Isolation, purification, culture and characterisation of myoepithelial cells from normal and neoplastic canine mammary glands using a magnetic-activated cell sorting separation system.

Isolation, purification, culture and characterisation of myoepithelial cells from normal and neoplastic canine mammary glands using a magnetic-activated cell sorting separation system

R. Sanchez-Cespedes [a]*, L. Maniscalco [b], S. Iussich[b], E. Martignani[c], S. Guil-Luna[a], R. De Maria [b], J. Martin de las Mulas[a], Y. Millan[3]

'Department of Comparative Pathology, Veterinary Faculty, University of Cordoba, Cordoba, Spain [b] Department of Animal Pathology, School of Veterinary Medicine, University of Turin, Turin, Italy [c] Department of Veterinary Morphophysiology, School of Veterinary Medicine, University of Turin, Turin, Italy
Keywords: Canine
Mammary gland Myoepithelial cells Purification Culture

A B S T R A C T

Mammary gland tumours, the most common malignant neoplasm in bitches, often display myoepithelial (ME) cell proliferation. The aim of this study was to isolate, purify, culture and characterise ME cells from normal and neoplastic canine mammary glands. Monodispersed cells from three normal canine mammary glands and five canine mammary tumours were incubated with an anti-Thyl antibody and isolated by magnetic-activated cell sorting (MACS). Cells isolated from two normal glands (cell lines CmME-Nl and CmME-N2) and four tumours (cell lines CmME-Kl from a complex carcinoma, CmME-K2 from a simple tubulopapillary carcinoma, and CmME-K3 and CmME-K4 from two carcinomas within benign tumours) were cultured in supplemented DMEM/F12 media for 40 days. Cell purity was >90%. Tumour-derived ME cell lines exhibited heterogeneous morphology, growth patterns and immunocyto-chemical expression of cytokeratins, whereas cell lines from normal glands retained their morphology and levels of cytokeratin expression during culture. Cell lines from normal glands and carcinomas within benign tumours grew more slowly than those from simple and complex carcinomas. This methodology has the potential to be used for in vitro analysis of the role of ME cells in the growth and progression of canine mammary tumours.

Introduction

In the normal mammary gland (NMG), the ductal and lobular system is lined by two cell layers, an inner or luminal layer and an outer layer composed of myoepithelial (ME) cells bordering the basal lamina. Mammary gland tumours are the most frequent malignant neoplasm in dogs and are known for their structural complexity and disputed histogenesis (Misdorp, 2002; Sorenmo, 2003). Malignant canine mammary tumours with ME cell proliferation (complex and mixed carcinomas) have a better prognosis than luminal-epithelial type simple carcinomas (Misdorp et al., 1999). In humans, ME cells play a role in cancer progression, as well as in the suppression of tumour growth and invasion (Sternlicht et al., 1997; Sternlicht and Barsky, 1997).

Isolation of enriched populations of epithelial and ME cells from mammary glands of several species, including rats, mice, rabbits, cattle and humans, has been achieved using density gradient centrifugation (Kraehenbuhl, 1977; McGrath et al., 1985; Zavizion et al., 1992), fluorescence-activated cell sorting (FACS; Sleeman et al., 2006; Keller et al., 2010; Rauner and Barash, 2012) and immunomagnetic cell separation methods (Clarke et al., 1994; Gomm et al., 1995). The latter include Dynabeads combined with ME cell-specific markers and magnetic-activated cell sorting (MACS), which have been used to separate human breast ME cells (Clarke et al., 1994; Gomm et al., 1995).

Thymocyte differentiation antigen 1 (Thyl, CD90) has been used as a marker for the isolation and/or in vitro identification of ME cells from NMGs in mice (Lennon et al., 1978; Kim and Clifton, 1993) and humans (Gudjonsson et al., 2002, 2005). Thyl is an N-glycosylated glycophosphatidylinositol (GPI)-anchored conserved cell surface protein, originally identified as a thymocyte antigen (Barclay et al., 1976). In humans, Thyl is expressed by fibroblasts, neurones and blood stem cells (Williams and Cagnon, 1982; Crawford and Barton, 1986; Craig et al., 1993; Saalbach et al., 1999). Expression of Thyl by microvascular endothelial cells promotes invasion of malignant melanoma cells (Saalbach et al., 2002). Thyl also plays a role in cell adhesion, proliferation and differentiation (Yamazaki et al., 2009).

The aim of this study was to isolate, purify and culture ME cells from normal and neoplastic canine mammary glands through selection for Thy 1 using MACS and to characterise the purified cells morphologically and immunophenotypically.

Materials and methods

Case selection

Five spontaneous mammary tumours and three NMGs were collected with the owner's permission from five bitches during surgery at the Department of Animal Pathology, University of Turin, Italy (Table 1). The three NMGs were from unaltered mammary glands in three of the bitches with tumours. Tissue samples from four tumours (cases 1, 2, 3 and 4) and two NMGs (cases 1 and 4) were used for ME cell isolation, purification, culture and characterisation, and for histological classification and immunophenotyping tumours, while tissue samples from one tumour and one NMG (case 5) were used for the same purposes, except that ME cells obtained were not cultured.

Histological classification and immunophenotyping of tumours

Tissue samples from tumours were routinely processed and stained for histological classification (Misdorp et al., 1999) and immunophenotyping using the avidin-biotin-peroxidase complex (ABC) method (Vector Laboratories) with monoclonal mouse anti-cytokeratin (CK) 5 antibody (clone PCK103; isotype IgGi; Euro-Diagnostica; diluted 1:10), polyclonal rabbit anti-CK14 antibody (Covance Research; diluted 1:500) and monoclonal mouse anti-human calponin antibody (clone CALP; isotype IgGi; Dako; diluted 1:400) (Vos et al., 1993a,b; Espinosa de los Mont-eros et al., 2002; Ramalho et al., 2006).

Immunohistochemical expression ofThyl

Fresh samples of NMGs and mammary tumours were frozen in liquid nitrogen and cryostat sections were prepared according to the method of Hellmen (1992). Blocks of tissue were covered with optimal cutting temperature (OCT) cryo-embed-ding media (Sakura) and stored at -70 °C. Cryostat sections (5 µm thickness) were cut at -20 °C and fixed in acetone at -20 °C for lOmin. Endogenous peroxidase activity was blocked by incubation with 0.05% phenyl-hydrazine (Sigma) in phosphate buffered saline (PBS; pH 7.2) for 40 min. Sections were covered with 10% normal rabbit serum in PBS for 30 min prior to incubation with monoclonal mouse anti-Thyl antibody (clone 5E10; isotype IgGi; BD Pharmingen; diluted 1:20) for 1 h at room temperature. The reaction was developed using Envision (Dako), with 3,3-diaminobenzidine tetrahydrochloride (Sigma) as the chromogen, and nuclei were counterstained with Mayer's haematoxylin. The primary antibody was replaced with mouse IgGi (Dako) at the same dilution as a negative control.

Isolation, purification, culture and characterisation of myoepithelial cells

Preparation of monodispersed canine mammary cell suspensions - Fresh mammary tissue (approximately 0.5 cm3) was transported from the operating room on ice in Dulbecco's modified Eagle's Medium/Nutrient Mixture F12 Ham (DMEM/F12; Sigma-Aldrich) supplemented with 5% fetal calf serum (FCS), 5000 IU/mL penicillin and 5 mg/mL streptomycin. Tissue samples were transferred to glass Petri dishes and cut into small pieces (about 1 mm3). Disaggregated tissue was digested for 3.5 h at 37 °C under gentle rotation in DMEM/F12 supplemented with 300U/mL collagenase and 100U/mL hyaluronidase (StemCell Technologies). Following enzyme digestion, the supernatant containing the fat layer was decanted and the remaining organoids and single cells were transferred to centrifuge tubes, then the sample was centrifuged at 80 g for 30 s to remove blood vessels and fibroblasts. The supernatant was discarded and the cell pellet was washed three times with DMEM/F12 supplemented with 1% FCS.The remaining pellet was enriched for epithelial organoids according to the method of Stingl et al. (2001). A single cell suspension was obtained by sequential dissociation of fragments of mammary tissue by gentle pipetting for 1-3 min in 1-5 mL prewarmed trypsin-ethylene diamine tetraacetic acid (EDTA; Sigma-Aldrich). After addition of 10 mL cold Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich) supplemented with 2% FCS, the suspension was centrifuged at 350 g for 5 min. The supernatant was removed and the pellet was treated with prewarmed 5 mg/mL dispase and 1 mg/mL DNase I (Stem-Cell Technologies) for 1 min. The reaction was stopped in the same manner. The cell suspension was filtered through a 40 µm pore nylon mesh filter (BD Biosciences) to remove remaining cell aggregates and centrifuged at 350 g for 5 min, after which the supernatant was discarded. Viable cells were counted using a haemocytometer after staining with Trypan blue.

Isolation and purification of myoepithelial cells - Monodispersed cells were resus-pended in MACS buffer containing PBS, 0.5% bovine serum albumin (BSA) and 2 mM EDTA, then incubated with the rat anti-canine Thyl monoclonal antibody (clone YKIX337.217; isotype IgG_{2b}; AbD Serotec; diluted 1:125) for 30 min at 4 °C. Samples were washed by adding 1-2 mL MACS buffer per 1 x 10^7 cells and centrifuged at 300g for lOmin.Magnetic beads conjugated to anti-rat IgG MicroBeads (Miltenyi Biotec) were incubated with the labelled monodispersed cells at 1 x 10^7 beads/ mL for 15 min at 2-8 °C. Cells were washed with 2 mL buffer and centrifuged at 300g for 10min. The supernatants were aspirated and the pellets were resus-pended in 500 µL buffer. Bead labelled ($Thyl^+$) cells were separated from unlabelled (Thyl") cells using a MACS Separator (Miltenyi et al., 1990). Viable cells were counted using a haemocytometer after staining with Trypan blue.

Culture of purified myoepithelial cells - Purified ME cells from NMGs 1 and 4 and mammary tumours 1-4 were cultured in tissue culture flasks, plates, dishes and un-coated chamber slides (Lab-Tek II Chamber Slide System, 8-well glass slide, Nalge Nunc). DMEM/F12 supplemented with 1% FCS, 2 mM glutamine, 1 µg/mL hydrocortisone, 5 µg/mL insulin, 10 ng/mL epidermal growth factor (EGF), 100 U/mL penicillin, 0.1 mg/mL streptomycin and 50 ng/mL amphotericin B was added to all ME cell vessels (Gomm et al., 1997) and incubated at 37 °C in a 5% CO2 humidified incubator. Media were changed every 2-3 days (Gomm et al., 1995). At confluency, cells were washed with PBS, incubated with 0.25% trypsin-EDTA (Sigma-Aldrich) for 2-5 min (Hellmen, 1992) and reseeded at split ratios of 1:2-l :3 for 40 days. Cells were examined using an inverted phase contrast microscope (Nikon Eclipse TS1000
).

Table 1
Clinical and pathological features of dogs with mammary tumours used for isolation of myoepithelial cells.

Case	Breed	Age (years)	Sex	Location of tumour	Size of tumour (cm)	Histological classification of tumour	Location of normal mammary gland
1	Hovawart	10	Female	IV right	4	Complex carcinoma	I right
2	Labrador retriever	10	Female	III right	1.5	Simple tubulopapillary carcinoma	–
3	Dachshund	10	Female	V right	2.3	Carcinoma within benign tumour	–
4	Yorkshire terrier	10	Female	II left	0.8	Carcinoma within benign tumour	V left
5	Mixed	9	Female	IV left	1.2	Complex carcinoma	I left

Table 2
Antibodies used in the immunocytochemical study.

Antigen	Antibody type	Clone	Isotype	Dilution	Source	Cases in which the antibody was used
Thy1	Monoclonal	YKIK337.217	IgG_{2b}	1:50	AbD Serotec	1, 2, 3, 4, 5
CK5	Monoclonal	PCK103	IgG_1	1:10	Euro-Diagnostica	1, 2, 3, 4
CK14	Polyclonal	–	–	1:500	Covance Research	1, 2, 3, 4, 5
CK19	Monoclonal	RCK108	IgG_1	1:100	Dako	1, 2, 3, 4
Vimentin	Monoclonal	V9	IgG_1	1:50	Dako	1, 2, 3, 4
SMA	Monoclonal	HHF35	IgG_1	1:100	Dako	1, 2, 3, 4
Calponin	Monoclonal	CALP	IgG_1	1:400	Dako	1, 2, 3, 4

Thy1, thymocyte differentiation antigen 1; CK, cytokeratin; SMA, smooth muscle α-actin.

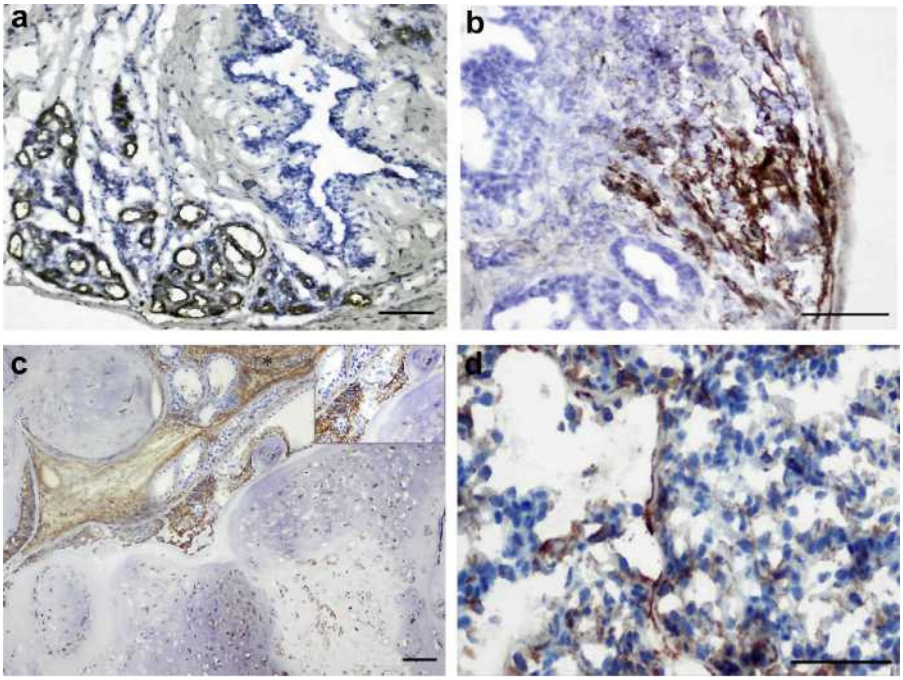

Fig. 1. Photomicrographs illustrating immunohistochemical expression of Thyl in a normal canine mammary gland (NMG) and in canine mammary tumours, (a) Expression of Thyl in frozen tissue from a NMG (case 4). A single layer of positive ME cells is present around alveoli, while periductal cells are negative, (b) Expression of Thyl in frozen tissue from a complex carcinoma (case 1). Spindle-shaped cells forming a nest within the bulk of the tumour cells are positive for Thyl. Isolated linear (membranous pattern) structures are also visible (arrows), (c) Expression of Thyl in frozen tissue from a carcinoma within a benign mixed tumour (case 3). Spindle-shaped (asterisk) and round cells are positive for Thyl, while epithelial cells lining tubules are unreactive. $Thyl^+$ round cells are present either as solid nests close to or within hyaline cartilage (inset), (d) Expression of Thyl in frozen tissue from a simple tubulopapillary carcinoma (case 2). Spindle cells forming a single layer around neoplastic epithelial tubules are positive for Thyl, while epithelial cells are unreactive. Scale bars = 100 µm. Mayer's counterstain.

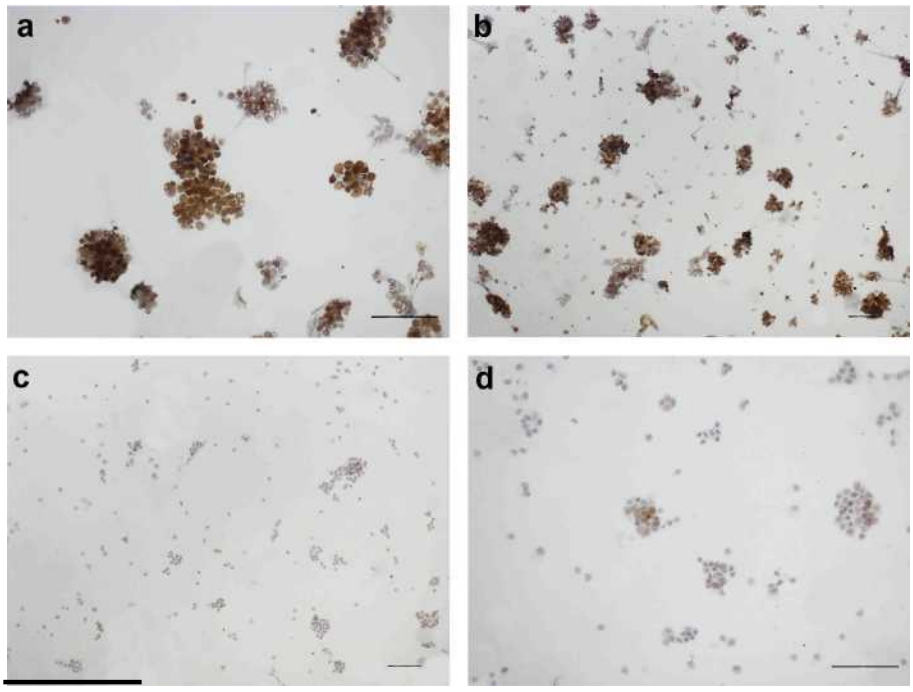

Fig. 2. Photomicrographs illustrating immunocytochemical expression of markers in cells isolated from a normal canine mammary gland (case 5). (a) Expression of Thyl in 94% of cells selected by MACS, (b) Expression of CK14 in 96% of cells selected by MACS, (c) Expression of Thyl in 2% of unselected cells, (d) Expression of CK14 in 4% of unselected cells. Scale bars = 100 µm. Mayer's counterstain.

Immunocytochemical characterisation of purified myoepithelial cells (Thyl) and unselected cells (Thyl~)*

Immunocytochemistry was performed on aliquots of uncultured $Thyl^+$ and Thyl~ cells from case 5 to assess $Thyl^+$ cell purity and also on $Thyl^+$ cells from cases 1-4 cultured on uncoated chamber slides at cell passages 0 (PO, primary isolation) and 1 (PI). Cells were fixed with acetone at -20 °C for lOmin (Wojcik et al., 1999), then stained with primary antibodies against Thyl, CK5, CK14 and CK19, vimentin, smooth muscle α-actin (SMA) and calponin (Table 2) using the ABC method. The primary antibody was replaced with the same immunoglobulin isotype at the same dilution as a negative control.

To evaluate uncultured immunostained ME cells, images were captured by photomicrography (x40 objective; NIS-Elements version F2.30 software, Nikon) from four neighbouring, non-overlapping fields. $Thyl^+$, $Thyl^-$, $CK14^+$ and CK14~ cells were counted with a digital pen tablet (Volito 2, Wacom) to determine the percentage of positive cells. To evaluate cultured immunostained ME cells, positive and negative cells were counted at a magnification of x400 by two pathologists and results were expressed as the percentage of positive cells in relation the total number of cells: high (5=60% positive cells), moderate (30-60%), low (10-30%) or negative (<10%).

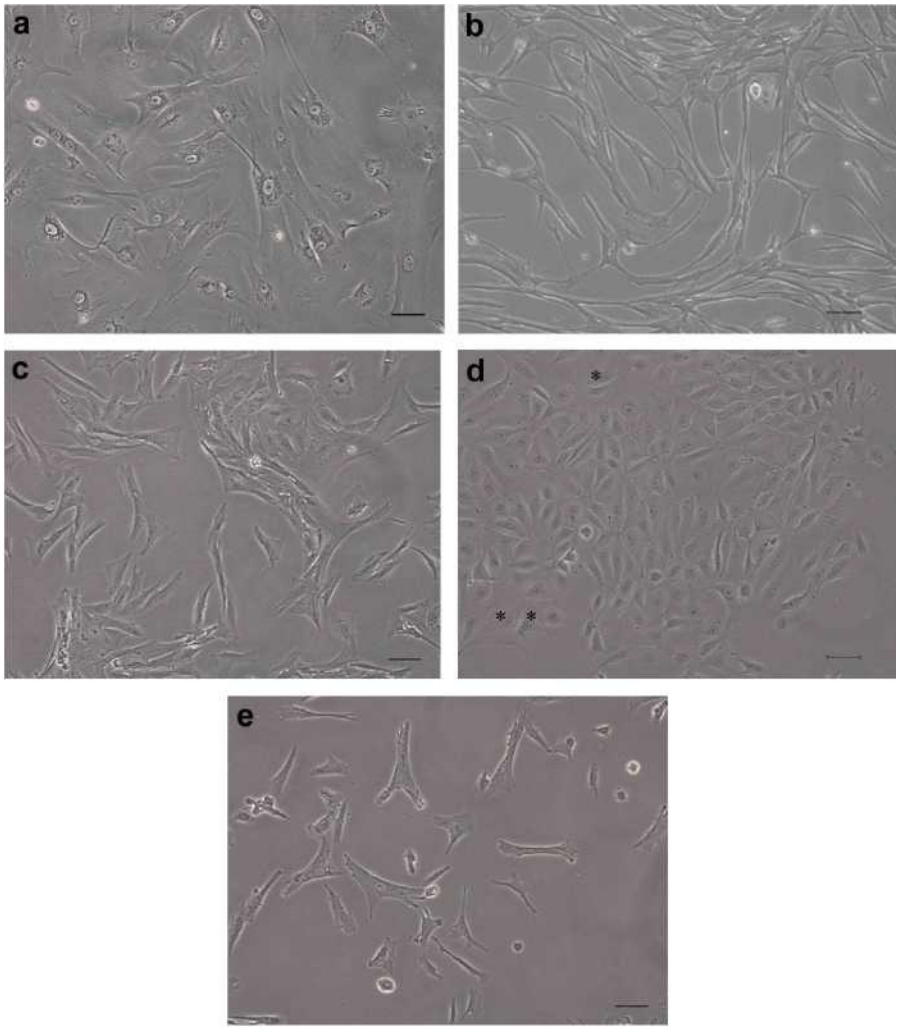

Fig. 3. Phase contrast photomicrographs of cell lines derived from normal (N) or neoplastic (K) canine mammary tissue on primary isolation (passage PO) or day 1 of culture, (a) CmME-N2 at PO, showing polyhedral cells with round to ovoid nuclei, one or more prominent nucleoli and perinuclear processes, (b) CmME-Kl at PO, showing elongated cells in contact with each other, (c) CmME-K2 at culture day 1, showing polyhedral and stellate cells, (d) CmME-K3 at culture day 1, showing small, polygonal cells forming compact islands attached to the bottom of the flask. Some cells are multinucleated (asterisks), (e) CmME-K4 at PO, showing a heterogeneous cell population with abundant, granular cytoplasm and round to ovoid nuclei with prominent nucleoli. Scale bars = 100 μm.

Table 3
Passages and days for each cell line and histological classification of the tissue of origin.

Cell line	Case number	Days in culture				Histological classification of the tissue of origin[a]
		Passage 0 (P0)	Passage 1 (P1)	Passage 2 (P2)	Passage 3 (P3)	
CmME-N1	1	0	19	33	–	Normal mammary gland
CmME-N2	4	0	30	–	–	Normal mammary gland
CmME-K1	1	0	14	19	33	Complex carcinoma
CmME-K2	2	0	14	19	33	Simple tubulopapillary carcinoma
CmME-K3	3	0	35	–	–	Carcinoma in benign tumour
CmME-K4	4	0	32	–	–	Carcinoma in benign tumour

[a] Misdorp et al. (1999).

Results

Histological classification and immunophenotyping of tumours

Two complex carcinomas (cases 1 and 5), one simple tubulo-papillary carcinoma (case 2) and two carcinomas within benign tumours (cases 3 and 4), along with NMGs from cases 1, 4 and 5, were used in this study. CK5, CK14 and calponin were expressed by neoplastic cells in all tumours and flattened or spindle-shaped cells forming a single, complete layer around normal ducts and acini in NMGs (see Appendix A: Supplementary Figs. 1-8).

Immunohistochemical expression ofanti-Thyl antibody in normal and neoplastic canine mammary gland tissues

Thyl antigen was expressed in normal and neoplastic canine mammary tissues as faint membranous and stronger cytoplasmic immunostaining. In NMGs, cells forming a single layer around most acini expressed Thyl, while duct epithelial cells were unreac-tive (Fig. la). Thyl was expressed by spindle-shaped, polygonal and round cells forming fascicles or nests in complex carcinomas (Fig. lb) and carcinomas within benign tumours, as well as by spindle-shaped cells forming a single layer around isolated neoplastic

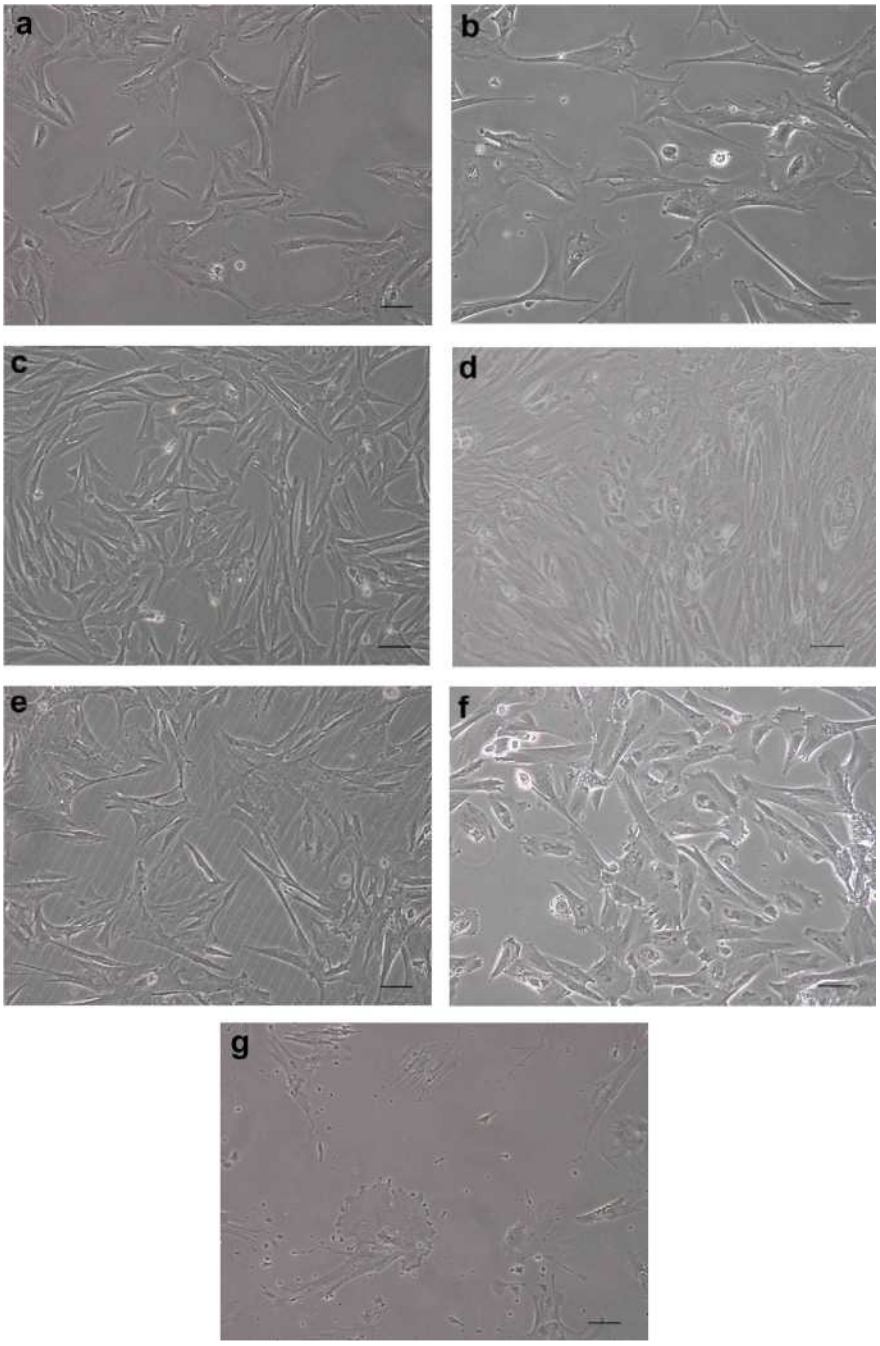

Fig. 4. Phase contrast photomicrographs of cell lines derived from normal (N) or neoplastic (K) canine mammary tissue after cell culture, (a) CmME-Nl at day 40, showing polyhedral to elongated myoepithelial cells, (b) CmME-N2 at PI, showing increased amounts of cytoplasm and less uniform appearance, but retention of polyhedral morphology, (c) CmME-Kl at day 40, showing cells with polyhedral morphology, (d) CmME-K2 at PO (at culture day 7), showing cells that are elongated, or small and polygonal, with round nuclei and prominent nucleoli, forming compact islands. Cells with intermediate morphology are also visible, (e) CmME-K2 at day 40, showing polyhedral morphology, (f) CmME-K4 at PI, showing increased size of cells, ratio of nucleusxytoplasm and perinuclear processes, (g) CmME-K3 at day 40, showing marked anisocytosis. Scale bars = 100 μm.

epithelial tubules in the simple tubulopapillary carcinoma (case 2; Fig. lc and d). Staining was non-specific or absent in negative control sections of NMGs and tumours (see Appendix A: Supplementary Figs. 9 and 10).

Immunocytochemical characterisation of purified uncultured myoepithelial cells (Thyl$^+$) and unselected cells (Thyl^)

ME cells were purified and isolated from canine mammary gland tumours and normal tissue by MACS using an anti-Thyl antibody. Thyl (Fig. 2a) and CK14 (Fig. 2b) were expressed by 94% and 96% of cells positively selected by MACS from the NMG of case 5, and 93% and 94% of cells positively selected by MACS from the tumour of case 5, respectively, although the intensity of the reaction was stronger in cells from NMGs (see Appendix A: Supplementary Fig. 11). Conversely, Thyl (Fig. 2c) and CK14 (Fig. 2d) were expressed by 2% and 4% of unselected cells from the NMG of case 5, and 3% and 3% of unselected cells from the tumour of case 5 (see Appendix A: Supplementary Fig. 12).

Morphological and immunocytochemical characterisation of purified and cultured myoepithelial cells

ME cells isolated and cultured from NMGs of cases 1 and 4 were designated CmME-Nl and CmME-N2, respectively, while cell lines from mammary tumours of cases 1, 2, 3 and 4 were designated CmME-Kl, CmME-K2, CmME-K3 and CmME-K4, respectively. ME cells from NMGs (Fig. 3a), CmME-Kl (Fig. 3b) and CmME-K2 (Fig. 3c) were polyhedral, stellate or spindle-shaped. ME cells from CmME-K3 (Fig. 3d) were small, polygonal and formed compact islands. ME cells from CmME-K4 (Fig. 3e) were polyhedral, stellate or spindle-shaped.

Purified ME cells (0.6 x 10^7 cells) were cultured for 40 days, with a variable number of cell passages (Table 3). The morphology of ME cell lines from tumours, but not from NMGs (Fig. 4a and b), changed over time. For example, ME cells from CmME-Kl were elongated at PI, then gradually became less elongated and more polyhedral towards the end of culture (Fig. 4c). ME cells from CmME-K2 were elongated at P0 (Fig. 4d), then gradually changed towards a more uniform polyhedral morphology towards the end of culture (Fig. 4e). ME cells from CmME-K4 had an increase in size, ratio of nucleusxytoplasm and formation of perinuclear processes (Fig. 4f). ME cells from CmME-K3 were the only cells exhibiting morphological changes consistent with senescence, which appeared after 25 days in culture (Fig. 4g).

The immunophenotype of cultured purified cells (Thyl$^+$, CK5$^+$, CK14$^+$, CK19~, vimentin*, SMA$^+$ and calponin*) was maintained in cultured ME cells from both NMGs and tumours, although the pattern of CK5 and CK14 expression was heterogeneous (Table 4; Fig. 5a-h; see Appendix A: Supplementary data).

Discussion

Using positive selection by MACS with a rat anti-canine Thyl primary monoclonal antibody, ME cells with a purity >90% were isolated from normal and neoplastic canine mammary glands. These cells were cultured for 40 days and characterised morphologically and immunophenotypically. This is the first time that pure populations of primary ME cells from normal and neoplastic canine mammary glands have been cultured. Previously, a canine mammary tumour ME cell line was established from a cultured spontaneous carcinoma and cells were characterised by morphology, growth patterns on plastic surfaces of culture vessels, ultra-structural features, cloning efficiency in soft agar, tumorigenicity in athymic nude mice and hormonal receptor status, but were not immunophenotyped (Wolfe et al., 1986).

Techniques to separate cells using magnetisable microparticles have been used for the purification and depletion of various cell types because they combine specificity and a high level of purification with economy and ease of performance (Gomm et al., 1995). Human breast ME cell populations >90% purity have been obtained using Dynabeads in combination with antibodies against CD10 (Clarke et al., 1994; Gomm et al., 1995), p4-integrin (Gordon et al., 2003) and Thyl (Gudjonsson et al., 2002). ME cell populations separated using Dynabeads were of high purity, but beads could not be removed from cells. Using the MACS method, ME cells can be separated to high purity, with the advantage that the microbeads are biodegradable and do not compromise viability or cell function in subsequent cell cultures (Clarke et al., 1994). Similarly, biodegradable microbeads were used in the present study.

Currently, there is limited availability of specific and sensitive markers for surface antigens expressed by canine mammary gland ME cells. CD10 is a sensitive marker for canine mammary ME cells, but is relatively non-specific (Sanchez-Cespedes et al., 2013). P-cadherin is present on canine ME cells, but is also present in luminal epithelial cells in 40-60% of canine mammary tumours (Gama et al., 2004). In the present study, an anti-canine Thyl antibody was selected for sorting canine mammary ME cell populations (Cobbold and Metcalfe, 1994). Immunocytochemically, >90% isolated cells from one normal and one neoplastic mammary gland

Table 4
Immunocytochemical study of myoepithelial cell lines derived from normal and neoplastic canine mammary glands.

Cell line	Case number	Immunocytochemical study (days in culture)	Cell passage	Expression of antigens						
				Thy1	CK5	CK14	CK19	Vimentin	SMA	Calponin
CmME-N1	1	17	P0	3	1	1	0	3	3	3
		28	P1	3	1	1	0	3	3	3
CmME-N2	4	18	P0	3	2	2	0	3	3	3
		35	P1	3	2	2	0	3	3	3
CmME-K1	1	13	P0	3	3	3	0	3	3	3
		17	P1	3	1	1	0	3	3	3
CmME-K2	2	13	P0	3	2[a]	2[a]	0	3	3	3
		17	P1	3	2[a]	2[a]	0	3	3	3
CmME-K3	3	20	P0	3	3	3	0	3	3	3
		38	P1	3	3	3	0	3	3	3
CmME-K4	4	18	P0	3	1[a]	2[a]	0	3	3	3
		35	P1	3	1[a]	2[a]	0	3	3	3

Thy1, thymocyte differentiation antigen 1; CK, cytokeratin; SMA, smooth muscle α-actin. 0, negative (⩽10% positive cells); 1, low level (10–30% positive cells); 2, moderate level (30–60% positive cells); 3, high level (⩾60% positive cells).

[a] There was a decrease in the percentage of positive cells without changing the level of expression.

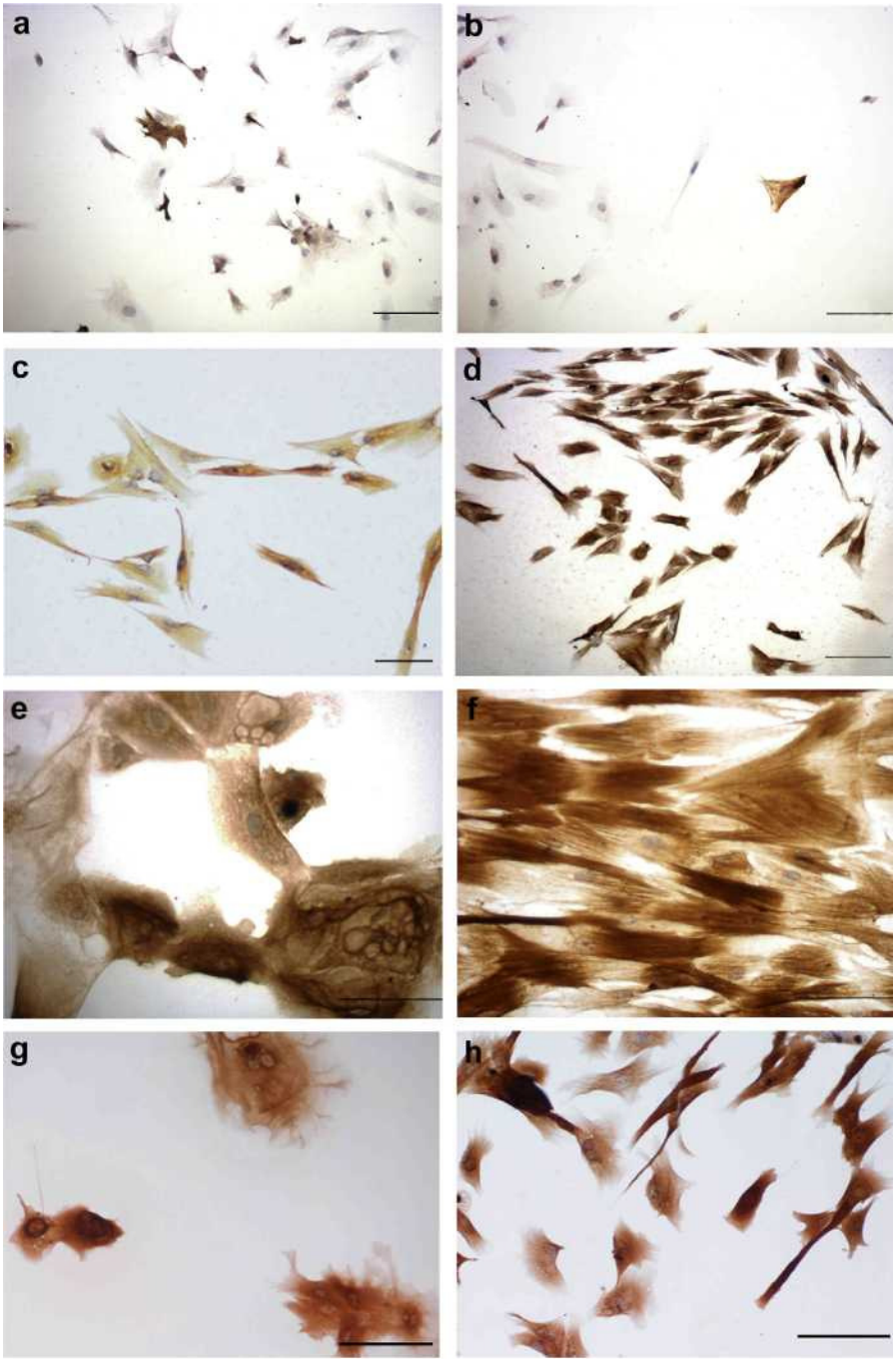

Fig. 5. Photomicrographs demonstrating immunocytochemical expression of markers by cell lines, (a) Expression of CK14 by CmME-N1 at passage 1 (PI), (b) Expression of CK5 by CmME-N1 at PI. (c) Expression of calponin by CmME-N2 at PI. (d) Expression of calponin by CmME-K1 at PO. (e) Expression of CK5 by CmME-K1 at PO. (f) Expression of calponin expression by CmME-K2 at PI. (g) Expression of CK14 by CmME-K3 at PO. (h) Expression of calponin by CmME-K4 at PO. Scale bars = 100 µm. Mayer's counterstain.

expressed Thyl immediately after immunomagnetic purification, a percentage similar to that reported for isolated cells expressing the ME cell lineage-specific marker CK14 (Martin de las Mulas et al., 2004; Gama et al., 2008). Furthermore, >95% Thyl~ cells from the same tissues were negative for both Thyl and CK14, suggesting that population selected as $Thyl^-$ was not of ME origin.

Using immunohistochemistry, Thyl expression was observed in neoplastic cells of varying morphology, as well as cells surrounding alveoli, but not ducts, in NMGs, as previously observed in histological sections of canine mammary tumours (Martin de las Mulas et al., 2004; Sanchez-Cespedes et al., 2013). Thyl expression has not been described in canine mammary glands previously. In the present study, we show that the staining pattern for Thyl matches that reported in human breast cancer, where Thyl is strongly expressed in basal cells, while expression was low or absent in luminal cells in 80% of cases (Donnenberg et al., 2010). However, Polyak and Hu (2005) noted that not all ME cells express all ME markers and our observations suggest that alveolar ME cells are Thyl positive, while ductal ME cells are Thyl negative.

ME cells isolated from NMGs in the present study were polyhedral, stellate or spindle-shaped, similar to benign ME cells from explants derived from a human salivary adenoma (Martinez et al., 2012). The morphology of ME cells isolated from tumours in the present study was variable and included the polyhedral, stel-

late or spindle-shaped morphology in cell lines CmME-Kl (derived from a simple tubulopapillary carcinoma), CmME-K2 (derived from a complex carcinoma) and CmME-K4 (derived from a carcinoma within a benign tumour), and small polygonal cells forming compact islands in CmME-K3 (derived from a carcinoma in a benign tumour). These findings suggest that ME cells from canine mixed mammary tumours are morphologically less similar to ME cells from NMGs than ME cells from simple and complex carcinomas. Furthermore, the changes observed in the morphology of cell lines derived from canine mammary tumours over time were not observed in NMG-derived cell lines. ME cells from CmME-K3 displayed morphological changes consistent with senescence after 25 days (enlarged, flattened cells with increased granularity), but additional investigations would be required to confirm senescence (Kuilman et al., 2010).

Cultured ME cells from both NMGs and tumours displayed a ME cell immunophenotype before and after PI, defined by expression of CK5, CK14, calponin, SMA and vimentin, and lack of expression of the luminal epithelial cell marker CK19 (Vos et al., 1993a,b; Espinosa de los Monteros et al., 2002; Ramalho et al., 2006; Gama et al., 2010). Vimentin is considered to be a mesenchymal marker and the co-expression of vimentin and other intermediate filaments, reported mainly in cultured tumours, is seen as an in vitro phenomenon (Hellmen, 1992). However, canine ME cells in vivo co-express vimentin and cell lineage-specific markers in both normal and neoplastic mammary glands (Hellmen, 1992).

In vivo studies have shown that CK5 and CK14 are canine ME cell markers (Vos et al., 1993a,b; Ramalho et al., 2006). In the present study, the pattern of immunocytochemical expression of these markers was heterogeneous. Low or moderate expression of both cytokeratins was observed in CmME-Nl and Cm-ME-N2, respectively, and did not change over time. In contrast, passage number was associated with a decrease in the percentages and/or levels of CK14 and d<5 expression most tumour cell lines, the exception being CmME-K3. Heterogeneous cytokeratin expression in canine mammary tumours, also noted by Hellmen (1992), could be attributed to phenotypic diversity (Lichtner et al., 1987), or may be related to the stage of tumour differentiation (Gustafsson et al., 1989). However, cultured purified ME cells from human breast cancer maintained their intermediate filament profile over different periods of time, depending on the culture media used (Pechoux et al., 1999).

Conclusions

A pure population of mammary ME cells from canine NMGs and tumours was isolated and cultured. Cell lines from normal glands retained their morphology and level of cytokeratin expression during culture, whereas tumour-derived cell lines exhibited alterations in morphology and cytokeratin expression with passage in culture. This methodology opens up the possibility of in vitro analysis of the role of ME cells in the growth and progression of canine mammary tumours.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

This work was supported by Grant Numbers P07-CVI-02559 and BIO287 of the Plan Andaluz de Investigacion (PAIDI), Conse-jeria de la Educacion y Ciencia, Junta de Andalucia, and by Grant

Number AGL2011-25553 of the Ministerio de Ciencia e Innovacion, Spain. The technical support of Dr Rafael Zafra is greatly appreciated.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.Org/10.1016/j.tvjl.2013.03.005.

References

Barclay, A.N., Letarte-Muirhead, M., Williams, A.F., Faulkes, R.A., 1976. Chemical characterisation of the Thy-1 glycoproteins from the membranes of rat thymocytes and brain. Nature 14, 563-567.
Clarke, C, Titley, J., Davies, S., OHare, M.J., 1994. An immunomagnetic separation method using superparamagnetic (MACS) beads for large-scale purification of human mammary luminal and myoepithelial cells. Epithelial Cell Biology 3,38-46.
Cobbold, S., Metcalfe, S., 1994. Monoclonal antibodies that define canine homologues of human CD antigens: Summary of the first international canine leukocyte antigen workshop (CLAW). Tissue Antigens 43, 137-154.
Craig, W., Kay, R., Cutler, R.L., Lansdorp, P.M., 1993. Expression of Thy-1 on human hematopoietic progenitor cells. Journal of Experimental Medicine 177, 1331-1342.
Crawford, J.M., Barton, R.W., 1986. Thy-1 glycoprotein: Structure, distribution, and ontogeny. Laboratory Investigation 54,122-135.
Donnenberg, V.S., Donnenberd, A.D., Zimmerlin, L, Landreneau, R.J., Bhargava, R., Wetzel, R.A., Basse, P., Brufsky, A.M., 2010. Localization of CD44 and CD90 positive cells to the invasive front of breast tumors. Cytometry. Part B: Clinical Cytometry 78, 287-301.
Espinosa de los Monteros, A.E., Millan, M.Y., Ordas, J., Carrasco, L, Reymundo, C, Martin de las Mulas, J., 2002. Immunolocalization of the smooth muscle specific protein calponin in complex and mixed tumours of the mammary gland of the dog: Assessment of the morphogenetic role of the myoepithelium. Veterinary Pathology 39, 247-256.
Gama, A., Paredes, J., Albergaria, A., Gartner, F., Schmitt, F., 2004. P-cadherin expression in canine mammary tissues. Journal of Comparative Pathology 130, 13-20.
Gama, A., Alves, A., Schmitt, F., 2008. Identification of molecular phenotypes in canine mammary carcinomas with clinical implications: Application of the human classification. Virchows Archiv 453,123-132.
Gama, A, Alves, A., Schmitt, F., 2010. Expression and prognostic significance of CK19 in canine malignant mammary tumours. The Veterinary Journal 184, 45-51.
Gomm, J.J., Browne, P.J., Coope, R.C., Liu, Q.Y., Buluwela, L, Coombes, R.C., 1995. Isolation of pure populations of epithelial and myoepithelial cells from the normal human mammary gland using immunomagnetic separation with Dynabeads. Analytical Biochemistry 226, 91-99.
Gomm, J.J., Coope, R.C., Browne, P.P., Coombes, R.C., 1997. Separated human breast epithelial and myoepithelial cells have different growth factor requirements in vitro but can reconstitute normal breast lobuloalveolar structure. Journal of Cellular Physiology 171, 11-19.
Gordon, L.A., Mulligan, K.T., Maxwell-Jones, H., Adams, M., Walker, R.A., Jones, J.L, 2003. Breast cell invasive potential relates to the myoepithelial phenotype. International Journal of Cancer 106, 8-16.
Gudjonsson, T., Ronnov-Jessen, L, Villadsen, R., Rank, F., Bissell, M., Petersen, O.W., 2002. Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. Journal of Cell Science 115, 39-50.
Gudjonsson, T., Adriance, M.C., Sternlicht, M.D., Petersen, O.W., Bissell, M.J., 2005. Myoepithelial cells: Their origin and function in breast morphogenesis and neoplasia. Journal of Mammary Gland Biology and Neoplasia 10, 261-272.
Gustafsson, H., Virtanen, I., Thornell, L.E., 1989. Glial fibrillary acidic protein and desmin in salivary neoplasms. Expression of four different types of intermediate filament proteins within the same cell type. Virchows Archiv B: Cell Pathology Including Molecular Pathology 57, 303-313.
Hellmen, E., 1992. Characterization of four in vitro established canine mammary carcinoma and one atypical benign mixed tumor cell lines. In Vitro Cellular and Developmental Biology 28, 309-319.
Keller, P.J., Lin, A.F., Arendt, L.M., Klebba, I., Jones, A.D., Rudnick, J.A., DiMeo, T.A., Gilmore, H., Jefferson, D.M., Graham, R.A., Naber, S.P., Schnitt, S., Kuperwasser, C, 2010. Mapping the cellular and molecular heterogeneity of normal and malignant breast tissues and cultured cell lines. Breast Cancer Research 12, R87.
Kim, N.D., Clifton, K.H., 1993. Characterization of rat mammary epithelial cell subpopulations by peanut lectin and anti-Thy-1.1 antibody and study of flow-sorted cells in vivo. Experimental Cell Research 207, 74-85.
Kraehenbuhl, J.P., 1977. Dispersed mammary gland epithelial cells. I. Isolation and separation procedures. Journal of Cell Biology 72, 390-405.
Kuilman, T., Michaloglou, C, Mooi, W.J., Peeper, D.S., 2010. The essence of senescence. Genes and Development 24, 2463-2479.
Lennon, V.A., Unger, M., Dulbecco, R., 1978. Thy-1: A differentiation marker of potential mammary myoepithelial cells in vitro. Proceedings of the National Academy of Sciences of the United States of America 75, 6093-6097.
Lichtner, R.B., Moskwa, P.S., Nicolson, G.L, 1987. Heterogeneous expression ofcytokeratins in metastatic mammary adenocarcinoma cells in vitro and in vivo.
Invasion and Metastasis 7, 367-383. Martin de las Mulas, J., Reymundo, C, Espinosa de los Monteros, A., Millan, Y., Ordas, J., 2004. Calponin expression and myoepithelial cell differentiation in canine,feline and human mammary simple carcinomas. Veterinary and Comparative
Oncology 2, 24-35. Martinez, E.F., Demasi, A.P., Napimoga, M.H., Arana-Chavez, V.E., Altemani, A., de Araujo, N.S., de Araujo, V.C., 2012. In vitro influence of the extracellular matrixin myoepithelial cells stimulated by malignant conditioned medium. Oral
Oncology 48,102-109. McGrath, M., Palmer, S., Nandi, S., 1985. Differential response of normal rat mammary epithelial cells to mammogenic hormones and EGF. Journal of CellPhysiology 125,182-191. Miltenyi, S., Miiller, W., Weichel, W., Radbruch, A., 1990. High gradient magnetic
cell separation with MACS. Cytometry 11, 231-238. Misdorp, W., 2002. Tumors in Domestic Animals, Fourth Ed. Iowa State Press/ Blackwell Publishing Company, Ames, IA, USA, pp. 575-606. Misdorp, W., Else, R.W., Hellmen, E., Lipscom, T.P., 1999. Histological classification
of the mammary tumours of the dog and the cat. In: Shulman, F.O. (Ed.), WorldHealth Organization International Histologic Classification of Tumours of
Domestic Animals. Armed Forces Institute of Pathology, Washington, DC, USA,pp. 16-29. Pechoux, C, Gudjonsson, T., Ronnov-Jessen, L, Bissell, M.J., Petersen, O.W., 1999.
Human mammary luminal epithelial cells contain progenitors to myoepithelialcells. Developmental Biology 206, 88-99. Polyak, K., Hu, M., 2005. Do myoepithelial cells hold the key for breast tumor
progression? Journal of Mammary Gland Biology and Neoplasia 10, 231-247. Ramalho, L.N., Ribeiro-Silva, A., Cassali, G.D., Zucoloto, S., 2006. The expression of
p63 and cytokeratin 5 in mixed tumors of the canine mammary gland providesnew insights into the histogenesis of these neoplasms. Veterinary Pathology 43,
424-429. Rauner, G., Barash, I., 2012. Cell hierarchy and lineage commitment in the bovine
mammary gland. PLoS ONE 7, e30113. Saalbach, A., Wetzig, T., Haustein, U.F., Anderegg, U., 1999. Detection of humansoluble Thy-1 in serum by ELISA. Fibroblasts and activated endothelial cells are
a possible source of soluble Thy-1 in serum. Cell and Tissue Research 298,307-
315. Saalbach, A., Hildebrandt, G., Haustein, U.F., Anderegg, U., 2002. The Thy-1/Thy-1ligand interactions is involved in binding of melanoma cells to activated Thy-1-

positive microvascular endothelial cells. Microvascular Research 64, 86-93. Sanchez-Cespedes, R., Suarez-Bonnet, A., Millan, Y., Guil-Luna, S., Reymundo, C,
Herraez, P., Espinosa de Los Monteros, A., Martin de las Mulas, J., 2013. Use of
CD10 as a marker of canine mammary myoepithelial cells. The VeterinaryJournal 195,192-199. Sleeman, K.E., Kendrick, H., Ashworth, A., Isacke, CM., Smalley, M.J., 2006.
CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. Breast Cancer
Research 8, R7. Sorenmo, K., 2003. Canine mammary gland tumours. Veterinary Clinics of North
America. Small Animal Practice 33, 573-596. Sternlicht, M.D., Kedeshian, P., Shao, Z.M., Safarians, S., Barsky, S.H., 1997. The human myoepithelial cell is a natural tumor suppressor. Clinical Cancer
Research 3,1949-1958. Sternlicht, M.D., Barsky, S.H., 1997. The myoepithelial defense: A host defense against cancer. Medical Hypotheses 48, 37-46. Stingl, J., Eaves, C.J., Zandieh, I., Emerman, J.T., 2001. Characterization of bipotent
mammary epithelial progenitor cells in normal adult human breast tissue.
Breast Cancer Research and Treatment 67, 93-109. Vos, J.H., van del Ingh, T.S., Misdorp, W., Molenbeek, R.F., van Mil, F.N., Rutteman, G.R., Ivanyi, D., Ramaekers, F.C., 1993a. Immunohistochemistry with keratin,vimentin, desmin, and alpha-smooth muscle actin monoclonal antibodies in
canine mammary gland: Normal mammary tissue. Veterinary Quarterly 15,
102-107. Vos, J.H., van del Ingh, T.S., Misdorp, W., Molenbeek, R.F., van Mil, F.N., Rutteman,
G.R., Ivanyi, D., Ramaekers, F.C., 1993b. Immunohistochemistry with keratin,vimentin, desmin, and alpha-smooth muscle actin monoclonal antibodies in
canine mammary gland: Malignant mammary tumours. Veterinary Quarterly
15, 96-102. Williams, A.F., Cagnon, J., 1982. Neuronal cell Thy-1 glycoprotein: Homology with immunoglobulin. Science 216, 696-703. Wojcik, S.F., Capen, C.C., Rosol, T.J., 1999. Expression of PTHrP and the PTH/PTHrP
receptor in purified alveolar epithelial cells, myoepithelial cells, and stromal fibroblasts derived from the lactating rat mammary gland. Experimental Cell
Research 248, 415-422. Wolfe, L.G., Smith, B.B., Toivio-Kinnucan, M.A., Sartin, E.A., Kwapien, R.P.,
Henderson, R.A., Barnes, S., 1986. Biologic properties of cell lines derived from canine mammary carcinomas. Journal of the National Cancer Institute 77, 783-
792. Yamazaki, H., Nishida, H., Iwata, S., Dang, N.H., Morimoto, C, 2009. CD90 and CD110
correlate with cancer stem cell potentials in human T-acute lymphoblastic leukemia cells. Biochemical and Biophysical Research Communications 383,
172-177. Zavizion, B., Politis, I., Gorewit, R.C., 1992. Bovine mammary myoepithelial cells. 1.
Isolation, culture, and characterization. Journal of Dairy Science 75, 3367-3380.