Establishment and characterization of a new feline mammary cancer cell line, FkMTp

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Abstract Studies on tumours in domestic animals are believed to greatly contribute to a better understanding of similar diseases in humans. Comparative studies have shown that feline mammary carcinomas share important features with human breast cancers, including a similar biological behaviour and histological appearance. In the present study we have established and characterized at different cellular levels one feline mammary cancer cell line, FkMTp, derived from a cat mammary carcinoma. The FkMTp cell line revealed to be a promising resource and tool to study tumour microevolution and all the mechanisms and processes involved in carcinogenesis from the tumour (primary culture) to the immortalized cell line. Several assays were conducted to assess the growth behaviour, differentiated morphology, anchorage independent growth in soft agar, wound-healing invasion and migration of the cell line across time (from the primary culture until the 160th passage). FkMTp revealed increased levels of anchorage independence, migration and invasion according to the course of time as well as different numbers of ploidy. These results demonstrate and validate the *in vitro* tumorigenicity of the FkMTp cell line. During the cell line establishment, it was cryopreserved approximately every six passages,

including the tumour primary culture, allowing now the possibility to access almost any specific *momento* of the tumour progression.

Keywords Model cell line · Feline mammary carcinoma · Growth behaviour · *In vitro* tumorigenicity · Anchorage/invasion/migration

Introduction

Cancer is a complex biological process that demands comprehensive experimental systems for its study. Oncology has undergone a considerable progress, moving from simple to complex networks of studies involving numerous models. Breast cancer has been described as a molecularly heterogeneous disease and is the most frequent malignant neoplasia among women worldwide (Boyle and Howell 2010). Genetic studies of breast cancer rely on the use of primary tumours, paraffin-embedded samples or cell lines. The latter (breast cancer cell lines) present the great advantage of being readily available, especially when an adequate characterization of these cellular models can easily be accessed by researchers (Snyder et al. 2001). Characterization of cell lines has shown to provide valuable insights regarding the degree of complexity of the polygenetic aetiology of breast cancer as well as the biological mechanisms beneath this disease (Vargo-Gogola and Rosen 2007; Ferreira et al. 2012; Louzada et al. 2012). Regarding breast

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cancer, no cell lines of hyperplasia, ductal carcinoma in situ (DCIS), or lines from the crucial step of early invasion from DCIS to invasive carcinoma are available (Hughes et al. 2008). The availability of reliable cell lines would be essential to study processes like invasion, a “step” that initiates the metastatic process, involving the participation of numerous biomolecules interacting in a variety of intricate cellular functions including altered cell adhesion, proteolysis or migration (Steeg 2006).

A significant fraction of breast cell lines available for research are descendant from transplantable or chemically induced rodent models (Hansen and Khanna 2004). Nevertheless these models lack many aspects of human cancers (Allred and Medina 2008). Feline mammary cancer (FMC) is the third most common neoplasm in cat (Uyama et al. 2005) with about 85–93 % of the cases being malignant and reported to occur in cats from 9 months to 23 years of age (mean, 10–12 years of age) (Misdorp 2002; Zappulli et al. 2005; Burrai et al. 2010). These tumours are similar to human breast cancer in the age of onset, incidence, histopathology, biologic behaviour, pattern of metastasis (Weijer et al. 1972; MacEwen 1990; Burrai et al. 2010), as well as in revealing poor prognosis; making it attractive as models for breast cancer research (Weijer et al. 1972; Misdorp and Weijer 1980; Minke et al. 1990; Uyama et al. 2005). Although the establishment of cell lines from feline mammary cancer has been conducted, the number of successfully established ones is limited (Elshire 1984; Norval et al. 1985; Minke et al. 1991), suggesting that the obtainment of FMC cell lines is difficult compared to those of other model organisms (Weijer et al. 1972; Misdorp and Weijer 1980; Uyama et al. 2005).

On this report we present and describe the establishment of a novel spontaneously immortalized FMC cell line named FkMTp. This cell line was derived from a 7-years-old cat with a mammary carcinoma and we believe it is able to overcome the gaps/limitations of the cell lines described above once it will allow studying the multiple and different processes involved along the microevolution of cancer and carcinogenesis. A detailed characterization of the FkMTp was performed regarding growth behaviour, morphology, anchorage independence growth in soft agar, wound-healing, invasion and migration abilities and in vitro tumorigenicity. We are confident that this work has

contributed to the validation of this cell model and to its use in future studies as a powerful and effective tool to study the complex breast carcinogenesis processes.

Methods

Cell line establishment and culture conditions

The tissue used to cell culture was a lesion characterized by proliferative luminal epithelial cells arranged in papillae, among a moderate fibrotic stroma. Neoplastic cells showed spheroid, large, central, euchromatic nucleus, with a central acidophilic nucleolus and scarce eosinophils, and a pale cytoplasm. There were approximately three mitotic figures per high-power field. It was identified an intense, multifocal and diffuse inflammatory infiltrate, composed by numerous lymphocytes and macrophages and a lower number of plasmacytes. The histological findings were compatible with a papillary carcinoma.

Under sterile conditions, the tumour tissue was rinsed twice with growth medium and cut with a razor into small pieces of approximately 1 mm³ in size. Appropriate amounts of fine tissue fragments were seeded into 25 cm² flasks. The primary culture was maintained in complete DMEM medium supplemented with 10 % fetal bovine serum (FBS), 1 % L-glutamine and 1 % of Penicillin–Streptomycin (all from Gibco, Life Technologies) at 37 °C in a humidified atmosphere containing 5 % CO₂. Cells were then harvested and subcultured. Culture was passaged at confluence of 75 % using 0.25 % trypsin (1×) with EDTA in Hanks’ balanced salt solution (Gibco, Life Technologies, Grand Island, NY, USA). During subcultivation the number of times of medium replacement was made regarding the requirements of the FkMTp cell line. Initially, medium was replaced about twice a week, but with the increase in the number of passages, this replacement has become more frequent, reaching a maximum of five times a week. These cells grew without interruption for over 23 months (one hundred sixty passages) following the initiation of the primary culture. Along establishment the line was cryogenically preserved every six passages (Fig. 1).

Cryogenic preservation of the cells was always performed at exponential growth and at approximately 75 % confluence. For this, cells were brought into

Fig. 1 Methodology of FkMTp cell line establishment

suspension using 0.25 % trypsin (1×) with EDTA in Hanks' balanced salt solution (Gibco, Life Technologies) and re-suspended in a volume of fresh complete DMEM medium supplemented with 10 % fetal bovine serum (FBS), 1 % L-Glutamine and 1 % Penicillin-Streptomycin (all from Gibco, Life Technologies). The cell suspension was centrifuged at 800 rpm for 10 min and then cells were re-suspended in 1 ml of freezing medium (60 % DMEM medium, 1 % L-Glutamine, 1 % Penicillin-Streptomycin, 20 % fetal bovine serum (FBS) and 20 % glycerol) and transferred to cryoprotective ampoules that were labelled with the cell line name, passage number, cell line growth medium and date. Ampoules were placed in a freezing container that was sequentially transferred during the day to temperatures decreasing from 4 °C, to −20 °C, to −80 °C and finally stored in a liquid nitrogen storage vessel.

Growth rate

Growth curves were assessed as described elsewhere (Provencher et al. 2000). Briefly, on day zero, 1×10^5 cells were seeded onto six well culture plates. For each dish, cells were collected daily during 6 days. On the day of counting, the cells on the dish were dislodged using 0.25 % trypsin (1× with EDTA in Hanks' balanced salt solution) (Gibco, Life Technologies). An aliquot of cell suspension was diluted and the cells' concentration assessed by trypan blue dye (Sigma, St.

Louis, MO, USA) exclusion using a hemocytometer. Each experiment was performed in triplicate for each harvest and repeated once. Doubling time was calculated according to the slope of the linear portion of the growth.

Dynamic changes in cell morphology and actin filament organization

The FkMTp cells grown on glass coverslips were washed three times with PBS1× at 37 °C, fixed with 3.7 % formaldehyde in PBS for 10 min. After washing in the same buffer three times for 5 min, cells were permeabilized with 0.1 % Triton X-100 in PBS1× for 5 min and again washed in PBS1× three times for 5 min. Cells were then blocked with 1 % BSA in PBS1× for 30 min. For filamentous actin (F-actin) detection, cells were incubated with fluorescent phalloidin (Rhodamin phalloidin-Molecular probes, Invitrogen) for 30 min. Coverslips were mounted on slides with Vectashield antifade solution with 4'-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA).

Anchorage independent growth in soft agar

For anchorage independent growth assay, six well culture plates were used. Agar (Noble agar, Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was melted and kept in a water bath at 50 °C. To

make the bottom layer, one part of the melted 1.2 % agar was mixed with $2\times$ DMEM supplemented with 40 % FBS. This final mixture (0.6 % agar) was then added to wells at 1 mL/well and allowed to gel at room temperature. To prepare the top layer (0.3 % soft agar), 0.6 % agar was mixed with $2\times$ DMEM supplemented with 40 % FBS. To this final mixture (0.3 % soft agar) it was added the cellular suspension (1×10^4 cells/mL). Finally, this mixture was deposited in the bottom layer, allowed to gel at room temperature and incubated in a humidified CO₂ incubator at 37 °C (Freshney 2005). After 3 weeks of growth, the colonies formed were stained with crystal violet, photographed under an inverted microscope and measured using the Image J open source image processing program (version 1.45). Colonies with a diameter between 38 and 75 µm were considered medium, below 38 µm were considered small and above 75 µm, large. This assay was carried out in passages 0, 21, 35, 42, 49, 56, 63, 98, 112, 126, 147 and 160 in triplicate. Five fields of each assay were then photographed and the colonies formed were counted (in a total of 40 colonies in each assay).

Migration assay

The cells were dislodged by 0.25 % trypsin ($1\times$) with EDTA in Hanks' balanced salt solution (Gibco, Life Technologies) and resuspended in complete DMEM medium (but supplemented with 1 % FBS). Then, 100 µL of cell suspension at 2×10^4 cells/mL was seeded on the upper chamber of transwell filters (8 µm pore) (VWR, Radnor, PA, USA). In the lower chamber complete DMEM medium with 5 % of FBS was added. Cells were incubated at 37 °C for 24 h. Non migrating cells that remained on the upper surface of the filter were removed, and the remaining cells on the lower surface were fixed with 100 % methanol, stained with Giemsa and counted under a Zeiss Axiovert 200 microscope. Each assay was carried out in triplicate (Ueda et al. 2003; Ouellet et al. 2008).

In vitro invasion assay

The Matrigel invasion assay was carried out according to the manufacturer's instructions (Becton–Dickinson, BD). Polycarbonate membranes (8 µm pore size) of the upper compartment of transwell culture chambers

were coated with 0.4 µg/mL matrigel. Cells were trypsinized and resuspended in complete DMEM medium (supplemented with 1 % FBS). The cell suspension at 2×10^4 cells/mL was placed in the upper compartment of the Boyden chamber, and the lower compartment was filled with medium with 5 % FBS. Cells were incubated at 37 °C for 24 h. Following incubation, membranes were methanol fixed and stained with Giemsa. Non-invading cells were removed with a cotton swab, while invading cells on the underside of the membrane were counted using an inverted microscope. The experiments were performed in duplicate.

Wound-healing assay

Migration potential was evaluated using the starch assay method as described elsewhere (Bossolasco et al. 2006; Ouellet et al. 2008). Briefly, the cells were plated onto 6 well culture plates and once the cell confluence reached about 90 %, wounds were created using a 200 µL plastic tip. In order to evaluate cell migration into the wound, cells were photographed at 0, 8, 24 and 48 h after creating the wound. The assay was performed twice in triplicate.

Conventional cytogenetics (chromosome analysis)

Chromosome harvest was performed every seven passages. Metaphase chromosomes were obtained by treating the cells with colcemid (10 µg/mL, Invitrogen, Life Technologies) followed by hypotonic treatment (1:1 of 0.05 M potassium chloride:sodium citrate 0.9 % at 37 °C). Fixation was done with methanol and acetic acid (3:1), and washed 3–4 times with the fixative solution. Fixed cultures were stored at –20 °C. The suspension was dropped onto microscope slides, and stained with 2 % Giemsa. The growth rate was calculated in all the passages of the FkMTp cell line and the following assays: anchorage independent growth in soft agar, wound-healing assay, migration and in vitro invasion were performed in passages 0, 21, 35, 42, 49, 56, 63, 98, 112, 126, 147 and 160. In what refers to chromosome number and modal karyotype of the cell line, these were determined at passages 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, 98, 105, 112, 119, 126, 133, 140, 147 and 154.

Image, capture, processing and analysis

Cells were observed using a Zeiss Axiovert 200 microscope with P.A.L.M Image Browser. Chromosomes were observed using a Zeiss Axioplan 2 microscope, actin filament organization was observed using a Zeiss AxioImager Z1 microscope, and images were captured by an AxioCam MRm digital camera with LSM 510 software (version 4.0 SP2). Digitized photos were prepared in Adobe Photoshop (version 7.0); image optimization included contrast and colour adjustments that affected the whole image equally.

Results

Cell culture: characterization of the established cell line

Morphology/growth characteristics

The primary culture was highly heterogeneous (Fig. 2a) with populations of epithelial and fibroblastoid cells. With subsequent passages, selection towards a more homogeneous population of cells occurred and the cell line acquired morphological “fibroblast-like” characteristic cells. These grew without interruption for over 23 months, and 160 passages after establishment of the primary culture. Based on phase-contrast microscopy, it was possible to observe that the morphology of cells maintained as “fibroblast-like” (Fig. 2b). At subconfluency, the cells assumed multipolar or bipolar shapes and were well spread on the flask surface, but at high confluence they

became bipolar and less well spread. Additionally, they formed characteristic parallel arrays and whorls visible to the naked eye. Concerning growth profile, the results of the growth curves and population-doubling times revealed that the cell line acquired stability between the 112th and 126th passages, where the doubling time of the population was 24 h (Fig. 2c).

Dynamic changes in cell morphology and actin filament organization

Reorganization of actin cytoskeleton is the primary mechanism of cell motility and is essential to most types of cell migration (Yamazaki et al. 2005). The dynamics of cell morphological changes in the FkMTp cell line was assessed by analysing F-actin structure during the course of time. This assay was performed at passages 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, 98, 105, 112 and 119. At passage 0, cells were mostly cuboidal shaped and organized in compact islets (Fig. 3a, b), after seven passages cells in these islets became more loosely arranged (Fig. 3c, d) and at passage 14 they started to elongate (Fig. 3e). These changes progressed gradually to a spindle-shaped morphology with cells arranged in parallel, what was evident in passage 28 (Fig. 3f), although cells elongated even further between this passage and passage 49 (Fig. 3g). With subsequent passages, the FkMTp cell line preserved a spindle-shaped morphology with cells arranged in parallel (Fig. 3h). In the first passages (0, 7) cells displayed a marginal actin bundle which is typical of epithelial cells and internal straight actin bundles. Also they were predominantly organized in cortical bundles tightly associated with cell–cell

Fig. 2 Cellular morphology and growth curve of FkMTp cell line. **a** Morphological appearance of FkMTp at passage zero. Heterogeneous populations of cells with obvious epithelioid and fibroblastoid shaped cells; **b** morphology appearance of FkMTp at passage 160. An evolution towards a more homogeneous

population of fibroblastoid shaped cells. **c** Growth curve of FkMTp cell line at passage 112. 100,000 cells were plated onto 6 well cultured plates. Cells were trypsinized and counted daily during 6 days; experiments were performed two times in triplicate. Scale bar = 150 μ m

Fig. 3 Morphological changes and actin cytoskeleton remodeling during FkMTp cell line establishment. F-actin is stained with rhodamin phalloidin (red) and cell nuclei with DAPI (blue). **A** Passage 0 ($\times 20$) showing FkMTp cells with cuboidal shape and organized in compact islets; **B** passage 0 ($\times 63$) FkMTp cells revealed a marginal actin bundle and straight actin bundles; **C** passage 7 ($\times 10$) FkMTp cells showed islets becoming more loosely arranged; **D** passage 7 ($\times 40$) FkMTp cells in more

detail; **E** FkMTp cell ($\times 63$) from passage 14 revealed an initial cytoskeleton elongation; **F** passage 28 ($\times 40$) FkMTp cells showed an evident elongated shaped; **G** FkMTp cells ($\times 40$) from passage 49 revealed a spindle-shaped morphology arranged in parallel; **H** passage 119 ($\times 40$) of FkMTp cells showing cells bundled into thick contractile stress fibres at the ventral cell; **a–h** F-actin shown in black and white for better contrast. Scale bars = 50 μm . (Color figure online)

adhesions. With the course of time F-actin became assembled in actin stressed fibres. The switch in actin filament organization observed was slow and progressive with increase of the number, width, and length of the actin filaments that occurred with changes in cell morphology.

Anchorage independent growth in soft-agar

This assay is used to measure the ability of cells to grow in an anchorage independent manner. This assay was performed at passages 0, 21, 35, 42, 49, 56, 63, 98, 112, 126, 147 and 160 (Fig. 4). Apparently, between

passages 0 and 63 there was noticed some degree of instability, observed by the oscillations of the colonies' size, which can reflect a major or minor capacity of cells to grow in an anchorage independent manner. From passage 98 on we observed an increasing number of medium size colonies and a significant increase of large colonies, suggesting a greater ability to grow in anchorage independence. It seems that at passage 112, the FkMTp cell line acquired stability, once the behaviour of cells didnot experience alterations in the following analysed passages (126, 147 and 160). Passages 112, 126, 147 and 160 showed an absence of small colonies and a prevalence of large colonies.

Fig. 4 Soft-agar colony assay. FkMTp cell line was assayed for growth in soft agar during the course of time as a measure of anchorage-independent growth. **a** Example of a small colony (at passage zero); **b** example of a medium colony (at passage 98);

d

Passage	Soft-agar colonies		
	Small %	Medium %	Large %
0	26,3	68,4	5,3
21	71,4	28,6	0
35	65	35	0
42	67,6	29,4	3
49	48,1	37	14,9
56	67,9	32,1	0
63	87,5	8,3	4,2
98	13,6	50	36,4
112	0	23,1	76,9
126	0	37,5	62,5
147	0	38,1	61,9
160	0	10	90

c example of a large colony (at passage 160); **d** percentage of small, medium and large colonies in each passage; **e** graphical representation of **d**. Scale bar = 75 µm

Migration assay and in vitro invasion assay

The chemoinvasion assay was used to quantify the invasive potential of the FkMTp cells. Using the modified Boyden Chambers, we have measured the ability of the cells to migrate and invade through matrigel membranes (Fig. 5). We noticed that migrating and invasive cells exhibited mostly the same

behavioural pattern over the time course, with the number of migrating cells being higher than the one of invasive cells. Exception goes to passage 21, where the number of invasive was higher than the one of migrating cells, and at passage 49 where no difference was observed between the values of cell migration and cell invasion. The number of migrating and invasive cells was higher in passages 21, 63 and 126.

Fig. 5 Cell migration and invasion assay in several passages of FkMTp cell line. Cell migration and invasion assay was performed in order to evaluate and measure the capability of migration and invasion of different passages of the FkMTp cell

line; **a** number of migrating cells versus invasive cells; **b** illustrative image of FkMTp migrating cells (passage 126); **c** illustrative image of FkMTp invasive cells (passage 126). Scale bar = 150 μ m

Wound-healing assay

To further characterize the new cell line FkMTp, the migration potential was assessed by the wound-healing assay (Fig. 6). Eight hours over the wound formation, migration of cells to a position close to the wound was observed in all tested passages. It was possible to identify an increase in the amount of migrating cells in passage 21 when compared to passage 0. After this, the number of migrating cells decreased until passage 42, where a progressive increase was again noticed. Afterwards no significant differences were observed in the next passages, and all were almost able to completely heal the wound within 24 h, with exception of passage 35. Nonetheless, 48 h after the wound formation, cells from all passages were capable to heal the wound completely.

Chromosomal analysis

Chromosomes of 100 random metaphase plates were analysed at passages 0, 7, 14, 21, 28, 35, 42, 49, 56, 63,

70, 77, 84, 91, 98, 105, 112, 119, 126, 133, 140, 147 and 160. The distribution of chromosome numbers is shown in Fig. 7. There were diverse aneuploidies and the chromosome number widely varied from 26 to 252. However the modal value was 38 for passages 0, 7, 14, 21, 28, 35, 42, 140, 147 and 154 (the standard chromosome number of the species). For passages 49 and 56 the modal number was 37 and for passages 63, 70, 77, 84, 91, 98, 105, 112, 119, 126 and 133 it was 39 (Fig. 7). It was observed that for some of the passages these modal numbers were observed in up to 50 % of the cells. Passages 21, 28 and 49 were the ones presenting a larger incidence of aneuploidy (57, 53 and 76 %, respectively).

Discussion

The establishment of feline mammary cancer (FMC) cell lines seems to be difficult when compared to those of other animal species and the reason(s) for this difficulty remains unknown (Uyama et al. 2005). In

Fig. 6 Study of FkMTp cells motility during time course by the wound-healing assay. Cells were plated onto 6 well culture plates and at near confluence, wounds were generated. Cells were photographed at 0, 8, 24 and 48 h. Scale bar = 150 μ m

1984, Elshire reported a failure in establishing FMC cell lines from 14 cat mammary carcinomas. One year later, in 1985, Norval et al. was able to establish the first FMC cell line from one of 30 cats with FMC. Later, in 1991, Minke et al. (1998) reported the establishment of four cell lines out of 135 cats with feline mammary cancer. Muleya et al. (1998) reported the establishment of one cell line in 1998. Finally, the last reported cell lines were published by Uyama et al. (2005). They established eight feline mammary adenocarcinoma cell lines from five out of 13 individual cats (the eight established cell lines were from cats with either primary or metastatic lesions of spontaneous feline mammary adenocarcinoma).

A major opportunity to increase our knowledge regarding the biology of breast cancer is associated with the availability of experimental model systems that recapitulate the several forms of this disease (Snyder et al. 2001). Recent studies have described the genetic characterization of breast cancer cell lines, showing their value in the investigation of the role of genomic alterations in cancer progression and as a resource for the discovery of new breast cancer genes (Kao et al. 2009; Kadota et al. 2010; Louzada et al. 2012).

In this study we present and report the establishment of a FMC cell line named FkMTp derived from a 7-years-old cat with a mammary carcinoma and its characterization, as referred before. This cell line was cryogenically preserved at every six passages.

As we expected, the primary culture of the FkMTp as well as the early passages presented a heterogeneous population of cells (fibroblast and epithelial like cells) (Fig. 2a). This heterogeneity could be observed by a simple morphological examination, similar to what Sarrió and colleagues described in 2008 (Sarrió et al. 2008), referring that breast cancer is a heterogeneous disease, which includes a wide range of histologic subtypes.

However, with subsequent passages, a selection towards a more homogenous population of the cell line was observed, presenting morphological characteristics of “fibroblast-like” cells (Fig. 2b). Fibroblasts are prototypical mesenchymal cells that exist in many tissues and are activated during repair processes as the ones of wound healing (Kalluri and Zeisberg 2006; Kalluri 2009). In breast carcinomas, about 80 % of stromal fibroblasts are thought to acquire an activated phenotype (Sappino et al. 1988; Lebret et al. 2007). Lebret et al. (2007) found that cancer-associated

A

FkMTp Passagens	% of FkMTp cells with							Different chromosome number
	2n=35	2n=36	2n=37	2n=38	2n=39	2n=74	2n=76	
14		5	8	70		5	5	10
21	6	9	28	43				15
28		11	15	47	7			19
35		10	25	50	5			13
42		9	25	44	10			10
49		20	30	25	5	6		16
56			40	15	37			8
63		5	21	28	33			13
70	5	7		18	53			14
77		6	10	30	45			14
84			14	23	55			12
91		5	7	39	54			11
98			5	23	68			9
105				5	86			8
112			5	14	74			10
119				20	67			12
126				19	76			7
133				25	55			11
140			9	52	31			10
147			7	67	11			14
154			17	56	10			14

Fig. 7 Cytogenetic analysis (ploidy) of the FkMTp cell line **a** variability of number of chromosomes for analysed passages; **b** schematic representation, number of chromosome per cell for each passage; **c** representative metaphase of FkMTp cell line

fibroblasts (CAFs) and fibroblasts from breast reduction specimens were able to induce epithelial-to-mesenchymal transition in PMC42-LA cells, a breast

cancer cell line that exhibits stem cell-like properties. Another study referred that fibroblasts isolated from the interface zone located within 5 mm of the cancer,

as well as CAFs and normal breast fibroblasts were able to induce epithelial-to-mesenchymal transition in MCF7 cells (Gao et al. 2010; Soon et al. 2013). Our results suggest that during the establishment of the cell line, probably an epithelial-to-mesenchymal transition has occurred. An essential developmental process by which cells of epithelial origin lose epithelial characteristics and polarity, and acquire a mesenchymal phenotype with characteristic gene-expression changes, increased potential for motility, and in the case of cancer cells, acquisition of migratory and invasive properties, loss of cell–cell adhesion and cell polarity, metastasis, and resistance to chemotherapy (Gupta et al. 2009; Taube et al. 2010; Soon et al. 2013).

Increasing evidence supports and reinforces the results obtained in the present work, where we observed a switch of polarized epithelial cells to a fibroblastoid phenotype, an outcome from epithelial to mesenchymal transition (EMT). Recent studies suggest that fibroblasts play a significant role in cancer initiation and progression (Elenbaas and Weinberg 2001; Tlsty and Hein 2001; Franco et al. 2010). Cancer-associated fibroblasts have the ability to induce a greater degree of EMT in MCF7 breast cancer and MCF10A breast epithelial cell lines, indicating that CAFs can contribute to a more malignant breast cancer phenotype and their role in resistance to therapy should therefore be considered when treating breast cancer (Soon et al. 2013).

The FkMTp cell line reached stability between passages 112 and 126, with a doubling time of these cell line populations of approximately 24 h (Fig. 2c). The FkMTp cells also maintained their properties without evidence of senescence after been thawed from liquid nitrogen approximately for about 6 months.

Regarding cytoskeleton we have assessed the dynamic changes in cell morphology and actin filament organization in the cell line over time. Actin cytoskeleton remodelling promotes morphological changes and cell migration during EMT and is also required for metastatic cancers to spread from primary tumours. Therefore, the factors controlling remodelling are potentially key targets for therapeutics thus restricting cancer progression (Haynes et al. 2011). We have observed progressive changes in cell morphology during the first passages of FkMTp cell line (passages 0, 14, 21, 28, 35, 42) (Fig. 3). These changes

were accompanied by reorganization of actin filaments from marginal and straight actin bundles, which are typical for epithelial cells (Ayollo et al. 2009) to cells bundled into thick contractile stress fibres at the ventral cell surface, typically found in transdifferentiated mesenchymal cells (Haynes et al. 2011). These results are in accordance with Haynes et al. (2011) and reinforce the evidences that EMT process occurred during the FkMTp cell line establishment and suggest that this process occurred during the firsts passages of the FkMTp.

Figure 4 reveals that all the analysed passages formed colonies in soft-agar. Passage 0 presented about 68 % of the colonies displaying a medium size. In passages 21, 35, 42, 49, 56, 63 the higher number of colonies detected were of small size. At passage 98 colonies of medium size were again observed and after passage 112, large colonies (the largest) were the prevalent ones, with no observable small colonies, indicating again a certain acquisition of stability.

In routine subculturing, the FkMTp cells seemed to develop a transformed phenotype that was further confirmed on soft agar colony formation testing, indicating that the FkMTp cells developed transformation capacity at every passage and were capable to form colonies after 3 weeks of culture in soft agar (Fig. 4). This suggests that the FkMTp cell line is indeed a transformed cell line, without the need of a substratum to grow. The anchorage independent growth in soft agar assay was then used to measure the ability of the FkMTp cells to grow in an anchorage independent manner, often regarded as one of the hallmarks of tumorigenicity (Fiebig et al. 2004; Hughes et al. 2008). Soft agar growth has become one of the most important and most widely used in vitro criterion to detect cell transformation, and its reliability and correlation with in vivo tumorigenicity are of considerable interest (Ke et al. 2004).

Cells that become anchorage independent have the potential to migrate through the body, colonize other tissues and to grow metastatically (Gassmann and Haier 2008). The acquisition of anchorage independent growth is directly associated to EMT in the literature, which enhances the results observed in the FkMTp cells during morphology analysis (Guadamillas et al. 2011).

Using the chemoinvasion assay, it was possible to observe that both migration and invasiveness ability were present at all analysed passages of the FkMTp

(Fig. 5). The invasiveness capacity of cells was higher in passages 21 and 35, being the values between migration and invasion very close in passages 126 and 147. In all other analysed passages, the value of migration was higher than that of invasiveness (0, 49, 56, 63, 98 and 112). These two properties associated, migration and invasiveness, can lead to the most lethal aspects of breast cancer, the process of tumour invasion and subsequent metastatic spread of the disease from the site of origin to distant organs (Steeg 2006; Hughes et al. 2008). Metastasis is a multi-step process involving both migration and invasion (Fidler 2003; Hughes et al. 2008) and it is generally accepted that the cells must display both migratory and invasive capacities to metastasize (Wang et al. 2005; Hughes et al. 2008).

The migration potential of the FkMTp cells was assessed by mechanical disruption in a wound-healing assay (Keese et al. 2004). Figure 6 depicts the results obtained in this revealing that all passages analysed had the ability to cicatrize the wound, meaning that all exhibited migratory cells. During the course of time migration of cells (cicatrisation of wounds) increased in the late passages, 112, 126, 147, 160, being the time required to completely heal the wound approximately 24 h. These results corroborate the ones obtained in the other assays performed. The migration activity of cells in vitro is thought to be related to several cellular in vivo behaviours, including the directed migration of cells during differentiation and the aberrant invasive activities of metastatic tumour cells (Keese et al. 2004).

Thereby our results suggest that the first passages of the FkMTp cell line exhibit reactive stromal fibroblasts which probably led to an epithelial-to-mesenchymal transition, resulting in the acquisition and increase of migratory and invasive properties as revealed by the migration, in vitro invasion and wound-healing assays. A time course molecular analysis of the FkMTp cell line may confirm these results as specific key genes must be involved in the profound differences found in the sequential passages over time. However, as the cat is not properly sequenced, neither experimental tools are available to perform this type of analysis (as for human or other genome models) an enormous amount of preliminary work has to be performed before this type of study is feasible.

The preliminary cytogenetic analysis of the FkMTp revealed a prevalence of cells with near diploid

karyotypes and low levels of ploidy in all analysed passages (0–160) (Fig. 7). Nevertheless, this cell line contained cells with a wide range of chromosome numbers, the so called “cytogenetic noise”, but at a lower percentage when compared with the near diploid cells. It was proposed that aneuploidy might be an early event in cancer evolution, which induces a *quasi-stable* karyotypic state that is balanced by selection towards tumorigenesis (Nicholson and Duesberg 2009; Schwartzman et al. 2010). This is in accordance with what we observed in the FkMTp cell line, where aneuploidy was observed in the early passages (0–55/65 passage), but an apparent *quasi-stable* karyotype in the later passages (105–160). Aneuploidy and chromosomal instability frequently coexist, underlying very complex and diverse aneuploid karyotypes in high-grade tumours. Aneuploidy and chromosomal instability have both been recognized as hallmarks of cancer (Hanahan and Weinberg 2000, 2011; Potapova et al. 2013) and some authors have referred that aneuploidy has a driving role in adaptive evolution by providing a fitness advantage under specific circumstances and thus a driver of positive selection in tumorigenesis (Pavelka et al. 2010; Gordon et al. 2012). These findings support our results once the cell line here presented shows a high level of chromosomal instability in the early passages, as previously mentioned. This theory can explain and substantiate the fact that the FkMTp cell line was able to survive and possibly to transform and achieve immortalization. Therefore we suggest that chromosomal instability and aneuploidy observed during the FkMTp cell line establishment were a driving force to induce the *quasi-stable* karyotypic state observed in the later passages.

There are evidences that suggest that the relationship between aneuploidy and chromosomal instability can be envisioned as a “vicious cycle,” where one potentiates the other. This vicious cycle can lead to a rapid broadening of the karyotype diversity in a population, providing the substrate for natural selection (Potapova et al. 2013).

Conclusions

The FkMTp cell line presents a number of valuable advantages making this cell line unique when compared to other cell lines described so far. First,

it has the uniqueness of the primary cultures derived directly from tumours, there is detailed pathologic analysis available allowing comparing the characteristics of the cultured cells to those of the original tumour. This is a significant advantage, once, cancer cell lines derived from earlier stage and lower grade disease seems to be the more promising models. In comparative studies made between cancer cell lines derived from earlier stage tumours and the original tumour tissues showed fair concordance in several parameters. This shows that this type of cells is more representative of the original tumour, reflecting more accurately the events that occur in cancer cells *in vivo*. Second, the FkMTp cell line has the advantages of infinite and continuous cell lines, in the way they are easy to handle and represent an unlimited self-replicating source that can be grown in almost infinite quantities. Also the experimental procedures for these cell lines are standardized and the results reproducible, enabling the validation of any experimental assay. Moreover, these *in vitro* cancer cell models are unconstrained by the practical and ethical considerations necessary in *in vivo* models. One of the most valuable feature of the FkMTp cell line when compared with all other cell lines (commercial, in cell banks) is the fact that during the cell line establishment, it was cryopreserved approximately every six passages, including the zero passage (tumour primary culture), allowing the possibility to access almost any passage and the analysis of specific moments (*momento*) in the tumour progression. This strategy provided a bank of *momentos* of the *in vitro* tumour progression. Each of these *momentos* can now be thawed and analysed, allowing a considerable decrease of possible faults caused by genotypic and phenotypic drift during continuous culturing, and substantially increase the reliability of the results in the various studies to be carried subsequently.

The different assays performed clearly demonstrate that the FkMTp is a cancer cell line with *in vitro* tumorigenicity. This cell line revealed to be a promising resource to deeply study the diverse processes involved in tumourigenesis, once it allows following the *in vitro* tumour progression from the primary culture over time (at least until passage 160). For these reasons, FkMTp represents a unique cell line offering excellent opportunities as a reliable tumour model.

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