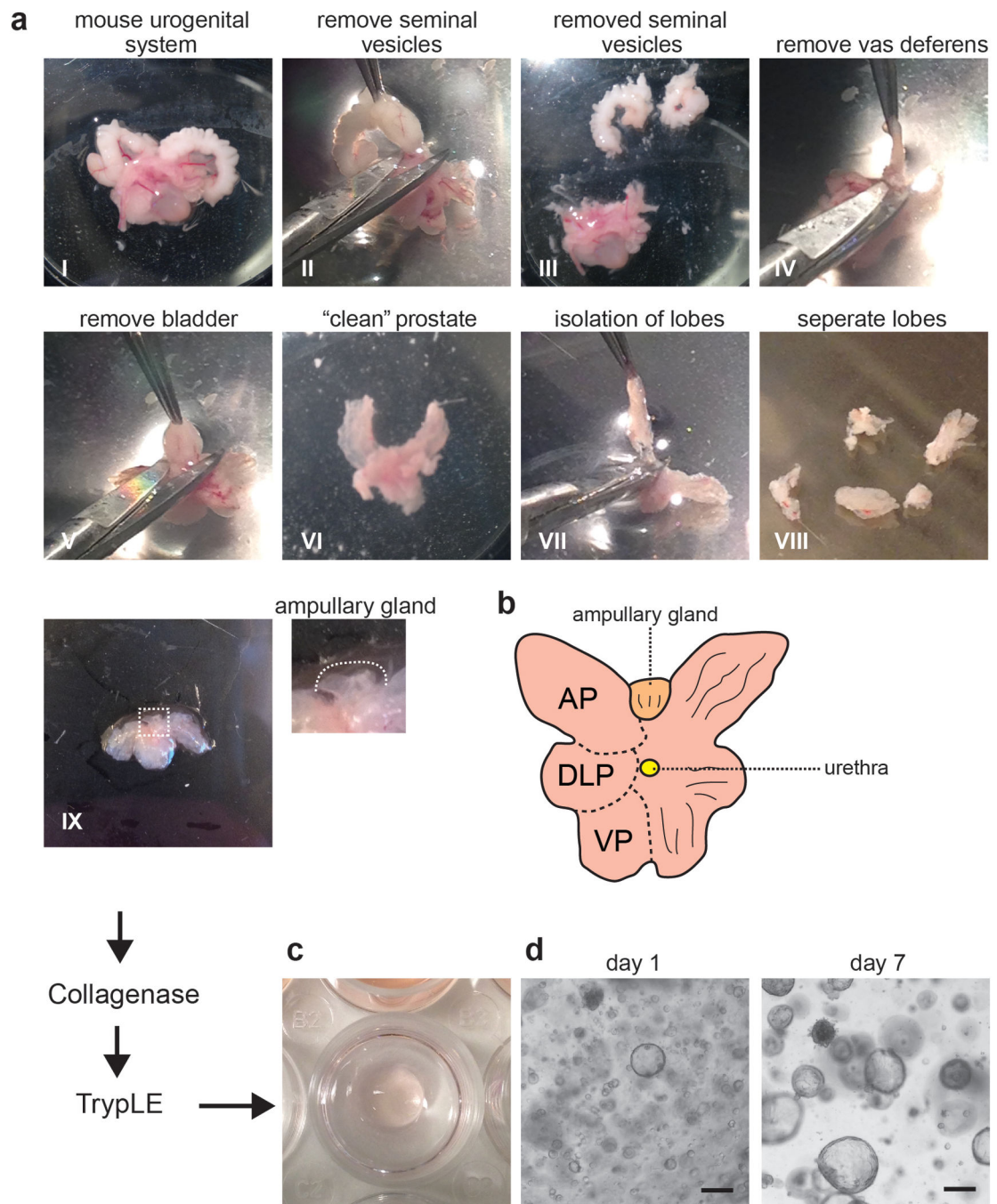


Figure 1. Establishment of mouse prostate organoid cultures

A) Overview of the isolation of the prostate from the mouse urogenital system (for a detailed isolation protocol see²⁵). The procedure refers to pictures I – IX.

B) Schematic representation of the anatomy of the mouse prostate. AP, anterior prostate; DLP, dorsolateral prostate; VP, ventral prostate.

C) Example of how to plate the matrigel disc in a well of a tissue culture plate.

D) Representative pictures of organoids growing from mouse prostate tissue 1 and 7 days after plating.

Scale bars, 100 micron.

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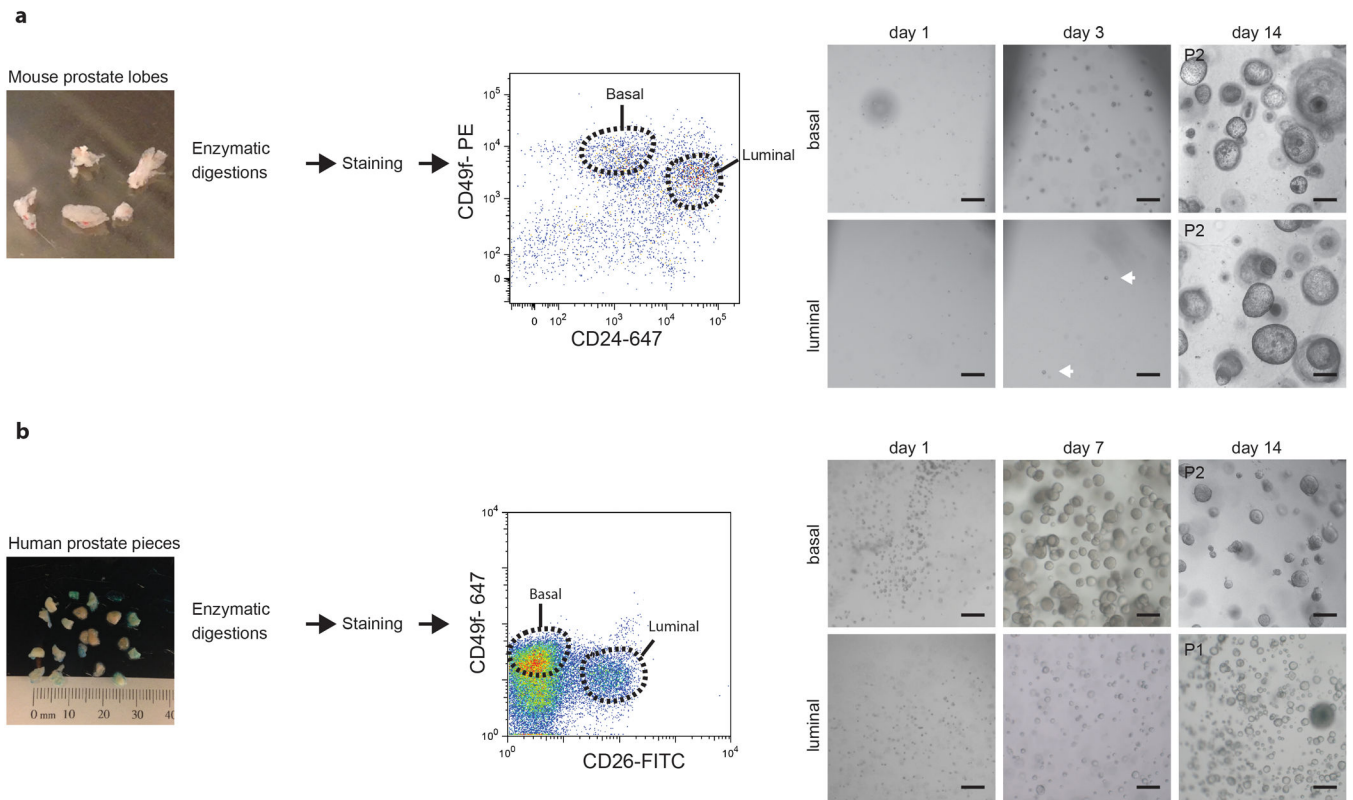


Figure 2. Establishment of mouse and human luminal and basal cell-derived prostate organoid cultures

A) Overview of the establishment of luminal and basal-cell derived mouse prostate organoid cultures using FACS. Cells are sorted based on expression of Cd24 (luminal cells) and Cd49f (basal cells). Scale bars, 100 micron.

B) Overview of the establishment of luminal and basal-cell derived human prostate organoid cultures using FACS. Cells are sorted based on expression of CD26 (luminal cells) and CD49f (basal cells). Scale bars, 100 micron.

Table 1

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 mM
SB202190		10 μM
DHT	1 nM	1 nM
Y-27632 *	10 μM	10 μM

* , Y-27632 is only added to the medium during establishment of the culture and after passaging the organoids using TrypLE.

Table 2

Plate format and culture medium volumes used for organoid culturing.

Plate	Matrigel volume	Number of Matrigel discs	Medium
96-well	10 μ l	1	100 μ l
48-well	20 μ l	1	250 μ l
24-well	40 μ l	1	500 μ l
12-well	40 μ l	3–5	1000 μ l
6-well	40 μ l	10–15	2000 μ l

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