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Research Article

# Isolation of stem-like cells from spontaneous feline mammary carcinomas: Phenotypic characterization and tumorigenic potential

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ABSTRACT

Current carcinogenesis theory states that only a small subset of tumor cells, the cancer stem cells or tumor initiating cells (TICs), are responsible for tumor formation and progression. Human breast cancer-initiating cells have been identified as CD44-expressing cells, which retain tumorigenic activity and display stem cell–like properties. Spontaneous feline mammary carcinoma (FMC) is an aggressive cancer, which shows biological similarities to the human tumor counterpart.

We report the isolation and phenotypic characterization of FMC-derived stem/progenitor cells, showing *in vitro* self-renewal, long-lasting proliferation and *in vivo* tumorigenicity. Twenty-one FMC samples were collected, histologically classified and characterized for the expression of Ki67, EGFR, ER-α and CD44, by immunohistochemistry. By culture in stem cell permissive conditions, we isolated, from 13 FMCs, a CD44-positive subpopulation able to survive and proliferate *in vitro* as mammospheres of different sizes and morphologies. When injected in NOD/SCID mice, FMC stem-like cells initiate tumors, generating cell heterogeneity and recapitulating the original histotype. In serum-containing medium, spheroid cells showed differentiation properties as shown by morphological changes, the loss of CD44 expression and tumorigenic potential.

These data show that stem-defined culture of FMC enriches for TICs and validate the use of these cells as a suitable model for comparative oncology studies of mammary biology and testing therapeutic strategies aimed at eradicating TICs.



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## Introduction

Naturally-occurring tumors in domestic animals represent an opportunity for comparative oncology studies for their translation potential to human pathology [1]. Cancer is the second most frequent cause of death in humans and the first one in dogs and cats [2]. Considering the incidence by site, mammary gland cancer is the most frequent (32%) in women, the first (52%) in bitches and the third (17%) in queens [2–4]. As compared to experimental cancers in murine models, spontaneous tumors of pets represent better models of human cancer since they share similar environmental risk factors, develop in immuno-competent organisms, offer a large population samples, and, finally, the shorter overall lifespan of domestic animals, associated with a more rapid cancer progression, allows a more rapid response time than humans [1]. In addition, several biological, morphological and clinical features of animal tumours can be identified in the same cancer types in humans [5].

Almost 80% feline mammary cancers are malignant, histologically classified as adenocarcinomas, associated with an aggressive clinical course and rapid metastasization, [6] and surgery is the most widely used treatment.

In addition, the lack of oestrogen dependency in most feline mammary carcinomas (FMC) [7] suggests that they could be a suitable model for hormone-independent human breast cancer [8]. Finally, similarly to the human counterpart, FMCs show overexpression of p53, cyclin A [9], epidermal growth factor receptor 1 (EGFR) and 2 (HER2) [10,11], suggesting that similar tumorigenesis mechanisms may be active in human breast cancer and FMC.

Accumulating evidence in human cancer biology supports the hypothesis that tumors are initiated and driven by a subpopulation of cells responsible of tumor cell hierarchy and heterogeneity, sharing several features with somatic normal stem cells (stem-like cells), such as self-renewal and pluripotency. Thus they have been named "cancer stem cells" (CSCs) or, due to their tumorigenic potential, "tumor-initiating cells" (TICs). TIC identification and characterization has important diagnostic, prognostic and therapeutic implications since, according to the CSC theory, effective anticancer therapy requires the complete elimination of TICs from the tumor mass, to block the indefinite regeneration of the cancer cell population and overcome chemo- and radio-resistance [12].

Candidate TICs have been identified in both haematological and solid human neoplasms [12]. Breast cancer is the first human solid tumor in which TICs were identified as cells expressing high levels of CD44 and low (or none) CD24 [13]. Since then, TICs were identified in a wide variety of neoplasms, including prostate [14], colon [15] and pancreas [16] carcinomas, glioblastoma [17] melanoma [18], and, possibly, pituitary adenomas [19]. Several experimental approaches have been developed to obtain in vitro cultures efficiently enriched in stem/progenitor cells from human mammary gland tumors [20]: $CD44^{+}/CD24^{-/low}$ cell sorting [21], Hoechst-dye effluxing side population by FACS analysis [22] and *in vitro* cultures in growth factor-enriched medium (stem cell permissive) as non-adherent spherical clusters, named mammospheres. Mammospheres were obtained growing dispersed tumor cells in selective culture conditions, to favor mammary stem/progenitor proliferation of cells retaining self-renewal and tumorigenicity in mice [23,24]. Shifting TICs in serum-containing medium, they differentiate, adhere to substrate in monolayer and acquire epithelial lineage marker expression [21]. On the other hand, TIC-enriched cultures display increased resistance to radiation [25] and chemotherapeutic drugs [26]. Some established human breast cancer cell lines also contain a subpopulation of cell that share some characteristics with stem cells from tumor samples [27]. However, the $CD44^{+}/CD24^{-/low}$ phenotype is not a unique and definitive breast TIC profile [28], since CD44 negative cells from pleural effusion also generate mammospheres *in vitro* and tumors when transplanted in immunodeficient mice [29].

Mechanisms of tumor development seem to be similar in human and companion animals, but CSC research in pets is currently in early stage of development [30]. Canine stem/progenitor cancer cells have been recently isolated in hepato- and cholangio-cellular carcinomas [31], prostatic intraepithelial tumors [32], glioblastoma [33], lung adenocarcinoma [34] and osteosarcoma cell lines [35]. Furthermore, cells with stem cell-like properties were isolated from canine normal and neoplastic mammary gland [36] and mammary carcinoma cell lines [37]. To date, only putative TICs have been identified from FMC cell lines by spherogenesis assay [38] while no studies rely on isolation of TICs from feline surgical tissues.

The availability of CSC derived from spontaneous tumors of cats and dogs offers many advantages for the biological and pharmacological characterization of these cells; the bioptic material reflects the natural setting of tumor cells, allows a relatively rapid follow-up of clinical cases, and retains the individual heterogeneity of drug responses, thus representing a reliable pre-clinical model to assay drug targeting to TICs [39].

Most molecular bases of tumorigenesis and altered intracellular pathways are common in humans and companion animals [40], thus, comparative oncology could be useful approach in CSC field [41]. The isolation of canine and feline TICs and the study of their biology represents a good pre-clinical model of cancer, whose deficiency often hampers the translation of cancer biology findings into clinical oncology practice [1].

Current experimental models (cancer cell lines, transgenic and xenograft rodent tumors), despite their long-lived contribution to cancer studies, are not completely able to reproduce the high individual heterogeneity within each tumor histotype and among patients [5]. Thus, pets may represent a new tool to bridge rodent experimental models to human cancer stem cells.

In this study, exploiting human breast cancer stem cell knowledge, we identified and cultured putative TICs from fresh FMC tissues, displaying phenotype and biology features of CSCs: expression of CD44, ability to grow as mammospheres, self-renew under stem cell-permissive culture conditions, differentiation when shifted in serum-containing medium *in vitro*. The formation of sub-cutaneous FMC xenografts in NOD/SCID mice confirmed the *in vivo* tumorigenic potential of isolated cultures.

This is the first report demonstrating the presence of TICs in FMC, supporting the relevance and the feasibility of studying pet spontaneous malignancies in order to elucidate the mechanisms of tumor biology.

## Materials and methods

### *FMC samples*

Twenty-one tissue samples from FMC resections were provided by the local network of free-lance veterinary doctors. Tumor