Received: 11 April 2019 | Revised: 20 June 2019 | Accepted: 27 June 2019

DOI: 10.1111/cas.14118

ORIGINAL ARTICLE

Cancer Science WILEY

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

Abbreviations: 2D , two-dimensional; 3D , three-dimensional; BC , bladder cancer; COX , cyclooxygenase; CSCs , cancer stem cells; PC , prostate cancer; PCA , principle component analysis; SMA , smooth muscle actin.

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Clinical Trial register and clinical registration number: 0016012 (Institute Animal Care and Use Committee of Tokyo University of Agriculture and Technology approval).

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Funding information
Japan Society for the Promotion of Science, Grant/Award Number: 17K15370

characteristics and gene expression profiles, and could be an important research model for this disease. Cancer patient-derived organoid culture can recapitulate organ structures and maintains the gene expression profiles of original tumor tissues. In a previous study, we generated 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.

KEYWORDS
biomarker, bladder cancer, dog, organoid, RNA-seq

1 | INTRODUCTION

Bladder cancer comprises approximately 1-2% of all naturally occurring cancers in dogs, a similar rate to that found in human.[1,2] While more than 90% of BC found in dogs consist of intermediate- to high-grade invasive urothelial carcinomas,[3-5] 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.[4,6-8]

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.[9] 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,[10-13] suggesting that dog BC could be a relevant model for muscle-invasive human BC.[10,14] 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.[15,16]

In a previous study, we established a culture method for dog prostate cancer (PC) organoids using urine samples from PC diseased dogs.[17] The organoids recapitulated the tumor microenvironment of dog PC tissues and showed tumorigenesis in vivo. Furthermore, this model could be used to investigate sensitivity of anticancer drugs.

Conversely, dog BC organoids had never been established. We therefore collected urine samples from BC–diseased dogs and cultured these samples using the previously described urine-derived organoid culture method. Here, for the 1st time, dog BC organoids were generated and demonstrated that BC organoids could be useful for analysis of tumorigenesis and to determine the sensitivity of anticancer drugs. We also identified novel diagnostic marker candidates (*MMP28*, *CTSE*, *CNN3*, *TFPI2*, *COL17A1*, and *AGPAT4*) by analyzing RNA-seq data from urine-derived BC organoids.

2 | 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.[17] Anticancer drugs used were as

TABLE 1 Sample information

Case ID	Age (y old)	Breed	Sex	Sample date	Muscle-invasive or not	Prior therapy	Other information
BC17004	11	ShiTzu	Male (not castrated)	12-10-2017	Muscle-invasive	Piroxicam, Enrofloxacin	This sample was used as Or 1
BC18004	12	Miniature Dachshund	Female (spayed)	02-05-2018	Muscle-invasive	Alendronate, Prednisolone	This sample was used as Or 2
BC18005	11	Mix	Female (spayed)	11-05-2018	Muscle-invasive	Piroxicam, Misoprostol	This sample was used as Or 3
BC18006	12	Miniature Dachshund	Female (spayed)	26-06-2018	Muscle-invasive	Prednisolone, Lansoprazole, Orbifloxacin	This sample was used as Or 4

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 (Bioss); 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.[17] 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 trypsination. 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 CO_2 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.[17,18] 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, CK7;1:100, CK20;1:200, UPK3A;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-*Prkdc*^{scid} 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 mice. 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 cmpfastq_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 Primers for real-time quantitative PCR analysis

	Primer	Sequence
CK5	Forward	5′-CAAGGTCCTGGACACCAAGT-3′
	Reverse	5′-ATGCTGTCCAGCTGTCTCCT-3′
DSG3	Forward	5′-CCTTGGGTTGTTGCAGTTTT-3′
	Reverse	5′-ATCGATCCCGAGGCTTATCT-3′
GATA3	Forward	5′-GTCCCTCCAGCCCTTTCTAC-3′
	Reverse	5′-GGCAAACGTCATTTTGCTTT-3′
ERBB2	Forward	5′-CCCCGAGAGTATGTGAAGGA-3′
	Reverse	5′-ACTTCCAGATGGGCATGAAG-3′
MMP28	Forward	5′-GAGGCGTAAGAAACGCTTTG-3′
	Reverse	5′-ATTGCTCCACAGTTGGAAGG-3′
CTSE	Forward	5′-CAGACCTTTGTGAACGCAGA-3′
	Reverse	5′-GTGGTCATAGCCTCCGAAAA-3′
CNN3	Forward	5′-TCCCAAAATGCAAACTGACA-3′
	Reverse	5′-CGCAGTACTTGGGGTCGTAT-3′
TFPI2	Forward	5′-AACGCCAACAACTTCGAAAC-3′
	Reverse	5′-AGCAGAGCACAGTCCCTCAT-3′
COL17A1	Forward	5′-CGGGAGATCCAGCAGTACAT-3′
	Reverse	5′-GACGCAGATACTGCCTCACA-3′
AGPAT4	Forward	5′-AATTCCTGTGCCATCTCGTC-3′
	Reverse	5′-CACCACTCCAGCAACATCAC-3′
GAPDH	Forward	5′-AACTCCCTCAAGATTGTCAGCAA-3′
	Reverse	5′-CATGGATGACTTTGGCTAGAGGA-3′
CK15	Forward	5′-AGACAGTGGACGGGAAAGTG-3′
	Reverse	5′-ACCCTCTGAAAGCAGGGACT-3′
TGM2	Forward	5′-TCAGAAAGGATGGGATGAGG-3′
	Reverse	5′-AGGTGGGGTTTCACACTCAG-3′
GJB2	Forward	5′-CCCATCTCTCACATCCGACT-3′
	Reverse	5′-CAAAGATGACCCGGAAGAAA-3′
IL1R2	Forward	5′-AGACGAGAATGTGGGTCCAG-3′
	Reverse	5′-TCTGGCAGTGCAGATGTAGG-3′
COL5A2	Forward	5′-CCTCAGGGAATTGATGGAGA-3′
	Reverse	5′-CTGCCCTTGTAAGCCTTGAG-3′
CDH3	Forward	5′-GGAGCCTATCACCTGCCATA-3′
	Reverse	5′-TGTCCGGAGAGGAGAGAGAA-3′
CHST4	Forward	5′-TGCTCAAGGAGGTACGCTTT-3′
	Reverse	5′-TAGCGTTCCCTCAGTGCTTT-3′
ADORA2B	Forward	5′-AAGCTGTTGCCTCGTGAAGT-3′
	Reverse	5′-AGCCAGCACAGAGCAAAAAT-3′

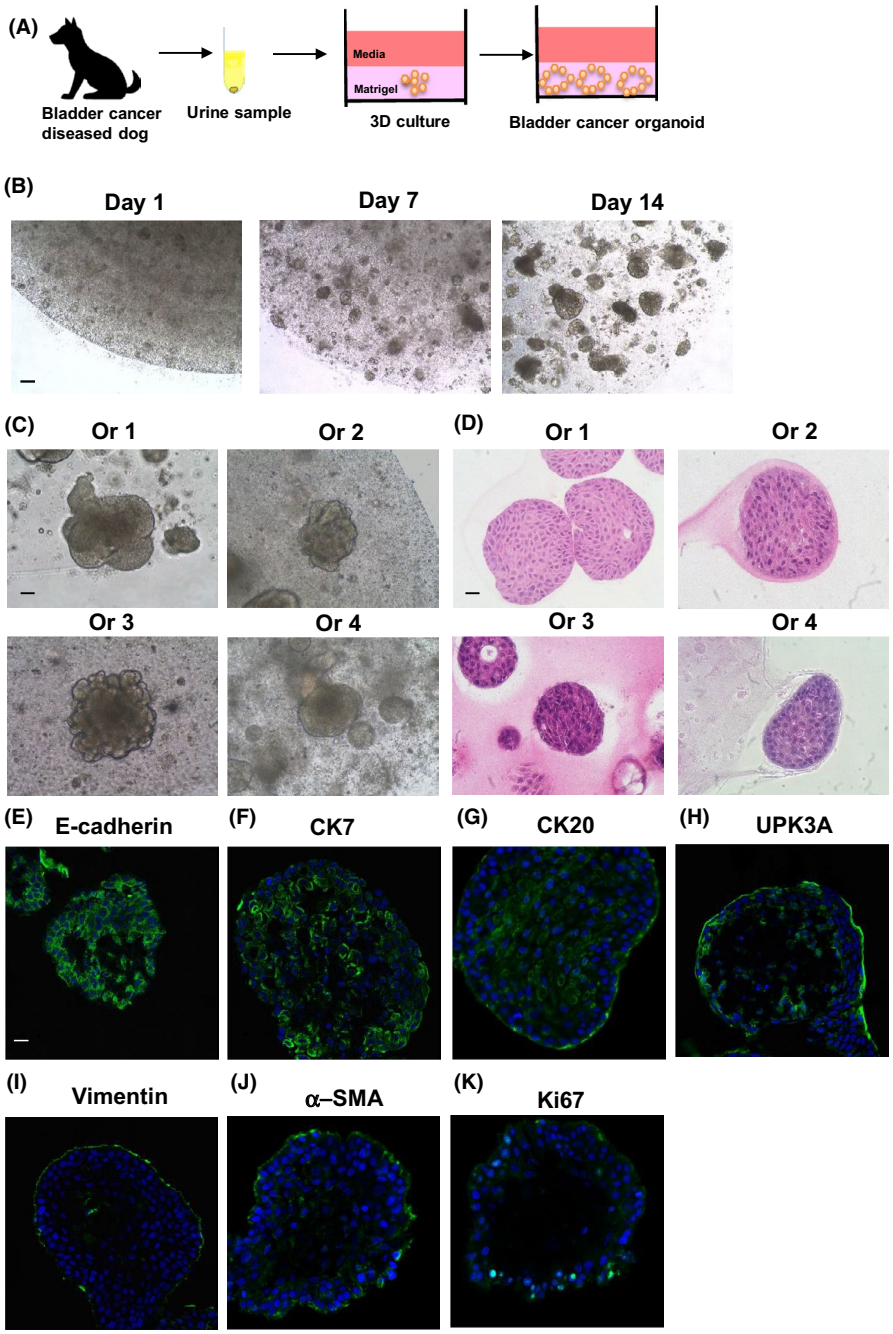

FIGURE 1 Generation of dog BC organoids. (A) Schematic experimental design of a procedure for generation of dog BC organoids using urine samples. (B) Images of a representative growing process were taken at days 1, 7, and 14 after seeding the cells. Scale bar: 500 μm. Representative images for phase-contrast (C) and hematoxylin and eosin (H&E) staining (D) (Culture days 14-21) of each dog-derived organoids (Ors 1-4) were shown. Scale bar: 200 μm (C), 50 μm (D). Expression of an epithelial cell marker, E-cadherin (E), urothelial cell markers, CK7 (F), CK20 (G), and UPK3A (H), a fibroblast cell marker, vimentin (I), a myofibroblast marker, α-smooth muscle actin (SMA) (J), and a proliferating cell marker, Ki67 (K) in the organoids. Representative photomicrographs were shown (n = 4). Scale bar: 50 μm (E-K).

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.[17] 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

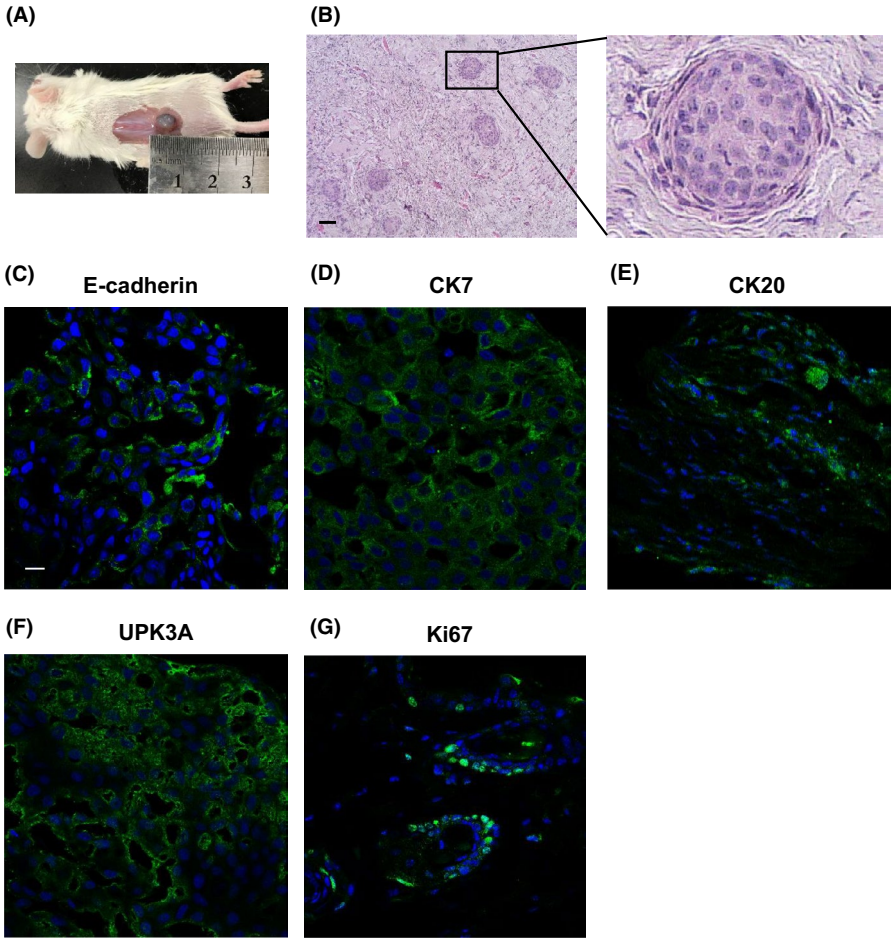

FIGURE 2 Tumorigenesis induced by BC organoids. The trypsinized BC organoid cells were subcutaneously injected into the back of NOD/SCID mice (n = 4). At 6 wk later, the formed tumors were stained with H&E and immunofluorescence recorded. (A) Observation of BC organoid injection-induced tumor formation. (B) Representative images with H&E staining of the tumor tissues were shown. The enlarged image is shown on the right. Scale bar: 100 μm. E-cadherin (C), CK7 (D), CK20 (E), UPK3A (F), and Ki67 (G) expression is shown. Representative photomicrographs are shown (n = 4). Scale bar: 50 μm.

biotinylated secondary antibody/PBS (1:500) for 30 min. Slides were treated with a solution of ABC kit (Vector Laboratories) for 30 min and then with a solution of DAB for 3-5 min. Images were obtained using a light microscope (BX-52).

2.13 | Western blotting

Western blotting was performed as described previously.[26] 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,[1,27] 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.[17] 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 (Figure 1C), and a similar histology to dog urothelial carcinomas reported previously[27] (Figure 1D). For the immunofluorescence assay, expression of an epithelial cell marker, E-cadherin, was observed in all organoids (Figure 1E). Expression of urothelial cell markers, CK 7 (Figure 1F), CK20 (Figure 1G), and UPK3A, (Figure 1H) was also

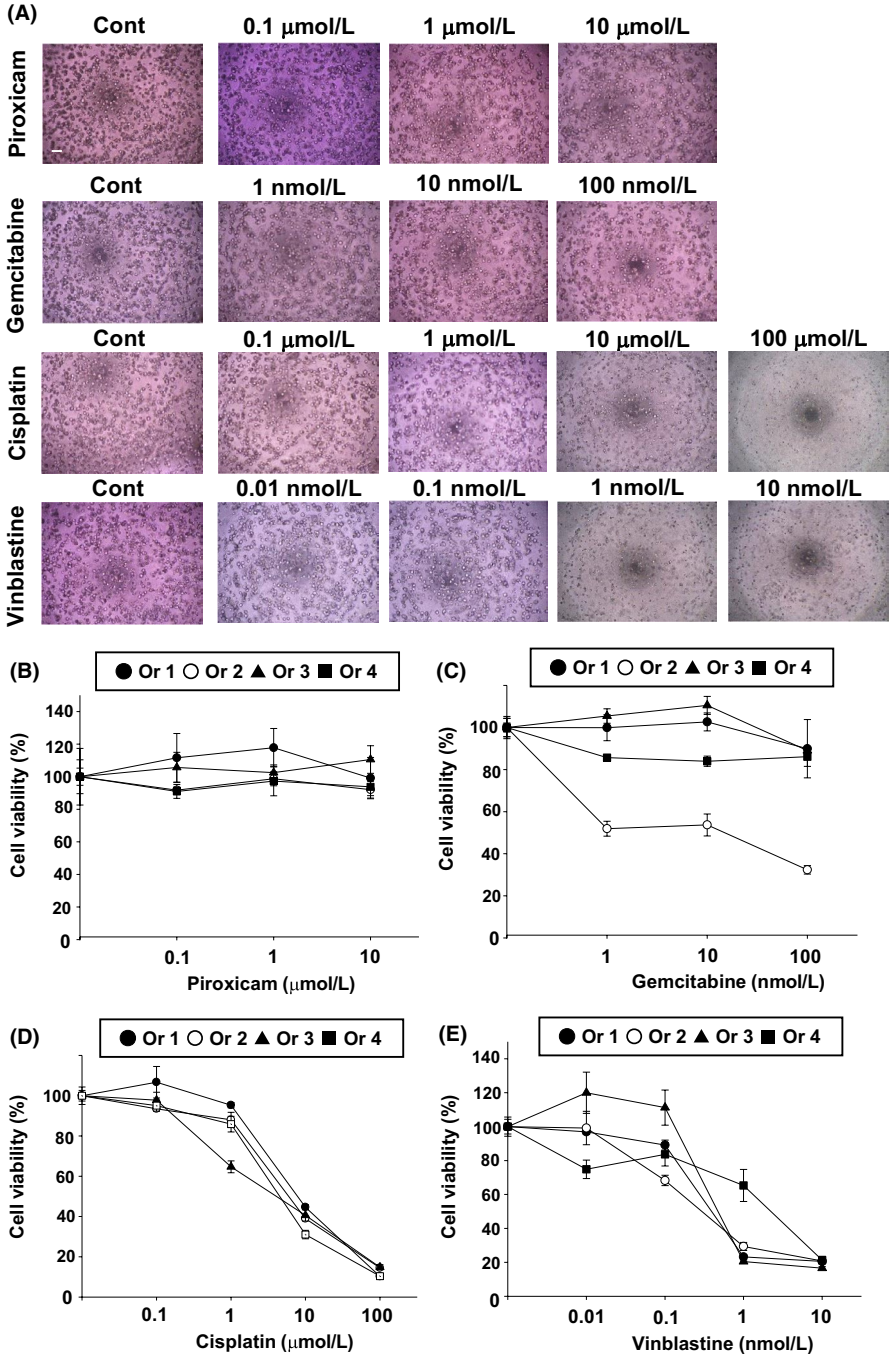

FIGURE 3 Effects of anticancer drugs on BC organoids. After BC organoids were trypsinized and seeded into Matrigel, they were treated with piroxicam (0.1-10 μmol/L), gemcitabine (1-100 nmol/L), cisplatin (0.1-100 μmol/L) or vinblastine (0.01-10 nmol/L) for 3 d (n = 6 each for four organoids [Ors 1-4]). (A) Representative phase-contrast images of BC organoids treated with piroxicam, gemcitabine, cisplatin or vinblastine are shown. Scale bar: 500 μm. (B-E) Cell viability was assessed using an alamarblue assay, and 100% represents cell viability for each control. Results are expressed as mean ± SEM.

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.

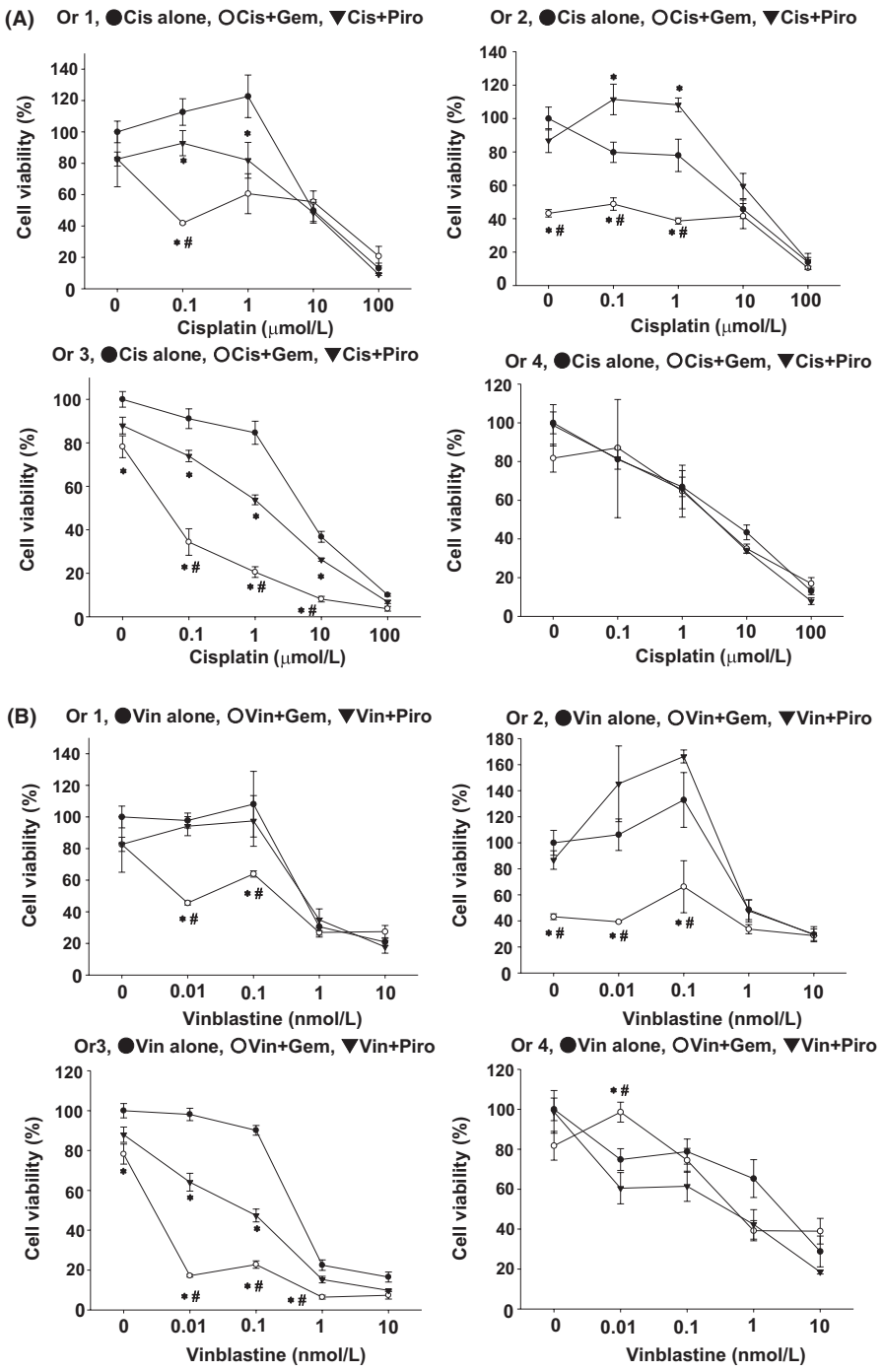

FIGURE 4 Effects of combination treatment with anticancer drugs on BC organoids. After BC organoids were trypsinized and seeded into Matrigel, they were treated with cisplatin (A) or vinblastine (B) in the presence or absence of piroxicam (10 μmol/L) or gemcitabine (100 nmol/L) for 3 d (n = 3-6 each for four organoids [Ors 1-4]). Cell viability was determined using an alamar blue assay; 100% represents cell viability of each control. Results were expressed as mean ± SEM $^{*}P \leq 0.05$ vs Cis alone; $^{\#}P \leq 0.05$ vs Cis + Piro (A). $^{*}P \leq 0.05$ vs Vin alone; $^{\#}P \leq 0.05$ vs Vin + Piro (B).

3.3 | Effects of anticancer drugs on BC organoids

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.[18,19] 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, expect 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.[28-32] 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.[33,34] 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.[17] 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.[35] Therefore, systemic medical therapies, including chemotherapy agents (cisplatin, vinblastine, and gemcitabine) and COX inhibitors (piroxicam), are considered to be mainstay treatments.[13,35] 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.[13] The direct effects of these drugs on patient-derived BC cells, however, have never been investigated. In the present study, for the 1st

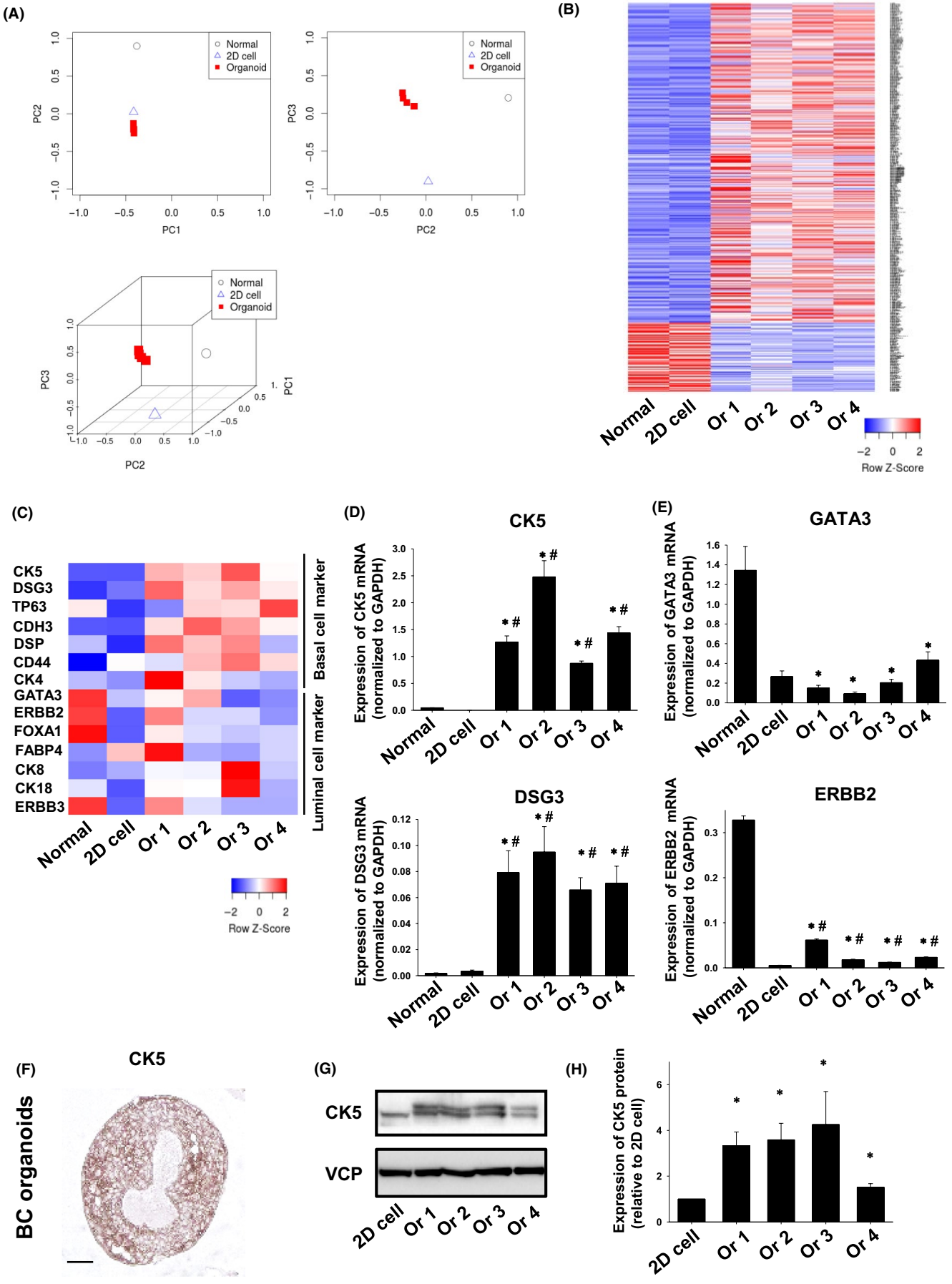
(A)
Normal
2D cell
Organoid
PC1
PC2
PC3
(B)
Normal
2D cell
Or 1
Or 2
Or 3
Or 4
Row Z-Score
(C)
CK5
DSG3
TP63
CDH3
DSP
CD44
CK4
GATA3
ERBB2
FOXA1
FABP4
CK8
CK18
ERBB3
Basal cell marker
Luminal cell marker
Row Z-Score
(D)
CK5
Expression of CK5 mRNA (normalized to GAPDH)
DSG3
Expression of DSG3 mRNA (normalized to GAPDH)
(E)
GATA3
Expression of GATA3 mRNA (normalized to GAPDH)
ERBB2
Expression of ERBB2 mRNA (normalized to GAPDH)
(F)
CK5
BC organoids
(G)
CK5
VCP
2D cell
Or 1
Or 2
Or 3
Or 4
(H)
Expression of CK5 protein (relative to 2D cell)

FIGURE 5 RNA sequencing analysis of BC organoids. (A) PCA plot of normal bladder tissues, 2D BC cell lines, and BC organoids. (B) 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. (C) Heatmap analysis of basal cell- and luminal cell-related 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 $^{*}P \leq 0.05$ vs Normal; $^{\#}P \leq 0.05$ 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 $^{*}P \leq 0.05$ vs 2D cell.

TABLE 3 Top 30 upregulated genes in dog bladder cancer organoids compared with normal bladder tissues

	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	FBLIM1	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	DDIT4	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

time, dog BC organoids were generated (Figures 1,2) and showed that piroxicam had no effect on cell viability of dog BC organoids (Figure 3A,B). In contrast, treatment with cisplatin or vinblastine decreased cell viability of BC organoids in a dose-dependent manner, similarly to the clinical use of cisplatin[28,36] and vinblastine[37,38] in the treatment of BC dogs. Interestingly, gemcitabine had no effect on most strains of dog BC organoids, except for the 2nd dog BC organoids (Figures2 and 3C). To clarify the difference

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	405.2	294.471 941
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

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.[13] The pattern of combination chemotherapy such as with cisplatin and piroxicam,[28,29,39] carboplatin and piroxicam,[40] vinblastine and piroxicam[41] has been reported. For example, piroxicam enhanced the antitumor activity of carboplatin[40] and cisplatin[28,29,39] in dog BC. Piroxicam also significantly increased the activity of vinblastine in dog BC.[41] However, renal toxicity is especially problematic when cisplatin is combined with piroxicam.[28] 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.

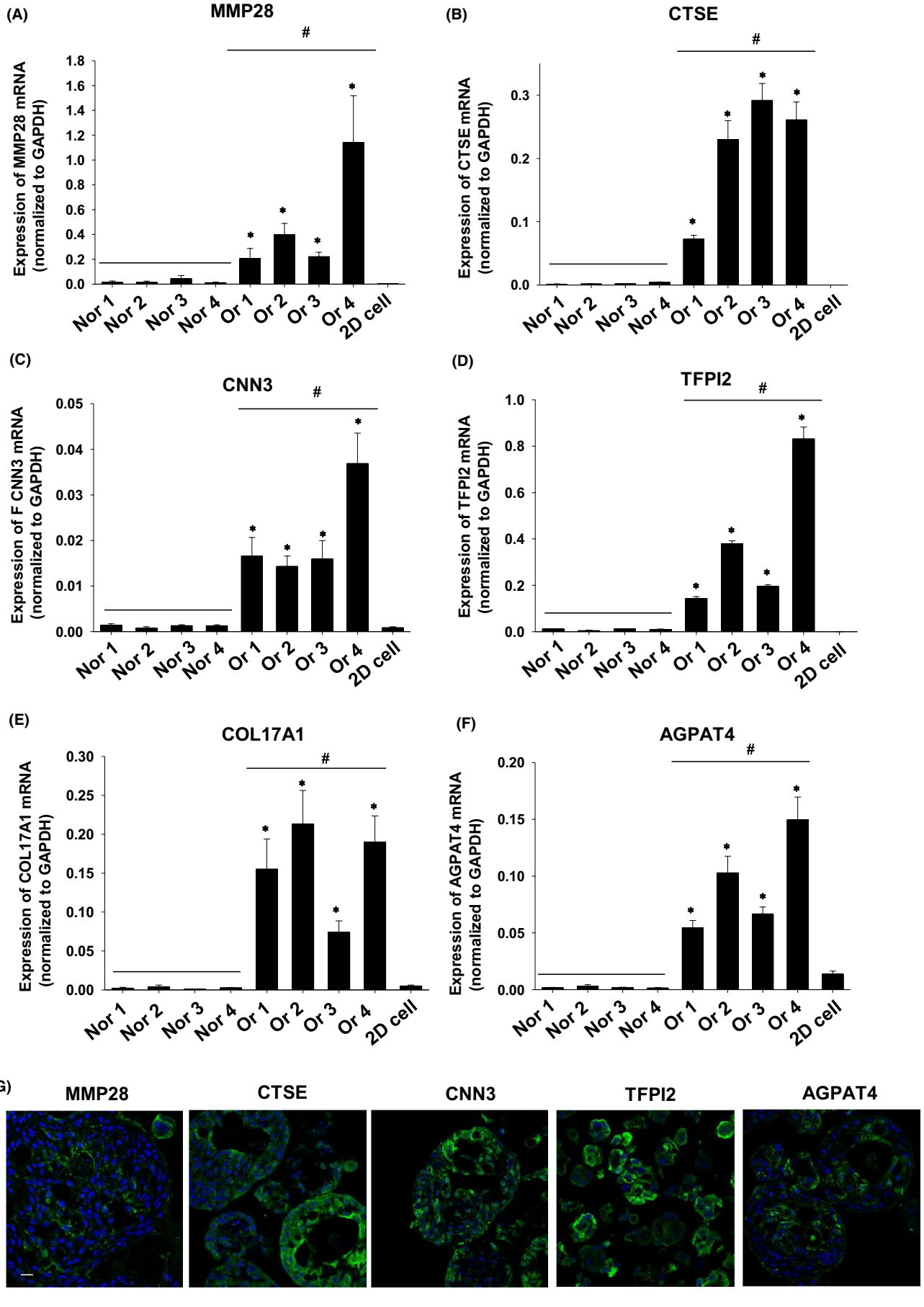
(A)
MMP28
Expression of MMP28 mRNA (normalized to GAPDH)
(B)
CTSE
Expression of CTSE mRNA (normalized to GAPDH)
(C)
CNN3
Expression of F CNN3 mRNA (normalized to GAPDH)
(D)
TFPI2
Expression of TFPI2 mRNA (normalized to GAPDH)
(E)
COL17A1
Expression of COL17A1 mRNA (normalized to GAPDH)
(F)
AGPAT4
Expression of AGPAT4 mRNA (normalized to GAPDH)
Nor 1
Nor 2
Nor 3
Nor 4
Or 1
Or 2
Or 3
Or 4
2D cell
(G)
MMP28
CTSE
CNN3
TFPI2
AGPAT4
BC organoids

FIGURE 6 Search for novel diagnostic makers by using BC organoids. Expression of *MMP28* (A), *CTSE* (B), *CNN3* (C), *TFPI2* (D), *COL17A1* (E), and *AGPAT4* (F) mRNA in normal bladder tissue (Nors 1-4), BC organoids (Ors 1-4), and 2D BC cell lines was determined by quantitative real-time PCR (n = 4). Expression level of each gene was quantified based on the ratio of expression level to that of GAPDH. Results are expressed as mean ± SEM $^{*}P \leq 0.05$ vs 2D cell; $^{\#}P \leq 0.05$ vs Normal. Protein expression of MMP28, CTSE, CNN3, TFPI2, and AGPAT4 in BC organoids (G). Representative photomicrographs are shown (G; n = 4). Scale bar: 50 μm.

Understanding how tumors arise requires identification of the cancer cell origin.[42] Although it is widely assumed that CSCs may arise from normal stem cells undergoing gene mutations[43] via a complex mechanism,[44] the origin of CSCs is still unclear.[44,45] 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.[33,46,47] 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.[33,46] Furthermore, it was demonstrated that tumor-propagating cells isolated from BC have a basal-type characteristics in human[48-51] and mice,[42] 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;[52] it is expressed in many normal tissues and has a role in the production of cytokines and growth factors.[53,54] CTSE is an aspartic endopeptidase belonging to the cathepsin family of proteases and functions mainly to liberate peptide epitopes for antigen presentation.[55,56] CNN3 is involved in the regulation of cell migration[57,58] and the placentation process.[59] TFPI2 encodes a Kunitz-type serine proteinase inhibitor[60] with inhibitory function against tumor growth and metastasis;[61,62] protecting the extracellular matrix of cancer cells from degradation and tumor invasion.[63] COL17A1 encodes collagen XVII (COL17), a transmembrane protein that is an essential component of type I hemidesmosomes and acts as a cell-matrix adhesion molecule.[64] AGPAT4 functions mainly to convert lysophosphatidic acid to phosphatidic acid, the 2nd step in de novo phospholipid biosynthesis.[65] In a previous study, expression levels of MMP28 were associated with human high-grade BC.[66] Overexpression of CTSE is associated with several types of cancer including human noninvasive BC.[67] 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.

ACKNOWLEDGMENTS

We thank Dr. Kuo and Dr. Salahudeen for kindly providing Wnt, Noggin and R-spondin-producing cells.

DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

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

How to cite this article: Elbadawy M, Usui T, Mori T, et al. Establishment of a novel experimental model for muscle-invasive bladder cancer using a dog bladder cancer organoid culture. *Cancer Sci*. 2019;110:2806–2821. https://doi.org/10.1111/cas.14118