Establishment of a novel experimental model for muscle-invasive bladder cancer using a dog bladder cancer organoid culture

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Abstract
In human and dogs, bladder cancer (BC) is the most common neoplasm affecting the urinary tract. Dog BC resembles human muscle-invasive BC in histopathological
INTRODUCTION

Bladder cancer comprises approximately 1-2% of all naturally occurring cancers in dogs, a similar rate to that found in human. While more than 90% of BC found in dogs consist of intermediate- to high-grade invasive urothelial carcinomas, low-grade, superficial transitional cell carcinoma (TCC) is very rare. Diagnosis of dog BC is usually at the late stage, and is then often impossible to treat. Although several experimental models of BC exist, including carcinogen-induced mouse models and genetically engineered mice, they do not completely reflect the characteristics of invasive or metastatic human BC. Interestingly, naturally occurring dog BC closely mimics human muscle-invasive BC in its cellular and molecular characteristics including histopathological characteristics, biological behavior, local cancer invasion, distant metastases, molecular features, response to chemotherapy, and prognosis, suggesting that dog BC could be a relevant model for muscle-invasive human BC. Therefore, identification of diagnostic markers and investigation of the mechanisms involved in dog BC might be beneficial not only for the veterinary clinic but also for human patients with muscle-invasive BC.

Three-dimensional (3D) culture (organoids), derived from self-renewing stem cells, typically recapitulates in vivo architecture, functions, and genetic and molecular signatures of the parent tumors. This technique holds great promise for use in medical research into the development of new personalized therapy, especially for cancer.

In a previous study, we established a culture method for dog prostate cancer organoids using urine samples, however dog BC organoids had never been produced. Therefore we aimed to generate dog BC organoids using urine samples and check their histopathological characteristics, drug sensitivity, and gene expression profiles. Organoids from individual BC dogs were successfully generated, expressed urothelial cell markers (CK7, CK20, and UPK3A) and exhibited tumorigenesis in vivo. In a cell viability assay, the response to combined treatment with a range of anticancer drugs (cisplatin, vinblastine, gemcitabine or piroxicam) was markedly different in each BC organoid. In RNA-sequencing analysis, expression levels of basal cell markers (CK5 and DSG3) and several novel genes (MMP28, CTSE, CNN3, TFPI2, COL17A1, and AGPAT4) were upregulated in BC organoids compared with normal bladder tissues or two-dimensional (2D) BC cell lines. These established dog BC organoids might be a useful tool, not only to determine suitable chemotherapy for BC diseased dogs but also to identify novel biomarkers in human muscle-invasive BC. In the present study, for the 1st time, dog BC organoids were generated and several specifically upregulated organoid genes were identified. Our data suggest that dog BC organoids might become a new tool to provide fresh insights into both dog BC therapy and diagnostic biomarkers.

MATERIALS AND METHODS

2.1 Materials

To generate dog BC organoids, cells from urine samples were mixed with Matrigel (BD Bioscience) and cultured with stem cell-stimulated medium, as described previously. Anticancer drugs used were as...
follows: piroxicam; gemcitabine; vinblastine (Cayman); and cisplatin (WAKO). Antibody sources used were as follows: E-cadherin (R&D System); CK7: Ki67 (Novus); CK20; UPK3; MMP28; TFPi2; AGPAT4 (Bios); vimentin (Sigma-Aldrich); α-smooth muscle actin (SMA) (DAKO); CK5; CNN3 (GeneTex, Inc.); and CTSE (Bioworld Technology, Inc.). Fluorescent secondary antibodies used were as follows: Alexa Fluor™ 488 donkey anti-goat IgG; Alexa Fluor 488™ goat anti-rabbit IgG; Alexa Fluor 488™ goat anti-mouse IgG; (Thermo Fisher Scientific Inc.); Biotinylated goat anti-mouse IgG (Vector Laboratories, Inc.). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cayman); and HRP-conjugated anti-mouse IgG (Millipore).

### 2.2 | Cell culture

Dog two-dimensional (2D) urothelial carcinoma cell lines were purchased from COSMO BIO CO., Ltd, and cultured in Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc.).

### 2.3 | Generation of urine sample-derived BC organoids

Between February 2017 and October 2018, 17 urine and blood samples were collected from dogs diagnosed with BC based on their clinical symptoms and cytological examinations (Figure S1). Sample collection was a cooperation between the Animal Medical Center, Faculty of Agriculture, Tokyo University of Agriculture and Technology (Tokyo, Japan), the Department of Small Animal Clinical Science, Joint Faculty of Veterinary Medicine (Tokyo, Japan), the Laboratory of Veterinary Radiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University (Osaka, Japan), Laboratory of Veterinary Clinical Oncology, Faculty of Applied Biological Sciences, Gifu University (Gifu, Japan), Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, University of Tokyo (Tokyo, Japan) and Laboratory of Small Animal Surgery 2 School of Veterinary Medicine, Kitasato University (Aomori, Japan). Written informed consent for this study was obtained from all dog owners, and the study was conducted under direction from the Institute Animal Care and Use Committee of Tokyo University of Agriculture and Technology approval (Approval number: 0016012). In total, 12 urine samples were successfully expanded using the 3D organoid culture method, as described previously. Among these, four organoid lines (Ors 1-4) were used for experiments; dog information is listed in Table 1.

### 2.4 | Passaging of BC organoids

After 7-14 d of culture, BC organoids were passaged into a new well at a ratio of 1:3-4. To dissolve Matrigel, 5 mmol/L EDTA/phosphate-buffered saline (PBS) was added to each well and the culture plate was placed on ice for 90 min. The cell suspension was collected into a 15-mL tube and then centrifuged at 600 g for 3 min. Cell pellets were washed with PBS and then trypsinized at 37°C for 5 min. Vigorous pipetting was performed and 100 μL of FBS was added to the tube to neutralize trypsinization. Cell pellets were collected by centrifugation and were mixed with new Matrigel on ice. Matrigel-containing cells were dropped into a 24-well plate, solidified in a CO₂ incubator at 37°C for 30 min, and then culture medium was added to each well.

### 2.5 | Collection of normal bladder tissue

Four healthy female beagle dogs (3 y old) were obtained from the Research Institute for Animal Science in Biochemistry and Toxicology (Tokyo, Japan). These canines were accorded recommendations listed in the “Guide for the Care and Use of Laboratory Animals” approved by the Faculty of Agriculture, Tokyo University of Agriculture and Technology. A catheter was inserted through the urethra, normal bladder tissues were isolated by scratching the mucosal membrane of the bladder and then used for RNA preparation. Blood samples were also collected and used for RNA preparation. The study was conducted under the approval of the Institute Animal Care and Use Committee of Tokyo University of Agriculture and Technology (Approval number: 30-55).

### 2.6 | Hematoxylin and eosin staining of BC organoids

Hematoxylin and eosin (H&E) staining of BC organoids was performed as described previously. Bladder cancer organoids were fixed in 4% paraformaldehyde (PFA) at room temperature for 2-3 h, and then
embedded in paraffin. After deparaffinization, 4-μm-thick sections were stained with H&E. Images were obtained using a light microscope (BX-52; Olympus).

2.7 | Immunofluorescence staining of BC organoids

Immunofluorescence staining of BC organoids was performed as described previously.17,18 Organoid cells were fixed in 4% PFA for 1 h, dehydrated with 30% sucrose solution at 4°C overnight, then embedded in OCT compound. Frozen sections were made and blocked with 1.5% normal goat serum (NGS)/PBS at room temperature for 1 h. Subsequently, sections were incubated with a primary antibody (E-cadherin; 1:200, vimentin; 1:200, α-SMA; 1:200, ki67; 1:100, MMP28; 1:200, CTSE; 1:200, CNN3; 1:200, TFPI2; 1:200, and AGPAT4; 1:200) at 4°C overnight, and then with a secondary antibody for 1 h and finally observed using a confocal microscope (LSM 800; ZEISS).

2.8 | Mouse xenograft assay

Mouse xenograft assay of BC organoids was performed as described previously.17 C.B-17/IcrHsd-Pkdicd mice were obtained from Japan SLC. Male immunodeficient mice (6 wk old) were housed under specific pathogen-free conditions. Here, 1 × 10^6 cells of BC organoids were subcutaneously injected into the back of each mouse. At 6 wk later, the organoid-derived tumors were isolated and used for H&E and immunofluorescence staining. All studies involving mice were conducted according to the Guide to Animal Use and Care, Tokyo University of Agriculture and Technology, and approved by the ethics committee (Approval number: 29-92).

2.9 | Cell viability assay of BC organoids

Cell viability assay of organoids was performed as described previously.17,19 Taking into consideration the therapeutic dose in clinic and pharmacokinetics in dogs,10,20-25 we determined the appropriate concentration to be used for each drug in the cell viability assay. Briefly, 5 × 10^3 cells of each BC organoid were seeded into 10 μL Matrigel in a 96-well culture plate and incubated for 24 h. Next, cells were treated with anticancer drugs at variable concentrations for 3 d. Cell viability was examined using an alamar blue kit (Thermo Fisher Scientific Inc.). Fluorescence (emission wavelength; 585 nm) was read on a microplate reader (TECAN).

2.10 | RNA-sequencing analysis

Total RNA was extracted from normal bladder tissues from healthy dogs, BC organoid samples and 2D urothelial carcinoma cell lines using the NucleoSpin kit (TaKaRa Bio Inc.) according to the manufacturer’s instructions. Total extracted RNA (10 ng) for each sample was used to generate the sequencing libraries. RNA-seq was performed at the Research and Education Center for Prevention of Global Infectious Disease of Animals, Tokyo University of Agriculture and Technology (Tokyo, Japan). A Ribo-Zero Human Kit (Illumina) and a TruSeq Stranded Total RNA Library Prep Kit (Illumina) were used for library preparation, followed by sequencing (7.5 million single-end reads) on an Illumina MiSeq instrument. Initial quality control of RNA-seq data (FASTQ) for each sample was performed using cutadapt (version 1.8.3) and cmgfastq_pe.pl software. Reads were mapped to the reference genome (CamFam 3.1) using the STAR (version 2.5.1b) software. PCA was performed

### Table 2

| Primer  | Sequence               |
|---------|------------------------|
| CK5     | Forward 5′-CAAGGTCTCTGGACACCAAGTG-3′ |
|         | Reverse 5′-ATGCCTGGCAGCCTGTGCTC-3′ |
| DSG2    | Forward 5′-CCTTGGGTGGTTGACGTTTTT-3′ |
|         | Reverse 5′-ATGCATCCCGAGCCTACTCT-3′ |
| GATA3   | Forward 5′-GTCCTCCAGGCTTTTCTCCT-3′ |
|         | Reverse 5′-GGCAAAACGTCATTGTTTCTT-3′ |
| ERBB2   | Forward 5′-CCCCAGAGATATGTTGAGAG-3′ |
|         | Reverse 5′-ACCTCCAGATGGGCAAGT-3′ |
| MMP28   | Forward 5′-GAGGCCTGAAAGACCGTTCCT-3′ |
|         | Reverse 5′-TGTGGTCATACCCTGCGAAA-3′ |
| CTSE    | Forward 5′-CCAGACCTTTTGGAAGCCAGA-3′ |
|         | Reverse 5′-TGCAGAAGTGGGGTGACTGAT-3′ |
| CNN3    | Forward 5′-TCCAAAAATGCAAACACTGCA-3′ |
|         | Reverse 5′-CGAGAAGTGGGGTGACTGAT-3′ |
| TFPI2   | Forward 5′-AAGCCCAAACACTGTCGAC-3′ |
|         | Reverse 5′-AAGCCCAAACACTGTCGAC-3′ |
| COL17A1 | Forward 5′-CGGAGATCCACAGACATAT-3′ |
|         | Reverse 5′-GAGCAGAATCTGCAGCACA-3′ |
| AGPAT4  | Forward 5′-AATTCCTGTGCACTCTGCTC-3′ |
|         | Reverse 5′-CACCCTCACACTGCTCACA-3′ |
| GAPDH   | Forward 5′-AACACTCTCAAGATGTTGACAGA-3′ |
|         | Reverse 5′-CATGGATGACTTTGGCTAGAGGA-3′ |
| CK15    | Forward 5′-AGACACTGGGAGGAAATG-3′ |
|         | Reverse 5′-ACCTCTGAGAGGACTGAC-3′ |
| TGM2    | Forward 5′-TCGAAAGGATGGGGAGGAGG-3′ |
|         | Reverse 5′-AGGTTGGGTTGTCACACTTACG-3′ |
| GJB2    | Forward 5′-CCCATCTCTCCACATCAGC-3′ |
|         | Reverse 5′-CAAAGATGACCAGCCGAAAGG-3′ |
| IL1R2   | Forward 5′-AGACGAGGAAATGTTGAGGTC-3′ |
|         | Reverse 5′-TCTGGCAGTCGATGTGAGG-3′ |
| COL5A2  | Forward 5′-CCTCAGGGAAATGATTGAGGA-3′ |
|         | Reverse 5′-CTGCCCTTGTAAGCCTTGGAG-3′ |
| CDH3    | Forward 5′-GGAACCTCATTACCTGCAT-3′ |
|         | Reverse 5′-GTTCGGGAGAAGGAGGACTA-3′ |
| CHST4   | Forward 5′-TGCTCAAGGGAGTCGCGCTT-3′ |
|         | Reverse 5′-TGGCTTTCCTCTGGCTTCT-3′ |
| ADORA2B | Forward 5′-AAGCTGTTGCCCTGCTGAAATG-3′ |
|         | Reverse 5′-AGCCAGCAGACAGCAGAATA-3′ |
to display differences between samples. Fragments per kilobase of transcript per million mapped reads were normalized using the trimmed mean of M value method.

2.11 | Quantitative real-time polymerase chain reaction

Total RNA was extracted from normal bladder tissues, organoid samples, 2D urothelial carcinoma cell lines, and blood samples using the NucleoSpin kit (Takara Bio Inc) according to the manufacturer’s instructions. First-strand cDNA was synthesized using a QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative real-time PCR was performed using a QuantiTect SYBR I Kit (QIAGEN) and a StepOnePlus Real-Time PCR System (Applied Biosystems). The ΔΔCq method was used for quantification. Specific primers used for dog CK5, DSG3, GATA3, ERBB2, MMP28, CTSE, CNN3, TFPI2, COL17A1, AGPAT4, GAPDH, CK15, TGM2, GJB2, IL1R2, COL5A2, CDH3, CHST4, and ADORA2B are listed in Table 2.

2.12 | Immunohistochemical staining of organoids

Immunohistochemical staining of BC organoids was performed as described previously. After deparaffinization, sections were treated with 1% peroxidase for 30 min, then blocked with 1.5% NGS/PBS for 30 min. Sections were then incubated with primary antibodies (CK5; 1:200) at 4°C overnight followed by incubation with
2.13 | Western blotting

Western blotting was performed as described previously. Protein lysates were obtained by homogenizing normal bladder tissues, organoid samples, and 2D urothelial carcinoma cell lines with Cell Lysis Reagent (Sigma-Aldrich) containing 1% protease inhibitor cocktail (Sigma-Aldrich). Loading proteins (10 μg) were separated by SDS-PAGE (7.5%) and transferred to a nitrocellulose membrane (Wako). After blocking with 0.5% skimmed milk, the membranes were incubated with primary antibody (CK5; 1:500, VCP; 1:500) at 4°C overnight followed by incubation with the secondary antibody (1:10 000 dilution, 1 h) and ECL Prime (GE Healthcare). Results were obtained with FujiFilm LAS-3000 and quantified using ImageJ densitometry analysis software (National Institutes of Health).

2.14 | Statistical analysis

Data shown are means ± SEM. Statistical evaluations were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's test. P-values ≤0.05 were considered to be statistically significant.

3 | RESULTS

3.1 | Generation of dog BC organoids

Urine sample-derived dog PC organoids were generated by our group in a previous study. As most dogs with BC also exhibit invasion and metastasis, we hypothesized that urine cells from BC diseased dogs would be useful for organoid culture. Urine samples from BC diseased dogs were collected and used to generate urine-derived organoids (Figure 1A). Urine cells were cultured using the method reported previously. Cells from the urine samples from each BC diseased dog gradually formed organoids (Figure 1B) and were continually propagated by serial passage. The efficacy in establishing continually propagated organoid lines from BC dogs was approximately 70% (12 lines from 17 attempted samples). We observed that urine-derived organoids from each sample had spheroidal structures and a similar histology to dog urothelial carcinomas reported previously (Figure 1C). For the immunofluorescence assay, expression of an epithelial cell marker, E-cadherin, was observed in all organoids (Figure 1E). Expression of urothelial cell markers, CK7 (Figure 1F), CK20 (Figure 1G), and UPK3A, (Figure 1H) was also observed.
observed in each organoid strain (Figure S2). Similarly to previously found for urine sample-derived dog PC organoids, we observed that a fibroblast cell marker, vimentin, (Figure 1I) and a myofibroblast marker, α-SMA, (Figure 1J) were expressed around the organoids. Expression of a proliferation cell marker, Ki67, (Figure 1K) was also observed in the organoids. These results suggested that urine sample-derived organoids from BC diseased dogs could recapitulate the characteristics of dog BC tissues.

3.2 Tumorigenesis induced by BC organoids

We next examined the ability of dog BC organoids to form tumors in vivo. Injection of the organoids into immunodeficient mice successfully generated tumors within 6 wk (Figure 2A). Cell clusters resembling the structure of organoids were observed in the BC organoid injection-derived tumor tissues (Figure 2B). As the xenografted tumor tissues contained many extracellular components, it was implied that BC organoids secreted cytokines to promote the proliferation of mesenchymal cells which resulted in tumor tissue occupation of mesenchymal components. Immunofluorescence staining was performed to identify the cell components of the tumor tissues. E-cadherin- (Figure 2C), CK7- (Figure 2D), CK20- (Figure 2E), UPK3A- (Figure 2F), and Ki67-positive cells (Figure 2G) were observed in the tumor tissues. These findings suggested that urine-derived dog BC organoids could reproduce tumor tissues in vivo and show similar features to the organoid cells.
To demonstrate the use of BC organoids as a preclinical model for checking the sensitivity of anticancer drugs, we performed a 96-well Matrigel cell viability assay, as described previously. Treatment with a nonselective cyclooxygenase (COX) inhibitor, piroxicam, had no effect on the cell viability of each BC organoid (Figure 3A,B). Treatment with a pyrimidine antimetabolite, gemcitabine, had no effect on the cell viability of organoids, except for the 2nd BC organoid (Or 2) (Figure 3A,C). Treatment with a DNA-damaging agent, cisplatin, or a microtubule inhibitor, vinblastine, decreased the cell viability of organoids in a dose-dependent manner (Figure 3A,D,E).

3.4 Effects of combination treatment with anticancer drugs on BC organoids

In the veterinary clinic, multiple anticancer drug therapies are used to treat BC diseased dogs. However, it is difficult to predict which combination therapies will be effective for each BC diseased dog. We therefore investigated the response to several patterns of co-treatment with anticancer drugs using BC organoids. In three out of four BC organoids (Ors 1, 2, and 3), co-treatment with gemcitabine and cisplatin significantly improved responses compared with cisplatin alone, while it had no additional effects on one organoid (Or 4) (Figure 4A). In two out of four BC organoids (Or 1
and Or 3), co-treatment with piroxicam and cisplatin significantly improved response compared with cisplatin treatment alone, while it had no additional effects on other organoids (Ors 2 and 4) (Figure 4A). In three out of four BC organoids (Ors 1, 2, and 3), co-treatment of gemcitabine with vinblastine significantly improved response compared with vinblastine treatment alone, while this had no additional effect on one organoid (Or 4) (Figure 4B). In one out of four BC organoids (Or 3), co-treatment with piroxicam and vinblastine significantly improved response compared with vinblastine treatment alone, while it had no additional effect on other organoids (Or 1, 2, 4) (Figure 4B). These results suggested that response to co-treatment with anticancer drugs is different in each BC organoid, and that cell viability testing using BC organoids might become a useful tool for measuring differences in the sensitivity of combination therapies.

### 3.5 | RNA-sequencing analysis of BC organoids

We next investigated the difference between gene expression profiles from normal bladder tissues, 2D BC cell lines, and BC organoid samples using RNA-seq analysis. Expression of each sample was clearly separated in a PCA plot (Figure 5A). Differential expression analysis showed that several genes were differentially regulated in BC organoid samples compared with normal bladder tissues or 2D BC cell lines (Figure 5B). In muscle-invasive human BC, a basal-like subtype showed a more aggressive phenotype than a luminal-like subtype. However, the main subtype of dog BC remains unclear. We therefore compared the expression levels of basal or luminal cell markers in BC organoids using a heat map (Figure 5C). Expression of basal cell markers, including CK5, p63, CD44, and DSG3, was upregulated in BC organoids compared with normal bladder tissues or 2D BC cell lines, while expression of luminal cell markers, including GATA3, FABP4, ERBB2, and FOXA1, was not upregulated. To validate the RNA-seq data, we performed quantitative real-time PCR. Expression of CK5 and DSG3 mRNA was significantly upregulated in the BC organoids compared with normal bladder tissues or 2D BC cell lines (Figure 5D), while expression of GATA3 and ERBB2 mRNA was significantly downregulated compared with normal bladder tissues (Figure 5E). We further observed that CK5 protein was expressed in BC organoids as determined by immunohistochemistry (Figure 5F). Furthermore, we confirmed by western blotting that the expression of CK5 in BC organoids was significantly increased compared with 2D cells (Figure 5G, H). These results indicated that the main type of dog BC might be a basal-like subtype, as is the case for human muscle-invasive BC.

### 3.6 | Searching for novel diagnostic makers using BC organoids

To explore novel diagnostic marker genes using dog BC organoids, we analyzed RNA-seq data and picked up the top 30 highly upregulated genes in dog BC organoids compared with normal bladder tissues (Table 3) or 2D BC cell lines (Table 4). To validate the RNA-seq data, we prepared an additional three strains of normal bladder tissue samples and performed quantitative real-time PCR. Expression of MMP28 (Figure 6A), CTSE (Figure 6B), CNN3 (Figure 6C), TFPI2 (Figure 6D), COL17A1 (Figure 6E), and AGPAT4 (Figure 6F) mRNA was significantly upregulated in the BC organoid group compared with the normal bladder tissue group. Interestingly, the mRNA expression levels of these genes did not change in blood samples from each group (Figure S3). We also confirmed that expression levels of these genes were quite low in the 2D BC cell lines and significantly lower compared with each BC organoid (Figure 6A-F). Other selected genes (CK15, TGM2, GJB2, IL1R2, COL5A2, CDH3, CHST4, and ADORA2B) were not upregulated in some BC organoids or upregulated even in normal bladder tissues (Figure S4). Finally, we observed that MMP28, CTSE, CNN3, TFPI2, and AGPAT4 proteins were expressed in BC organoids (Figure 6G).

### 4 | DISCUSSION

In the current study, for the 1st time, dog BC organoids were generated and several genes specifically upregulated in the organoids were identified. The main findings of the current study are as follows: The organoids from BC diseased dogs expressed urothelial cell markers, and showed urothelial carcinoma-like structures, which resembled the architecture of invasive type of BC in dogs (Figure 1). Injection of BC organoids into mice formed tumors (Figure 2). The responsiveness to combination treatment with anticancer drugs was markedly changed in each organoid (Figure 4). Many basal but not luminal cell markers were upregulated in the BC organoids (Figure 5). Several novel genes were specifically upregulated in the BC organoids (Figure 6). Collectively, our data suggest that dog BC organoids might become a new tool to provide new insights for both dog BC therapy and diagnostic markers.

Dog BC, also classified as TCC, is usually a high-grade invasive cancer. Treatments for dogs with BC, such as surgery, radiation therapy, medical therapy, and local intravesical therapy were conducted. As BC diseased dogs usually have severe conditions and it was difficult to perform surgery or tissue biopsy prior to treatment, in the present study we could not obtain tissue samples to compare histology between original tumor tissues and generated organoids, as shown in our previous dog PC organoid paper. Furthermore, surgical resection in dogs with BC is limited due to the trigonal location (difficult for surgical operation) and metastases at the time of diagnosis. Therefore, systemic medical therapies, including chemotherapy agents (cisplatin, vinblastine, and gemcitabine) and COX inhibitors (piroxicam), are considered to be mainstay treatments. In particular, oral piroxicam (0.3 mg/kg daily with food) is known to be a useful palliative treatment for dogs with BC, which provides excellent quality of life. The direct effects of these drugs on patient-derived BC cells, however, have never been investigated. In the present study, for the 1st
(A) Basal cell marker

(B) Luminal cell marker

(C) Expression of CK5 mRNA (normalized to GAPDH)

(D) Expression of GATA3 mRNA (normalized to GAPDH)

(E) Expression of ERBB2 mRNA (normalized to GAPDH)

(F) BC organoids

(G) Expression of CK5 protein (relative to 2D cell)

(H) Expression of VCP protein (relative to 2D cell)
Hierarchical clustering of differentially expressed genes in normal bladder tissues, 2D BC cell lines, and BC organoids. Genes shown in blue are downregulated, while genes shown in red are upregulated. Expression of basal cell markers (D) and luminal cell markers (E) in normal bladder tissues, 2D BC cell lines, and BC organoids were determined by quantitative real-time PCR (n = 4). Expression levels of CK5, DSG3 (D), GATA3, and ERBB2 (E) are quantified based on the ratio of their expression level to that of GAPDH. Results are expressed as mean ± SEM *P ≤ 0.05 vs Normal; #P ≤ 0.05 vs 2D cell. (F) Protein expression of CK5 in BC organoids. Representative photomicrographs are shown (F; n = 4). Scale bar: 100 μm. (G) Expression of CK5 in 2D BC cell lines and BC organoids was determined by western blotting. Equal protein loading was confirmed using total VCP antibody. (H) Expression level of CK5 was analyzed using an ImageJ software and quantified (n = 4). Results are expressed as mean ± SEM *P ≤ 0.05 vs 2D cell.

| Name      | Normal | Or 1  | Or 2  | Or 3  | Or 4  | Fold increase (relative to normal) |
|-----------|--------|-------|-------|-------|-------|-----------------------------------|
| 1 SLPI     | 0.6    | 683.0 | 5.7   | 5.5   | 251.4 | 421.176 752.3                    |
| 2 S100A2   | 8.4    | 2312.6| 2088.0| 2545.5| 1645.8| 255.162 300.6                    |
| 3 LOC489428| 1.1    | 643.5 | 34.0  | 281.9 | 7.3   | 215.324 602.4                    |
| 4 CTSE     | 1.1    | 93.5  | 128.5 | 502.2 | 235.1 | 213.691 455                      |
| 5 CHST4    | 0.6    | 269.4 | 34.0  | 28.0  | 25.2  | 158.861 991.5                    |
| 6 ADORA2B  | 0.6    | 15.8  | 88.8  | 107.5 | 135.9 | 154.983 282.1                    |
| 7 HK2      | 1.7    | 433.9 | 157.8 | 111.4 | 110.6 | 120.821 426.1                    |
| 8 FBLM1    | 0.6    | 12.4  | 80.3  | 70.9  | 104.9 | 119.614 246.5                    |
| 9 MMP28    | 0.6    | 51.8  | 84.1  | 38.2  | 88.7  | 117.052 030.2                    |
| 10 TFPI2   | 2.8    | 184.8 | 326.0 | 35.0  | 735.5 | 114.153 761.3                    |
| 11 COL5A2  | 2.2    | 53.0  | 189.9 | 353.5 | 261.2 | 95.502 436.34                    |
| 12 IL1R2   | 1.1    | 27.0  | 144.6 | 39.7  | 188.7 | 89.106 915.88                    |
| 13 RHCG    | 0.6    | 149.9 | 13.2  | 24.9  | 4.1   | 85.576 011.25                    |
| 14 CKMT1B  | 0.6    | 46.2  | 24.6  | 58.4  | 59.4  | 83.998 604.46                    |
| 15 CK6A    | 0.6    | 165.7 | 0.9   | 14.0  | 0.0   | 80.464 846.31                    |
| 16 CK5     | 38.7   | 3048.5| 2702.1| 4184.6| 2154.3| 78.051 089.03                    |
| 17 COL17A1 | 15.2   | 1056.0| 833.3 | 1360.4| 1074.7| 71.347 086.34                    |
| 18 CK14    | 0.6    | 112.7 | 10.4  | 29.6  | 4.9   | 70.189 688.7                     |
| 19 FSCN1   | 1.1    | 59.7  | 84.1  | 57.6  | 104.1 | 68.062 078.44                    |
| 20 AGPAT4  | 1.7    | 58.6  | 140.8 | 83.3  | 132.6 | 61.668 709.28                    |
| 21 CK16    | 8.4    | 1890.0| 41.6  | 10.9  | 56.1  | 59.353 708.39                    |
| 22 CDH3    | 1.7    | 82.3  | 132.3 | 106.7 | 74.0  | 58.691 222.64                    |
| 23 C9H17orf64| 1.1  | 129.6 | 21.7  | 48.3  | 30.1  | 51.165 373.21                    |
| 24 ALDOC   | 2.2    | 59.7  | 71.8  | 184.5 | 130.2 | 49.697 809.89                    |
| 25 CNN3    | 2.2    | 124.0 | 116.2 | 82.5  | 111.5 | 48.353 037.49                    |
| 26 ADM     | 3.9    | 251.3 | 110.5 | 191.6 | 171.7 | 46.142 754.19                    |
| 27 ALDH1A3 | 0.6    | 21.4  | 25.5  | 10.9  | 43.1  | 44.966 681.58                    |
| 28 DDT4    | 4.5    | 126.2 | 102.0 | 316.1 | 223.7 | 42.772 478.99                    |
| 29 PDPN    | 1.1    | 23.7  | 30.2  | 37.4  | 93.6  | 41.169 345.72                    |
| 30 HIP1    | 2.8    | 30.4  | 142.7 | 81.0  | 196.9 | 40.177 382.67                    |

Table 3: Top 30 upregulated genes in dog bladder cancer organoids compared with normal bladder tissues.
in gemcitabine sensitivity, the expression levels of genes involved in gemcitabine metabolism (dCK, CDA, RRM1, RRM2, and SLC29A1) were measured using RNA-seq (Figure S5). However, no correlation between the sensitivity and gene expression pattern in organoids was found. Further investigations using dog BC organoids are needed to measure drug sensitivity and to select a suitable medical therapy for each BC diseased dog.

In the treatment of dog BC, medical therapy is not usually curative, and resistance to one drug often develops. Once treatment is tolerated, dogs sequentially receive multiple different treatment protocols over the course of their disease. The pattern of combination chemotherapy such as with cisplatin and piroxicam, carboplatin and piroxicam, vinblastine and piroxicam has been reported. For example, piroxicam enhanced the antitumor activity of carboplatin and cisplatin in dog BC. Piroxicam also significantly increased the activity of vinblastine in dog BC. However, renal toxicity is especially problematic when cisplatin is combined with piroxicam. In a previous report, the combination of cisplatin and piroxicam led to effective remission rates, but caused renal, gastrointestinal, and bone marrow toxicities. Considering these studies, simultaneous treatment with multiple chemotherapeutic agents might be more toxic than curative. It also remains unknown whether it would be more appropriate to simultaneously combine multiple chemotherapy agents in dogs with BC. To solve these problems, we measured which anticancer drug combination treatment affected cell viability of BC organoids. Co-treatment with gemcitabine and cisplatin or vinblastine was more effective than piroxicam (Figure 4) suggesting that, in the future, use of a cell viability assay for BC organoids would predict the most effective combination treatment for dogs.

### Table 4: Top 30 upregulated genes in dog bladder cancer organoids compared with 2D cell lines

| Name       | 2D cell | Or 1  | Or 2  | Or 3  | Or 4  | Fold increase (relative to 2D cell) |
|------------|---------|-------|-------|-------|-------|-------------------------------------|
| 1 CK5      | 1.9     | 3048.5| 2702.1| 4184.6| 2154.3| 1628.263 966                        |
| 2 TGM2     | 0.6     | 544.3 | 1382.2| 242.2 | 1031.6| 1293.094 044                        |
| 3 GJB2     | 0.6     | 871.2 | 300.4 | 122.3 | 127.7 | 574.395 25                          |
| 4 CK15     | 1.9     | 1165.3| 646.2 | 1009.2| 295.3 | 419.679 583 7                       |
| 5 ANXA6    | 0.6     | 20.3  | 334.5 | 245.3 | 372.6 | 392.993 828 9                       |
| 6 CDH13    | 0.6     | 10.1  | 171.0 | 142.5 | 162.5 | 405.2 294 471 91                   |
| 7 NDUFA4L2 | 0.6     | 313.3 | 85.0  | 161.2 | 78.9  | 257.961 221 9                       |
| 8 CP       | 1.9     | 9.0   | 515.9 | 128.5 | 732.2 | 186.611 392 8                       |
| 9 COL5A2   | 1.2     | 53.0  | 189.9 | 353.5 | 261.2 | 173.245 696 5                       |
| 10 IL1R2   | 0.6     | 27.0  | 144.6 | 39.7  | 188.7 | 161.643 935 9                       |
| 11 CDH3    | 0.6     | 82.3  | 132.3 | 106.7 | 74.0  | 159.702 759 4                       |
| 12 CYP1B1  | 0.6     | 86.8  | 79.4  | 131.6 | 91.1  | 157.117 706 5                       |
| 13 AUTS2   | 0.6     | 105.9 | 115.3 | 58.4  | 102.5 | 154.392 274 5                       |
| 14 SEMA3G  | 0.6     | 38.3  | 69.9  | 86.4  | 168.4 | 146.699 493 7                       |
| 15 S100A12 | 0.6     | 353.9 | 1.9   | 0.0   | 1.6   | 144.404 975 4                       |
| 16 CHST4   | 0.6     | 269.4 | 34.0  | 28.0  | 25.2  | 144.091 383 5                       |
| 17 ADORA2B | 0.6     | 15.8  | 88.8  | 107.5 | 135.9 | 140.573 307 2                       |
| 18 NCCRP1  | 1.2     | 293.0 | 205.0 | 126.1 | 65.9  | 139.414 207 3                       |
| 19 CYP1A1  | 9.3     | 668.3 | 1584.4| 1497.4| 954.3 | 126.721 804 2                       |
| 20 MMP28   | 0.6     | 51.8  | 84.1  | 38.2  | 88.7  | 106.168 812 4                       |
| 21 SCEL     | 0.6     | 176.9 | 8.5   | 39.7  | 14.6  | 96.890 595 78                       |
| 22 C9H17orf64 | 0.6   | 129.6 | 21.7  | 48.3  | 30.1  | 92.816 278 39                       |
| 23 EGLN3   | 8.0     | 757.3 | 745.4 | 851.9 | 534.5 | 89.797 596 59                       |
| 24 FADS2   | 0.6     | 5.6   | 42.5  | 101.2 | 61.8  | 85.339 282 83                       |
| 25 GPR115  | 0.6     | 75.5  | 56.7  | 30.4  | 42.3  | 82.777 703 16                       |
| 26 SSPN    | 0.6     | 4.5   | 36.8  | 54.5  | 74.0  | 68.646 626 33                       |
| 27 DKK3    | 0.6     | 4.5   | 9.4   | 58.4  | 87.9  | 64.737 402 18                       |
| 28 LOC100685649 | 6.8     | 1382.8| 159.7 | 59.2  | 29.3  | 59.908 252 73                       |
| 29 ATP2C2  | 0.6     | 51.8  | 32.1  | 20.2  | 43.1  | 59.528 317 9                       |
| 30 FA2H    | 1.2     | 91.3  | 35.0  | 42.8  | 57.8  | 45.826 176 65                       |
Expression of MMP28 mRNA (normalized to GAPDH)

Expression of CTSE mRNA (normalized to GAPDH)

Expression of CNN3 mRNA (normalized to GAPDH)

Expression of TFPI2 mRNA (normalized to GAPDH)

Expression of COL17A1 mRNA (normalized to GAPDH)

Expression of AGPAT4 mRNA (normalized to GAPDH)

BC organoids

MMP28 CTSE CNN3 TFPI2 AGPAT4
Understanding how tumors arise requires identification of the cancer cell origin. Although it is widely assumed that CSCs may arise from normal stem cells undergoing gene mutations via a complex mechanism, the origin of CSCs is still unclear. In general, urothelial lesions are classified into basal and luminal types that have different gene expression patterns. Basal and luminal subtypes of BC show distinct clinical behaviors and responses to frontline chemotherapy. For example, muscle-invasive basal BC is more aggressive with shorter survival time, high metastasis, and is more sensitive to cisplatin-based chemotherapy compared with luminal cancers. Furthermore, it was demonstrated that tumor-propagating cells isolated from BC have a basal-type characteristics in human and mice, suggesting that the origins of muscle-invasive BC are mainly basal cells. As it remains unclear which subtype of cells contributes to the development of dog BC, we examined the expression pattern of basal and luminal cell markers in BC organoids (Figure 5). Expression of several basal cell markers including CK5 and DSG3 was upregulated in most BC organoids. Conversely, luminal cell marker expression, including GATA3 and ERBB2, was downregulated. These data suggest that the cell origin of dog BC organoids might be basal cells, as is the case with muscle-invasive human BC.

In the present study, we found that expression levels of MMP28, CTSE, CNN3, TFPI2, COL17A1, and AGPAT4 were specifically upregulated in dog BC organoids (Figure 6). MMP28 is a member of metalloproteinase family; it is expressed in many normal tissues and has a role in the production of cytokines and growth factors. CTSE is an aspartic endopeptidase belonging to the cathepsin family of proteases and functions mainly to liberate peptide epitopes for antigen presentation. CNN3 is involved in the regulation of cell migration and the placentaion process. TFPI2 encodes a Kunitz-type serine proteinase inhibitor with inhibitory function against tumor growth and metastasis; protecting the extracellular matrix of cancer cells from degradation and tumor invasion. COL17A1 encodes collagen XVII, a transmembrane protein that is an essential component of type I hemidesmosomes and acts as a cell-matrix adhesion molecule. AGPAT4 functions mainly to convert lysophosphatidic acid to phosphatidic acid, the 2nd step in de novo phospholipid biosynthesis. In a previous study, expression levels of MMP28 were associated with human high-grade BC. Overexpression of CTSE is associated with several types of cancer including human noninvasive BC. These studies suggested that genes identified in the present study may be promising biomarkers for dog BC. Our established dog BC organoid system could become in the near future a useful tool to identify further novel biomarkers of both dog and human BC.

In conclusion, for the 1st time, we produced dog BC organoids from urine samples. The organoids showed tumorigenesis in vivo and basal-like subtype. It was also suggested that several genes might become biomarkers to predict the development of high-grade BC. Further studies on the dog BC organoid contribution to the treatment and diagnosis of both dog and human BC.

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Additional supporting information may be found online in the Supporting Information section at the end of the article.