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# Characterization of OCT3/4, Nestin, NANOG, CD44 and CD24 as stem cell markers in canine prostate cancer

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ABSTRACT

The cancer cell population is heterogeneous, and cancer stem cells (CSCs) are important for tumor growth and maintenance. The CSC population is associated with different neoplastic characteristics, such as cell migration, resistance to apoptosis, radiation therapy and chemotherapy. To increase the knowledge of CSCs in canine prostate cancer (PC), we characterized CSC markers in canine PC tissues and tumorspheres. We performed immunohistochemistry of OCT3/4, Nestin, NANOG, CD44 and CD24 in 10 normal canine prostatic tissue samples, 10 prostatic hyperplastic (PH) tissue samples and 28 PC tissue samples. Then, we established two canine prostate cancer cell cultures and characterized the CSC profile of tumorspheres grown from these cultures. Normal and PH tissues were positive for Nestin, NANOG, CD44 and CD24 only in the basal cell layer. OCT3/4 was expressed in the luminal cells of normal and PH tissues. There was no significant difference in Nestin expression among the prostatic tissues. However, we found higher expression of NANOG and CD44 in canine PC tissues than that in normal and PH tissues. Tumorspheres from canine prostate cancer cells express OCT3/4, Nestin, NANOG and CD44, indicating that these markers may be potential cancer stem cell markers in canine PC. The results obtained can be useful to better characterize the stem cell population in canine prostatic cancer and to guide future studies in comparative oncology.

## 1. Introduction

Human prostate cancer (PC) is the most common cancer subtype in the western world and has a high mortality rate (Siegel et al., 2018). The greatest therapeutic challenge of human PC is the development of resistance to standard androgen-deprivation therapy (Cattrini et al., 2017). Studies performed on animal models of prostate cancer have increased the understanding of tumor biology, metastasis and therapeutic targets (Facina et al., 2018; McClurg et al., 2018), and dogs can provide an interesting spontaneous model for human PC (Fonseca-Alves, 2018).

Canine PC is a multifactorial disease with a heterogeneous cancer cell population, which may promote tumor proliferation, invasion and metastasis (Keller et al., 2013). Cancer stem cells (CSCs) maintain the capacity for cellular proliferation, survival and motility and thereby contribute to carcinogenesis in many tumors, including prostate cancer in men (Kleeberger et al., 2007). The presence of CSCs could explain the long life of cancer and its immortalization since CSCs are resistant to apoptosis and chemotherapeutic agents (Jaworska et al., 2015; Jeter et al., 2015; Klarmann et al., 2009). In human PC, neoplastic cells with stem cell properties can express different CSC markers, such as OCT3/4, Nestin, NANOG, CD44 and CD24 (Miyazawa et al., 2014). However, limited information is available on the existence of CSCs in canine PC. Since canine PC can be an important model for the human counterpart,

*Abbreviations:* CSC, cancer stem cells; PC, prostate cancer; OCT3/4, octamer-binding protein 3/4; CD44, cluster of differentiation 44; CD24, cluster of differentiation 24; PH, prostatic hyperplastic; MELK, maternal embryonic leucine zipper kinase; WHO, World Health Organization; DAB, 3,3′-diaminobenzidine; DPBS, Dulbecco's Phosphate Buffered Saline Modified; FBS, fetal bovine serum; EDTA, 2,2',2",2"'-(ethane-1,2-diyldinitrilo) tetraacetic acid; PBS, phosphate buffered saline; DAPI, 4′6-diamidine-2′-phenylindole dihydrochloride; DMEM, Dulbecco's Modified Eagle's Medium; EGF, epidermal growth factor; RNA, ribonucleic acid

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**Table 1**
Immunohistochemical protocol and antibody information for all markers.

| Antibody | Manufacturer | Dilution | Antigen Retrieval* | Secondary Antibody |
|---|---|---|---|---|
| CD44 | Santa Cruz Biotechnology | 1:50 | Sodium citrate buffer, pH 6.0, Pascal ® | Polymer Detection System (Envision, Dako) |
| CD24 | Santa Cruz Biotechnology | 1:50 | Sodium citrate buffer, pH 6.0, Pascal ® | Polymer Detection System (Envision, Dako) |
| NANOG | Abcam | 1:1000 | Sodium citrate buffer, pH 6.0, HIER (microwave) | Biotinylated antibodies (Vector Labs) |
| Nestin | Santa Cruz Biotechnology | 1:100 | Sodium citrate buffer, pH 6.0, HIER (microwave) | Biotinylated antibodies (Vector Labs) |
| OCT3/4 | Santa Cruz Biotechnology | 1:50 | Sodium citrate buffer, pH 6.0, Pascal ® | Polymer Detection System (Envision, Dako) |

* HIER = heat-induced antigen retrieval.

the characterization of canine PC is an important step for comparative studies.

The gene expression of CD133, CD44, C-KIT, CD34, ITGA6, OCT4, DDX5 and MELK (Moulay et al., 2013) and the protein expression of Survivin and Sox9 (Bongiovanni et al., 2018) were previously investigated in canine PC. CD117 gene and protein expression were also previously demonstrated. However, a loss of CD117 was found in PC samples, indicating that CD117 has no role in prostate tumorigenesis (Fonseca-Alves et al., 2017). However, these authors did not perform functional studies to associate staining with stem cell properties. Usui et al. (2017) established an organoid model of canine PC and demonstrated high CD44 expression, indicating CD44 as a cancer stem cell marker in canine PC.

OCT3/4 (also known as Oct4 and Pou5F1) is a transcriptional regulator of genes involved in maintaining an undifferentiated pluripotent state and is characterized by two isoforms: OCT4A (isoform 1), which is localized in the nucleus, and OCT4B (isoform 2), which is mainly localized in the cytoplasm (Lee et al., 2006; Monsef et al., 2009). In the human prostate, OCT4 isoform 1 is associated with self-renewal and pluripotency of CSCs (Lee et al., 2006).

Nestin is an intermediate filament involved in motility, cellular stress, signal transduction and myogenesis regulation (Hyder et al., 2014). Nestin has been reported in prostate cancer in association with cell migration, metastasis (Kleeberger et al., 2007) and the self-renewal of prostate CSCs (Kimbro and Simons, 2006; Mabjeesh and Amir, 2007), and it is considered a prognostic marker in other cancers (Hope et al., 2016; Bernal and Arranz, 2018; Bien-Möller et al., 2018).

NANOG is a divergent homeobox domain protein transcription factor that functions in association with OCT4 to form an embryonic stem cell identity (Gong et al., 2015; Amini et al., 2014). NANOG also plays an important role in cell proliferation, tumorigenicity, clonogenic growth, invasiveness and therapeutic resistance (Jeter et al., 2015).

CD44 is a cell surface marker and transmembrane glycoprotein involved in various cellular activities by binding with hyaluronan or other extracellular molecules (Yu et al., 2012; Moura et al., 2015). CSC properties have been explored in canine mammary gland tumors, and the CD44+/CD24- phenotype is a feature of tumors with more aggressive behaviors (Magalhães et al., 2013; Figueroa et al., 2015; Rybicka and Król, 2016).

The gene expression of the stem cell markers CD44, CD133, CD34, c-KIT, OCT4, ITGA6, MELK and DDX5 has been evaluated in different canine prostatic cell lines (Liu et al., 2007). However, no studies have characterized the stem cell population of tumorspheres or canine prostatic tissues. To identify potential cancer stem cells in canine PC, we analyzed the expression of OCT3/4, NANOG, Nestin, CD44 and CD24 in normal, hyperplastic and neoplastic canine prostate samples, as well as tumorspheres derived from two canine prostate cancer cell lines.

## 2. Materials and methods

### 2.1. Formalin-fixed paraffin-embedded tissue samples

This study was approved by the animal ethics committee of the School of Veterinary Medicine and Animal Science of the São Paulo State University (Protocol: 0004/2017). To evaluate the expression of stem cell markers in canine prostatic tissue, 48 formalin-fixed paraffin-embedded canine prostatic tissues were retrieved from the archives of the Veterinary Pathology Service, São Paulo State University and the School of Veterinary Science, The University of Queensland. Ten normal prostatic tissues, 10 pH tissues and 28 PC tissues were randomly selected according to previous histopathological diagnoses. The prostate samples were collected by incisional biopsies (4/28), partial prostatectomy (3/28) or necropsy (21/28). This tumor group was previously stained for PSA, AR, CK8/18, and uroplakin-III to confirm the luminal origin (Fonseca-Alves, 2018).

### 2.2. Clinical history and histopathology

Clinical records were available in 14 out of 28 PC cases. All PC slides (28/28) were reviewed by three pathologists (PEK, RLA, CP) to confirm the previous diagnosis. The histopathological classification was performed according to the human WHO classification of Tumors of the Urinary System and Male Genital Organs (Humphrey et al., 2016), which has been recently adapted to canine PC (Palmieri et al., 2014). The Gleason score was performed according to Palmieri and Grieco (Palmieri and Grieco, 2015).

### 2.3. Immunohistochemistry

Tissue sections (3 μm) were deparaffinized and treated as described in Table 1. For CD44, CD24 and OCT3/4, endogenous peroxidase was quenched with 8% hydrogen peroxide in methanol for 20 min, and nonspecific binding was blocked with 8% skim milk for 60 min, both at 27 °C. For NANOG and Nestin, peroxidase blocking was achieved using animal-free blocker 5x (Vector Laboratories, Burlingame, CA, USA) for 30 min at 27 °C. 3,3´-diaminobenzidine (DAB, Dako, Carpinteria, CA, USA) was used as a chromogen, and sections were counterstained with Harris' hematoxylin. Normal stomach tissue was used as a positive control for CD44, and germ cells from normal canine testes were used as positive controls for Oct3/4, NANOG, Nestin and CD24. For the negative controls, the primary antibody was replaced by Tris buffer. We only used antibodies with cross-reactivity with canine tissue. Immunostaining was qualitatively assessed based on the distribution of the protein (nuclear/cytoplasmic) and semiquantified and scored according to the following percentages of positive cells: 0, no positive cells; 1, ≥1% to ≤10% positive cells; 2, ≥11% to ≤25% positive cells; 3, ≥26% to ≤50% positive cells; 4, ≥51% to ≤75% positive cells; and 5, ≥76% positive cells.

### 2.4. Establishment of tumor-derived cell cultures

Cell cultures were established using fresh tissue from a 10-year-old, intact, mixed breed dog with nonmetastatic PC (cell line PC1) and an 11-year-old, intact, poodle dog with metastatic PC (cell line PC2). The tissue samples were washed three times with Dulbecco's Phosphate Buffered Saline Modified (DPBS) (Sigma, Portland, OR, USA) containing 1% of 100 U/mL penicillin G and 100 mg/mL streptomycin (Sigma, Portland, OR, USA). Tissue fragments (2 mm) were dissociated by incubation in 0.5% collagen type IV (Sigma, St. Louis, MO, USA) for three hours at 37 °C in 5% $CO_2$. The cells were suspended in culture

**Table 2**
Immunohistochemical expression of the stem cell markers in canine prostatic tissues.

| Score | OCT3/4 | | | CD44 + /CD24- | | | Nanog | | | Nestin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normal | BPH | PC | Normal | BPH | PC | Normal | BPH | PC | Normal | BPH | PC |
| 0 | 0 | 0 | 46.4% (13/28) | 0 | 0 | 0 | 40% (4/10) | 20% (2/10) | 21.4% (6/28) | 80% (8/10) | 80% (8/10) | 82.2% (23/28) |
| 1 | 0 | 0 | 46.4% (13/28) | 0 | 0 | 0 | 60% (6/10) | 80% (8/10) | 39.2% (11/28) | 20% (2/10) | 0 | 10.7% (3/28) |
| 2 | 0 | 0 | 7.2% (2/28) | 0 | 0 | 35.7% (10/28) | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 35.7% (10/28) | 0 | 0 | 14.2% (4/28) | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 28.6% (8/28) | 0 | 0 | 17.8% (5/28) | 0 | 0 | 0 |
| 5 | 100% (10/10) | 100% (10/10) | 0 | 0 | 0 | 0 | 0 | 0 | 7.1% (2/28) | 0 | 20% (2/10) | 7.1% (2/28) |

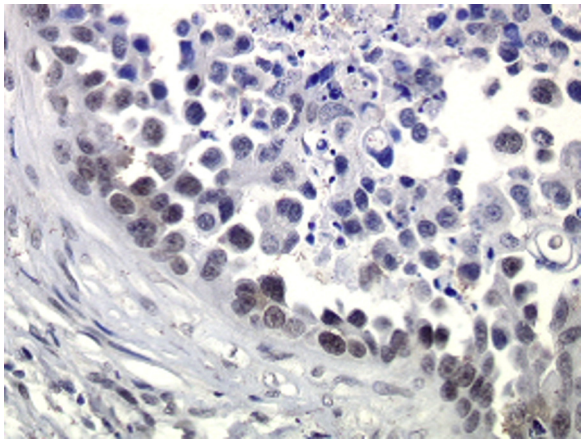

**Fig. 1.** Immunohistochemical analysis of OCT3/4 in canine prostatic carcinoma. Note positive nuclear expression in cancer cells that are in contact with the basal cell membrane. Harris' hematoxylin counterstaining, 200×.

medium (PrEBM, Lonza, Basel, Switzerland) with 1% of 100 U/mL penicillin G and 100 mg/mL streptomycin (Sigma, Portland, OR, USA) and then incubated at 37 °C in 5% $CO_2$ in culture medium (PrEBM, Lonza, Basel, Switzerland) supplemented with 10% inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 1% of 100 U/mL penicillin G and 100 mg/mL streptomycin (Sigma, Portland, OR, USA). The culture medium was discarded and replaced with fresh medium every 48 h.

To remove fibroblast contamination, selective trypsinization by incubation in cold (4 °C) 0.25% trypsin with EDTA for 2 min at room temperature was performed at passage 2. The remaining adherent cells were detached by incubation in 0.25% trypsin with EDTA for 5 min in a humidified atmosphere of 5% $CO_2$ at 37 °C and then resuspended in PrEGM™ medium (Lonza, Basel, Switzerland) containing 10% FBS and 1% penicillin-streptomycin solution. All cell cultures were grown until passage 10.

### 2.5. Cell phenotype characterization by immunofluorescence

Cell phenotype was assessed by immunofluorescence using the following primary antibodies: anti-mouse monoclonal pan-cytokeratin (1:300) (Invitrogen, Carlsbad, CA, USA), anti-mouse monoclonal vimentin (1:300) (Invitrogen, Carlsbad, CA, USA), anti-rabbit polyclonal prostate specific antigen (PSA) (1:1200) (Biorbyte, Cambridge, UK), anti-rabbit polyclonal androgen receptor (AR) (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse monoclonal uroplakin-III (1:300) (Fitzgerald, Acton, MA, USA) and anti-mouse monoclonal p63 (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunofluorescence was performed on round sterile 1.5 coverslips in 12-well plates (Sigma, Portland, OR, USA). A total of $1 \times 10^5$ cells were seeded in 250 µL of culture medium (PrEBM, Lonza, Basel, Switzerland) supplemented with 10% FBS and were incubated in a humidified atmosphere of 5% $CO_2$ at 37 °C. When adherent cells reached over 50% confluence, the medium was removed, and the cells were washed three times with DPBS and fixed with cold methanol (4 °C) for 15 min, followed by permeabilization with 0.25% Triton-X (Sigma, Portland, OR, USA). The cells were incubated for 45 min with 3% bovine serum albumin in DPBS, which was used as a blocking solution. The cells were then incubated overnight at 4 °C with the primary antibodies as described above. Alexa Fluor 594 (BioLegend, San Diego, CA, USA) was applied at a 1.5 µg/mL dilution in PBS for 60 min at room temperature for all antibodies, except for the rabbit polyclonal anti-PSA antibody that was detected using Alexa Fluor 484 (Invitrogen, Carlsbad, CA, USA) at a 2 µg/mL dilution in PBS. The slides were counterstained with 4′-6-diamidino-2-phenylindole (DAPI; Sigma, Portland, OR, USA)

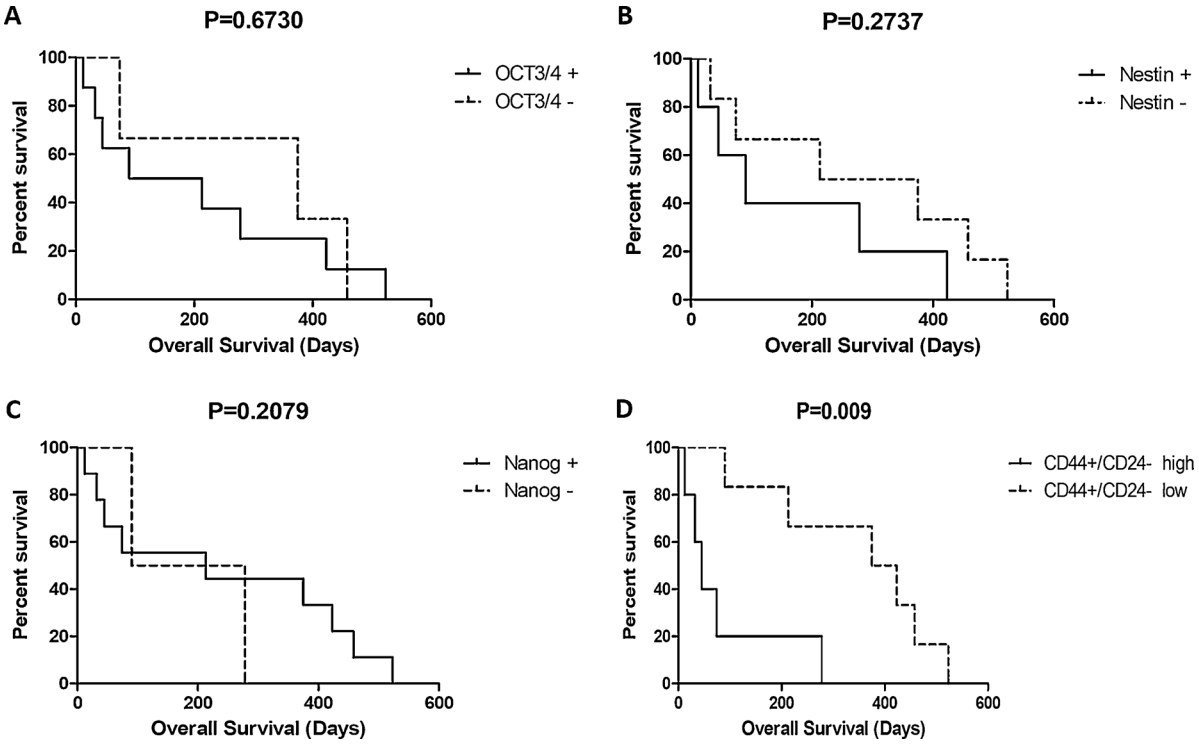


**Fig. 2.** Survival analysis of canine patients with prostate cancer according to stem cell marker expression. No significant difference was observed in patients showing high expression of OCT3/4 (A), Nestin (B) and NANOG (C). D: Patients with a higher number of $CD44^{+}/CD24^{-}$ cells experienced shorter survival.

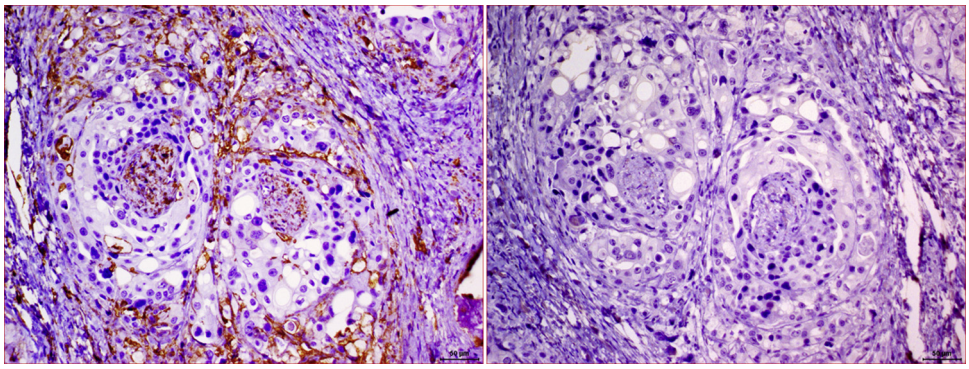

**Fig. 3.** Immunohistochemical expression of CD44 and CD24 in canine prostatic carcinoma. Note the same tumor area showing diffuse membranous CD44 expression (A) and negative CD24 expression by cancer cells (B). Harris' hematoxylin counterstaining, 200×.

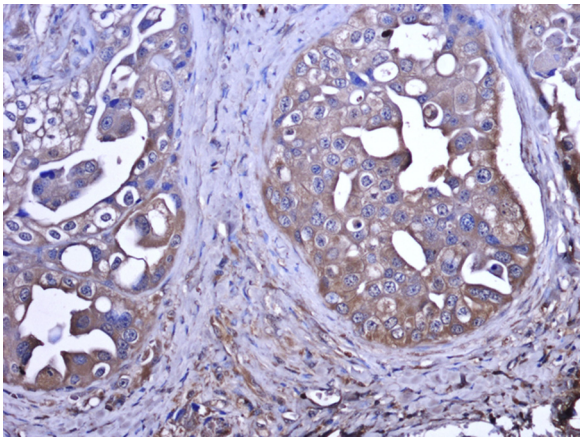

**Fig. 4.** Immunohistochemical expression of NANOG in canine prostatic carcinoma with a solid pattern. Note diffuse positive expression by cancer cells. Harris' hematoxylin counterstaining, 200×.

and evaluated under a laser scanning confocal microscope (Leica Biosystems, Wetzlar, Germany).

### 2.6. Characterization of tumor cells by flow cytometry

Flow cytometry was performed to confirm the PC1 and PC2 prostatic phenotype by means of an LSRFortessa model flow cytometer (BD Biosciences, USA), and the same primary antibodies for anti-pan-cytokeratin, -PSA and -AR were used, as listed above. A secondary goat anti-mouse antibody conjugated with Alexa Fluor® 594 (Biolegend, San Diego, CA, USA) and a secondary goat anti-rabbit antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA, USA) were used. The fluorescence reaction was analyzed using BD FACSDiva™ software, accounting for 10,000 events. Gratama et al. (1998) was used as a reference for the flow cytometry controls. For each analysis, cell auto fluorescence was evaluated, and a negative control for unspecific binding of the secondary antibody was included.

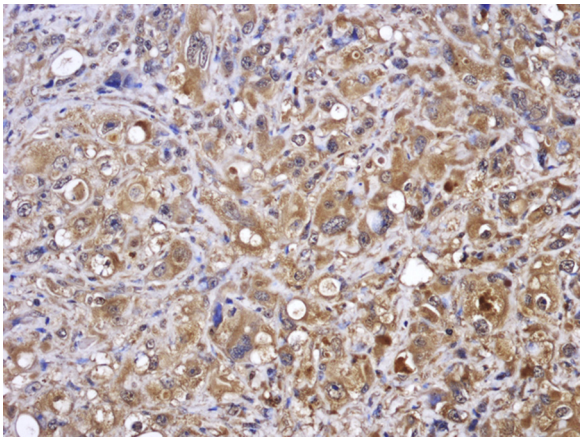

**Fig. 5.** Immunohistochemical expression of Nestin in canine prostatic carcinoma with a cribriform pattern. Note diffuse positive cytoplasmic expression in cancer cells and some stromal cells. Harris' hematoxylin counterstaining, 200×.

### 2.7. Tumorsphere formation assay (prostaspheres)

The sphere formation assay was performed following the protocol described by Liu et al. (Liu et al., 2007). Briefly, cell suspensions of PC1 and PC2 were seeded on a 24-well ultralow attachment plate (Inc., Corning, NY, USA) with DMEM medium (Lonza, Basel, Switzerland) containing a 1:50 dilution of B-27 supplement (Life Technologies), 4 µg/mL heparin (Sigma, Portland, OR, USA), 20 ng/mL EGF (Sigma, Portland, OR, USA) and 20 ng/mL fibroblast growth factor (Sigma, Portland, OR, USA) in a humidified atmosphere of 5% $CO_2$ at 37 °C. PC1 and PC2 cells were cultured for 12 days.

### 2.8. Single-cell suspension assay

To investigate the ability of tumorspheres to self-renew through secondary sphere formation, prostaspheres were mechanically dissociated, and then suspended cells were chemically digested into single-cell suspensions, as previously reported by Liu et al. (Liu et al., 2012). The cells were then plated on an ultralow attachment 24-well plate (Inc., Corning, NY, USA) with methylcellulose medium at a density of 1 cell/well.

### 2.9. Expression of stem cell markers in the tumorspheres

The tumorspheres from PC1 and PC2 cells were detached using 0.25% trypsin with EDTA, followed by mechanical dissociation using a pipette tip, and they were cultured in a 24-well plate in duplicate for at least three doubling times. The medium was subsequently removed, and the cells were washed with cold (4 °C) DPBS and lysed with 20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, 5 mM EDTA, 250 mg/mL sodium vanadate and 10 mg/mL leupeptin (RIPA Lysis Buffer, Millipore Co., Bedford, MA, USA). Proteins were extracted from the supernatant and quantified as described by Bradford (1976). Equal amounts of protein (50 µg) from each culture were heated at 95 °C for 5 min in sample loading buffer, subjected to SDS–PAGE and then transferred to nitrocellulose membranes (Sigma Chemical Co., St. Louis, MO). The blots were blocked with 5% bovine serum albumin in TBS-T (10 mM Tris−HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20) for 1 h and then incubated overnight with OCT3/4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:500 dilution; Nestin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:100 dilution; NANOG (Abcam, Cambridge, UK) at a 1:1000 dilution; CD44 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:400 dilution; and CD24 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:500 dilution. Goat anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody, the blots were visualized via chemiluminescence (Amersham ECL Select Western Blotting Detection Reagent, GE Healthcare).

### 2.10. Statistical analysis

The chi-square or Fisher exact test was used to compare the immunohistochemical scores between the categorical variables (normal versus PH or normal/ PH versus PC). For statistical purposes, the survival analysis of OCT3/4, Nestin and NANOG expression was conducted in two large categories: positive versus negative samples. Since all tumors contained cells with a $CD44^+/CD24^-$ phenotype, a median of the expression for all samples with available survival data was performed, considering the following two groups of samples: $CD44^+/CD24^-$ low (samples with $CD44^+/CD24^-$ cells lower than the survival median) and $CD44^+/CD24^-$ high (samples with $CD44^+/CD24^-$ higher than the survival median). Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA). $P < 0.05$ was considered statistically significant.

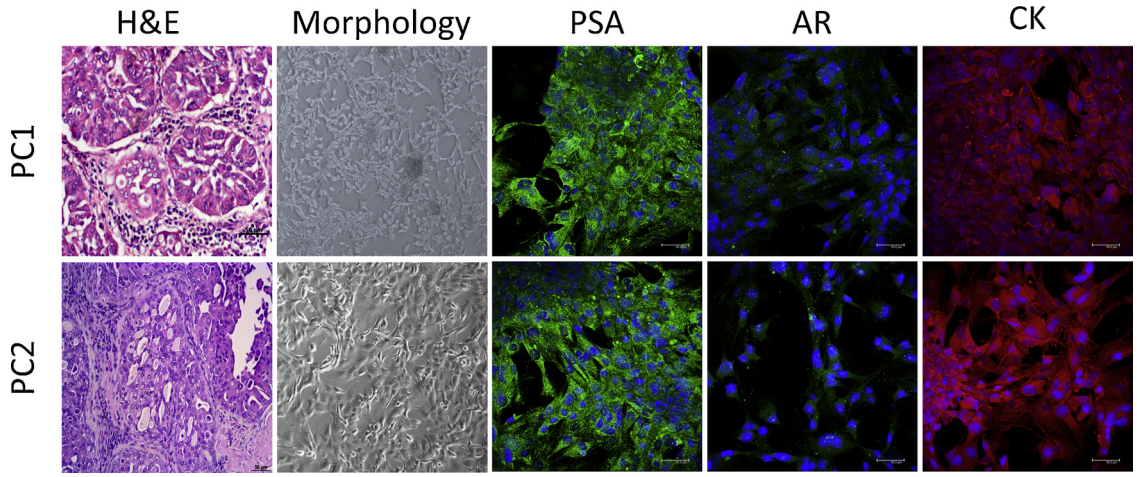


**Fig. 6.** Characterization of canine prostate cancer cell cultures. Both primary tumors had a cribriform growth pattern, and cells grew as attached monolayer cultures. Both cell cultures were positive for PSA and pan-cytokeratin and were negative for AR.
Blue: Alexa Fluor; Green: PSA; Red: Pan-cytokeratin (AE1/AE3).

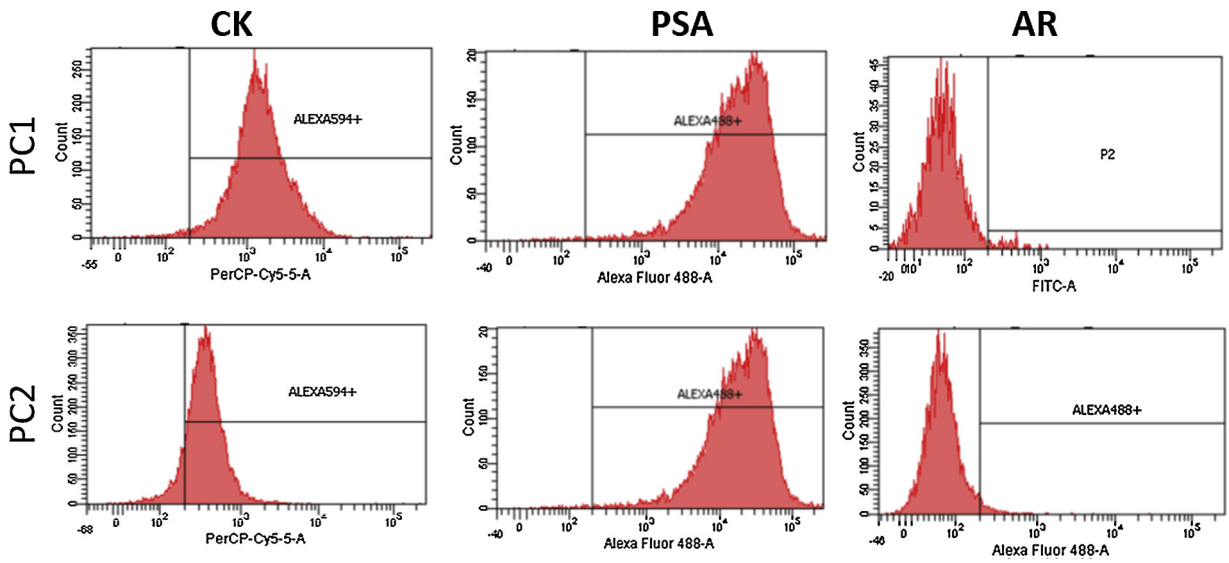


**Fig. 7.** Flow cytometry analysis of canine prostatic cell cultures PC1 and PC2. Both cell cultures were positive for pan-cytokeratin (CK) and prostate specific antigen (PSA) and negative for androgen receptor (AR).

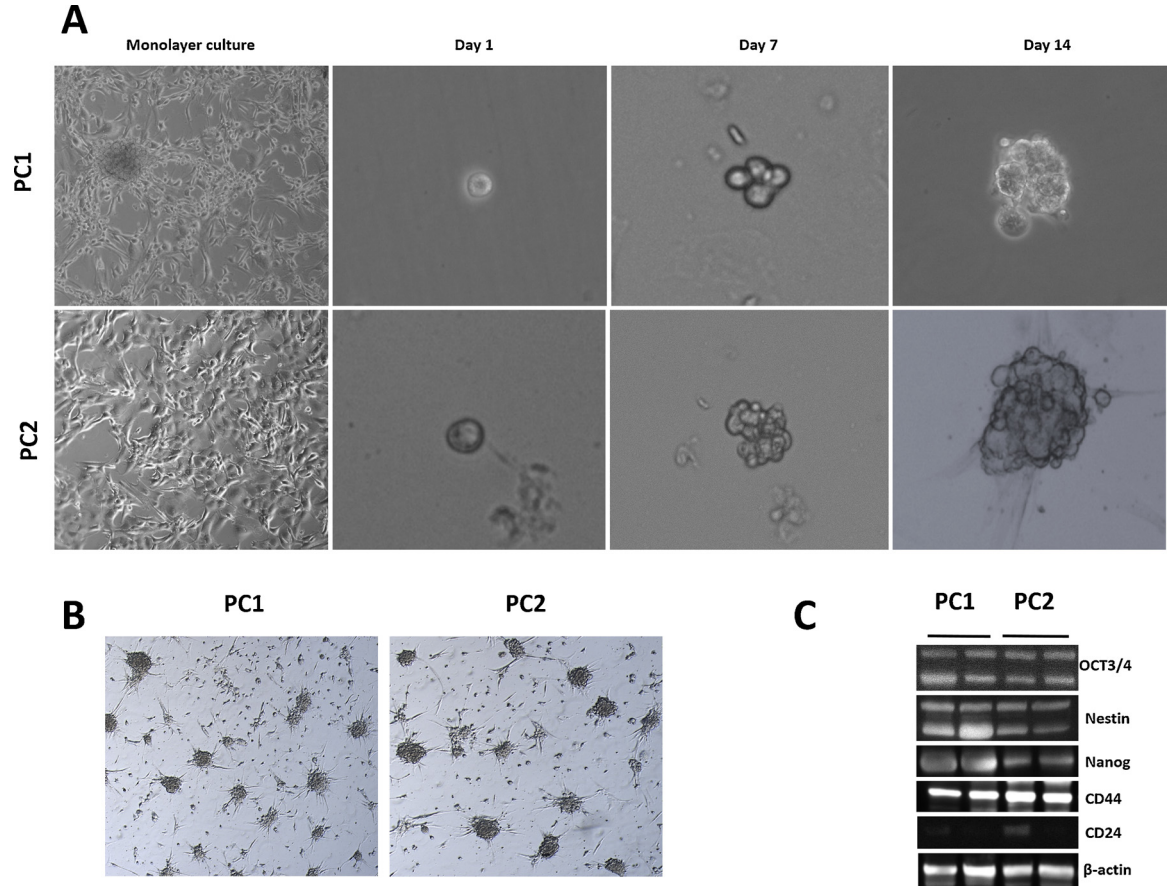


**Fig. 8.** Tumorsphere formation assay and characterization of stem cell markers. A: The morphology of cell cultures PC1 and PC2 in a monolayer culture and during tumorsphere formation. A single cell (Day 1) was able to divide and grow in a sphere morphology (Day 7). At day 14, both cell cultures were able to form spheres. B: Self-renewal assay showing the formation of numerous new spheres after single sphere suspension. C: Positive expression of OCT3/4, Nestin and NANOG by Western blot analysis of the tumorspheres. We also identified a $CD44^{+}/CD24^{-}$ phenotype in prostaspheres from both cell cultures.

## 3. Results

### 3.1. Clinical and histopathologic analyses

Based on the clinical records, metastases were present in 14 out 28 PC cases. Six out of 14 (42.9%) of these patients had metastases at the time of diagnosis, and the most commonly affected sites were the lung (4/6), bone (3/6) and intestine (2/6). Eight animals underwent chemotherapy (8/14), and, in three of them (3/14), only a surgical procedure was performed. The remaining three animals (3/14) received

only palliative treatments. The main clinical signs in the dogs with metastases were dysuria (9/14), tenesmus (6/14), lameness (4/14) and urinary incontinence (2/14). Unfortunately, the clinical outcome was available only in 11 out of 28 (39.23%) patients. The median survival time was 229 days (range 12–458 days).

Regarding tumor histology, the predominant tumor pattern was cribriform with comedonecrosis (9/28), followed by solid PC (7/28). Other histological patterns observed were papillary (5/28), small acinar (4/28), signet ring (1/28) and mixed (cribriform with solid areas) (4/28). All of the tumors had a Gleason score equal to or greater than 7.

### 3.2. Immunohistochemical expression of stem cell markers in canine prostatic tissue

The immunohistochemical results are summarized in Table 2. Positive nuclear OCT3/4 expression was observed in all normal prostate (10/10) and PH tissues (10/10), with a score of 5. Fifteen (15/28) canine PC samples were OCT3/4 positive with a score of 1 (13/28), and two samples (2/28) had a score of 2 (Fig. 1). OCT3/4 expression was significantly decreased in PC tissues compared to that in normal prostate ($P = 0.0001$) and PH ($P = 0.0001$) tissues. The overall survival time was similar in animals with either OCT3/4-positive or OCT3/4-negative tumors (Fig. 2A).

Regarding CD44 and CD24, cells with the $CD44^+/CD24^-$ phenotype were only present in PC samples (Fig. 3), with a statistically significant difference compared to normal ($P = 0.001$) and PH ($P = 0.001$) samples. PC-affected animals with a lower number of $CD44^+$/CD24- cells experienced a longer survival time than PC-affected animals who showed a higher number of $CD44^+$/CD24- cells (Fig. 2D).

Nuclear and cytoplasmic NANOG expression was observed in normal, hyperplastic and neoplastic tissues Fig. 4. Six out of 10 normal prostate samples, 8 out of 10 PH samples and 22 out of 28 PC samples showed NANOG-positive cells (Fig. 6) (Table 2). There were no significant differences among the three groups regarding NANOG expression. The overall survival rate was similar in animals with either NANOG-positive or NANOG-negative tumors (Fig. 2C).

Only 2 out of 10 normal prostate samples showed Nestin expression, with a score of 1 in the luminal cells. A Nestin score of 5 was found in two PH samples (2/10). Nestin was observed in 5 out of 28 PC samples (2/5 with a score of 5, 3/5 with a score of 1). All Nestin-positive tumors had a Gleason score of 10. Nestin was confined to the nucleus in normal and PH samples, while nuclear and cytoplasmic expression was observed in PC samples (Fig. 5). Statistically significant differences in cytoplasmic Nestin were observed between PC and normal prostate ($P = 0.001$) and PH ($P = 0.01$) samples. The overall survival rate was similar in animals with either Nestin-positive or Nestin-negative tumors (Fig. 2B).

### 3.3. Establishment and characterization of tumor-derived cell cultures

Both cell lines (PC1 and PC2) were from primary tumors with a cribriform pattern (Gleason score 10) (Fig. 6). The cells grew in a monolayer culture with a polygonal cell morphology (Fig. 6). At passage 10, both cell cultures expressed pan-cytokeratin and PSA (Fig. 6) and were negative for AR, uroplakin-III, vimentin and p63 by immunofluorescence. Additionally, we evaluated the expression of pan-cytokeratin, PSA and AR by flow cytometry, and both cell cultures were positive for pan-cytokeratin and PSA and were negative for AR (Fig. 7).

### 3.4. Tumorsphere formation, self-renewal and expression of stem cell markers

In medium containing 10% serum (DMEM with 10% FBS), both primary cell cultures showed adherent growth with a polygonal cell morphology (Fig. 8A – monolayer culture). In serum-free medium supplemented with growth factors, single cells began to form clumps with progressively increased cell density (Fig. 8A – day 1). At day 7, both cell cultures demonstrated sphere-like growth. At day 14, both cell cultures formed spheres from a single cell (Fig. 8A). In addition, PC1 and PC2 cell cultures demonstrated self-renewal capacity through secondary sphere formation (Fig. 8B). PC1 and PC2 cells contained OCT3/4-, Nestin- and NANOG-positive cells and showed a $CD44^+/CD24^-$ phenotype (Fig. 2C).

## 4. Discussion

The present study identified a group of cells that express OCT3/4, $CD44^+/CD24^-$, NANOG and Nestin in canine PC tissues, with the potential to be a cancer stem cell population. No previous literature has shown a strong link between the different CSC markers and stem cell properties in canine PC. Moulay et al. (Moulay et al., 2013) evaluated the gene expression of several stem cell markers in canine monolayer prostatic cell lines and, among them, identified high transcript levels of CD44. However, these authors did not perform a stem cell assay to confirm CD44 expression as a CSC marker. To address this gap in the literature, in addition to the evaluation of CSC markers in prostatic tissue (normal, PH and PC), we performed a tumorsphere assay (considered a stem cell assay) to identify a cancer stem cell population and characterize OCT3/4, CD44, NANOG and Nestin as stem cell markers in canine PC.

CSCs represent an immortal tumor cell population with self-renewal properties that is responsible for tumor heterogenicity. There is limited information concerning the stem cell population in canine tumors. In our study, we confirmed that two canine primary cell cultures form tumorspheres with self-renewal potential, indicating a cancer stem cell population in both cell cultures. However, some markers were expressed in tumorspheres from both cell cultures but were not expressed in the canine prostatic tissues. For example, OCT3/4 was expressed in the tumorspheres but had lower expression in canine PC tissues. In our study, OCT3/4 expression was observed in the luminal cells of normal and hyperplastic canine prostatic tissues. Since luminal prostatic cells are adult, well-differentiated cells, we suggest that OCT3/4 might play a role in canine prostate maintenance instead of representing a signature of cancer stem cells. In human PC, the role of OCT4 as a CSC marker is still controversial, with inconsistent reports of up- and downregulation of the OCT4 protein (Monsef et al., 2009; Resende et al., 2013). Resende et al. (Resende et al., 2013) demonstrated downregulation of OCT4 in PC samples in humans, correlating positive OCT4 expression with a good prognosis. In canine PC, weak OCT4 expression was reported in canine prostate cancer-derived cell lines by RT-qPCR and flow cytometry (Moulay et al., 2013).

Nestin expression was first described in neuroepithelial stem cells (Cattaneo and McKay, 1990; Lendahl et al., 1990), particularly during the development of neural stem cells (Cattaneo and McKay, 1990). Well-differentiated cells express Nestin only when tissue regeneration occurs (Wiese et al., 2004). We identified Nestin expression in prostaspheres from both cell lines, and Nestin expression was already demonstrated in human PC cell lines (Guzmán-Ramírez et al., 2009) and in five (5/28) PC tissue samples. The original tumors used for deriving the two cell cultures, as well as the five Nestin-positive PC samples, were characterized by a cribriform pattern and a Gleason score of 10. Further investigations on a larger number of representative samples may be required to evaluate a possible association among Nestin expression, malignant behavior and histologic subtype in canine PC.

NANOG is a master transcription factor that is responsible for the self-renewal properties of embryonic cells (Jeter et al., 2015). In our study, tumorspheres and most of the PC samples were NANOG-positive. The inhibition of NANOG expression by NANOG short-hairpin RNA causes loss of stem cell properties in human PC cell lines (Jeter et al., 2009), confirming that NANOG expression is required for a stem cell phenotype in human PC. The same authors have also demonstrated a positive correlation between NANOG levels and the $CD44^+$ phenotype.

The prostaspheres derived from our PC1 and PC2 cells had concomitant expression of NANOG and CD44, suggesting the existence of a stem cell population that is also CD24-, as described in other solid tumors with CSC-like features (Magalhães et al., 2013; Figueroa et al., 2015; Rybicka and Król, 2016). CD44 expression is associated with a more proliferative, tumorigenic and metastatic phenotype of PC cells both *in vitro* and *in vivo* (Patrawala et al., 2006). In our study, a larger number of $CD44^+/CD24^-$ cells was observed in canine PC cases with a higher Gleason score and a shorter survival time, thus suggesting the potential prognostic value of these markers.

## 5. Conclusion

Canine prostate cancer does express a population of cells with a stem cell phenotype, confirmed by *in vitro* tests on tumorsphere formations. This new information can be useful for future studies in comparative oncology, as well as studies on the role of CSCs in prognosis (survival time), tumor chemoresistance and metastatic potential in canine PC.

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