**Original Full Paper**

# Establishing primary tumor cell lines in dogs: Challenges and Ki-67 correlation

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## Abstract

The incidence of malignant neoplasms in dogs has significantly increased in recent decades. Due to their aggressive nature and poor prognosis, the search for new, patient-specific treatments has become a promising approach. The use of a patient's own tumor cell lines is a valuable tool for genetic analysis, treatment targeting, and serves as a translational strategy for the development of

new drugs. However, there are technical limitations and poorly understood factors that influence the success or failure of establishing cell lines from primary tumors. This study aimed to establish continuous tumor cell lines from canine patients using a simplified protocol. After 10 passages in culture, the cells were considered established and used for further assays. Of the 10 tumors collected, 6 cell lines were successfully established: oral melanoma (PM13C and PF11C), cutaneous lymphoma (BF11C), mast cell (DF13C), urothelial carcinoma (MF10C), and mammary carcinoma (PF12C). Statistical analysis using the Chi-squared test revealed that success in establishing the cell lines was correlated with the histopathological grade of malignancy and the clinical stage of the patients, but not with the Ki-67 proliferation marker. DF13C and PF11C cells were inoculated into a xenograft model and exhibited a neoplastic pattern, confirming the histogenesis of the lines. This study identifies key factors influencing the success or failure of establishing long-term cell lines from patient tumors, contributing valuable data that is currently scarce in the literature and limiting the integration of this protocol into routine laboratory practices.

**Keywords**: cancer, molecular markers, lineages, translational research, tumorgenicity.

## Introduction

Cancer is a complex disease characterized by multiple genetic mutations and epigenetic alterations, which contribute to tumor heterogeneity and hinder effective therapy (17, 23, 35). In dogs, the prevalence of cancer has been steadily increasing, driving the search for new diagnostic methods and therapeutic approaches aimed at improving prognosis (26, 32, 34). As in human medicine, veterinary oncology has increasingly embraced individualized treatment strategies, allowing for patient-derived models that guide therapy in a personalized manner (29). In this context, accurate diagnosis is essential, as it determines therapeutic strategies and provides a deeper understanding of tumor characteristics.

Ki67 is a nuclear protein biomarker expressed during cell division, directly associated with tumor cell proliferation. It has been widely used as a proliferation marker in histopathological assessments (36). As a reliable indicator of tumor growth, its expression is often correlated with disease stage and metastatic potential. In general, Ki67 levels are higher in aggressive, poorly differentiated tumors compared to normal tissues. Moreover, elevated Ki67 expression can predict better responsiveness to certain treatments, serving as a predictive marker. The Ki67 index is also considered an independent prognostic factor for patient survival. Studies have shown that inhibiting its activity can halt cell division, highlighting its potential as a therapeutic target (18). Therefore, advanced investigations using cell and animal models are essential to further explore its diagnostic and therapeutic applications in cancer.

The use of in vitro models with patient derived tumor cells enables testing of individualized tumor responses to treatments and correlating them with the tumor's molecular profile there by supporting precision medicine strategies (37). In this regard, cell cultures have proven to be excellent experimental models for in vitro analyses, facilitating the study of molecular physiology, carcinogenesis, and the evaluation of mechanisms of action and proliferation processes (11, 16). Cell culture offers several advantages, including sample homogeneity and controlled environmental conditions (1). This method is widely used to assess molecular, biochemical, cellular, and histochemical parameters, making it a valuable tool for studying tumor behavior (6).

In veterinary oncology, especially in rare tumor types, the availability of canine tumor cell lines for individualized treatment approaches remains limited. New therapies for canine cancers are often extrapolated from human cancer cell line studies, resulting in a lack of treatment strategies that reflect the biological characteristics of canine tumors (10). Moreover, the success rate for establishing these cell lines is generally low, and the factors determining failure remain poorly understood (8). Additional barriers include relatively high costs and lengthy establishment times, which discourage laboratories from incorporating these protocols into routine practice.

In this study, six cell lines were successfully established from cancers of different origins in canine patients. The use of this simplified protocol may serve as a translational model for the development of new drugs and the implementation of individualized therapies. Correlations were made to identify the key determinants of success or failure in establishing continuous cell lines.

## Material and Methods

### *Ethical considerations*

The project was exempted from Unicamp Ethics Committee on the Use of Animals. Given the lack of standardization in veterinary clinical studies, the research was conducted in accordance with Resolution N°. 466/2012 and other resolutions from the National Health Council.

### *Tumor collection*

Tumors were collected from dogs of various ages and sexes, all with a prior diagnosis of malignant neoplasia. The surgeries were performed by the team at the Oncovida Veterinary Oncology Center in Campinas (SP), with documentation of the tumor locations and the clinical characteristics of the patients. The guardians authorized access to medical records and clinical exams through informed consent.

After resection, the samples were divided: one part was placed in a tube containing IMDM (Iscove's Modified Dulbecco's Medium, code 17633-10x1), supplemented with fetal bovine serum and antibiotics (100 IU/mL penicillin and streptomycin, A5955 - Sigma) (pH 7.4), and sent to the Laboratory of Advanced Therapies (LATERA/UNICAMP) for cell culture; the other part was fixed in 10% formaldehyde and sent to the VETPAT laboratory for histological analysis.

### *Histopathology and immunohistochemistry analysis*

Histological processing and the histopathology report were performed by a specialized veterinary laboratory (VETPAT), following the inclusion, microtomy, and staining protocols established by the laboratory. Images of the slides were captured using a photomicroscope [Nikon NIS Elements Advanced Research]. Immunohistochemistry was also conducted by VETPAT. The following antibodies were used in the immunohistochemistry protocol: anti-Ki-67 Dako/Agilent antibody #IR629 to assess cell proliferation in all tumors, anti-C-KIT (CD117) #A4502 for mast cell tumors, CD3 #IR503, CD18 antibody Abcam #ab131044, CD79A #IR62161, MUM1 #GA644, and C-KIT for lymphoma, and for salivary gland carcinoma, CK5/6 #IR780, AE1AE3 antibody CellMarque 313M-16, p63 #IR662, and CAM5.2 #452M-96. The quantifications were performed using the PathoZoom software from Smart In Media (smartinmedia.com).

***Cell culture***

To establish the cell lines, tumor samples were cut into 1–2 mm fragments, macerated, and filtered through a 70 µm strainer [Cell Strainer 70 µm - BD]. The suspension was centrifuged (1500 rpm, 5 min, 4°C), and the pellet resuspended in complete IMDM medium. Cells were seeded into 25 $cm^2$ culture flasks and incubated at 37°C with 5% $CO_2$ until ~90% confluence, with medium changes every 3 days (Fig. 1). Cells were passaged using a cell scraper, and after every 3 passages, $1 \times 10^6$ cells were frozen in IMDM with 10% DMSO [D2660, Sigma]. Upon reaching 10 passages, the cell line was considered established.

***Cytopathology of cell lines (Preparation and characterization of cell lines by cytocentrifugation)***

Morphological and immunocytochemical analyses were performed on the established cell lines using a cell centrifuge [Cytocentrifuge, Incibrás]. The strains, maintained in 25 $cm^3$ cell culture bottles, were transferred to a culture tube, and the resulting suspension was centrifuged (1500 rpm, 5 min, 4°C). The pellet was resuspended in 100 µL of complete IMDM medium. After homogenization, 10 µL of the cell suspension was removed and deposited into a plastic funnel,

allowing the cells to be concentrated in a designated area of the circular field on the slide. The cells were then centrifuged again (1000 rpm, 10 min) [Cytocentrifuge - Incibrás]. The slides were removed from the centrifuge and allowed to dry at room temperature. Four slides were prepared for each strain. First, one slide of each strain was stained using rapid panoptic stain [Laborclin, Code 620529], submerged in each dye with an up-and-down movement for 5 seconds, then rinsed with running water. After drying, cell morphology was analyzed. For immunolabeling, the following antibodies were used: melanoma #COKTEL Melanoma, mast cell tumor #C-KIT #TRYPTASE, lymphoma #CD3 #PAX5, and mammary carcinoma #AE1AE3 #ESTROGEN [same markers as used previously].

***Tumorigenicity test***

The experimental protocol was submitted to the Ethics Committee for the Use of Animals (CEUA/UNICAMP) and approved under number 6200-1/2023. Adult (8-10 weeks old) female and male RAG-/- mice (B6.129S7-Rag1tm1Mom) were obtained from the Institute of Biological Sciences, University of São Paulo (ICB/USP). The animals were housed in microisolator type cages in the Animal Facility of the Immunology Area, Department of Structural and Functional Biology, Institute of Biology/UNICAMP, with a 12/12 hour light/dark cycle and access to water and food ad libitum.

A cell suspension of $3 \times 10^5$ cells in 100 µL of Phosphate Buffered Solution (PBS 1M) was inoculated subcutaneously into the dorsal region of the mice (n = 4). The animals were monitored every 48 hours until the tumors formed. The animals were humanely euthanized between 30 and 50 days post-inoculation under intraperitoneal (i.p.) anesthesia with ketamine (300 mg/kg) [VETNIL] and xylazine (30 mg/kg) [Anasedan; CEVA]. The tumors were collected, measured, weighed, and stored in 4% paraformaldehyde (pH 7.4). Histopathological and immunohistochemical analyses were performed to assess tumor histogenesis.

### *Statistical analysis*

The analysis of the quantification of histopathology and immunohistochemistry was carried out using GraphPad prism, V, 5.0 (GraphPad, San Diego, CA). The level of significance was analyzed using the one-way ANOVA test, followed by Dunnet's multiple comparisons test. To study the correlation between variables, the Chi-square test was applied. The significance level adopted was 5% ($p<0.05$). Significance was represented by ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$.

## Results

### *Description of the tumor samples obtained and the lineages established*

Ten samples were obtained from patients with different types of tumors, and the corresponding cell lines were named SM12C, LF12C, LF13C, PF10C, PM13C, BF11C, DF13C, MF10C, PF11C, and PF12C (Figs. 2 and 3, Table 1). The histopathological diagnosis, epidemiological information, anatomical location of the tumors, and clinical staging of each patient are detailed in (Table 1).

All cells were cultured; however, the SM12C, LF12C, LF13C, and PF10C cell lines were lost during cultivation. After the fifth passage, the SM12C and LF12C cells underwent cell death, while the LF13C and PF10C strains, both derived from breast neoplasms, took about 24 days to reach confluence. After the first passage, these strains also underwent cell death (Table 2). The PM13C, BF11C, DF13C, MF10C, PF11C, and PF12C cell lines were successfully established for long-term culture (Fig. 3, Table 3).

### *Histopathological characteristics and clinical staging*

The histopathological characteristics of each tumor collected, along with their degree of malignancy and the clinical staging of the patients, are described and summarized in (Table 4). The degree of malignancy of the neoplasms was classified in this study as either low or high grade, based

on the cellular atypia observed in the histopathological examination. For mast cell tumor, the grade III classification, used in this study to designate high-grade tumors, was established by Kiupel (14). For mammary carcinoma, the classification followed the pre-established guidelines of the World Health Organization (WHO), adapted to distinguish low-grade (grades I and II) from high-grade (grade III) tumors.

***Immunohistochemical analysis (molecular characterization) of the tumors collected and correlations with the successful establishment of the lineages***

The cell proliferation marker Ki67 was used on all cell lines. This antibody binds to replicating cells, allowing the assessment of the neoplastic proliferation rate, and it is also a prognostic factor for canine neoplasms. Tumors diagnosed with a high proliferation rate (high Ki67 expression) may be associated with a higher recurrence rate. Specific markers for each tumor type were also used, as detailed below. The results of the molecular analyses for each tumor are summarized in Table 5.

Salivary gland carcinoma (SM12C) showed expression of the following markers: CK5/6, AE1AE3, and p63, but no CAM5.2 expression. Ki67 was positive in approximately 40% of the neoplastic cells, indicating a high rate of cell proliferation. Although the tumor was histologically aggressive and the patient had lymph node metastasis, it was not possible to establish this cell line. Possible causes include insufficient knowledge of the behavior of these tumor cells, lack of essential nutrients, and slower replication rates.

Solid ductal cystic apocrine carcinoma (LF12C) also could not be established. Ki67 was positive in approximately 20% of the neoplastic cells, a low value indicating low malignancy. Regarding breast tumors, two samples were obtained: a grade I mixed tumor breast carcinoma (LF13C), with Ki67 positive in only 8% of the neoplastic cells, considered a low percentage; and a grade II mixed tumor breast carcinoma (PF10C), with Ki67 positive in 30% of the neoplastic cells,

suggesting a high risk of recurrence for the patient. Despite the differences in Ki67 expression, neither of these two lines was established.

Among the oral cavity tumors, two oral melanoma cell lines were obtained, identified as PM13C and PF11C. In the PM13C lineage, Ki67 was positive in approximately 25% of the neoplastic cells, while in the PF11C lineage, the marker was positive in 30% of the neoplastic cells. In the latter case, the patient already had lymph node metastasis. Both oral melanoma lines were successfully established, with Ki67 positive in 90% of the neoplastic cells, a high percentage strongly associated with a higher rate of tumor recurrence. This lineage was established for long-term culture.

The cutaneous lymphoma (BF11C) exhibited mutations in CD3 and CD18, indicating the presence of T lymphocytes, which are considered the most aggressive in dogs. It did not express the MUM1 or C-Kit mutations. In grade III cutaneous mast cell (DF13C), Ki67 was positive in approximately 10% of the neoplastic cells, considered low despite the high histological grade. In urothelial carcinoma (MF10C), Ki67 was positive in approximately 40% of the neoplastic cells, indicating high proliferation and a high risk of recurrence. Finally, in grade I mixed breast carcinoma, Ki67 was positive in 15% of the neoplastic cells. Both of these lineages were successfully established.

***Correlation of success in establishing lineages with the histopathological classification and molecular profile of tumors, and the clinical staging of patients.***

The long-term success rate in establishing tumor cell lines was higher in patients at advanced stages and with lymph node metastasis. The vast majority of losses occurred in tumors with a low degree of malignancy and in patients with low clinical staging. The Chi-squared test showed that these correlations were statistically significant, as presented in Table 6. The correlation between success and failure in establishing the cell lines and the molecular profile of the tumors (Ki67 expression) however, no statistically significant difference was observed (Table 7).

***Morphological and Immunocytochemical Characterization of Established Cell Lines and Tumorigenicity in vivo model***

In this study, different antibodies were used to label various proteins associated with specific cell types, such as tumor cells, immune system cells, and cells related to breast cancer, melanoma, and bladder cancer (Fig. 4). Although the immunocytochemical tests conducted to detect tumor markers yielded negative results, an additional evaluation was carried out through the tumorigenicity assay. This assay was crucial for assessing the oncogenic potential of the sample, providing a more in-depth analysis of the possibility of tumor development. The absence of specific markers in the immunocytochemistry did not rule out the need for complementary tests, as the presence of tumors may be associated with molecular factors not detected by conventional immunocytochemical methods.

Tumorigenicity was assessed using the established cell lines PM13C, BF11C, DF13C, MF10C, PF11C, and PF12C. Only the PF11C and DF13C lines developed tumors. Mice that received the DF13C mastocytoma line developed tumor growth within 20 days (Fig. 5A-C). The melanoma line (PF11C) showed tumor growth between 30 and 40 days (Fig. 6A-C). Histologically, animals that received the DF13C transplant displayed a spindle-shaped and elongated morphology, suggestive of sarcoma (Fig. 5D). Immunohistochemistry with the C-KIT and Trypase antibodies was performed to assess histogenesis. After staining, the tissue showed weak positivity for tryptase and negative for C-KIT, confirming the diagnosis of mastocytoma (Fig. 5E-F). Animals that received PF11C exhibited an elongated and polygonal morphology (Fig. 6D), with positivity for the MELAN-A marker and negativity for HMB-45, confirming the melanoma pattern (Fig. 6E-F).

## Discussion

In veterinary medicine, cancer has gained significant attention as one of the leading causes of disease in dogs. Consequently, the pursuit of new treatment options, as well as targeted and individualized therapies, has become an ongoing effort (26, 33). Moreover, pet owners increasingly seek improved therapeutic approaches, prioritizing quality of life (4, 25, 31).

This study, ten canine tumors were collected with the aim of establishing continuous cell lines as translational models for the development of novel, patient-tailored chemotherapeutic strategies. Of the ten tumors collected, six long-term cell lines were successfully established. All tumors were thoroughly characterized based on their histopathological features and relevant patient information, such as clinical stage, presence of metastasis, or recurrence, enabling a broader assessment and increasing the potential for correlations between parameters.

The literature shows that success rates for establishing tumor-derived cell lines vary widely. Factors associated with higher success include high proliferative activity (elevated Ki-67), larger tumor volume, high mitotic index, necrosis, and high cellularity (27). Microbiological contamination remains a major barrier in primary cultures but can be overcome by techniques such as enzymatic digestion (16, 28). The parameters that define the success or failure of establishing cell lines from patient tumors have not yet been clearly described in the literature. Most studies report only successfully established cell lines, without addressing failed attempts (5, 9, 27, 38).

In our study, long-term cell line establishment success or failure showed a statistically significant correlation with tumor histopathological grade (classified in this study as high or low grade) and the patient's clinical stage, but not with the Ki-67 proliferation marker, which is widely regarded as a prognostic factor in both animals and humans. Ki-67 is a key marker for assessing tumor cell proliferation and malignancy (12) and is considered more accurate than mitotic figure counting (30). A high proliferation index can indicate a poorer prognosis (7, 13). However, despite its prognostic relevance, Ki-67 should not be used as a sole marker but rather in combination with other prognostic parameters.

Our findings suggest that proliferation alone is not a determining factor for successful cell culture establishment. Instead, other malignancy related characteristics such as cell adhesion, migration capacity, and immortality potential, including evasion of apoptotic mechanisms may play a more decisive role. These parameters could be used in the future to guide individualized chemotherapy recommendations. Studies have shown that cell lines derived from high Ki-67 tumors CMT-1026 and IPC-366 (2, 22) tend to maintain or even increase this index, reflecting the selection of highly proliferative clones. Direct comparisons (28) confirm that more aggressive tumor subtypes give rise to cultures with significantly higher proliferation rates. Clinically, high Ki-67 is associated with shorter survival in bitches with mammary carcinoma (3, 22), reinforcing its prognostic and predictive value for culture viability.

Morphological and immunohistochemical analyses were performed on the established cell lines to confirm and identify cell morphology. This method enables the detection of a specific tissue component in situ through antigen–antibody interaction (20). All markers tested were negative in the cultured cells, although marker negativity may reflect phenotypic changes induced by passaging and in vitro adaptation. This does not exclude the possibility of neoplasia, particularly when morphological changes and clonal selection occur over time (21, 24).

To address this, a tumorigenicity assay was performed on the established cell lines. Two of the six lines (PM11C and DF13C) developed tumors in vivo, similar to the findings of Lainetti (16), in which four out of ten lines exhibited in vivo tumor growth. Low-grade lines, such as PF12C (mammary carcinoma origin), did not develop tumors. These findings support the idea that, although neoplastic, not all established cell lines retain equivalent tumorigenic potential (15, 16). Tumor growth in tumorigenicity models depends on multiple factors, including tumor heterogeneity, immune system status, tumor grade, and inoculated cell concentration (2). The mice used in this study were Rag immunodeficient, lacking functional T and B cells but still producing innate immune cells such as Natural Killer cells, which may have impacted tumor growth and possibly explain the absence of tumor development in some animals (19).

In conclusion, this study successfully established six new canine tumor cell lines, showing that culture success is more closely related to tumor histopathological grade and clinical stage than to Ki-67 alone. Two lines retained in vivo tumorigenic potential, offering promising models for translational research and the development of individualized therapies in veterinary oncology.

## Conflict of Interest

The authors declare no competing interests.


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436

438 **Tables**

439

440 **Table 1.** Solid tumor samples obtained from canine patients.

| Cell Lineage | Histopathological diagnosis | Species | Sex | Age | Location | Staging clinical |
|---|---|---|---|---|---|---|
| **SM12C** | Salivary gland carcinoma | Canine | M | 12 years | Salivary gland | IV |
| **PF10C** | Breast carcinoma mixed grade II | Canine | F | 10 years | Mama M5 | I |
| **LF12C** | Cystic solid ductuolar apocrine ductal carcinoma | Canine | F | 12 years | Left lateral thorax | IV |
| **LF13C** | Breast carcinoma mixed grade I | Canine | F | 13 years | Mama M3 | I |
| **PM13C** | Oral melanoma | Canine | M | 13 years | Lymph node | IV |
| **BF11C** | Cutaneous Lymphoma | Canine | F | 11 years | Cutaneous | V |
| **DF13C** | Cutaneous mast cell grade III | Canine | F | 13 years | Cutaneous | II |
| **MF10C** | Urothelial carcinoma | Canine | F | 10 years | Bladder | II |
| **PF11C** | Oral melanoma | Canine | F | 11 years | Oral cavity | II |

| | | | | | | |
|---|---|---|---|---|---|---|
| **PF12C** | Breast carcinoma mixed grade I | Canine | F | 12 years | Mama M4 | III |

441 ** Each tumor collected was given an acronym for identification throughout the study. F = female;
442 M = male.

443

444

445 **Table 2.** Long-term non-established strains (Figure 2).

| | |
|---|---|
| **Lineage SM12C** | Histologically characterized as salivary gland carcinoma. It showed solid arrangements and acinar outlines of round cells with oval nuclei and very scarce cytoplasm and numerous figures of mitosis. In cell culture, adhesion and rapid confluence were observed in around 10 days. This may be related to the fact that this was a patient with metastasis (in the submandibular lymph node), with stage IV clinical staging, considered aggressive and very advanced. However, after the 5th passage, the cells began to die. |
| **Lineage LF12C** | Malignant cutaneous neoplasm and histologically classified as a solid cystic ductuolar apocrine carcinoma. The cells were basoloid, oval, with few mitosis figures, and the stroma was fibrous and scarce. The patient's clinical staging was stage II. After the 5th passage, the cells went into cell death. |
| **Lineage LF13C-2** | Malignant breast neoplasm characterized histologically as Grade I mixed tumor mammary carcinoma. It showed tubular proliferation of cells with low nuclear polymorphism. Cell adhesion to the plaque was slower compared to the other tumors obtained, with confluence observed in around 24 days. The cells died after the 2nd repique. The patient's clinical staging was stage I. |

| | |
|---|---|
| **Lineage PF10C** | Malignant breast neoplasm classified histologically as Grade II mixed tumor breast carcinoma. It showed tubular proliferation of cells with low nuclear polymorphism, scarce cytoplasm and osseous metaplasia. Cell adhesion was slower compared to the other tumors obtained, taking around 24 days for confluence. Cell death occurred after the first rebound. The patient's clinical staging was stage I. |

446

447

448 **Table 3.** Long-term established strains (Figure 3).

| | |
|---|---|
| **Lineage PM13C** | Histologically characterized as melanoma with lymph node metastasis, an aggressive neoplasm with a high degree of dissemination. This lineage took around 20 days to confluence, however, after the first recurrence, the intervals were 5 days for each passage. The patient's staging was stage IV. |
| **Lineage BF11C** | A malignant skin neoplasm invading the panniculus and musculature, histologically characterized as cutaneous lymphoma. It presented round cells with large nuclei, scarce cytoplasm and numerous mitosis figures. The lineage confluenced in 15 days, they were adherent cells and, after the first recurrence, the intervals were 6 days for each passage. This neoplastic manifestation is extremely aggressive. The patient's clinical staging was V. |

| | |
|---|---|
| **Lineage DF13C** | Skin neoplasm, histologically characterized as a cutaneous mastocytoma with a high degree of malignancy (grade III). The cells were round, with moderate nuclear pleomorphism, round nuclei and ample cytoplasm containing coarse intracytoplasmic granulation. In culture, the cells were adherent, with a spindle-shaped, well-elongated morphology. The cells confluenced in 15 days, and after the 1st re-challenge the intervals for each passage was 4 days. The patient's clinical staging was stage II. |
| **Lineage MF10C** | It originated in the urinary bladder and was histologically classified as urothelial carcinoma. It presented a neoplastic proliferation of urothelial epithelium with cells showing a papillary aspect superficially and in more solid arrangements deeply, with oval nuclei, multiple nucleoli and voluminous cytoplasm. The cells confluenced in 15 days and after the 1st reoccurrence the intervals for each passage were 4 days. The patient's clinical staging was stage II. |
| **Lineage PF11C** | The oral tumor was histologically characterized as a melanoma; it showed neoplastic proliferation consisting of melanocytes arranged in small, short cell bundles with a random, tangled arrangement and clusters located in the superficial, middle and deep chorion, infiltrating adjacent connective tissue. Cytoplasm with well-defined borders, elongated to polygonal and containing a variable number of melanocytic granules, rounded nuclei with evident nucleoli. In this lineage, the cells were adherent, fusiform and elongated, showing rapid proliferation. Its 90% confluence was achieved in 7 days and, after its 1st restart, the interval between passages was 4 to 7 days. The patient's clinical staging was stage II. |

| | |
|---|---|
| **Lineage PF12C** | Malignant breast neoplasm classified histologically as Grade I mixed tumor breast carcinoma. It presented tubular proliferation of cells with low nuclear polymorphism, large nuclei, central nucleolus and scarce cytoplasm and osseous metaplasia, tubular proliferation of cells with low nuclear pleomorphism, large nuclei, central nucleolus and scarce cytoplasm. Cell adhesion was slower compared to the other tumors obtained, taking around 24 days for confluence. Cell death occurred after the first rebound. The patient's clinical staging was stage I. |

449

450

451 **Table 4 -** Histopathological characteristics of the samples and degree of malignancy established according to clinical and histopathological observations.

452

| Cell Lineage | Histopathological diagnosis | Microscopic description | Cell | Nucleus | Cytoplasm | Mitosis figures | Degree of malignancy |
|---|---|---|---|---|---|---|---|
| **SM12C** | Salivary gland carcinoma | Round cells, oval nuclei, scarce cytoplasm, numerous mitosis figures | Round cells | Oval core | Scarce cytoplasm | Numerous mitosis figures | High degree |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **PF10C** | Breast carcinoma mixed grade II | Malignant neoplasm, characterized by papillary proliferation of cells with moderate nuclear pleomorphism, large nuclei, central nucleolus and scarce cytoplasm. Presence of cysts and myoepithelial proliferation. Forming tangles with a myxoid appearance. Free surgical margins. Adjacent lymph nodes free of malignancy. | Papillary cell proliferation | Broad core | Scarce cytoplasm | - | Low grade |
| **LF12C** | Cystic solid ductuolar apocrine ductal carcinoma | Basaloid cells, oval, hypercolored nuclei, pale cytoplasm, sparse mitosis figures, ductolar and sometimes solid arrangements, interdigitating melanocytes, scarce fibrous stroma, free margins. | Basaloid and oval cells | Hypercolor cores | Pale cytoplasm | Sparse figures | Low grade |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **LF13C** | Breast carcinoma mixed grade I | Malignant neoplasm, characterized by papillary proliferation of cells with moderate nuclear pleomorphism, large nuclei, central nucleolus and scarce cytoplasm. Presence of cysts and myoepithelial proliferation. Forming tangles with a myxoid appearance. Free surgical margins. Adjacent lymph nodes free of malignancy. | Tubular cell proliferation | Broad core | Scarce cytoplasm | - | Low grade |
| **PM13C** | Oral melanoma | Fragments showing malignant neoplasia diffusely invading the submucosa, characterized by organoid and fusocellular arrangements of cells with high nuclear pleomorphism, round nuclei with exuberant central nucleolus, numerous mitoses (13 figures in 10 fields/40x), | Organoid and fusocellular cell arrangements | Round cores | Large cytoplasm | 13 mitosis figures in | High degree |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | voluminous cytoplasm containing varied amounts of brownish pigment. Metastasis in adjacent lymph node. | | | | 10 40x fields | |
| **BF11C** | Cutaneous Lymphoma | Malignant neoplasm deeply invading the panniculus and musculature, characterized by round cells with large nuclei, some cleaved and evident nucleoli, scarce cytoplasm, numerous mitosis figures. | Round cells | large cores | scarce cytoplasm | Numerous mitosis figures | High degree |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **DF13C** | Cutaneous mastocytoma grade III | Malignant neoplasm, characterized by round cells, with moderate nuclear pleomorphism, round nuclei and ample cytoplasm containing coarse intracytoplasmic granulation - mitosis figures (10 fields/40x): 6. Presence of multinucleated/bizarre cells (10 fields/40x): 2; vascular invasion: not evident; level of invasion/infiltration: dermis/panicle/musculature; cranial, caudal, dorsal, ventral and deep surgical margins: free. | Round cells | Rounded cores | Large cytoplasm | 6 out of 10 40X fields | High degree |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **MF10C** | Urothelial carcinoma | Neoplastic proliferation of urothelial epithelium, cells with a papillary aspect superficially, solid arrangement deeply, oval nuclei, small multiple and irregular nucleoli, moderate anisokaryosis, voluminous cytoplasm, acidophilic, vascularized, 6 figures of mitosis in 10 fields of 400X, atypical figures, fibrous stroma, discrete and moderate. | Cells with a papillary appearance | Oval cores | Large cytoplasm | 6 mitosis figures in 10 40x fields | High degree |
| **PF11C** | Oral melanoma | Lined by mucous lining epithelium with areas of ulceration covered by dense serocellular crusts. In other fields, the mucosal lining epithelium shows areas of melanosis and in some fields atypical melanocyte nests are formed inside it. In the chorion, | Small short cell bundles with random arrangement | Rounded cores | Cytoplasm with well-defined edges | 8 figures of mitosis in 10 400x fields | High degree |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | there is evidence of neoplastic proliferation consisting of melanocytes arranged in small, short cell bundles with a random, tangled arrangement and clusters located in the superficial, middle and deep chorion, infiltrating adjacent connective tissue. The neoplastic cells show cytoplasm with well-defined borders, elongated to polygonal and containing a variable number of melanocytic granules. The nuclei are rounded, with nucleoli, sometimes evident but inconspicuous. Mitosis figures were observed, and the count of mitosis figures in 10 400X fields is equal to eight. Inflammatory infiltrate composed of lymphocytes and | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | plasma cells with the presence of neutrophils. Surgical margins compromised by tumor cells | | | | | |
| **PF12C** | Breast carcinoma mixed grade I | Malignant neoplasm, characterized by tubular proliferation of cells with low nuclear pleomorphism, large nuclei, central nucleolus and scarce cytoplasm. Presence of cysts and myoepithelial proliferation. Forming tangles with a myxoid appearance. | Cell with low pleomorphism | Large nuclei | Scarce cytoplasm | - | Baixo grau |

453 (-) No mitosis figures present

454

455

456 **Table 5.** Molecular characterization of established and non-established immunohistochemical
457 strains

| Cell lineage | Histopathological diagnosis | Established lineage | Immunohistochemistry |
|---|---|---|---|
| **SM12C** | Salivary gland carcinoma | No | CK5/6; AE1AE3 and p63; Ki67+ 40% cells; negative for CAM5 |
| **PF10C** | Breast carcinoma mixed grade II | No | Ki 67+ 30% cells |
| **LF12C** | Cystic solid ductuolar apocrine ductal carcinoma | No | Ki 67+ 20% cells |
| **LF13C** | Breast carcinoma mixed grade I | No | Ki67+ 8% cells |
| **PM13C** | Oral melanoma | Yes | Ki67+ 25% cells |
| **BF11C** | Cutaneous Lymphoma | Yes | CD3 e CD18<br>Ki67 + 90% of cells<br>Negative for MUM1 and C-Kit |
| **DF13C** | Cutaneous mastocytoma grade III | Yes | Ki67+ 10% cells |
| **MF10C** | Urothelial carcinoma | Yes | Ki67+ 40% cells |

| | | | |
|---|---|---|---|
| **PF11C** | Oral melanoma | Yes | Ki67 + 30% cells |
| **PF12C** | Breast carcinoma mixed grade I | Yes | Ki67 + 15% cells |

458

459

460 **Table 6.** Correlation between the ratios of success and failure in establishing long-term cell lines and
461 the histopathological grade of malignancy of tumors, patient staging, and tumor molecular profile
462 (ki67).

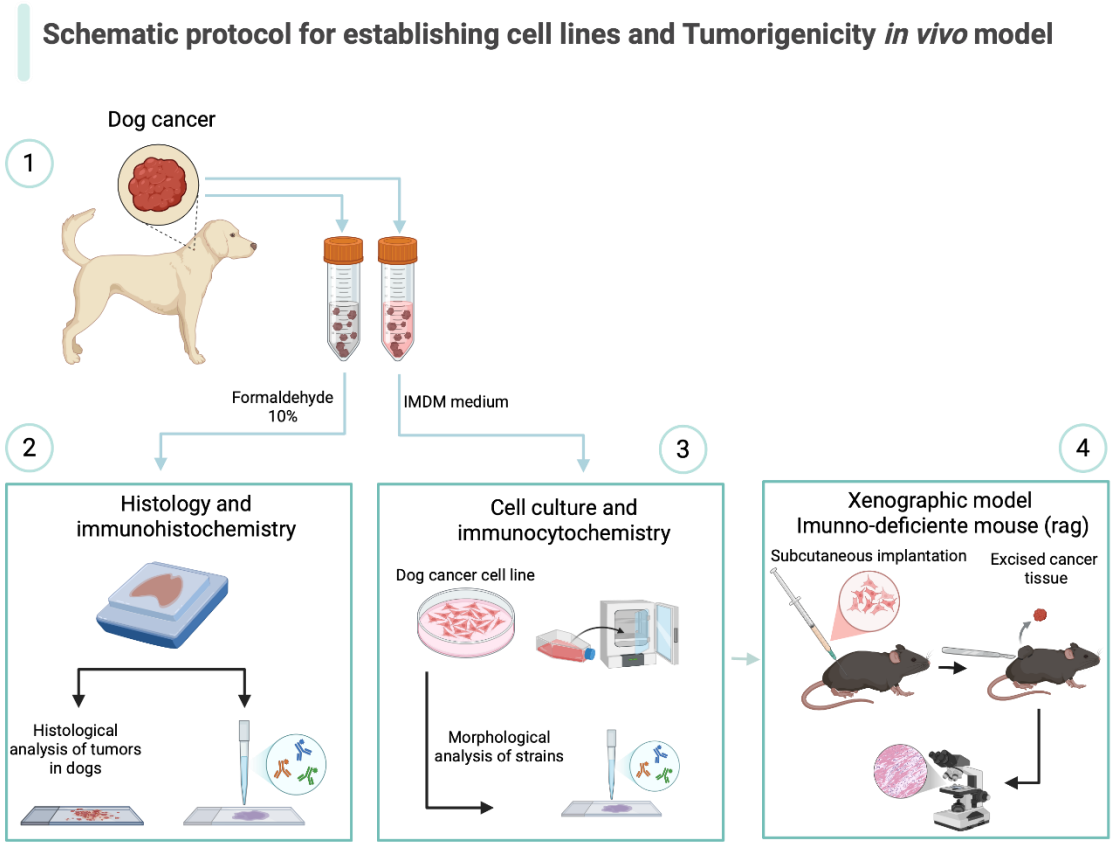
| | | **Lineages** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **Not Establisheds** | | **Establisheds** | | **Total** | | |
| | | n | % | n | % | n | % | **p value[1]** |
| **Ki67** | 0-20% | 2 | 33 | 2 | 33 | 10 | 100 | 0.0323 |
| | ≥ 20% | 2 | 33 | 4 | 66 | | | |
| **Clinical grade** | Low | 2 | 50 | 1 | 16 | 10 | 100 | <0.0001 |
| | High | 2 | 50 | 5 | 83 | | | |
| **Pathological grade** | I + II | 2 | 16 | 1 | 16 | 4 | 100 | <0.0001 |
| | III | 0 | 50 | 1 | 16 | | | |
| **Staging** | I | 2 | 0 | 0 | 0 | 10 | 100 | <0.0001 |
| | ≥ II | 2 | 50 | 6 | 100 | | | |

463 [1] Chi-square test.

**Table 7.** Correlation between the Ki67 molecular marker performed by immunohistochemistry, staging, histopathological grade and malignancy of the tumors.

| | | ki67 | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **0-20%** | | **≥20%** | | **Total** | | |
| | | n | % | n | % | n | % | **p value[1]** |
| **Clinical grade** | low | 2 | 50 | 1 | 16 | 10 | 100 | <0.0001 |
| | high | 2 | 50 | 5 | 83 | | | |
| **Pathological grade** | I + II | 2 | 50 | 1 | 16 | 4 | 100 | 0.0046 |
| | III | 1 | 25 | 0 | 0 | | | |
| **Staging** | I | 1 | 25 | 1 | 16 | 10 | 100 | 0.1606 |
| | ≥ II | 3 | 75 | 5 | 83 | | | |

[1] Chi-square test.




471

472 **Figure 1.** Figure 1. Schematic protocol for establishing cell lines and performing tumorigenicity in
473 an in vivo model 1) Tumors obtained by biopsy were placed in two labeled Falcon® tubes containing
474 complete IMDM culture medium (10% fetal bovine serum, penicillin, and streptomycin) and 4%
475 formaldehyde. 2) The paraformaldehyde-fixed samples were analyzed by histology and
476 immunohistochemistry. 3) The sample intended for cell culture was sectioned and treated with
477 complete IMDM for 10 minutes. The resulting suspension was centrifuged, and the precipitated cells
478 were resuspended in the same medium. The resulting cell suspension was then seeded in cell culture
479 flasks. The cultures were monitored daily (observed under a microscope) and kept in an incubator
480 at 37 °C and 5% CO2. After the lines were established, immunocytochemistry was performed. 4)
481 The cells were inoculated into immunodeficient animals and monitored until a palpable tumor
482 formed. After the development of the xenographic model, removal and histological and
483 immunohistochemical analysis were performed to confirm histogenesis.

484

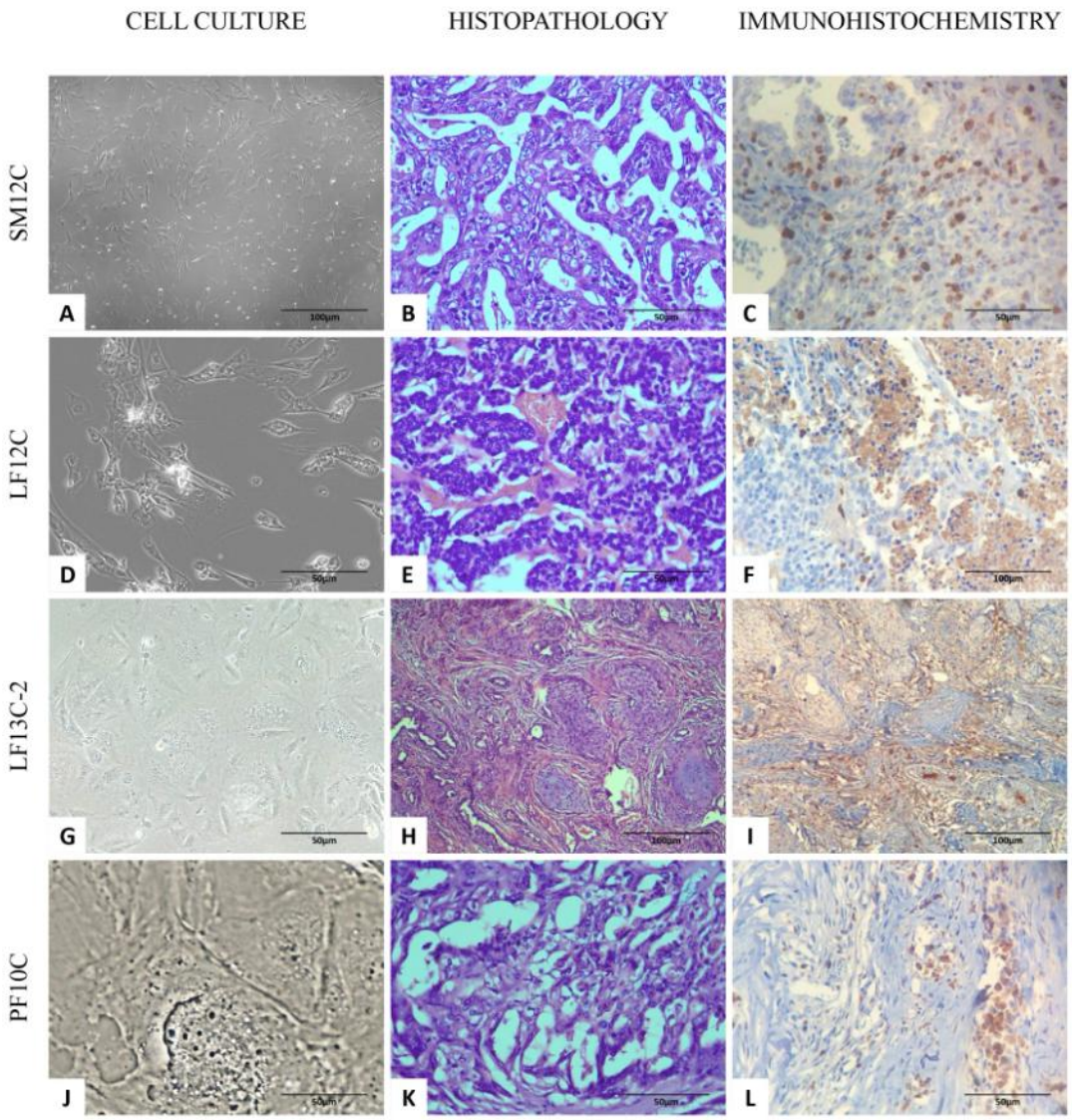



**Figure 2.** Non-established lineages. A) Salivary gland carcinoma (SM12C), morphology of the cells in culture, B) Histological sections of the tumor stained with hematoxylin and eosin for histopathological analysis and C) The cell proliferation marker Ki67 was positive in 40% of the cells. D) Apocrine ductuolar solid cystic carcinoma (LF12C) and morphology of the cells in culture, E) histology and F) Ki67 cell proliferation marker positive in 20% of the cells. G) Grade I mixed tumor breast carcinoma (LF13C-2) and cell morphology in culture, H) histology and Ki67 cell proliferation marker positive in 8% of the cells. J) Breast carcinoma in mixed tumor Grade II (PF10C) cell morphology in culture, K) histopathology and I) Ki67 cell proliferation marker positive in 30% of the cells.

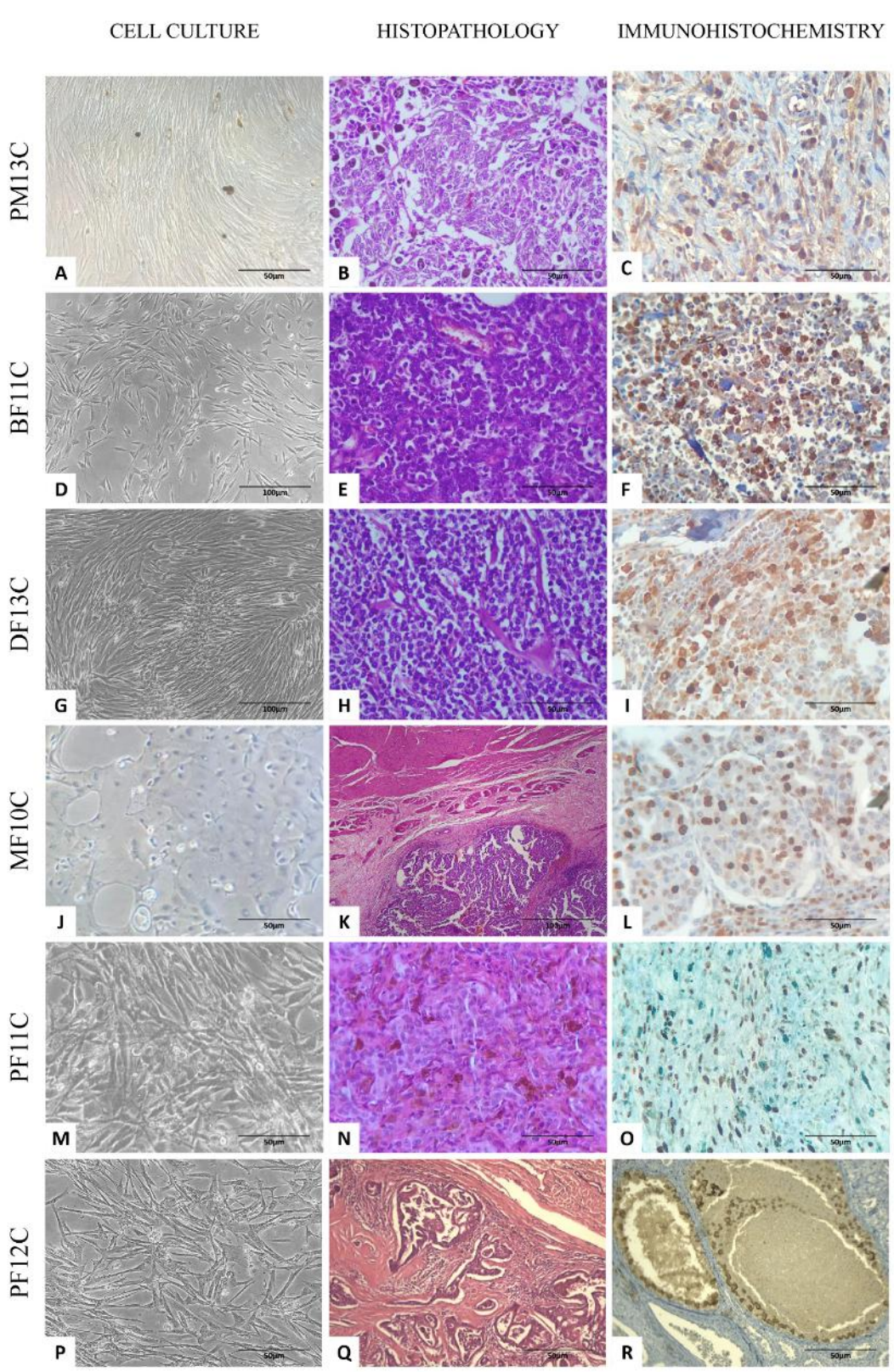



**Figure 3.** Established lineages. A) Oral melanoma (PM13C) cell morphology in culture, B) histology and C) cell proliferation marker Ki67 was positive in 25% of the cells. D) Cutaneous lymphoma (BF11C) cell morphology in culture, E) histology and F) cell proliferation marker Ki67 was positive

in 40% of the cells. G) Grade III cutaneous mast cell (DF13C) morphology in culture, H) histology and I) cell proliferation marker Ki67 was positive in 10% of the cells. J) Urothelial carcinoma (MF10C) cell morphology in culture, K) histology and L) Ki67 cell proliferation marker positive in 40% of the cells. M) Oral melanoma (PF11C) cell morphology in culture. N) histology and O) Ki67 cell proliferation marker positive in 30% of the cells. P) Grade I mixed breast carcinoma (PF12C) cell morphology in culture, Q) histology, R) Ki67 cell proliferation marker positive in 15% of cells.

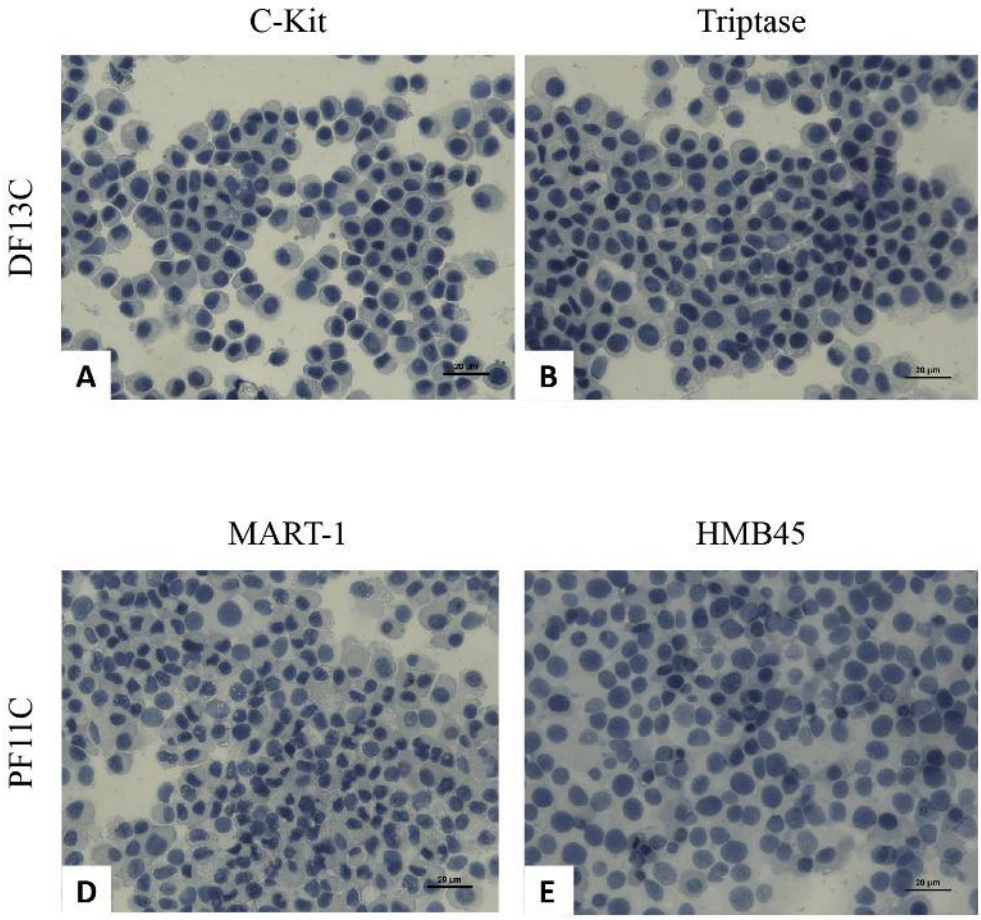



**Figure 4**. Immunolabeling of cells in culture. Cutaneous mast cell line (DF13C) after labeling with C-Kit A) and Tryptase B). Oral melanoma cell line (PF11C) labeled with MART-1 C) and HMB45 D). Both cell lines showed no positive labeling.

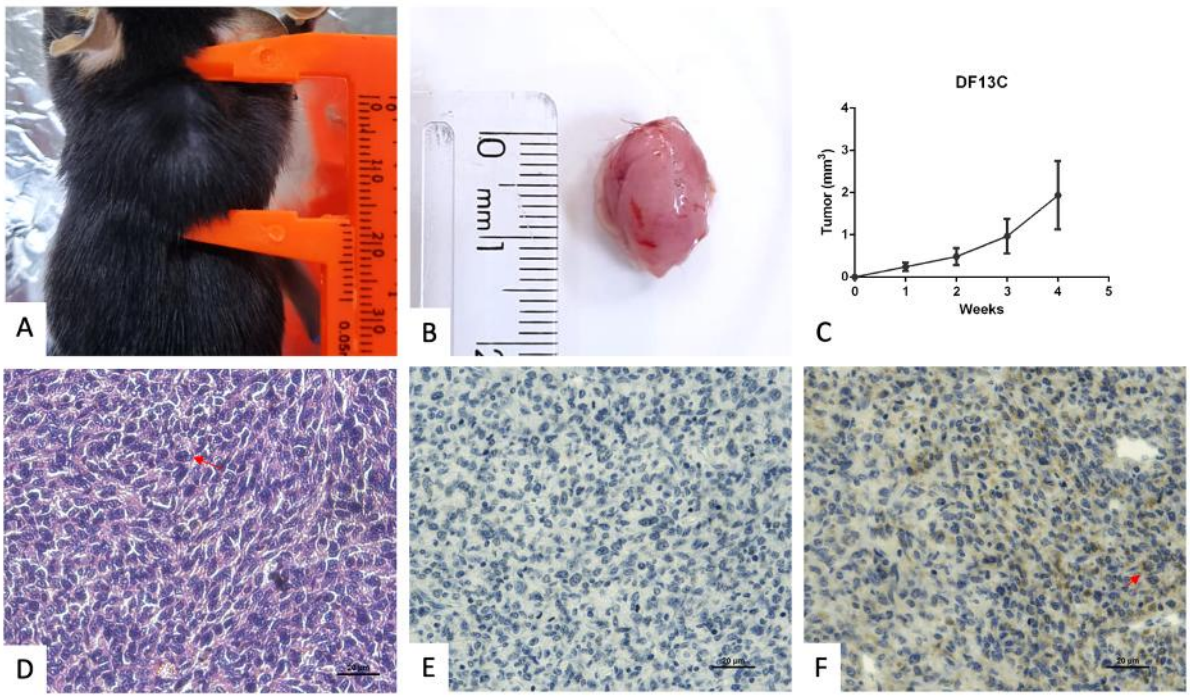
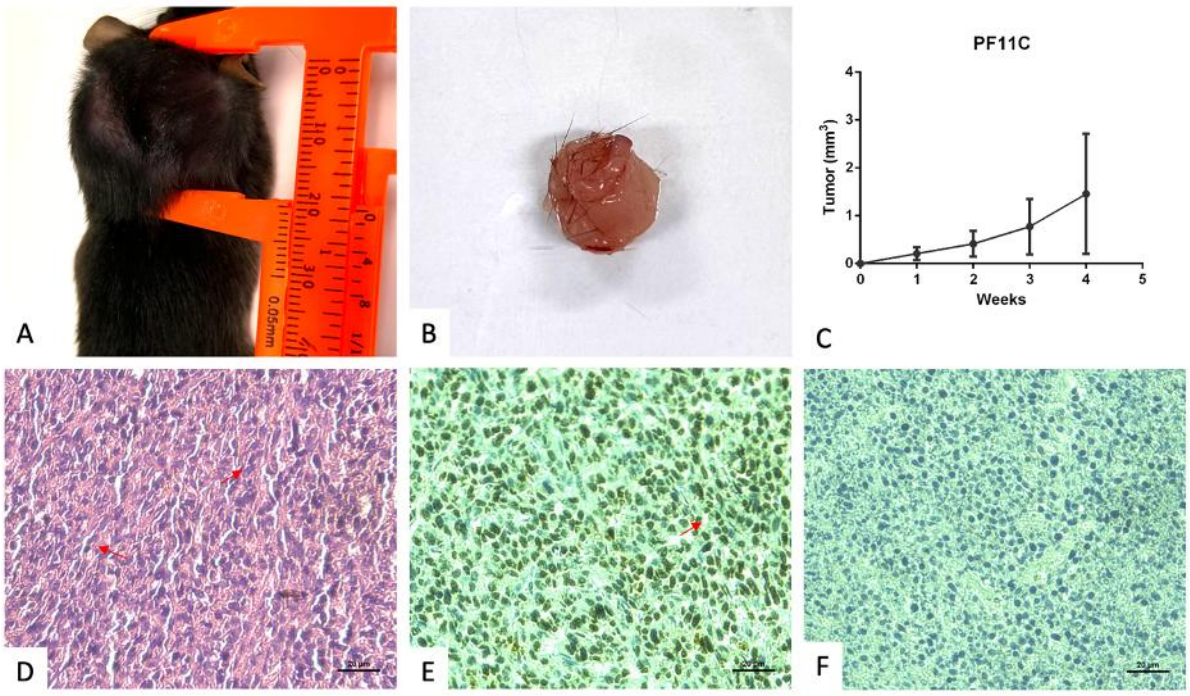



**Figure 5.** Mice inoculated with the DF13C strain. A) DF13C xenograft after 4 weeks. B) Solid tumor. C) Tumor growth curve in mm3. D) Histological section stained with HE showing fusiform morphology. E) Immunohistochemical staining with C-KIT showing weak staining. F) Immunohistochemical staining for tryptase showing weak positive staining.



519 **Figure 6.** Mice inoculated with the PF11C strain. A) PF11C xenograft after 4 weeks. B) Solid tumor.
520 C) Tumor growth curve in mm3. D) Histological section stained with HE showing ellipsoid
521 morphology. E) Immunohistochemical staining with MELAN-A showing weak positive staining. F)
522 Immunohistochemical staining with HMB-45 showing no staining pattern.
523