Establishment and characterization of canine mammary gland carcinoma cell lines with vasculogenic mimicry ability *in vitro* and *in vivo*

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ABSTRACT

Mammary neoplasm affects a population of intact and elderly female dogs and 50% are malignant. In order to study this disease, cell culture is as a promising preclinical model, creating the opportunity to deposit cell lines at a cell bank, allowing a great reproducibility of the assays and making the validation of the results more reliable. Another important aspect is the possibility to establish models for better understanding tumour characteristics, such as vasculogenic mimicry. Due the importance of cancer cell lines in preclinical models, this study aimed to establish and characterize primary cell lines from canine mammary gland tumours according to immunophenotype and tumorigenicity, and with its ability to form vasculogenic mimicry-like structures *in vitro* and *in vivo*. Cell cultures were evaluated for morphology, phenotype, vasculogenic mimicry and tumorigenicity abilities. We collected 17 primary mammary carcinoma and 3 metastasis and had a satisfactory result in 10 of them. All cell lines presented spindle shape

or polygonal morphology and expressed concomitant pan-cytokeratin and cytokeratin 8/18. Four cell lines had vasculogenic mimicry ability *in vitro* and two of them showed *in vivo* tumorigenic potential and forming VM in the xenotransplant tumour. Cell characterization of those lines will help to create a database for more knowledge of mammary carcinomas in dogs, including studies of tumour behaviour and new therapeutic targets.

Keywords: female dog, cell culture, mammary cancer, veterinary, oncology.

BACKGROUND

Intact and old female dogs are frequently affected by mammary gland tumours and more than 50% of the cases are malignant (Sorenmo 2003; Sleeckx et al. 2011). In women, breast cancer is the main cause of mortality and the most common cancer type diagnosed (Salehiniya, Ghoncheh, Pournamdar 2016). The occurrence of neoplasms in dogs are spontaneous and share some similar aspects to breast cancer in women, as histological classification, molecular targets and biological behaviour. For these reasons, canine mammary gland tumour is a natural model for human breast cancer (Abdelmegeed and Mohammed 2018).

Female dogs with mammary carcinoma do not have the same therapeutic outcome as women, since chemotherapy treatment is not as effective as in humans and doesn't increase the patient's survival time or influence local disease control (Tran, Moore, Frimberger 2016; Levi et al. 2016). In dogs, the golden standard treatment is radical mastectomy and chemotherapy is based on the human breast cancer literature (Sorenmo 2003; Hernandez-Aya and Gonzalez-Angulo 2013; Cassali et al. 2017). Thus, even performing chemotherapy in female dogs affected by mammary gland tumours, there is no standardized protocol (Levi et al. 2016). Besides that, we lack information regarding markers that can predict antitumor response, as occurs in human breast cancer treatment (Sawyers 2004, Blows et al. 2010). In this scenario, cancer cell lines represent a great opportunity for the evaluation of antitumour response.

Cell lines are an alternative and an experimental model *in vitro* to human breast cancer and canine mammary gland tumours, allowing the investigation of carcinogenesis process as proliferation, apoptosis and migration (Visan et al. 2016). Cell culture is considered an excellent preclinical model, essential for the identification and evaluation of drugs action mechanism, identification of genes involved in carcinogenesis, such as oncogenes and tumour suppressors, defining cell signalling pathways and how they contribute to tumour pathogenesis, discovering of new drugs and for the development process of antitumour drugs (Ruggeri, Camp, Miknyoczki 2014).

In canine and human patients affected by highly aggressive mammary neoplasms, neoplastic cells may be able to form vascular-like structures or channels, used to conduct plasma,

red cells and neoplastic cells during epithelial mesenchymal transition (Ge and Lou 2018). The capacity of tumour cells to create non-endothelial vascular channels is called vasculogenic mimicry (VM) (Delgado-Bellido et al. 2017). This VM process occurs by the influence of cancer stem cells that become endothelial-like cells and induce tumour neovascularization (Ge and Lou 2018). The vessels formed during VM are composed by tumour cells and endothelial cells, and then, gradually the cells are transformed into tumour cells only. The new generated vessels or channels are bonded to preexisting vessels (Delgado-Bellido et al. 2017; Ge and Lou 2018). VM has been studied as a mechanism of tumour nutrition besides angiogenesis and may be a possible explanation for metastasis (Sun et al 2006, Zhang, Zhang, Sun 2007; Delgado-Bellido et al. 2017; Ge and Lou 2018). The presence of these vessels can be associated with a more malignant tumour, higher histopathological grade, shorter survival time, higher capacity of invasion and metastasis (Delgado-Bellido et al 2017). In canine mammary gland tumours, the phenomenon has been studied in inflammatory mammary carcinomas and other tumour subtypes (Clemente et al. 2010; Barreno et al. 2019). In humans VM formation is a poor clinical prognostic characteristic (Delgado-Bellido et al. 2017).Thus, this study aimed to establish and characterize ten cell lines from canine mammary gland tumour (7) and three metastasis, according to immunophenotype, tumorigenicity and ability to form vascular-like structures *in vitro* and *in vivo*.

MATERIALS AND METHODS

Animals and experimental design

This study was performed in accordance with the National and International Recommendations for the Care and Use of Animals (National Research council 2011). All procedures were performed after receiving approval from the Ethics Committee on Animal Use (CEUA) of Veterinary Teaching Hospital of São Paulo State University (CEUA/UNESP, #0208/2016).

Reagents

All reagents used had high purity and were purchased from companies GE Healthcare (Uppsala, Seeden), Sigma-Aldrich (São Paulo, Brazil), Merck SA (São Paulo, Brazil), otherwise, they were cited. In addition, cell culture medium used were *Mammary Epithelial Cell Growth Medium* (MEGM™, Lonza Inc., Allendale, NJ, USA) and medium *Dulbecco's Modified Eagle Medium:F12* (DMEM/F12, Lonza Inc., Allendale, NJ, USA), fetal bovine serum (FBS, LGC Biotecnologia, Cotia, SP, Brazil), *Dulbecco's Phosphate Buffered Saline* (DPBS) (Sigma Aldrich,

St. Louis, MO, USA), antibiotic/antimycotic solution (TermoFischer Scientific, Waltham, MA, USA) and trypsin (0.25%, GBICO TermoFischer Scientific, Waltham, MA, USA).

Tumour samples and cell isolation

Twenty samples of mammary gland tumours were collected at the veterinary teaching hospital from Veterinary Medicine and Animal Science School, UNESP (between December 2016 and March 2017) for histopathological examination and cell culture. Tumour samples were formalin fixed and paraffin-embedded and another sample, placed in Mammary Epithelial Cell Growth Medium (MEGM™, Lonza Inc., Allendale, NJ, USA) media,brought immediately to cell culture. From the twenty samples, seventeen were primary tumours and three were respective metastasis. Cell lines were obtained using enzymatic dissociation as previously described in the literature (Costa et al. 2019). Briefly, tumour fragments with approximately 1 cm² were collected and dissociated with type IV collagenase (Sigma-Aldrich, St. Louis, MO, United States) for four hours at 37 °C, in a humidified atmosphere containing 5% CO_2. After this process the material was separated using a 75 µm mesh filter, centrifuged and washed with Dulbecco's Phosphate-Buffered Saline DPBS (Sigma-Aldrich, St. Louis, MO, United States) to remove excess collagenase. After cell isolation process, they were counted in a Neubauer chamber and the cell viability was evaluated by the Trypan blue technique. Plating was performed at a concentration of 10^4 cells / mL in 25 mL culture bottles with filter.

Samples identification, histological classification, and cell obtention technique are described in Supplementary Table 1. Identification of histological subtypes and tumour grade were based on the international classification of mammary gland tumours (Goldschimidt et al. 2011).

Molecular phenotype of primary tumours and respective metastasis

Immunohistochemistry (IHC) was performed with the tumour's samples from paraffin block, wherein 4 µm-thick sections were placed onto positively charged slides (StarFrost, Braunschweig, Germany) and then, deparaffinized. Antigen retrieval was done using citrate buffer (pH 6.0) in a pressure cooker (Pascal, Dako, Agilent Technologies, Santa Clara, CA, USA) and endogenous peroxidase was blocked with 8% hydrogen peroxide (Dinâmica Química Contemporânea, Indaiatuba, SP, Brazil) in methanol (Dinâmica Química Contemporânea, Indaiatuba, SP, Brazil) for 20 min. Nonspecific protein binding was blocked with 8% skim milk for 60 min, both at room temperature. Primary antibodies against HER2, ERα, PR, Ki-67, CK5/6 and EGFR and respective clones were diluted, and samples remained incubated according to Nguyen et al. (2018) (Supplementary Table 2). Antibody detection was achieved using a polymer

system (EnVision, Agilent Technologies, Santa Clara, CA, USA). 3,3'-Diaminobenzidine (DAB) (EnVision, FLEX, High pH, Dako, Agilent Technologies, Santa Clara, CA, USA) was used as a chromogen and tissue counterstaining was performed with Harris Haematoxylin. Four human mammary carcinoma samples were used as positive control being a negative, HER-2 1+, HER-2 2+ and HER-2 3+. Thus, based on this staining pattern we evaluated our samples and classified them. For Ki67, CK5/6 and EGFR, we used the adnexa glands from subcutaneous region as a positive internal control. For ER and PR, we used a canine uterine sample (Dos Anjos et al. 2019).

ERα and PR were considered positive when ≥ 10% of the nuclei were stained, CK5/6 and EGFR receptors when cytoplasmic staining was ≥ 10% and Ki-67 when more than 33.3% of the cells had stained nuclei. For HER-2 evaluation, a group of more than 500 cells were scored for labelling distribution (0: unlabelled or <10% labelled tumour cells; 1+:> 10% of labelled tumour cells with incomplete membrane labelling; 2+: moderate to > 10% strong tumour cells; 3+: complete and strong membrane labelling of > 10% tumour cells). In this classification, 0 and 1+ were considered negative and, 2+ and 3+ positive.

The immunophenotype was achieved according to the previous human classification (Nielsen et al. 2004; Blows et al. 2010). Briefly, the classification of different molecular phenotypes of canine mammary carcinoma is described as Luminal A, Luminal B, Triple negative or HER2 overexpressing. The Luminal A contemplates tumours HER2-, ER or PR + and Ki67 less than 33%. The Luminal B type is HER2-, ER or PR + and Ki67 more than 33%. Tumours HER 2, ER and PR negative and with EGFR and/or CK5/6 positive where considered Triple-negative basal-like and tumours HER 2, ER and PR negative and with EGFR and/or CK5/6 negative were considered Triple-negative non basal-like (Nielsen et al. 2004).

Cell Expansion

Cell culture was stablished from tumour fragments in MEGM™ medium containing 1% antibiotic/antimycotic solution and 10% FBS kept in a humid atmosphere containing 5% CO_2 at 37º C. When the cells reached 80% confluence, the medium was discarded, and the bottles were washed with sterile DPBS pH 7.2 to eliminate residual FBS. For detachment of the cells from the bottle, trypsin 0.25% was added at 37 °C, followed by a 5 min incubation period in a 5% CO_2 humid atmosphere and 37 ºC. Cells were cultured until 10th passage and used for cell phenotyping, karyotype, morphology and western blot.

Elimination of contaminating fibroblasts

To eliminate fibroblasts from primary cultures, selective cell trypsinization with cold trypsin (4°C) was performed according to the previous literature (Kisselbach et al. 2009) at passage 5 (P5). Briefly, cells were washed with DPBS at 4 °C to avoid direct thermal shock by the cold trypsin. Subsequently, 2 mL of cold trypsin (4 °C) were used at room temperature (27 °C) for 2 min. Then, supernatant was inactivated with complete medium containing 10% FBS in a 1: 1 ratio and trypsin was collected and discharged. Then, the flasks were washed with DPBS buffer to remove residual trypsin. Subsequently, cells were washed with DPBS solution at room temperature twice, and then washed with DPBS buffer at 37 °C once. After this procedure, 800μl of trypsin at 37 °C were added to the bottle and the cells were kept in a humid atmosphere at 37 °C in a humidified atmosphere containing 5% CO_2 for 5 min. After this period, the remaining cells were detached with manual mechanical impact and the trypsin was inactivated with complete medium (containing 10% FBS) in a 1:1 ratio. Then, cells were centrifuged for 5 min at 1200 rpm and resuspended in 5 ml of MEGM™ medium containing 10% FBS and 1% antibiotic and antifungal.

Cell Karyotype

Karyotype analysis was performed according to Moorhead et al. (1960). Initially the cells at P10 were cultured in DMEN/F12 medium, supplemented with FBS (10%) and phytohemagglutinin for 72 hours. After this period, the cells were evaluated under the inverted microscope and the mitotic spindle was interrupted by the addition of colchicine (16 µg / ml). Subsequent washes and centrifugations were performed at 4 °C to then fixed the slide material and stained by the Wright-Giemsa staining method for karyotype assembly. After karyotype preparation and staining, 70 different images were captured from each cell culture (n=10) and at least 20 metaphases of each culture were analyzed, according to the previously described by Gouveia et al. (2017).

Cell morphology and doubling time

The morphology of each culture was evaluated at P10 to characterize the cell phenotype. For this, 12 sterile well plates with sterile circular coverslips were used. After plate preparation, 500 μl of complete culture medium were added to each well and subsequently 10^3 cells were pipetted into the middle of the well, for 72 hours. After this period, cell density in each coverslip was verified. When the coverslips presented confluence above 50%, they were removed for cell morphology.

For the morphology technique the medium was removed, the coverslips washed with DPBS three times and then fixed in cold methanol (4 °C) for 30 min in a refrigerator (8 °C). After

methanol was removed, the cells were washed three times with PBS and immersed in 0.1% Triton-X solution for 10 min, at room temperature, for cell permeabilization. Subsequently, the permeabilizing solution was removed and the cells washed three times with DPBS. After that, cells were stained with hematoxylin and eosin (HE).

Cells were also evaluated for cell doubling time. The cell medium was discarded and the bottles were washed with sterile DPBS pH 7.2 to eliminate residual FBS. For detachment of the cells from the bottle, 0.25% trypsin was added at 37 °C, followed by a 5 min incubation period in a 5% CO_2 humid atmosphere and after this period, trypsin was inactivated with cell culture medium supplemented with 10% FBS and 1% antibiotic/antimycotic solution. Cells were centrifuged (450 g, 5 min) and after discharging the supernatant, resuspended in 1 mL DMEM/F12 medium with 10% FBS and 1% antibiotic/antimycotic solution. Cells were diluted in Trypan blue (Trypan blue solution, cod. T8154, Sigma-Aldrich, St. Louis, MO, USA) in a 1: 1 ratio and counted in a Neubauer chamber. For doubling time, the protocol described by Caceres et al. (2015) was followed. Briefly, $1x10^5$ were plated in 25cm² flasks and maintained with DMEN/F12 medium supplemented with 10% FBS and 1% antibiotic/antimycotic solution in triplicate. Every 24 hours cells were trypsinized and counted. This procedure was performed for 5 consecutive days to evaluate the exponential growth curve. The final number of cells for each culture was obtained by averaging three counts.

Cell Phenotype

The protocol was the same as cell morphology. When the coverslips presented confluence above 50%, they were removed for immunofluorescence (IF).

For the IF technique the medium was removed, the coverslips washed with DPBS three times and then fixed with cold methanol absolute (4 °C) for 30 min in a refrigerator (8 °C). After methanol was removed, the cells were washed three times with PBS and immersed in 0.1% Triton-X solution for 10 min at room temperature for cell permeabilization. Then, cells were blocked with commercial solution (Protein block, Dako, CA, USA) for 30 min at room temperature and primary antibodies were added to each well. We investigated pan-cytokeratin, cytokeratin 8/18 and vimentin expression. The information regarding the antibodies can be found in Supplementary Table 3. Cells were incubated with goat anti-mouse IgG secondary antibody (Alexa Fluor 647, Life Technologies, Corporation, Carlsbad, CA, EUA) and counterstained with DAPI (Sigma Aldrich, St. Louis, MO, EUA) at 1:10.000 dilution. As a negative reaction control, the primary antibodies tested during the procedure were omitted and replaced with TRIS buffer solution.

Vasculogenic mimicry formation *in vitro*

For the *in vitro* vasculogenic mimicry assay, the cells were cultured in three-dimensional (3D) conditions according to Salinas-Vera et al. (2019). After each cell culture achieved more than 80% of confluency, they were trypsinized and $50x10^3$ viable cell were cultured with DMEM in a 24-well plate with 250 μL of Matrigel (Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, *LDEV-Free, Corning, New York, NY, USA). The Matrigel was added in each well and air-dried for 30 min, at room temperature. The cells were added with DMEM medium and incubated in a humidified atmosphere with 5% of CO_2 at 37°C. Then, the cells were evaluated in an inverted microscopy every hour to determine vasculogenic mimicry *in vitro* formation. The experiment was performed in triplicate for each cell culture.

Tumour growth in immunodeficient mice

This experiment was approved by ethics committee to use laboratory animals from the experimental unity of Botucatu Medical School – UNESP (#1267/2018-CEUA). To evaluate cell culture tumorigenicity, a total of 12 nude mice (BALB/c nude, C.Cg-Foxn1nu line) were acquired from the Institute of Biomedical Sciences, University of São Paulo – USP and housed in individually ventilated caging. All procedures regarding feeding, humidity, temperature and light control were performed according to the literature (Cordeiro et al. 2018). For *in vivo* tumorigenicity evaluation, $1x10^6$ cells, from each cultured cell line, were inoculated on a different mouse, at subcutaneous inguinal mammary gland region (Fleming et al. 2010) and the subjects were accompanied once a week, for at least 60 days. After tumour growth, the visits occurred daily to measure tumour volume using a digital calliper. After achieving 3 cm^2, the subjects were submitted to humanely euthanasia and the material from the tumour was collected (formalin fixed andparaffin embedded) to confirm malignancy with histological evaluation. Then, pan-cytokeratin and vimentin immunohistochemistry were performed to confirm tumour phenotype. Immunohistochemistry analysis was performed as described above, using the antibodies mouse monoclonal anti-vimentin (Clone V3, Santa Cruz Biotechnology, Dallas, TX, USA) and mouse monoclonal anti-cytokeratin (Clone AE1/AE3, Santa Cruz Biotechnology, Dallas, TX, USA) at 1:300 dilution, overnight. The secondary antibodies, chromogen, counterstaining and negative controls were performed as described above. The epithelial component of a normal skin was used as positive control for pan-cytokeratin and the dermis was used as positive control for vimentin.

CD31 and periodic acid Schiff (PAS) double staining

The procedures for the CD31/PAS double staining were previously described by Kim et al. (2019). Briefly, immunohistochemistry with rabbit polyclonal anti-CD31 primary antibody (PECAM-1, Thermo Fischer Scientific, Waltham, MA, EUA) and a polymer system conjugated with peroxidase as first staining in the tumour from xenotransplant. Then, sections were counter stained with 0.5% PAS and Schiff. VM was characterized by endothelial-like structure by tumour cells containing red blood cells stained with PAS and negative for CD31 (Kim et al. 2019). Blood vessels will be identified by the CD31/PAS double staining.

RESULTS

Molecular phenotype of primary tumours and cell isolation

Of all the twenty tumour samples, ten were able to grow on cell culture and were evaluated by morphology (HE) and IHC (Supplementary table 1). Six were classified according to Nielsen et al 2004 as HER-2 overexpressing (UNESP-CM1, UNESP-CM9, UNESP-CM11, UNESP-CM60, UNESP-MM3 and UNESP-MM4), three as triple-negative basal-like (UNESP-CM5, UNESP-CM61 and UNESP-MM1) and one as triple negative non basal-like (UNESP-CM4).

The samples UNESP-CM2, UNESP-CM3, UNESP-CM6, UNESP-CM7, UNESP-CM8, UNESP-CM10 and UNESP-CM12, UNESP-CM13, UNESP-CM14 and UNESP-CM15 did not show *in vitro* expansion. The cell cultures UNESP-CM2 and UNESP-CM3 were cultured using enzymatic dissociation overnight and had no cellular growth after 72 hours in culture condition. UNESP-CM6 and UNESP-CM7 culture cells were made using explants and for both cell lines, fungal contamination discontinued the cellular growth. Thus, cells submitted enzymatic dissociation with type IV collagenase showed the best *in vitro* expansion (Supplementary table 1). UNESP-CM8, UNESP-CM10, UNESP-CM12, UNESP-CM13, UNESP-CM14 and UNESP-CM15 stopped growing and did not continue to expand to further passages.

Contaminating Fibroblast Elimination

Cell cultures prior P5 showed mixed morphology (spindle cells, polygonal cells, cells growing in groups, and rounded cells) (Figure 1A). After selective trypsinization at 4 °C, cells became more homogeneous and showed slow growth with spindle morphology (Figure 1B) and cells that did not show confluence above 50% after 30 days of culture were discharged. Cells from trypsinization at 37 °C showed homogeneous morphology with some cultures presenting uniform spindle morphology (UNESP-CM1, UNESP-CM4 and UNESP-MM1) or polygonal morphology (UNESP-CM5, UNESP-CM9, UNESP-CM11, UNESP-CM60, UNESP-CM61, UNESP-MM3

and UNESP-MM4) (Figure 1C). Cells were grown to passage 10 (P10), showing approximately 90% confluence 48 hours after passage.

Cell Karyotype

Of the 70 images captured, the best ones for chromosome counting were selected (Supplementary Figure 1A). It was possible to identify aneuploidies in different metaphases of different cell cultures. In cell cultures UNESP-CM4 and UNESP-CM11, hypoploidy of metaphase was identified, as well as hyperploid of cultures UNESP-CM1, UNESP-CM60 and UNESP-MM3. The representation of chromosomal alterations in each culture can be seen in Supplementary Figure 1B.

Cell morphology, phenotype and doubling time

In the morphological evaluation (Figure 2I and 2J), the primary and metastatic adenosquamous carcinoma tumour cells showed growth in monolayers, in fusiform pattern, basophilic nucleus and eosinophilic cytoplasm, with presence of mitosis. Grade III solid carcinoma cells showed multinucleated cells and colony formation (Figure 2K and 2L). All samples evaluated showed strong pan-cytokeratin (Figure 3A) and CK8/18 (Figure 3B) staining and were negative for p63 (Figure 3C). All cell cultures also showed vimentin positive cells (Figure 3D). The concomitant pan-cytokeratin and CK8/18 expression for all cell lines confirmed their epithelial phenotype. The primary culture cells, UNESP-CM1, UNESP-CM4, UNESP-CM5, UNESP-CM9, UNESP-CM11, UNESP-CM60 and UNESP-CM61 reached twice the number of cells cultured initially (doubling time) at 6.31, 5.1, 7.5, 8.95, 7.45, 11.79 and 13.06 hours, respectively. The UNESP-MM1, UNESP-MM3 and UNESP-MM4 metastasis cultures presented their doubling time at 25.41, 34.17 and 10.28 hours, respectively.

Vasculogenic mimicry *in vitro* and *in vivo*

The *in vitro* vasculogenic mimicry formation was identified in 4 out of 10 cell cultures. The cell lines UNESP-CM1, UNESP-CM9, UNESP-CM60 and UNESP-MM4 presented *in vitro* vasculogenic mimicry formation at four hours (Figure 4) until six hours and vasculogenic mimicry-like structures were disrupted from 6 hours.

One primary cell culture (UNESP-CM60) and its respective metastasis (UNESP-MM4) showed *in vivo* tumorigenicity (2 out of 10 cell cultures) (Supplementary Figure 2). The macroscopic growth was evident after 50 days after cell application and histology revealed a tumour with highly vasculogenic mimicry formation. Vasculogenic mimicry was characterized by

neoplastic cells forming PAS-positive tubules containing plasm or red cells (Figure 5). It was also observed internal positive controls, composed by blood vessels positively stained by PAS associated with CD31-positive staining by endothelial cells (Figure 5). Both cell lines presented neoplastic cells with evident nucleoli forming endothelial-like structures, mimicking capillaries (6A and 6D). These capillaries-like structures were positive for both pan-cytokeratin and vimentin, indicating (6B, 6C, 6D, 6E and 6F). Interestingly, the metastatic cell line (UNESP-MM4), also showed an intravascular growth (6A). It was possible to observe several blood vessels in tumour periphery with intravascular growth of cancer cells positive for pan-cytokeratin (6B).

DISCUSSION

We cultured and characterized *in* vitro and *in vivo* (xenotransplant animals) seven primary cell cultures from mammary gland tumours and three from metastatic disease. The technique applied was adequate to stablish the cell lines, since there is a need for *in vitro* and natural models for studies in veterinary and comparative oncology. We also found four cell lines that presented VM, an aggressive feature of mammary tumours.

Canine mammary gland tumours represent one of the most important tumours in intact female dogs and can be considered a model for studying human disease. In human medicine, several cell lines have been used as a preclinical model for understanding breast cancer (BC) development and progression or studying the antitumour effect of new drugs (Comsa, Cîmpean, Raica 2015; De et al. 2019; Lefort et al. 2019; Muhammed et al. 2019). Comparing with human BC, there are fewer canine mammary gland tumour cell line developed and studied (Hellmén et al. 2000; Osaki et al. 2016; Gentile et al. 2017; Cordeiro et al. 2018). To establish the primary cell cultures, we tried different methodologies aiming to identify the most effective in isolating the neoplastic epithelial mammary neoplastic cells. The cultures obtained through explant samples presented several problems in their establishment, mainly related to contamination during cultivation. Thus, we tried protocols based on the use of enzymatic dissociation. In this step we tried collagenase type I and II in different incubation time (3, 4, 12, 24 and 48 hours) with no success (data not shown). On the other hand, using 0.05% type IV collagenase showed better results. For type IV collagenase 0.05% with 24- and 48-hours incubation, we identified cell damage and death, with no cell growth (data not shown). For samples incubated using 0.05% type IV collagenase within 4 hours there was no cell damage and satisfactory cultures expansion. Therefore, we standardized the time to up to 4 hours for enzymatic dissociation.

After the different cell cultures were established, they presented a heterogeneous morphology, so selective trypsinization of cells was performed to eliminate fibroblasts and

stimulate cell clone formation (Geng et al. 2016). After initial expansion, cells were used for characterization at passage 10 (P10), since from lower passages they tend to have a heterogenous morphology and in higher passages can present a higher number of chromosomal alterations related to cell culture conditions (Turin et al. 2014; Muñoz et al. 2016). In addition, at passage 10 or above, we did not find contaminating fibroblasts in the cultures. To eliminate fibroblasts, we used two different strategies, using a culture medium that show specific epithelial cell growth factors and also did a selective trypsinization. Together, both strategies were effective in eliminating fibroblasts and selecting epithelial cells during cell expansion, that can be confirmed based on the expression of epithelial markers by neoplastic cells.

Cell lines presented a monolayer growth, similar size and morphology. They showed spindle-shape, high nucleus/cytoplasm ratio and a tight cell-cell adhesion. The high ratio between nucleus and cytoplasm size can usually be associated with malignant tumour behaviour (Chang et al. 2010). In haematoxylin and eosin staining, it was possible to observe basophilic nucleus and eosinophilic cytoplasm, with presence of mitosis and some cells appeared multinucleated and with colony formation. Besides that, our cell lines also expressed vimentin, same results found in MCF7 and HeLa cells cultured *in vitro* (Messica et al. 2017; Saunus et al. 2018). Since in culture conditions cells need to change their cytoskeleton for the flask attachment, it is common to observe vimentin expression either in epithelial cells (Liu et al. 2015). This expression was previous explained by post translational modification during cell culture conditions (Caceres et al. 2015). Doubling time of each culture was analysed and the shortest was from UNESP-CM4 (5.1 h) and the longest from cell UNESP-MM3 (34.17 h) at passage 10. These results are similar to those found by Cordeiro et al. (2018) in which the doubling times of two different cells lines from canine mammary gland tumour cultured *in vitro* were 26 and 42 hours and, in this case, one cell line was more malignant than other considering invasion potential and the *in vitro* tumorigenicity.

An interesting finding in this study was the *in vitro* to vasculogenic mimicry (VM) in four different cell lines. Tumour cells that present VM are highly malignant and are capable of penetrate endothelium, developing tumour invasion and metastasis (Zhang, Zhang, Sun 2007). Among our cell lines, two also showed vasculogenic mimicry ability *in vivo* (tumour from xenotransplant). The ability of VM formation is associated with aggressive melanoma cells and not with non-aggressive cells (Delgado-Bellido et al. 2017), and high tumour grade, invasion, metastasis and poor clinical prognosis in hepatocellular carcinomas (Sun et al. 2006). In inflammatory mammary tumours, both human and canine, VM is commonly described, possibly related to tumour aggressiveness and metastasis capacity (Clemente et al. 2010; Barreno et al. 2019). The VM results corroborate with the results from tumorigenicity assay where two cellular types, that were capable

of VM formation, produced tumours *in vivo*. When analysed in HE, the tumours from the xenotransplants had highly vasculogenic mimicry formation, showing the aggressivity of this neoplasia, *in vivo* and *in vitro*. The ability to form vasculogenic mimicry *in vitro* and *in vivo*, reinforce the use of these cells as a preclinical model for canine mammary gland tumours.

Among our tumour cell lines, four out of 10 showed tumorigenicity *in vivo*. This result was similar with previously described by Cordeiro et al. (2018), wherein only one in two different cell lines grew *in vivo*. Although the cells had resembling epithelial characteristics, as morphology, growth pattern, phenotype, protein expression and tumoursphere formation, the tumour did not grow *in vivo* (Cordeiro et al. 2018). The tumour growth *in vivo* depends on many factors, as the application choice location, cell concentration, tumour heterogeneity and the immune system of the animal (Mollard et al. 2011). In our case, this result may be explained by the animal model used in the study. The BALB-c nude mice lack thymus and is unable to produce T-cells being considered an immunodeficient mice. However, this nude model shows other immune cells, such as B-cells and natural killers that can affect the tumour growth *in vivo*. In our xenograft study, the histological evaluation revealed a high inflammatory infiltrate in all tumours (n=4), indicating that other immune cells were infiltrating the tumours. Thus, a nude mouse with no B-cell and Natural Killer cells can be accurate to evaluate *in vivo* tumorigenicity (Kariya et al. 2014). However, for using a more immunocompromised mice, it is necessary an accurate pathogen-free laboratory system. Unfortunately, our institution doesn't have the necessary structure for accommodating these immunocompromised mice. Thus, the major limitation of our study is the lack of structure to evaluate the cell tumorigenicity in a less immunogenic mice.

Some studies show that drug testing and behavioral study of tumors can be performed efficiently on mammary tumor cells cultured *in vitro* (Hsiao et al. 2014; Caceres et al 2015; Zhang et al. 2018). The establishment and characterization of new cell lines is significant and a useful cell model for studies of basic biology of tumors, their development and possible uses (Hsiao et al. 2014; Cáceres et al. 2015). In addition, cells characterized as triple negative have a higher importance due to their worse prognosis and can assist in understanding and study of these types of tumors in dogs and humans (Abdelmegeed, Mohammed 2018; Zhang et al. 2018).

Overall, our study stablishes new canine mammary gland tumour cell lines, that can increase the understanding of this disease in dogs. Besides that, our study provided a valuable information regarding cell lines that form VM *in vitro* and *in vivo* (Massimini et al. 2019). VM it is a potential prognostic and predictive markers in tumours; however, it is hard to find *in vivo* models to understand this phenomenon. Our study provides two canine mammary gland tumour cells with ability to form VM *in vivo* as a unique model for understanding this phenomenon.

CONCLUSION

In summary, we established and characterized 10 cell lines and xenografts from canine mammary gland carcinomas and metastasis. The cells cultured *in vitro* demonstrated morphological and phenotype similarities but had tumorigenicity differences. Furthermore, four cell lines were capable of vasculogenic mimicry ability in vitro and two of them showed *in vivo* tumorigenicity, both related to malignancy and aggressiveness. Thus, described cell lines will be able to be used in the future for clinical investigations, therapeutic targets, and for studying genes targets and pathways. The results showed similarities with BC in human demonstrating that the results obtained with these cell lines could be used for comparative oncology studies.

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The authors report no conflict of interest

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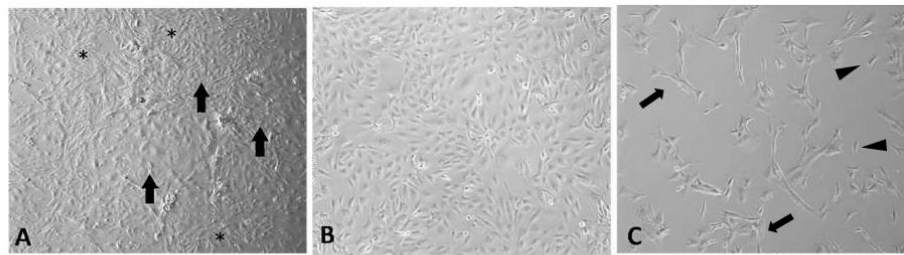
Figure 1. Mammary gland tumour cell culture (UNESP-CM9). A: Culture of mammary gland cancer cells at passage 5 (P5). Note the heterogeneity in cell morphology, with cells showing spindle morphology (asterisk) and other cell groups showing polygonal morphology (arrows). B: Cell culture after selective trypsinization with polygonal morphology (compatible with epithelial cells) and high cell density after 48 hours of culture. C: Culture of cells from cold trypsinization. Note the majority of the cell population with fusiform morphology (arrows) and few cells with polygonal morphology (arrowhead). Note low cell density after 30 days of cell culture.
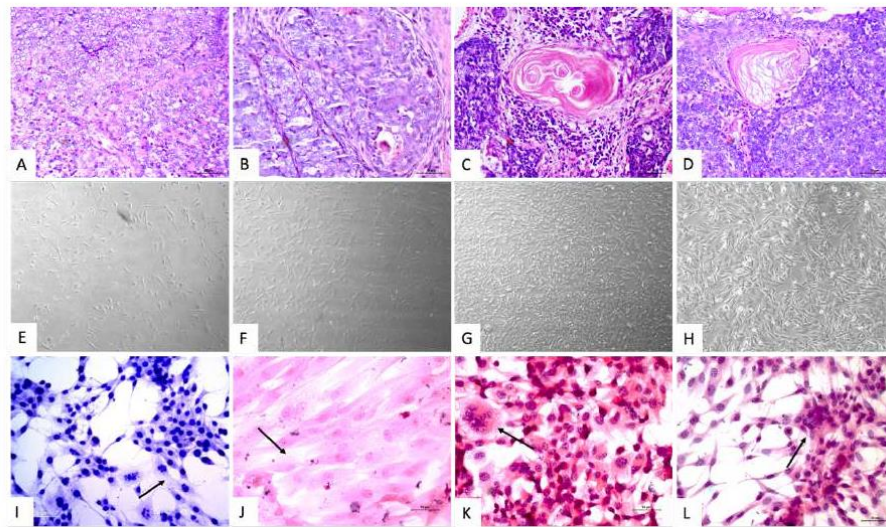
Figure 2. Histopathological evaluation (A, B, C and D) of tissue samples from canine primary and metastatic mammary tumours at 40x magnification. Grade III solid carcinoma (A) and its bone metastasis (B), and adenosquamous carcinoma (C) and its lymph node metastasis (D). *In vitro* (E, F, G and H) cell culture of primary (E and G) and metastatic (F and H) mammary tumour cells at 50x magnification. H&E staining (I, J, K and L) of cells grown *in vitro* at 200x magnification. In the figure the arrows indicate multinucleated cells (K), elongated cells with large cytoplasm (I and J) and colony formation (L).
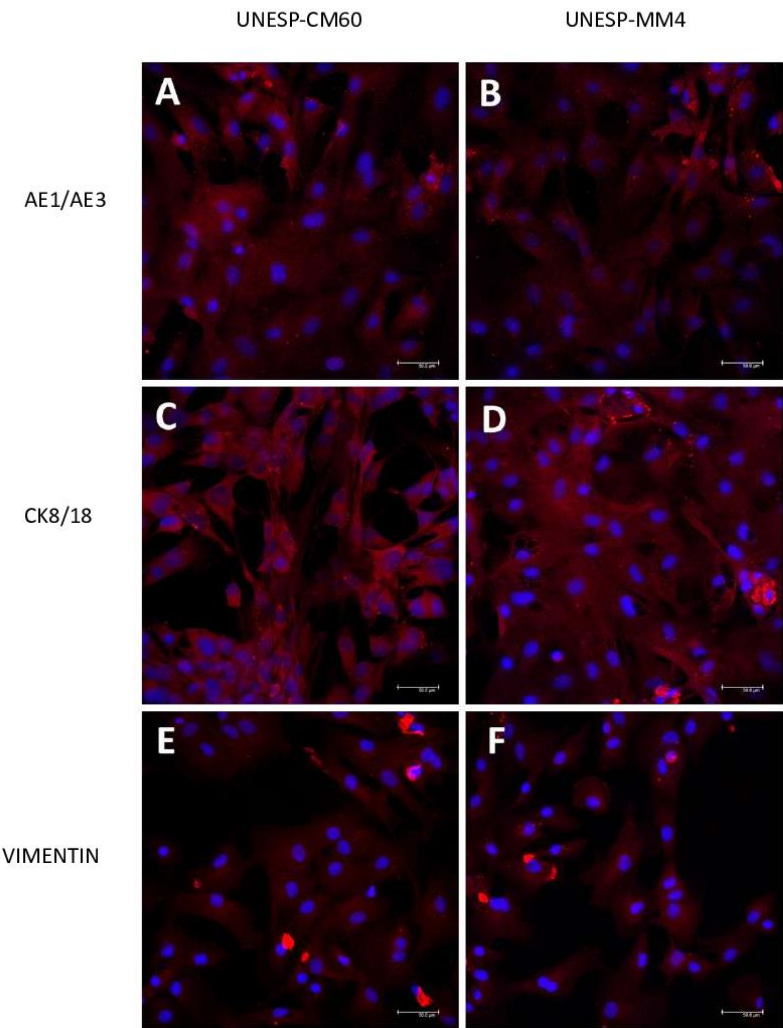
Figure 3. Immunofluorescence staining for pan-cytokeratin, CK8/18 and vimentin expression in canine mammary gland tumour cells. The first column represents the cell line UNESP-CM60 and the second column the cell line UNESP-MM4. It is possible to observe pan-cytokeratin cytoplasmic expression in UNESP-CM60 (A) and UNESP-MM4 (B) cell lines. Both cell lines also expressed CK8/18 (C and D) and vimentin (E and F).
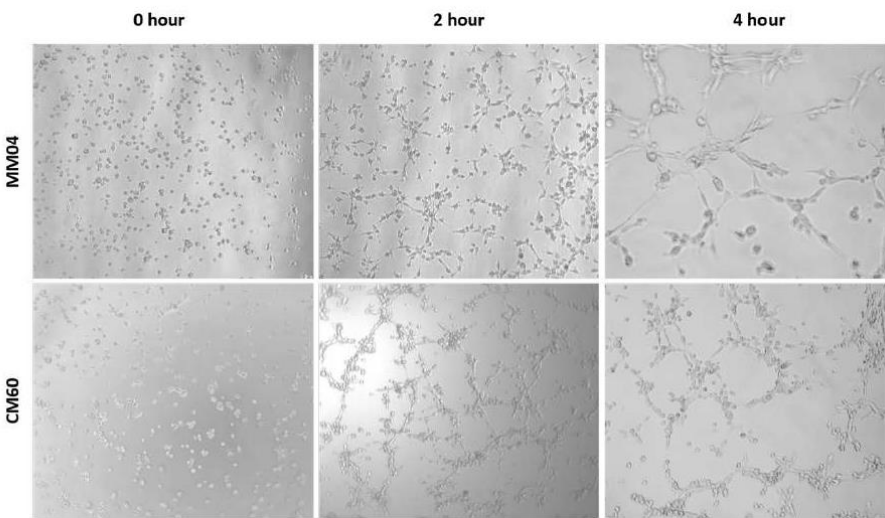
Figure 4. 3D experiment to evaluate the vasculogenic mimicry ability *in vitro* of cells from a primary carcinoma tissue (UNESP-CM60) and its metastasis (UNESP-MM4). Three different moments can be observed in both cell cultures. The tubular complete formation occurred at 4 hours for the two cell lines.
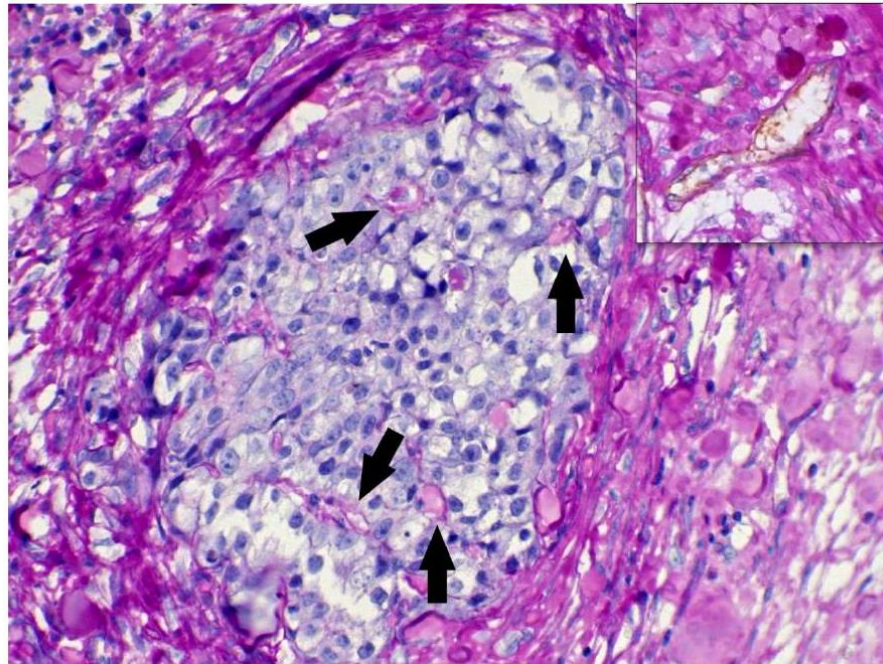
Figure 5. CD31/Periodic Acid-Schiff (PAS) double staining in the xenotransplant. It is possible to observe a group of neoplastic cells in a solid formation. Neoplastic cells are forming PAS-positive endothelial-like structures, characterized as vasculogenic mimicry. In the insert, it is possible to observe a blood vessel double stained for CD31/PAS. 400x
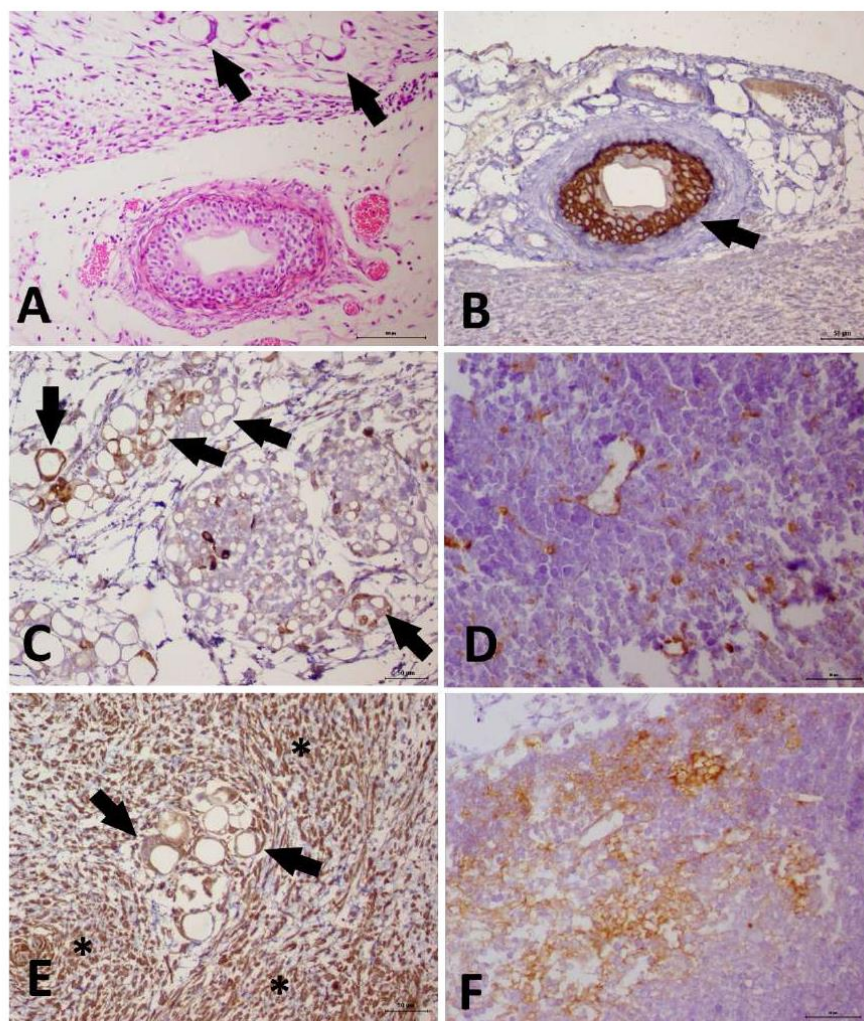
Figure 6. Histochemistry and immunohistochemistry analysis of a tumour growth (xenograft) from the cancer cell line MM4 and CM60. A: haematoxylin and eosin staining showing neoplastic cells forming vascular-like structures (arrows) and presence of a blood vessel showing tumour cells growing into the blood vessel from MM4 cell line. B: positive pan-cytokeratin expression in the neoplastic cells growing inside a blood vessel (arrow) from MM4 cell line. C: positive cytokeratin 8/18 expression by neoplastic cells forming vascular-like structures, confirming its epithelial origin. D: vascular-like structure from the xenotransplantation of CM60 cell line. Note a scatted cytokeratin 8/18 expression of neoplastic cells, including vascular-like structures, confirming its epithelial origin. E: Vimentin expression of tumour growth from MM4 cell line. Note the positive vimentin expression in neoplastic cells (arrows) stromal cells (asterisk). F:

Vimentin positive expression in tumour growth from xenotransplantation of CM60 cell line.