Experimental Methods for Establishing and Maintaining Canine Bladder Cancer Organoids

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Abstract

Dogs share many anatomical and physiological characteristics with humans and, in certain instances, represent a more reliable research model than rodents for some diseases, including several types of human cancer. Advances in adult stem cell research have accelerated the development of 3D organoid cultures in non-model animals, such as dogs. These advancements have the potential to significantly impact disease modeling, biomarker discovery, and drug development for both human and canine diseases, including bladder cancer (BC). In this Chapter, we present a standardized protocol for generating BC organoids from canines. The chapter describes the detailed methodology for establishing canine BC organoids from urine or tissue-derived stem cells. Additionally, it highlights the potential of these organoids to recapitulate the expression of key markers from their original tissues and their utility in various biomedical applications such as drug screening, gene mutation analysis, and toxicology. Finally, we discuss potential future research directions for canine BC organoids in translational research.

Advantages of Canine Adult Stem Cell (ASC)-Derived Organoids

Rodent models, particularly mice, have extensively been used to study human diseases due to their cost effectiveness, and ease of access to genetically engineered animals. Adult stem cell (ASC)-derived organoids are three-dimensional cellular aggregates grown in an extracellular matrix (ECM) and stem cell supporting media. These organoids have the ability to accurately recapitulate the epithelial architecture of the primary tissue and can self-renew, self-organize, and differentiate into all epithelial cell types found in the parent tissue. Recent advances in organoid technology have opened avenues for the development of novel and fundamental research such as disease modeling, drug testing, and future transplantation attempts (Artegiani and Clevers 2018; Gabriel et al. 2024; Abugomaa and Elbadawy 2020). ASC-derived organoids have been developed from multiple epithelial tissues and species, including mouse models, alongside ASC-derived organoids from healthy and diseased human tissues. However, the use of murine models in biomedical research does not always allow for an accurate prediction of the efficacy of compounds in a pre-clinical setting (Perlman 2016). This has been attributed to the fact that mouse models of human diseases often fail to accurately mimic the underlying human diseases. For chronic conditions, such as cancer, diabetes mellitus, Alzheimer's disease, inflammatory bowel disease, and cancer, it can be difficult to accurately model the human disease in rodents due to their short lifespan, different anatomy and physiology as well as vastly different diet and intestinal microbiome (LeBlanc et al. 2016; Coelho et al. 2018; Schneider et al. 2018).

The use of more diverse models, including dogs, has recently gained momentum. Dogs develop spontaneous diseases that are analogous to human diseases, which are typically more representative of chronic disease states than rodent models of diseases. One factor seen as advantageous for the biopharmaceutical industry is the fact that dogs have a relatively large body size, allowing for improved comparative pharmacological and dose-finding studies of compounds. Additionally, the longer lifespan of dogs compared to rodents, and their anatomy and physiology more closely resembling humans, are added benefits when using dogs. On the other hand, using the *in vivo* dog model, either by assessing

safety and toxicity in healthy dogs (on purpose-bred research dogs) or testing the efficacy of compounds in dogs with analogous diseases as humans (in canine clinical trials to gain pre-clinical efficacy for human trials), is expensive and ethically questionable if using healthy dogs for research. Hence, using ASC-derived organoids from epithelial tissues of both healthy dogs and dogs that develop spontaneous diseases closely resembling those in humans, could help bridge this divide (Mochel et al. 2017). Recently, there has been progress in developing larger bioarchives of canine organoid cell lines, which can be made available to researchers and eventually diminish the use of live dogs in biopharmaceutical research (Chandra et al. 2019; Gabriel et al. 2024; Allenspach et al. 2023; Elbadawy et al. 2019) and (https://caninecommons.cancer.gov/#/)

To this end, cancer has recently gained recognition as one of the diseases that can benefit from using dogs as a natural disease model. Dog and human cancers share numerous important characteristics, including a similar histological appearance, incidence ratio, clinical behavior such as the development of metastatic disease, immune response patterns, and genetic and histological heterogeneity within tumors and among patients (de Brot et al. 2018; Knapp et al. 2019). In dogs, BC comprises about 1.5–2% of all naturally occurring cancers, a rate similar to that documented in humans (Knapp et al. 2014; Antoni et al. 2017). In both species, BC occurs mainly at alder ages and with similar clinical symptoms including hematuria being the most common. Also, great resemblance was noted in the microscopic picture and prostate involvement ratio between canine and human BC (Knapp et al. 2014). Similar histologic variants of BC were shown between dog and humans such as plasmacytoid and rhabdoid types (Lin et al. 2018; Humphrey et al. 2016). Further, canine invasive BC replicate efficiently the local invasion and distant metastases (in frequencies and sites) of human invasive BC which are difficult in experimental models (Knapp et al. 2014). At the molecular level, similar basal and luminal subtypes were shown in human (Robertson et al. 2017; Choi et al. 2014) and canine invasive BC (Dhawan et al. 2018; Elbadawy et al. 2019). Additionally, the tumor suppressor pathway (P53) is inactivated in most of cases of human (Watanabe et al. 1994) and canine invasive BC (Hanazono et al. 2016; Dhawan et al. 2018). EGFR overexpression occurs in 73–75% of high grade BC in human (Chaux et al. 2012) and dogs (Dhawan et al. 2015). Cox-2 is also overexpressed in more than 80% of human (Bourn et al. 2019) and canine BC (Knapp et al. 2014). HER-2 (EGFR2/ERBB2/NEU) has been found to be significantly overexpressed in canine invasive BC (Millanta et al. 2018), as is the case in human invasive BC (Eriksson et al. 2017; Cimpean et al. 2017) when compared to non-neoplastic urothelium. The standard treatment options for invasive BC in humans and dogs are nearly similar and both species showed similar responses to treatments (Knapp et al. 2019). Canine organoid models in this context can serve as a preclinical screening tool to identify the optimal drugs or drug combinations for novel cancer drug candidates, which can then be tested in canine clinical trials before they are taken into human clinical trials.

Establishment of Canine BC Organoids

The diagnosis of BC in dogs is made by a combination of signalment, history, diagnostic tests and ultimately urine cytology or histology. A tissue biopsy from the bladder mass can be obtained by surgery

or cystoscopy, or in some cases using a urinary catheter. These techniques can confirm the state of BC as well as its subvariant, grade, invasiveness, and stage (Brambilla et al. 2022).

Methods of Deriving UC Organoids from Urine-Derived Stem Cells

Urine samples can be collected either by free catch or through the use of a catheter. A gentle scratch of the bladder mass with the catheter end can help collect additional tumor cells (Elbadawy et al. 2022; Elbadawy et al. 2019. Next, the urine sample is centrifuged at 300 g/5 min/4°C to remove the urine supernatant. The cell pellet should then be suspended in a cold preservation buffer or shipping medium (Table 1) (Sato et al. 2023) and immediately transferred to a cold environment (4°C) and then to the laboratory for processing. If the pellet is contaminated with red blood cells, which is common in BC, 5 mL of red blood cell lysis buffer (Roche; 11814389001) can be prewarmed at room temperature (RT) and mixed with the pellet on a nutator for 5 min at RT to remove RBCs. After this incubation, again centrifuge the tube at 300 g/5 min/4°C and discard the supernatant. If the pellet is large, it may contain debris such as mucous, or crystals. If this is the case, the pellet should be washed three times with cold PBS and centrifuged at 100 g/3 min/4°C to remove as much debris as possible. After the final wash, the pellet should be resuspended using an ECM such as Matrigel (Corning; 356255) (30 µl/well) on ice and seeded in 24-well plates (Corning; 3524). After solidifying, the gel at 37°C for ~ 30 min in the CO_2 incubator, the culture medium (Table 2) is added (500–750 µL/well) and changed three times weekly. Once organoids grow in size and density, BC organoids can be passaged at a ratio of 1:3–4 as previously described (Elbadawy et al. 2019; Elbadawy et al. 2021; Abugomaa et al. 2023).

Methods of Preparing UC Organoids from BC Tissue-Derived Stem Cells

Biopsy samples from a bladder mass can be obtained during cystectomy or transurethral biopsy via cystoscopy. Representative pieces (0.5-1 cm^3) from different tumor parts should be taken and inserted in sterile containers or tubes containing cold preservation buffer (Table 1) and transferred immediately to the laboratory. In a biosafety cabinet, small fragments from these parts are then dissected and transferred to a 6 cm culture dish. They are then washed three times with 5 mL of cold PBS. After washing, 2 mL of cold advanced Dulbecco's Modified Eagle's Medium (AdDMEM) (Gibco; 12634-010) is added to these fractions. The fragments are then mechanically cut into fine pieces as much as possible using sterile surgical blades or curved micro scissors. These fine fragments can either be plated in Matrigel, allowing for the cells to grow, or further enzymatically digested following the steps below.

The dissected tissue parts are then transferred into a sterile 15 mL tube containing pre-warmed (37°C) AdDMEM with a digestive enzyme such as Liberase TH (a mixture of collagenase type II and dispase II solution at 0.125 mg/mL of AdDMEM) and incubated in a shaking thermostatic water bath set at 37°C for up to 45 min with pipetting every 15 min. This allows enzymatic digestion and the release of stem cell clusters from tissue fragments. The release of stem cells can be confirmed under a bright field microscope. After digestion, the tube is centrifuged at 600 g/5 min/4°C, resuspended in 5 mL of PBS, and

mixed before being centrifuged again. If the pellet contains many red blood cells, RBC lysis buffer can be used as mentioned above. Cell clusters are then dissociated by mixing the pellet with 3–5 mL of pre-warmed (37°C) TrypLE™ Express (Gibco; 12604-021). The cell suspension is then passed through a 100 µm nylon cell strainer to a 50 mL tube containing 5 mL AdDMEM, which stops the digestion from Tryple Express. The strainer can be washed twice with 5 mL of PBS to collect more cellular fractions. The strained fraction is then centrifuged at 300 g/5 min/4°C and the pellet is washed twice with 5 mL of sterile PBS or AdDMEM. After the final wash, an appropriate pellet size is taken and mixed with an ECM such as Matrigel (30 µl/well) on ice, seeded in 24-well plates, and cultured as mentioned in the prior section. A general scheme for generating canine BC organoids from tissue biopsies or urine and their potential analyses and applications are shown in Fig. 1.

Necessary Culture Medium Supplements and Growth Factors

When expanding canine BC organoids, the media composition may contain growth factors important for stem cell expansion, such as Wnt-3a, R-spondin, and Noggin. These growth factors can be obtained using 2D cell cultures (e.g., L-WRN cells) (Miyoshi and Stappenbeck 2013) and harvesting their conditioned media, which contains an approximate concentration of these secreted proteins. Alternatively, recombinant proteins can be purchased at a known weight (Table 2). Our laboratory utilizes the latter method to attain a more accurate concentration, along with a combination of antibiotics and additives that may help support stem cell growth for BC organoids as well as for other tissues.

Over time, we have modified and optimized our organoid media to include a variety of additional media components that support various organoid cultures including EGF, Nicotinamide, Gastrin, A-83-01, SB202190, Y27632, and CHIR99021. The specific pathways and functions of these growth factors have been previously described (Rauth et al. 2021). Further details and concentrations of the current media composition used to cultivate canine BC organoids are listed in Table 2.

Passaging of BC Organoids

After 1–2 weeks of culture, BC organoids can be passaged into new wells at a ratio of approximately 1:3–4 wells depending on the size and density of the organoids. To dissolve the ECM, such as Matrigel, 5 mmol/L EDTA/PBS is added to each well and the culture plate is placed on ice for 90 min, during which gel domes are detached using a sterile 1 mL tip after 30 min. Alternatively, 500 µL of Cell Recovery Solution (Corning; 354270) can be added to each well, mixed by pipetting, and incubated on ice for 10–60 min to dissolve the Matrigel. The cell suspensions can then be collected into sterile 15 mL tubes and centrifuged at 100 g for 5 min at 4°C. Cell pellets can be washed with PBS or AdDMEM prior to dissociation or left in Cell Recovery Solution. To dissociate the organoids, ~ 500 µL of TrypLE Express is added to the tubes at 37°C for ~ 10 min depending on organoid plasticity. Vigorous pipetting is performed to dissociate the organoids into single cells and 10% of FBS or 5 mL AdDMEM is added to the tube to neutralize trypsinization. Cell pellets are collected by centrifugation (100 g/5 min/ 4°C) and mixed with

new Matrigel on ice and then plated into a 24-well plate at 30 µL/well. The plate is then solidified in a CO_2 incubator at 37°C for 30 min. Finally, 500 µL of warm culture medium (Table 2) is added to each well and changed three times weekly until the organoids become ready for passage (before organoids become too large or necrotic) or further analysis or harvesting (Elbadawy et al. 2019).

Cryopreservation and Reculture

To cryopreserve the organoids, remove the media and add 500 µL of Cell Recovery Solution (Corning; 354270) to each well. Mix the organoids with a pipette, collect them, and transfer them to a 15 mL tube. Incubate on ice for 10 min to assist in the degradation of excess Matrigel before spinning at 100 g/5 min/4°C. Discard the supernatant and resuspend the pellet in 6 mL of AdDMEM to remove the Cell Recovery Solution. Then, resuspend the pellet in 1 mL of cell banker solution, freezing medium[(a)], or Cryostor CS10 (BioLife Solutions; 210102) and immediately transfer it to a cryovial before placing it in a Mr. Frosty container (Nalgene; 5100-0001) filled with isopropanol and place in a 4°C fridge for 10 min. After this short incubation, move the Mr. Frosty to -80°C storage overnight with samples being transferred to vapor phase liquid nitrogen (-196°C) indefinitely.

To subsequently thaw samples, recover cryovials from liquid nitrogen storage and immediately place them in a 37°C water bath. After thawing the samples, transfer the cells in Cryostor to a tube containing 6 mL of AdDMEM and spin at 100 g/5 min/4°C. Remove the supernatant, add Matrigel to the pellet, and plate 30 µL of sample in each well of 24 well plates. Transfer to a 37°C and 5% CO_2 incubator for ~ 20–30 min, then add 500 µL of media to each well.

[(a)] A solution of 50% culture medium, 40% FBS, and 10% Dimethyl sulfoxide. We found that the recovery of the organoids after thawing was superior when using Cryostor CS10.

Characterization of Canine BC Organoids

To confirm the cell type of BC organoids, markers used to identify normal urothelium as well as markers used to identify urothelial carcinoma cells were utilized. Normal urothelium is composed of three distinct layers (Jafari and Rohn 2022). The basal or outermost layer is made up of two to three sublayers of small cuboid cells, while the intermediate or middle layers being made up of irregularly shaped cells, and the superficial layer that lines the apical side of the bladder epithelium is composed of umbrella cells. Each layer can be characterized using specific markers. For example, basal type BC organoids can be characterized using basal cell markers including cytokeratins (CK) 5, 6, and/or 14, and/or P63 (a homolog of P53), and CD44 (Elbadawy et al. 2019; Walz et al. 2023; Sahoo et al. 2022; Hanazono et al. 2016). Luminal type BC organoids express markers of terminal urothelial differentiation, such as those seen in umbrella cells (Uroplakins Ia, Ib, II, and III, and KRT20) (Mullenders et al. 2019; Elbadawy et al. 2019). Additionally, loss or decrease of E-cadherin expression and an increase of vimentin and alpha smooth muscle actin expression in BC organoids can indicate the cells undergoing epithelial–mesenchymal transition (EMT), which can occur during tumor progression and metastasis (Tambunan et al. 2022;

Monteiro-Reis et al. 2023; Mezheyeuski et al. 2020). Several methods can be used to characterize canine BC organoids including bright field imaging, histological staining using hematoxylin and eosin (H&E), RNA *in situ* Hybridization (ISH) and immunofluorescence (IF) (Fig. 2).

Bright Field microscopy

Bright-field microscopy (BF) can be used to observe the organoid morphology, growth and viability conditions during culture and to determine the morphological metrics of organoids such as diameter, length and area (Borten et al. 2018). BF microscopy also can be used to compare different culture conditions (such as healthy and disease models) and can help to carry out real-time and dynamic detection of live and dead cells (Fei et al. 2022). Representative BF images of three canine BC organoids are shown in Fig. 2A.

Histological Staining

To fix the organoids, the media is removed from each well and 500 µL of Formalin-acetic acid-alcohol is added as previously described (Gabriel et al. 2022). Alternatively, tissue samples are fixed in 10% formalin, with approximately a 1:10 ratio of tissue to formalin. After 24 h at RT, samples are changed to 70% ethanol before processing and paraffin-embedding. Next, the samples are stained with hematoxylin and eosin (H&E) for histological assessment. After the H&E staining process, full slides are scanned on an appropriate microscope such as the Leica Aperio GT 450 Scanner, and then analyzed using software, such as ImageScope (v12.4.3.5008). Representative H&E images of three canine BC organoids are shown (Fig. 2B).

RNA In Situ Hybridization

RNA *in situ* hybridization (RNA-ish) relies on mRNA sequences and can be made custom to the gene of interest. When antibodies had low homology to canines, custom RNA- ish probes were used. RNA-ish is performed according to the manufacturer's protocol to characterize mRNA expression for CD44 (ACDBio; 838991) in the canine BC organoids using the RNAscope® 2.5 HD Detection Kit (ACDBio, Ref. 3223600). Once mounted and allowed to dry overnight, slides are imaged the next day on an Olympus BX40 microscope and evaluated with a 60x objective. Representative ISH images of three canine BC organoids are shown (Fig. 2C).

Immunofluorescence

For deparaffinization, microscopy slides should be submerged in xylene twice for 10 min. Then, they should be transferred to 100% ethanol twice for 1 min with regular agitation. Slides are then allowed to dry on tissue paper for 5 min. Next, the slides undergo Heat Induced Epitope Retrieval (HIER) using either of two buffers (Citrate buffer - pH 6 or Tris/EDTA buffer - pH 9) heated to 75°C in a HybEZ II Oven for 2 h. The slides were then removed from the oven for approximately 15 min with the lid off before being rinsed twice in PBS for 2 min each time before receiving a final 10 min wash in PBS. For permeabilization, slides

should be placed in 0.25% Triton in PBS twice for 10 min each. After being rinsed in PBS three times, the slides are blocked in Casein in PBS for 1 h at RT. Then, the samples should have their primary antibody added and be incubated in a humidity tray overnight at 4°C. Primary antibodies used included UPK1A (Novus Biologicals; NBP2-79733), P53 (Genetex; GTX102965), and CK5 (United States Biological; 222068). The following day, the slides should be rinsed in PBS; and the corresponding secondary antibody should be added (UPK1A [anti-mouse, Invitrogen; R37114], P53 and CK5 [anti-rabbit, Thermo; A32731]) at a ratio of 1:500–1000 in PBS for 1 h at RT. The slides should be rinsed again after this. Finally, the slides should be incubated with DAPI (Sigma; D9542-1MG) at a ratio of 1:500–1000 in PBS for 20 min and washed three times for 10 min each in PBS, and then switched to distilled water. For mounting, Fluoroshield (Sigma, Cat no: F6182-20ML) can be used and the slides should be allowed to dry overnight. The imaging can then be done on a Stellaris confocal microscope with scalebars added using the Leica LAS AF Lite (v. 2.6.0 build 7266) software. Representative IF images of three canine BC organoids are shown in Fig. 2D.

Possible Applications of the Canine Organoid Model in BC research

The use of canine BC organoids as a pivotal tool for preclinical drug screening represents a novel and promising approach, with the potential to streamline research and development of novel therapeutic modalities for BC in both human and canine patients. At the preclinical stage of drug research and development, canine organoids could help in identifying key therapeutic targets, which are important for designing new treatment options for BC using a relatively high-throughput system. Drug candidates evaluated in this model could later be tested in vivo for proof-of-concept studies in canine patients with BC, utilizing resources from the NIH Comparative Oncology Program. These studies could then demonstrate proof-of concept efficacy and safety of drugs, enabling go/no go decisions before formal testing in human patients.

From a veterinary medicine perspective, organoids can accurately model the genetic heterogeneity and complexity inherent in BC, ensuring that therapeutic interventions are tailored to the specific genetic and molecular characteristics of individual tumors and enabling personalized medicine applications in canine patients (Elbadawy et al. 2019; Elbadawy et al. 2021; Abugomaa et al. 2023). Future development opportunities, including more complex versions of the canine model with the co-culture of immune cells, stromal cells, and endothelial cells incorporated into organoid cultures, will allow for emulation of the tumor microenvironment and will facilitate comparative studies of new modalities in BC immunotherapy.

Current Limitations of Organoids

Cancer-derived organoids have become a valuable tool in the field of oncology. They provide a more accurate representation of tumor biology and treatment response when compared to traditional two-dimensional cell cultures (Kapalczynska et al. 2018). However, despite their benefits, there are still limitations and challenges when using organoids for these purposes.

These include:

Tumor Heterogeneity and Subclonal Diversity. Tumors are composed of a diverse range of cell types, including cancer cells, stromal cells, immune cells, and endothelial cells. While cancer-derived organoids can capture some of this complexity, they may not fully replicate the tumor microenvironment and its various cell types, which are crucial for understanding tumor behavior and treatment response (Fang et al. 2023; Jiang et al. 2023). Additionally, tumors often have multiple subclones with distinct genetic and phenotypic profiles (Jamal-Hanjani et al. 2015). Capturing this subclonal diversity can be challenging in organoids, which may limit their effectiveness in accurately modeling tumor evolution and resistance mechanisms (Fang et al. 2023; Jiang et al. 2023).

Lack of Immune System Components. The tumor microenvironment plays a significant role in cancer progression and treatment response (Wang et al. 2023). However, most organoid cultures do not include immune components, making it difficult to evaluate immunotherapies and understand immune evasion strategies and resistance to chemotherapy (Neal et al. 2018).

Vascularization. Tumors have abnormal blood vessel networks that can affect drug delivery and hypoxia-induced pathways (Dewhirst and Secomb 2017). Organoids often lack a complete vascular network, which limits their ability to model the complex interactions between tumor cells and their surrounding vasculature (Zhao et al. 2021).

Drug Penetration and Metabolism. The dense extracellular matrix of organoids may hinder drug diffusion *in vitro*, potentially leading to an inaccurate assessment of drug efficacy and pharmacodynamics (Karolak, Poonja, and Rejniak 2019). Furthermore, organoids may not fully replicate the metabolic activities of tumors, thereby affecting the metabolism of therapeutic agents and possibly skewing results regarding a drug's effectiveness or toxicity (Richiardone, Van den Bossche, and Corbet 2022).

Future Research Directions

To overcome these limitations, research is focused on:

Co-Culture Models. Research efforts are being made to integrate immune cells, stromal cells, and endothelial cells into organoid cultures in order to better mimic the complex tumor microenvironment (Luckett and Ganesh 2023; Yuan, Li, and Yu 2022). In particular, co-culturing organoids with other cell types or utilizing microfluidic devices can enhance the modeling of tumor-stroma and tumor-immune interactions (Duzagac et al. 2021).

Developing Vascularized Organoids. Advances in bioengineering aim to incorporate vascular networks within organoids in order to study drug delivery and hypoxia-related responses (Strobel, Moss, and Hoying 2023).

Developing New Culture Techniques

Application of microfluidic devices (Organ-On-A-Chip) capable of providing a gravity-driven flow that imitate a fluid stream in the bladder tumor and its perimetrical tissues can ease the oxygen supply and nutrient/waste exchange, resulting in a marked decrease of cell death, support cell expansion as well as functional maturation, thereby recapitulating prominent features of BC in a much more precise and reproducible manner (Duzagac et al. 2021).

Standardization of Protocols. Establishing standardized culture conditions and protocols can help improve the reproducibility and scalability of organoids derived from cancer (Zhao et al. 2022).

Despite these challenges, cancer-derived organoids represent a significant advancement in cancer research. They offer a more relevant model for studying tumor biology and testing existing therapeutics, as well as novel drug candidates (alone, or in combination with other therapeutic modalities, such as radiation therapy). As the field progresses, these models are expected to become even more sophisticated and valuable for translational research and personalized medicine.

Abbreviations

AdDMEM	Advanced Dulbecco's Modified Eagle's Medium
ASC	Adult stem cells
BC	Bladder cancer
BF	Bright-field
ECM	Extracellular matrix
H/E	Hematoxylin and eosin
IF	Immunofluorescence
PBS	Phosphate-buffered saline
UC	Urothelial Carcinoma
RNA-ish	RNA *in situ* hybridization
RT	Room Temperature

Declarations

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Tables

Tables 1 to 2 are available in the Supplementary Files section

Figures

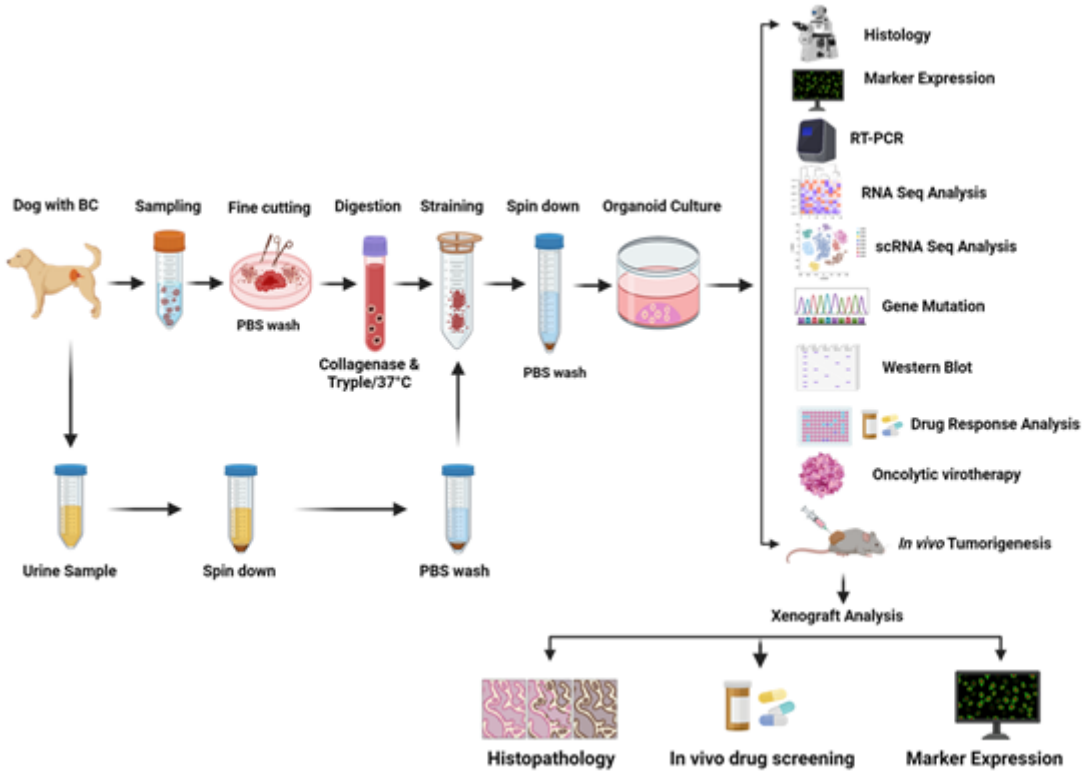

Figure 1

General workflow for generating canine bladder cancer organoids from tissue biopsies or urine. Potential analyses and applications of these patient-specific organoids are listed.

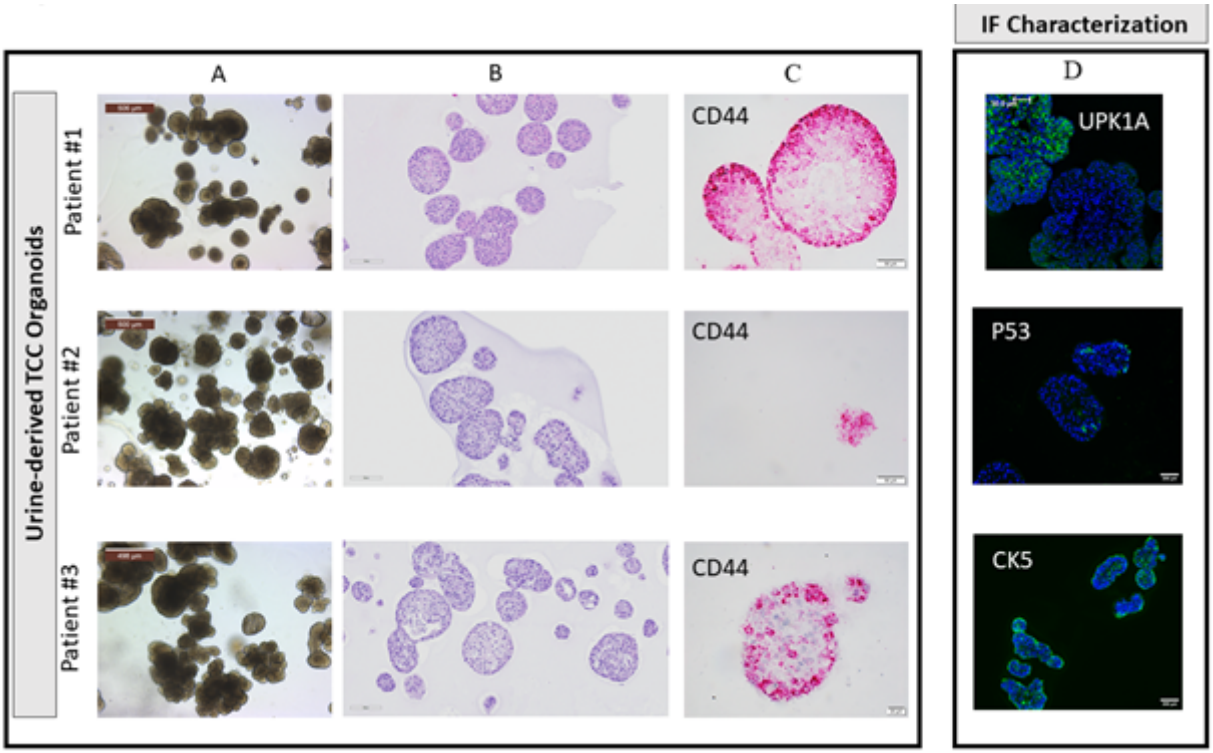

Figure 2

Morphological and histological characterization of three urine-derived canine bladder cancer organoid lines. (A) Bright field, (B) hematoxylin & eosin (H&E) and (C) RNA *in situ* hybridization (RNA-*ish*) corresponding to three urine-derived canine UC organoid lines. Additionally, representative (D) immunofluorescence (IF) taken from various organoid lines are shown (Of note: P53 and CK5 did not utilize HIER during processing). Organoid images were captured using a Leica Dmi1, a Stellaris confocal, or an Echo Revolution microscope. Image scalebars are in μm as indicated.

Supplementary Files

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