Establishment of a dog primary prostate cancer organoid using the urine cancer stem cells

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Dog spontaneously develop prostate cancer (PC) like humans. Because most dogs with PC have a poor prognosis, they could be used as a translational model for advanced PC in humans. Stem cell-derived 3-D organoid culture could recapitulate organ structures and physiology. Using patient tissues, a human PC organoid culture system was established. Recent study has shown that urine cells also possess the characteristic of stem cells. However, urine cell-derived PC organoids have never been produced. Therefore, we generated PC organoids using the dog urine samples. Urine organoids were successfully generated from each dog with PC. Each organoid showed cystic structures and resembled the epithelial structures of original tissues. Expression of an epithelial cell marker, E-cadherin, and a myofibroblast marker, α-SMA, was observed in the urine organoids. The organoids also expressed a basal cell marker, CK8, and a luminal cell marker, CK8. CD49f-sorted basal cell organoids rapidly grew compared with CD24-sorted luminal cell organoids. The population of CD44-positive cells was the highest in both organoids and the original urine cells. Tumors were successfully formed with the injection of the organoids into immunodeficient mice. Treatment with a microtubule inhibitor, docetaxel, but not a cyclooxygenase inhibitor, piroxicam, and an mTOR inhibitor, rapamycin, decreased the cell viability of organoids. Treatment with a Hedgehog signal inhibitor, GANT61, increased the radiosensitivity in the organoids. These findings revealed that PC organoids using urine might become a useful tool for investigating the mechanisms of the pathogenesis and treatment of PC in dogs.

Prostate cancer (PC) is the most common cancer in men worldwide.1 Because dogs are known to spontaneously develop PC, like humans, and have some similarities in the pathogenesis of the disease,2 they may be a suitable translational model for advanced PC in humans. Human PC mainly exhibits low grade and slow progression, which hardly leads to the cause of death.3 In contrast, dog PC possesses an aggressive nature and exhibits local invasion and widespread metastasis.4 Prostate-specific antigen (PSA) protein in the blood is used for diagnosis of PC in humans.5 However, it is not available for the diagnosis of dog PC due to the lack of PSA produced from the prostate cells.6 The prevalence of dog PC is 0.2–0.6%7 and the survival time after definitive diagnosis is quite short (from weeks to months).8 Therefore, the mechanisms of the causes of dog PC remain unclear and an appropriate treatment protocol for dog PC has never been established.

3-D organoid culture is derived from self-renewing stem cells, which accurately recapitulate in vivo architecture, functions and genetic signatures. It also could be useful for cancer research and personalized therapy.9 Recently, prostate organoid culture systems were established from primary prostate and advanced PC tissues.10 In addition, recent studies demonstrated that urine cells could be used for the bladder repair.11 Urine cells possess the capacity of multipotent differentiation12 and express stem cell markers, such as CD44 and CD29, after culturing in the media.13 Nevertheless, organoid culture using urine cells from PC patients has never been conducted.

In the present study, we cultured the cells of urine samples from dogs with PC using the 3-D organoid culture method. Then, we, for the first time, established the system of urine-derived organoid culture and demonstrated that the organoids could be useful for the analysis of the cell components, structures, origins and tumorigenesis of dog PC as well as the application of chemotherapy and radiotherapy for dog PC.

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Materials and Methods

Materials. To generate organoids, cells of urine samples were cultured with modified media as described previously. The components were as follows: Advanced DMEM with 50% Wnt, Noggin and R-Spondin conditioned medium; GlutaMax; B-27 supplement; 100 μg/mL Primocin (Thermo Fisher Scientific, Waltham, MA, USA); 1 mM N-Acetyl-l-cysteine; 10 mM nicotinamide (Sigma-Aldrich, St. Louis, MO, USA); 50 ng/mL mouse EGF (PeproTech, Rocky Hill, NJ, USA); 500 nM A83-01 (Adooq Bioscience, Irvine, CA, USA); 3 μM SB202190; and 10 μM Y-27632 (Cayman, Ann Arbor, MI, USA). Anti-cancer drugs were as follows: rapamycin (Sigma-Aldrich); piroxicam; docetaxel; GANT61 (Cayman). Antibody sources were as follows: E-cadherin; glioma-associated oncogene homolog (GLI)-2 (R&D System, Minneapolis, MN, USA); CD45 (Abgent, San Diego, CA, USA); vimentin (Sigma-Aldrich); α-smooth muscle actin (SMA) (DAKO, Glostrup, Denmark); CK5; androgen receptor (AR); GLI-1; PTCH1 (GeneTex Irvine, CA, USA); CD44 (Bethyl Laboratories, Montgomery, TX, USA); ki67 (DAKO); CK8 (Biolegend, San Diego, CA, USA); COX-2 (Santa Cruz, Dallas, TX, USA); phosphorylation of Akt (Cell Signaling, Beverly, MA, USA); total Akt; β-III tublin; p53 (Cell Signaling); and actin (Sigma-Aldrich). Secondary antibodies were as follows: Alexa Fluor 488 goat anti-mouse IgG; Alexa Fluor 488 goat anti-rabbit IgG; Alexa Fluor 488 donkey anti-goat IgG (Thermo Fisher Scientific Inc); HRP-conjugated anti-rabbit IgG; HRP-conjugated anti-mouse IgG (Cayman); and HRP-conjugated anti-mouse IgG (Millipore, Temecula, CA, USA). Fluorescence-activated cell sorting (FACS) antibodies were as follows: FITC-conjugated CD44 (1M7); allopheocrucyanin (APC)-conjugated CD49f (G0H3) (Biolegend); FITC-conjugated CD133 (13A4) (eBioscience, San Diego, CA, USA); and FITC-conjugated CD24 (M1/69) (BD Bioscience, San Jose, CA, USA). Isotype control antibodies were as follows: APC-conjugated anti-rat IgG and FITC-conjugated anti-rat IgG (Biolegend).

Urine sample from prostate cancer dog. Urine samples were mainly obtained from dogs at the Department of Small Animal Clinical Science, Joint Faculty of Veterinary Medicine, Yamaguchi University Graduate School of Medicine (Yamaguchi, Japan). The rest of the samples were given by The Laboratory of Veterinary Radiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University (Osaka, Japan), the Laboratory of Veterinary Clinical Oncology, Faculty of Applied Biological Sciences, Gifu University (Gifu, Japan), the Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, The University of Tokyo (Tokyo, Japan), and the Laboratory of Small Animal Surgery 2 School of Veterinary Medicine, Kitasato University (Aomori, Japan). All dogs were diagnosed with prostate tumors. The urine samples were used for the organoids culture. Eight samples of urine samples from eight dogs were attempted to produce organoids (Table 1). Five organoids were successfully generated and expanded rapidly. Organoids from one sample grew so slowly that they were discarded. The rest of the samples were contaminated. Among them, we showed the data for four samples in this study. Written informed consent for this study was obtained from all the dog owners.

Generation of urine sample organoids. The urine samples from dogs with PC were centrifuged at 600 g for 3 min. After the pellets were washed with cold HEPES buffered saline (HBS) and centrifuged at 600 g for 3 min, they were mixed with Matrigel (BD Bioscience) on ice and seeded on 24-well plates. After solidifying the gel at 37°C for 30 min, the media was added and cultured. Organoids were passaged every 7–14 days by using a 1.5-mM EDTA/HBS solution at 1:2–4 split.

Cell culture. Dog mammary tumor cells, CIP-p and CIP-m, and dog osteosarcoma cells, C-HOS, were cultured in RPMI-1640 supplemented with 10% FBS (Thermo Fisher Scientific) as described previously.

H&E staining of organoids. After the organoids were fixed with 4% paraformaldehyde (PFA) at 4°C overnight, they were embedded in paraffin. After deparaffinization, 4 μm-thick sections were stained with H&E as described previously. The images were obtained using a light microscope (BX-53; Olympus, Tokyo, Japan).

Table 1. Sample information

| Case ID | Age (year old) | Breed | Sample Date | Stage | Prior Therapy | Organoid growth | Other information |
|---------|----------------|-------|-------------|-------|---------------|-----------------|------------------|
| PC16001 | 11             | Chihuahua | 09-12-2016 | T3N1M1 | Lansoprazole, | Good            | This sample was used for experiment. |
|         |                |        |             |       | Enrofloxacin, |                 |                  |
|         |                |        |             |       | Firocoxib,    |                 |                  |
|         |                |        |             |       | Cephalexin,   |                 |                  |
|         |                |        |             |       | Robenacoxib, |                 |                  |
| PC16002 | 13             | Yorkshire Terrier | 16-12-2016 | T3N0M0 | Piroxicam     | Contaminated    | This sample was used as D1. |
| PC17001 | 13             | Yorkshire Terrier | 11-01-2017 | T3N0M0 | Piroxicam     | Good            |                  |
| PC17002 | 14             | Miniature Dachshund | 04-03-2017 | T3N1M0 | Piroxicam, Amoxicillin, | Good            |                  |
|         |                |        |             |       | Famotidine,   |                 |                  |
|         |                |        |             |       | Carbazolechrome sodium sulfonate, |                 |                  |
|         |                |        |             |       | Tranexamic acid |                 |                  |
| PC17003 | 10             | Toy Poodle | 24-03-2017 | T3N2M1 | Firocoxib, Potassium Clavulanate | Good            | This sample was used as D3. |
| PC17004 | 13             | Labrador Retriever | 17-04-2017 | T2N0M0 | Piroxicam     | Bad             |                  |
| PC17005 | 14             | Mix     | 20-04-2017 | T3N1M0 | Piroxicam     | Good            |                  |
| PC17006 | 14             | Miniature Dachshund | 20-04-2017 | T2N0M0 | Antibiotics   | Contaminated    |                  |

Modified from Owen LN. 1980. TNM Classification of Tumours in Domestic Animals. Geneva: World Health Organization. T0: no evidence of a primary tumor, T1: Superficial papillary tumor, T2: tumor invading the prostate wall, with induration, T3: tumor invading neighboring organs; N0: no regional lymph node involvement, N1: regional lymph node involved, N2: regional lymph node and juxtaregional lymph node involved; M0: no evidence of metastasis, M1: distant metastasis present.
Immunofluorescence staining of organoids. Immunofluorescence staining of organoids was performed as described previously. After the organoids were fixed with 4% PFA for 1 h and dehydrated with 30% sucrose solution at 4°C overnight, they were embedded in OCT compound. The frozen sections were made and blocked with 1% BSA/PBS at room temperature for 1 h. They were then incubated with a primary antibody (E-cadherin; 1:100, CD44; 1:100, AR; 1:100, vimentin; 1:200, α-SMA; 1:200, CD45; 1:50, ki67; 1:100) at 4°C overnight. After incubation with a secondary antibody (1:500 or 1:1000) at room temperature for 1 h, they were observed with a confocal microscope (LSM 800; ZEISS, Copenhagen, Germany).

Immunohistochemical staining of organoids. Immunohistochemical staining of organoids was performed as described previously. After the deparaffinized sections were treated with 3% peroxidase for 15 min, they were blocked with 1% BSA/PBS at room temperature for 1 h. They were then incubated with primary antibodies (CK5; 1:100, CK8; 1:100; ki67; 1:100) at 4°C overnight. They were washed three times with PBS for 5 min. After incubation with secondary antibodies (1:500) at room temperature for 1 h, they were washed three times with PBS for 5 min. They were observed using a light microscope (BX-53).

Flow cytometry. After the organoids were trypsinized for 15 min, 2 × 10^5 cells were collected into 96-well plates. After the cells were washed with FACS buffer (2% FBS/PBS), they were stained with antibodies (CD24; 1:50, CD49f; 1:50, CD44; 1:100, CD133; 1:100) for 30 min. APC-conjugated anti-rat IgG and FITC-conjugated anti-rat IgG antibodies were used as isotype control. Cells were incubated with propidium iodide before flow cytometric analysis to exclude dead cells. The samples were analyzed using BD Accuri C6 (BD Biosciences) as described previously. The cell population data were analyzed using BD Accuri software (BD Bioscience) and determined using a standard plate reader (Beckman Coulter, Brea, CA, USA).

Western blotting. Western blotting was performed as described previously. Protein lysates were obtained by homogenizing the cells with Triton-based lysis buffer (50 mM Tris·HCl [pH 8.0], 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM Na2VO4, 20 mM sodium pyrophosphate, and Roche Complete protease inhibitor mixture). Loading proteins (10–20 µg) were separated by SDS-PAGE (10%) and transferred to a nitrocellulose membrane (Wako, Osaka, Japan). After incubation with 3% BSA or 0.5% skim milk, the membranes were incubated with primary antibodies (COX-2; 1:500, P-Akt; 1:500, total Akt; 1:200, total actin; 1:500, β-H tublin; 1:200, p53; 1:200, PTCH1; 1:200, GLI-1; 1:200, GLI-2; 1:200) at 4°C overnight. The membranes were incubated with secondary antibodies (1:10,000 dilution, 1 h) and ECL Pro (PerkinElmer, Freiburg, Germany). The results were visualized using LAS-3000 (Fujifilm, Tokyo, Japan) and quantified using Image gel densitometry analysis software (National Institutes of Health, Bethesda, MD, USA).

Radiation exposure. After the organoids were trypsinized for 15 min and filtered using a 70-µm cell strainer (Falcon), 5 × 10^4 cells of the tumor organoids were seeded into 10 µL of Matrigel on a 96-well culture plate and incubated for 24 h. They were then treated with radiation at 2–8 Gy using an X-ray generating system (MX-80 Labo; MedXtec Japan, Chiba, Japan). The X-ray was delivered at a dose rate of 0.22 Gy/min (40 kVp and 0.06 mA). After 4 days, cell viability was examined by cell counting using alamarBlue kit (Thermo Fisher Scientific).

Statistical analysis. Data are shown as means ± SEM. Statistical evaluations were performed by using Student’s t-test between two groups. Values of P < 0.05 were considered statistically significant.

Results
Generation of urine-derived organoids. Because most dogs with PC exhibit invasion and metastasis, we hypothesized that the cells from urine samples are useful for PC organoid culture. To prove this, we collected the urine samples from dogs with PC and generated urine-derived organoids (Fig. 1a). After collecting and washing the cells with HBS solution without any digestion treatment, they were seeded into Matrigel and cultured in stemness-stimulated media containing Wnt, EGF, Noggin and R-spondin as reported previously. We observed that the cells in the urine sample from each dog gradually formed organoids at days 7–14. After passing several times, the organoids continually increased over 28 days (Fig. 1b). We also observed that the structures of urine-derived organoids resembled their original tumor epithelium (Fig. 1c). To identify the cell components of urine sample-derived
organoids, we performed immunofluorescence staining. Expression of an epithelial cell marker, E-cadherin, was observed exclusively in the organoids (Fig. 1d). In contrast, expression of a cancer stem cell marker, CD44, was heterogeneously observed (Fig. 1e). Although AR signaling is essential for PC initiation and progression in humans, AR expression is down-regulated in dog PC tissues. As expected, AR expression was faintly observed in the organoids (Fig. 1f). Interestingly, expression of a fibroblast cell marker, vimentin (Fig. 1g), and a myofibroblast marker, α-SMA (Fig. 1h) was observed in the surroundings of organoids. Expression of a leukocyte marker, CD45 (Fig. 1i), and a proliferating cell marker, ki67, (Fig. 1j) was also observed in the organoids. These results suggest that urine-derived organoids could recapitulate the tumor microenvironment of dog PC tissues.

**Cell origins of urine-derived prostate cancer organoid.** Prostate epithelial cells consist of basal, luminal and a few neuroendocrine cells. Basal cells are regarded as stem cell components and are located at the basement membrane; they are identified by expression of CK5. The luminal cells are the secretory components of the gland, which express CK8. To clarify which cells regulate tumor progression in dog PC, we performed the sorting of basal and luminal cells (Fig. 2a). We first observed that CK5 and CK8 were expressed in the urine-derived organoids (Fig. 2b). In the flow cytometry experiments, we also checked the population of a basal cell marker, CD49f (36.1%), and a luminal cell marker, CD24 (45.5%), in the organoids (Fig. 2c). After isolating basal and luminal cells from the organoids by using CD49f and CD24 antibodies, the same number (2.5 ± 0.5%) and ALDH-active cells in the organoids was significantly higher than that of CD24-sorted organoids (Fig. 2d). The CD49f-sorted organoids grew rapidly, while the CD24-sorted organoids hardly grew (Fig. 2e). The organoid-forming efficiency of CD49f-sorted organoids was significantly higher than that of CD24-sorted organoids (Fig. 2e). The CD49f-sorted organoids had solid sphere structures. In contrast, CD24-sorted organoids had glandular structures (Fig. 2f). These results correspond with human prostate sorted cell-derived organoids as reported previously and imply that basal cells mainly regulate tumor initiation and progression of dog PC.

**Population of cancer stem cell markers in the urine-derived prostate cancer organoids.** In the PC tissues, several cancer stem cell markers, such as CD44 and CD133, were expressed and regulate tumor progression. Active ALDH-expressing prostate cancer cells are also associated with tumor formation ability. To clarify the populations of cancer stem cell markers in the organoids and the cells in the urine, we performed flow cytometry (Fig. 3a). The population of CD44, CD133-positive and ALDH-active cells in the organoids was significantly higher than that of the cells in urine (Fig. 3b-d). In the urine-derived organoids, the population of CD44-positive cells was the highest (Fig. 3b, 34.0 ± 2.6%) compared with CD133-positive cells (Fig. 3c, 3.1 ± 0.5%) and ALDH-active cancer stem cell markers.
Fig. 2. Cell origins of urine-derived prostate cancer organoids. Schematic experimental design of an analysis of the cell components of the organoids (a). After the cultured urine-derived organoids were tripped, they were sorted for basal and luminal cells by flow cytometry using specific cell markers, CD49f and CD24. The sorted cells were cultured and used for experiments. Expression of a basal cell marker, CK5, and a luminal cell marker, CK8, in the organoids (b). Representative photomicrographs are shown (n = 3). Scale bar: 50 μm. Expression of a basal cell marker, CD49f, and a luminal cell marker, CD24, in the organoids (c). After the organoids were sorted, they were treated with antibodies. The number of organoids was counted (e, n = 6). *P < 0.05 versus CD49f organoid. Representative images of flow cytometry are shown (f). Culture day 14. Scale bar: 50 μm.

cells (Fig. 3d, 6.4 ± 0.8%). Similarly, CD44-positive cells in the urine were the highest (9.6 ± 1.3%) compared with CD133-positive cells (Fig. 3c, 3.1 ± 0.4%) and ALDH-active cells (Fig. 3d, 0.5 ± 0.2%). In the normal urine, CD44 positive-cells existed but were lower (Fig. 3c, 2.4 ± 0.5%) than those of dogs with PC. These findings imply that CD44 is essential for the generation of urine-derived organoids.

Tumorigenesis by urine-derived prostate cancer organoid. We next examined whether urine-derived organoids form tumor in vivo (Fig. 4a). Within 8 weeks, tumors (>1 cm in diameter) were successfully formed following injection of the organoids into immunodeficient mice (Fig. 4b) but there was no metastasis. We also observed that both granule and solid-like cell structures exist in the tumor tissues (Fig. 4c). This mixed tumor histology was also observed in a previous report on the injection of human PC organoids. To identify the cell components of the tumor tissues, we performed immunofluorescence and immunohistochemical staining. Expression of E-cadherin was observed in the tumor tissues (Fig. 4d). Expression of α-SMA was observed in the surroundings of granule-like cells (Fig. 4e). CK5 (Fig. 4f), CK8 (Fig. 4g) and ki67 (Fig. 4h)-positive cells were also observed in the granule-like cells. In contrast, expression of AR and PSA was not observed in the tumor tissues (data not shown). These findings suggest that urine-derived organoids reproduce tumor tissues in vivo, which exhibit similar features of original tumor tissues of dogs with PC.

Effects of anti-cancer drugs on urine-derived prostate cancer organoids. Because most dogs with PC are diagnosed at advanced stages and the tumors are highly metastatic, the prognosis is poor. Although most dogs were treated with COX inhibitors, which extended their survival period to some extent, remission was not achieved in any cases. To find an effective therapy for dogs with PC, we examined whether anti-cancer drugs for human PC have effects on the cell viability of dog PC organoids. To examine the responsiveness of organoids to anti-cancer drugs, we performed a 96-well Matrigel cell viability assay as described previously (Fig. 5a). Treatment with a COX inhibitor, piroxicam, had no effect on cell viability of organoids in each dog culture (Fig. 5b,c). PTEN is a tumor suppressor gene, negatively controlling activation of PI3K/Akt signal, which regulates cell growth, survival and division. Inhibition of PTEN is also associated with progression and recurrence of PC. Rapamycin inhibits mTOR activity, a signal downstream of PI3K/Akt, which leads to the inhibition of proliferation and survival in cancer cells. Docetaxel is a microtubule stabilizing taxane that prevents formation of mitotic spindles, which leads to mitotic arrest and cell death through activation of p53. In men with PC, treatment with docetaxel improves survival times. Although treatment with rapamycin decreased cell viability of organoids in each culture at the concentration of 0.01–0.1 μM in a dose-dependent manner, high concentration of rapamycin (1, 10 μM) had no additional effects, and proliferating cells of organoids remained (Fig. 5b,c). In contrast, docetaxel decreased cell viability of organoids in a dose-dependent manner (Fig. 5b,c). Interestingly, dog 1’s (D1) organoids exhibited more resistance to docetaxel compared with D2 and D3 (Fig. 5c). To check the expression level of the target protein of anti-cancer drugs, we performed western blotting (Fig. 5d, e). The expression level of COX-2 was higher in the organoids compared with other dog cancer cells (mammary tumor cells, CIP-p and CIP-m, and osteosarcoma cells, C-HOS) (Fig. 5d,f). In contrast, the phosphorylation level of Akt was lower than in other cells, which might cause the resistance to rapamycin (Fig. 5d). In D1 organoids, expression of β-III tubulin was higher than in D2 and D3 organoids. In contrast, expression of p53 was lower (Fig. 5e,f).
decreased p53 are correlated with resistance to docetaxel. D1 organoids might be resistant to docetaxel owing to upregulation of β-III tubulin and downregulation of p53 expression.

Effects of irradiation on urine-derived prostate cancer organoids. Radiotherapy has been attempted for dogs with PC. We therefore checked whether irradiation has effects on cell viability of the organoids. To examine the responsiveness of organoids to irradiation, we performed a 96-well Matrigel cell viability assay after 4 days of irradiation (Fig. 6a). Treatment with irradiation decreased the cell viability of organoids in each culture in a dose-dependent manner (Fig. 6b,c). The Hedgehog signal plays a significant role in the course of body development, which is also involved in PC development and resistance to therapy. Hedgehog signals are activated by the binding of Hedgehog ligands and the receptor, PTCH1. Activated Hedgehog signals allow GLI-1 proteins to translocate to the nucleus and promote transcription of target genes, such as GLI-2. To compare the expression level of these Hedgehog signal-related proteins between these organoids, we performed western blotting (Fig. 6d). The expression level of PTCH1, GLI-1 and GLI-2 was lower in D1 organoids compared with D2 and D3 organoids. We therefore investigated the combination of a GLI-1 inhibitor, GANT61, and irradiation in D3 organoids, expressing higher level of GLI-1 expression. Treatment with GANT61 significantly increased radiosensitivity of the organoids at 2–6 Gray (Gy) (Fig. 6e). These results suggest that urine organoids could be useful for checking the radiosensitivity of PC in dogs. It is also shown that Hedgehog signals might mediate the resistance of dog PC to radiotherapy.

Discussion

In the present study, we, for the first time, established dog PC organoids by using a urine sample from PC diseased dogs. The major findings of the present study are as follows: (i) urine-derived organoids consist of epithelial cells, myofibroblasts, cancer stem cells and a few leukocytes (Fig. 1); (ii) basal cells in the organoids play a significant role in organoid formation.
and growth (Fig. 2); (iii) the population of CD44-positive cells in the organoids is the highest compared with CD133-positive cells and ALDH-active cells (Fig. 3); (iv) injection of the organoids into NOD/SCID mice was successful in forming tumors (Fig. 4); (v) Treatment with docetaxel but not piroxicam and rapamycin decreased the cell viability of organoids (Fig. 5); and (vi) treatment with GANT61 increased the sensitivity of radiation in the organoids (Fig. 6). Collectively, our results indicate that dog PC organoids might help in investigating the mechanisms of tumor growth and also provide new insights for dog PC treatment.

The cell origins of PC are still debated. In human PC tissues, luminal phenotype characterized by atypical glands and an absence of basal cells was observed, suggesting that prostate cancer originates from luminal cells. In PC mouse models, it was shown that both basal and luminal cells serve as original cells for PC. In the present study, we showed that the basal cell-sorted organoids grow rapidly compared with luminal cell-sorted organoids (Fig. 2). A recent study also demonstrated that xenograft experiments using human c-Myc/Akt-expressing basal cell organoids showed more aggressive histology than luminal cell organoids. This finding corresponds with the data in the present study. We therefore suppose that basal cells might be the origin and mainly mediate the progression of PC in dogs.

CD44 is a transmembrane glycoprotein that plays a role in cell adhesion. Its expression correlates with cancer metastasis and poor prognosis. Several studies show that the population of CD44-positive cells of PC cells is associated with tumor-initiating cells. Liu et al. (2011) show that CD44 is negatively regulated by microRNA (miR)34a in PC. They also show that overexpression of miR34a inhibits PC metastasis through the decrease in the expression of CD44. In the present study, we showed that the population of CD44 is the highest among stem cell markers in both urine-derived organoids and the original cells in urine samples. Interestingly, the population of each stem cell marker-positive cell in the organoids was higher than that of cells in urine samples (Fig. 3b–d). It was also shown that the population of CD44 in the PC dog-derived urine cells was higher than in normal cells (Fig. 3b,e). These results suggest that CD44 expression might mainly regulate the initiation and progression of dog PC organoids. In addition, miR34a might become novel therapeutic targets for dog PC metastasis.

In human PC patients, androgen deprivation is used as the primary therapy. However, hormonal therapy is not likely

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**Fig. 4. Tumorigenesis by urine-derived prostate cancer organoids.** Schematic experimental design of a xenograft experiment of urine-derived organoids (a). The tripesinized cells were subcutaneously injected into the back of NOD/SCID mice (n = 6). After 8 weeks, the formed tumor was observed and removed for H&E, immunohistochemistry and immunofluorescence staining. Observation of organoid-injected tumor formation (b). Representative images for H&E staining of the tumor tissues are shown (c). The enlarged image is shown on the right. Scale bar: 100 μm. Expression of E-cadherin (d), α-SMA (e), vimentin (f), CK5 (g), CK8 (h) and ki67 (i). Representative photomicrographs are shown (n = 4). Scale bar: 100 μm (c, d, f–h), 50 μm (e). Image of α-SMA (green) was merged with E-cadherin (red). Arrows show the representative positive cells (e). Each enlarged image is shown at the bottom (f–h).
to be effective in most dogs because androgen plays a relatively minor role in PC in dogs. (8, 46) In the present study, urine-derived organoids hardly expressed AR (Fig. 1f), which corresponds with a previous report on AR expression in dog PC tissues. (25) Because the dogs were usually treated with COX inhibitors, such as piroxicam or carprofen. While COX inhibitors extended the survival periods to some extent, (25) the direct effects on PC cells remain unknown. In the present study, we demonstrated that piroxicam had no effects on the cell viability of dog PC organoids (Fig. 5b,c). In contrast, docetaxel decreased cell viability in a dose-dependent manner (Fig. 5b,c). Docetaxel inhibits depolymerization of tubulin through binding, which ultimately leads to apoptosis via activation of p53. (33) Prostate tumor cells bearing wild-type p53 are more sensitive to docetaxel treatment compared to those with mutant or null p53. (33) In the present study, p53 expression was correlated with the sensitivity of docetaxel (Fig. 5e). We therefore speculate that docetaxel treatment might be more effective for the dogs with PC bearing wild-type p53.

In the present study, we investigated the radiosensitivity of urine-derived PC organoids. The cell viability was decreased in a dose-dependent manner (Fig. 6b). In a previous study, the cell viability of human PC cell lines was decreased by 2–6 Gy irradiation for 4 days. (47) We therefore suppose that the radiosensitivity of dog PC organoids was almost the same level as them. Hedgehog signals are involved in PC development, progression and resistance to therapy. (34, 46) Previous studies showed that Hedgehog signals are involved in radiation resistance of hepatocellular and pancreatic cancer cell lines. (49, 50) It is also reported that a GLI-1 inhibitor, GANT61, improved radiosensitivity in a pancreatic cancer cell lines. (49, 50) Although radiotherapy was conducted in dogs with PC, the combination effects of GANT61 on dogs with PC were still unclear. We therefore investigated whether the combination of GANT61 and irradiation has effects on the cell viability of dog PC organoids. As expected, GANT61 increased the radiosensitivity in Hedgehog signal-related proteins expressing organoids (Fig. 6d,e). Because treatment with GANT61 at 2 Gy was more effective than at 4 and 6 Gy, we speculate that high exposure of radiation affects activity of Hedgehog signals, which might lead to the low sensitivity to GANT61. Further investigations using urine-derived dog PC organoids might
help to explore a promising therapeutic strategy for enhancing the radiation response of dogs with PC.

In conclusion, for the first time, dog PC organoids were produced using urine samples. The organoids recapitulated the tumor microenvironment and showed tumorigenesis in vivo. It was also suggested that basal cells of the organoids and CD44-positive cells mediate organoid formation and growth. Further studies on the urine sample-derived organoid culture system could contribute to the treatment of PC in dogs.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Establishment of cancer organoid using urine

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