

Disodium Cromolyn and Anti-podoplanin Antibodies Strongly Inhibit Growth of BHK 21/C13-derived Fibrosarcoma in a Chick Embryo Chorioallantoic Membrane Model

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Abstract. *Aim: To characterize baby hamster kidney fibroblast (BHK 21/C13) cells and test the effects of antibodies against podoplanin and disodium cromolyn on BHK 21/C13 cell line-derived tumors grown on chick embryo chorioallantoic membrane (CAM). Material and Methods: BHK 21/C13 cell-derived fibrosarcomas developed in hamsters were implanted on CAM and treated with anti-podoplanin antibodies and disodium cromolyn. BHK 21/C13 cell immunophenotype was assessed. Results: Fibrosarcoma cells were positive for vimentin, CD117, smooth muscle actin, vascular endothelial growth factor epidermal growth factor receptor, homeobox prospero gene 1 and negative for platelet-derived growth factor B, neuron-specific enolase, S100, CD34, Ewing sarcoma and podoplanin. CAM-grown fibrosarcomas were highly sensitive to disodium cromolyn and anti-podoplanin antibodies. Conclusion: Immunophenotyping BHK 21/C13 cells and their response to drugs represent the first step in revealing cell line utility and a reliable tool for experimental cancer research.*

Experimental models are still powerful tools for medical research. *In vitro* and *in vivo* models are designed to help researchers in their work for understanding disease

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mechanisms and for the discovery and testing of new therapeutics which by their future clinical application may improve the prognosis and survival of patients with various diseases (1-3). Despite researchers' efforts to create a perfect experimental model for each disease, several issues remained unresolved (4). Ethics rules become more and more strict regarding the use of animals in experimental models (5, 6) and thus the development of alternative experimental models which lack the use of animals is a real challenge for the scientific world. Even though in the future microfluidic systems such as tumor/organ on a chip model (7, 8) or 3D-printed organs may be used to develop new experimental models (9, 10), cell lines will remain the main component of any experimental model. Countless options for the use of cell lines are available today. There are normal and tumor cell lines used for various purposes from vaccine production (11) to testing cell toxicity of different agents (12, 13).

Baby hamster kidney fibroblasts (BHK-21/C13) is a well-known cell line sensitive to various viruses such as herpesvirus (14), hepatic (15) or rabies virus (16), but not polyoma virus. These cells are able to undergo malignant transformation, with fast growing behavior due to rapid proliferation and invasion of adjacent tissues when they are subcutaneously injected into hamsters. A giant fibrosarcoma-like tumor without evidence of lymphatic or distant metastasis may develop at the site of inoculation. Few studies in the field of tumor experimental models used BHK-21/C13 cell line to obtain fibrosarcomas and even fewer to test the effects of different therapeutic agents.

The BHK-21/C13 cell line has not been characterized regarding their phenotype except for some old articles which reported the expression of fibroblast growth factor by BHK-21/C13 cells (17) and the effects of vascular permeability factor on their proliferation and migration (18, 19). Regarding BHK-21/C13 cell response to different therapeutic

agents, previous research was mainly focused on the inhibitory effects of potential or certified antiviral agents (20, 21). Other drugs with different properties such as antiallergic, antitumor or antiangiogenic actions, have not been tested yet on BHK-21/C13 cell-derived sarcomas as far as we are aware.

BHK-21/C13 cells have an unknown phenotype. Therefore, we proposed to immunohenotype tumor derived from BHK-21/C13 cells for markers with a potential prognostic role or which could be used as therapeutic target. Here we designed a combined experimental model [in hamsters and chick embryo chorioallantoic membrane (CAM)] which allowed us to study the ability of BHK-21/C13 cells to develop sarcomas and the reaction of chick embryo CAM-implanted sarcomas to bevacizumab, disodium cromolyn and anti-podoplanin antibodies, three therapeutic agents with controversial effects on tumor tissues.

Materials and Methods

Ethics and animal protection. All procedures involved animals were performed according to the present international guidelines recommended by European Union (Directive 2010/63/EU). The Ethics Commission of Victor Babes University of Medicine and Pharmacy approved all the laboratory and experimental procedures (No.5 /5821/26.05.2016).

Cell lines and culture procedure. BHK-21/C13 cell line was provided by American Type Culture Collection (Manassas, VA, USA). The cells were cultured according to the manufacturer's protocol and following the method previously described by Lalosevic et al. (22).

Inoculation and tumor development in Syrian hamsters. Cultured BHK-21/C13 cells were subcutaneously inoculated into 10 Syrian hamsters (6 weeks old, weight of 250 g). Two weeks later, a well-developed tumor mass was detected at the inoculation site. The tumor was characterized by fast growing behavior and invasiveness into the surrounding tissues without local or distant metastases.

Tissue processing and routine staining. Three weeks after initial inoculation, the hamsters were sacrificed and tumors were removed. Fresh tumor tissue of about 2 mm were collected for the future implant procedure on chick embryo CAM model. Remaining tumor was formalin fixed for about 24 hours and then paraffin embedded according to routine protocol. Three-micrometer sections were made and one of these was stained with hematoxylin and eosin method for morphological assessment. Based on evaluation of quality slide, specimens were selected for immunohistochemistry. The same tissue processing was also applied for the specimens collected from chick embryo CAM specimens (treated and untreated).

Chick embryo CAM model, and drug delivery template. Twenty-two fertilized chicken eggs were prepared for the development of the experimental model. Briefly, the eggs were incubated at 37°C for 72 hours in incubators with controlled temperature and humidity. On the fourth day of incubation, 2 ml of albumen was removed from each specimen and the CAM was made visible by making a window in

the superior part of the egg shell. On the seventh day of incubation, the chick embryo CAM was ready for implantation of BHK-21/C13-derived fibrosarcoma tissue collected from the tumor previously obtained from the hamster model. Two-milimeter-thick tumor piece was implanted inside a silicon ring previously fixed on the chick embryo CAM. Eggs were organized into three groups for testing therapeutic agents (bevacizumab, disodium cromolyn and anti-podoplanin antibodies). A control group (four eggs) received saline solution, while fibrosarcomas implanted in another 18 eggs were treated with bevacizumab, disodium cromolyn or anti-podoplanin antibodies (2 µl each day for 5 days on six specimens each, at a concentration of 100 µg/ml). Treated and control specimens were observed daily under stereomicroscopy and, at the end of day 5 of the treatment, the chick embryo CAM was fixed *in ovo* with 10% buffered formalin for 1 hour. It was then paraffin-embedded and examined by immunohistochemistry following the protocol described below. A brief overview of the procedure from hamster inoculation to chick embryo CAM implantation is summarized in Figure 1.

Immunohistochemistry. Three-micrometer sections were loaded in a Bond Max Autostainer previously scheduled to perform a simple immunostaining procedure with step by step program provided by the manufacturer (Leica Microsystems, Medist Life Sciences, Bucharest, Romania). Briefly, this program included a dewaxing step followed by antigen retrieval and incubation with primary antibodies (30 minutes at room temperature) selected for the present study and detailed in Table I. Visualization of the final product for each antibody was performed using Bond Refine Detection System Brown specific for Bond Max Autostainer. The workflow also included an automated mounting procedure using Leica Permanent Mounting (Leica Microsystems).

Image acquisition and data interpretation. An Axio Zoom A2 Research Microscope (Zeiss, Munchen, Germany) was used for the evaluation of routine and immunohistochemically stained slides. This system allowed us to capture and process microscopic images and to assess blood vessel changes around and inside the treated and untreated specimens.

Results

The tumor expanded rapidly into the tissue of the Syrian hamsters. Two weeks after initial inoculation, a macroscopically well-defined apparently encapsulated tumor mass was observed but when we tried to remove it, we found that it was highly and deeply invasive into surrounding tissues (Figure 1a and b). Microscopically, the specimens collected from hamster had a fibrosarcoma morphology, being composed of closely packed spindle-shaped highly mitotic cells, and scant cytoplasm, including elongated nuclei and variable nucleoli (Figure 1c). Specimens harvested from this tumor were implanted onto the surface of chick embryo CAM. Fibrosarcoma tumor volume increased rapidly and the tumor became highly vascularized (Figure 1d) with the acquisition of blood vessels from adjacent chick embryo CAM. Blood vessel acquisition was confirmed by stereomicroscopy (Figure 1e) and by the assessment of histological specimens (Figure 1f).

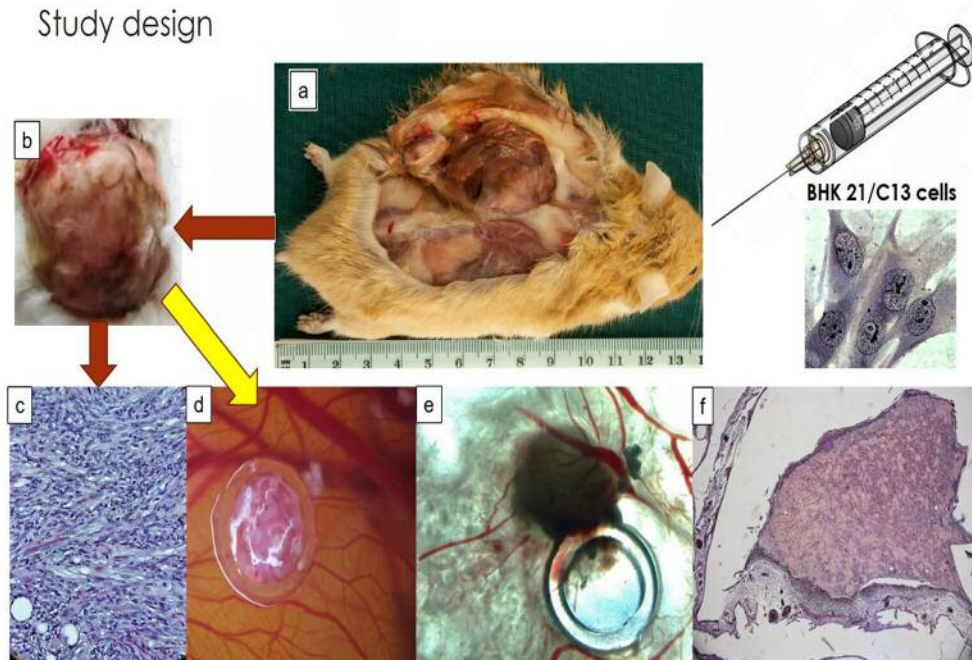


Figure 1. Experimental study design. Two weeks after subcutaneous inoculation of BHK 21/C13 cells in Syrian hamsters, a huge tumor mass was clinically and macroscopically detected, with a well-vascularized surface and deeply infiltrative behavior (a). Small biopsies from the tumor mass (b) were collected, some of them being paraffin-embedded for histopathological evaluation (c). Fresh tissue from the tumor was implanted on chick embryo chorioallantoic membrane (CAM) (d, e) and treated with three different drugs. Tissues were processed by routine histopathological methods followed by staining with hematoxylin and eosin for preliminary evaluation of tumor biopsies from CAM (f). Original magnification, c: $\times 200$, f: $\times 100$.

Table I. Antibodies, dilutions and working system used for immunohistochemistry.

Antibody	Manufacturer	Clone	Dilution	Working system
Vimentin	Novocastra	V9	Ready to use	Fully automated with Bond Autostainer, Bond Polymer Refine Detection System, diaminobenzidine (Leica Microsystems, UK)
CD34	Novocastra	QBEnd10	Ready to use	
Podoplanin	Dako Cytomation	D2-40	Ready to use	
S100 protein	Novocastra	Polyclonal	Ready to use	
CD117	Novocastra	C KIT	Ready to use	
SMA	Novocastra	1A4	Ready to use	
Ki67	Novocastra	MIB 1	Ready to use	
PROX1	Reliatech	Polyclonal	1:400	
VEGF	Dako Cytomation	VG 1	Ready to use	
EGFR	Dako Cytomation	Polyclonal	Ready to use	
PDGF BB	Reliatech	Polyclonal	1:200	

SMA: Smooth muscle actin, PROX1: homebox prospero gene 1, VEGF: vascular endothelial growth factor, EGFR: epidermal growth factor receptor, PDGF-BB platelet-derived growth factor B.

Accurate immunophenotyping of the fibrosarcomas grown in hamsters was performed before implantation on chick CAM. As expected, all tumor cells were positive for vimentin. Vimentin expression was accompanied by a strong immunoreaction for CD117 in the tumor cells with a relatively high proliferation rate as evaluated by Ki67. CD34

expression was restricted to the endothelial level of tumor blood vessels, while tumor cells were totally negative. Smooth muscle actin (SMA)-positive tumor cells were present inside fibrosarcoma, isolated or in an island-like distribution around tumor blood vessels, giving the appearance that they emerged from perivascular cells. Two

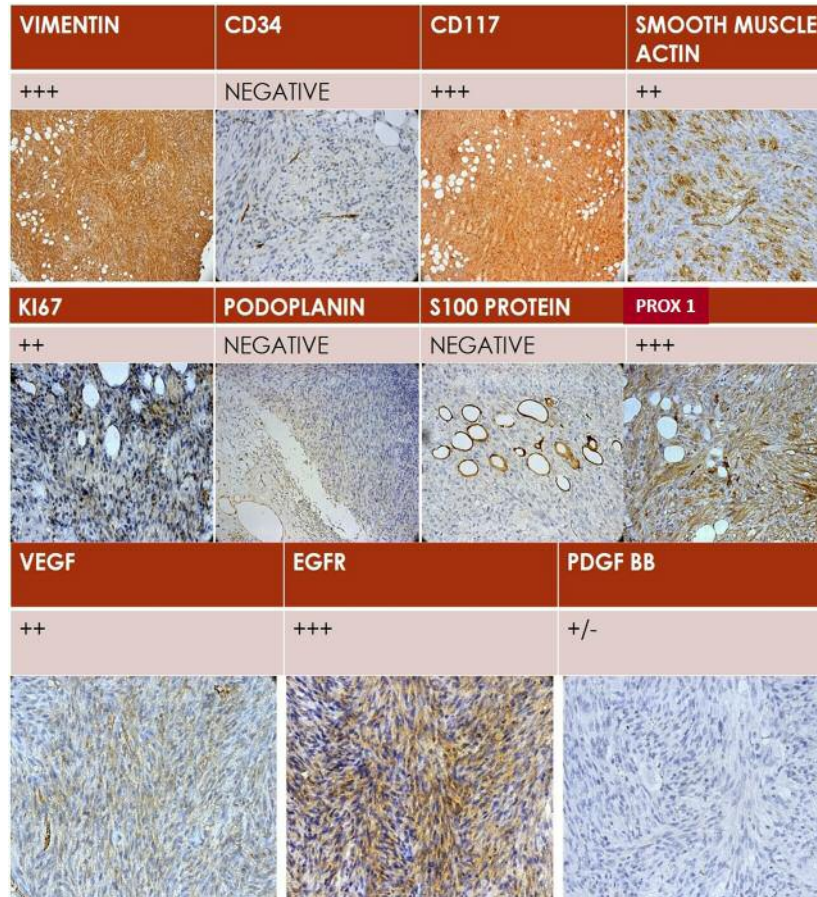


Figure 2. Immunoprofile of BHK 21/C13 cell-derived fibrosarcoma. CD34: Transmembrane phosphoglycoprotein specific for endothelial cells; CD117: mast/stem cell growth factor receptor, proto-oncogene c-Kit or tyrosine-protein kinase KIT; PROX1: homebox prospero gene 1; VEGF: vascular endothelial growth factor; EGFR: epidermal growth factor receptor; PDGF BB: platelet-derived growth factor BB. Original magnification, $\times 200$.

lymphatic markers, podoplanin (D2-40) and homebox prospero gene 1 (PROX1) have significant value in the evaluation of any malignancy for the assessment of tumor lymphangiogenesis and of tumor cells expressing podoplanin or PROX1. BHK-21/C13 cell-derived fibrosarcoma was characterized by a podoplanin-negative/PROX1-positive tumor cell immunophenotype. Podoplanin highlighted lymphatic vessels in the peritumoral tissue by labeling of lymphatic endothelial cells. No intratumoral lymphatic vessels were detected inside the tumor mass. PROX 1 immunoexpression was restricted to fibrosarcoma cells with nuclear and cytoplasmic pattern. Amongst three growth factors, vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) and platelet derived growth factor BB (PDGF BB), EGFR had the highest expression with a consistent distribution inside the cytoplasm of fibrosarcoma tumor cells. VEGF highlighted tumor blood vessel endothelium with a high intensity and

with a weak to moderate and inconstant expression inside tumor cells. Staining for PDGF BB was mostly negative, with scattered cells around tumor blood vessels showing a scant positive reaction (Figure 2).

During the next step of evaluation of BHK-21/C13 cell-derived fibrosarcoma on chick embryo CAM, the implanted tumors were treated with three different drugs: disodium cromolyn (a well-known mast cell stabilizer), bevacizumab (Avastin) and anti-podoplanin antibodies. We focused on their effects on tumor cells and vascular network. Fibrosarcoma cells reacted to each of these drugs in a specific and different manner. Disodium cromolyn, induced massive necrosis of the fibrosarcoma grown on chick CAM, but did not influence the blood vessels surrounding the tumor implant (Figure 3a and b). Despite the total necrosis observed for the initial implant, disodium cromolyn favored migration of tumor cells along the blood vessels of the CAM. This suggested an increase of tumor cell invasiveness

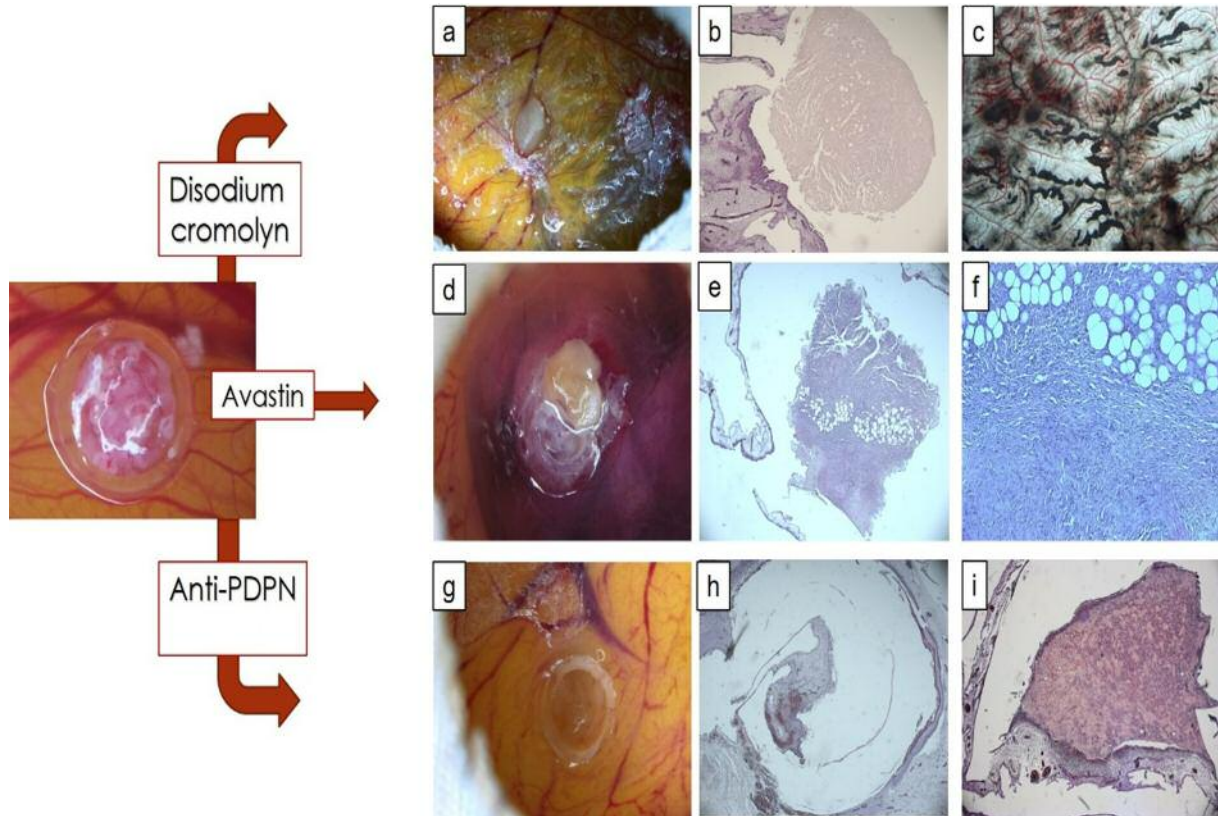


Figure 3. Comparative assessment of chick chorioallantoic membrane (CAM) implants of BHK 21/C13 cell-derived fibrosarcoma treated with disodium cromolyn (a-c), Avastin (bevacizumab) (d-f) or anti-podoplanin (PDPN; g-i). Macroscopic view of the specimens revealed strong differences regarding tumor mass and vascularization (a, d, g). The strongest inhibition of tumor mass was reported for anti-PDPN antibodies (h), followed by disodium cromolyn (b) and Avastin (e). Despite the strong effect of disodium cromolyn on fibrosarcoma implanted on CAM, this drug was responsible for the appearance of distant metastases (c). A high inflammatory infiltrate was also noted for implants treated with anti-PDPN antibodies. Lipomatous differentiation around the implant (f). Original magnification, a-e, g-i: $\times 100$, f: $\times 200$.

and migration after disodium cromolyn treatment. In contrast, bevacizumab had no discernable effects on tumor cells, despite the previous observation of VEGF expression in fibrosarcoma tumor cells. Weak lipomatous changes inside the implanted tumor were observed (Figure 3d-f). The highest regression of implanted fibrosarcoma was noted for the specimens treated with anti-podoplanin antibodies. The tumor volume was dramatically reduced macroscopically (Figure 3g) and microscopically (Figure 3h) after anti-podoplanin treatment. Compared with the other two drugs, anti-podoplanin treatment was followed by activation of the chick CAM immune system (not active under normal conditions) followed by a strong inflammatory response inside and around the implanted fibrosarcoma (Figure 3i).

Regarding the effects of the three drugs on the vascular network around the fibrosarcoma implants, it was noticed that disodium cromolyn stimulated the development of peritumoral blood vessels (accompanied by extensive migration of tumor

cells along them), while bevacizumab partially induced a decrease of their density around the implant. Anti-podoplanin antibodies also reduced the number of peritumoral blood vessels, despite a high degree of inflammatory infiltrate noted around and inside the implant.

Inflammation was present for specimens treated with disodium cromolyn and anti-podoplanin antibodies but was lacking from the specimens treated with bevacizumab. The highest inflammatory infiltrate was noted for anti-podoplanin-treated specimens followed by those treated with disodium cromolyn, where scattered small patches of inflammatory cells were noted between peritumoral blood vessels of the CAM.

Discussion

BHK 21/C13 are versatile cells, subclone 13 being derived from the kidneys of five unsexed, 1-day-old hamsters. These cells have a high proliferative rate, as we also observed in

our study during development of fibrosarcoma both in hamster and chick CAM model. They are highly adherent cells used in molecular biology and virus-related studies, especially to produce veterinary rabies vaccines (22). But BHK 21/C13 cell line is not exclusively used for vaccine production. In the early 1970s, BHK 21/C13 cell cultures were used for testing different therapeutic agents such as colchicine (23), bleomycin (24) and other chemotherapeutic drugs (25). During these tests, it was observed that BHK 21/C13 cells are highly resistant, few cytostatic drugs being effective. This may suggest that more targeted therapies should be tested on such types of cell lines. We proposed and tested here for the first time the effects of bevacizumab, disodium cromolyn and anti-podoplanin antibodies on fibrosarcoma developed from BHK 21/C13 cells in chick embryo CAM model. Despite their therapeutic resistance to drugs, fibrosarcoma cells implanted on chick CAM showed a high sensitivity for two out of the three agents used in the present studies, rarely disodium cromolyn and anti-podoplanin. Disodium cromolyn has been tested on other aggressive malignant cells such as melanoma (26), exhibiting similar effects regarding tumor cell necrosis and stimulation of vascular network development. Compared with the effects on malignant melanoma cells, disodium cromolyn seems to favor the invasiveness and migration of fibrosarcoma tumor cells in close association with blood vessel development. No data have been reported to our knowledge regarding the potential mechanism of this migration of tumor cells along the tumor blood vessels mediated by the action of disodium cromolyn in fibrosarcoma cells, nor for other malignant cells.

Podoplanin-negative fibrosarcoma cells were highly sensitive to anti-podoplanin antibodies. This discrepancy may be due to high glycosylation of podoplanin in tumor cells previously reported in glioblastoma cell line LN229 (27). This aberrant glycosylation of podoplanin may give negative immunohistochemical findings despite podoplanin presence inside the fibrosarcoma cells due to aberrant glycosylation not being recognized by usual anti-podoplanin clone D2-40. This may be an explanation for massive necrosis of tumor cells in specimens treated with anti-podoplanin.

The present study may be considered as the first attempt to characterize the immunophenotype of BHK-21/C13 cells. Of the 11 markers used in the present study to define the immunophenotype of this cell line, four of them had potential impact on the future use of these cells. Tyrosine kinase receptor CD117 (c-KIT), intensely expressed in BHK-21/C13 cell-derived fibrosarcoma, is a well-known target for imatinib mesylate and future studies may therefore be able to use these cells to test new drugs with a similar target. Currently the expression of this tyrosine kinase receptor in fibrosarcoma is questionable. The presence of CD117 in

fibrosarcomas was rarely reported in the literature (29). In cats with fibrosarcomas, the presence of CD117 was not correlated with survival or histological grade (29). Indirectly evidence suggested that rat kidney fibroblasts expressed a glycosylated form of CD117, namely s-KIT (30). Because of their early embryologic origin, it seems that BHK-21/C13 cells retain CD117 expression specifically for cells with pluripotency features. The persistence of CD117 in BHK-21/C13 fibroblasts supports the aggressiveness of these cells, this aspect being previously reported for CD117-positive fibroblasts-like stromal cells present in ovarian cancer stroma of patients with a unfavorable clinical outcome (31). Moreover, their ability to differentiate into smooth muscle actin-positive cells during fibrosarcoma development (reflected by the expression of SMA) supports the fact that these cells are not fully differentiated and still have an immature phenotype.

Aggressiveness and immaturity of BHK-21/C13 fibroblasts are also supported by the expression of PROX1 in tumor cells with both nuclear and cytoplasmic localization. During embryonic life, PROX1 is expressed in neural crests and is a marker of endodermal compartment. Renal development depends on PROX1 expression, especially at the level of Henle loop, most probably due the interaction between epithelial and stromal cells (32). In a zebrafish model, it seems that PROX1 has a dual role in renal development: after its initial roles in the specification of inter-renal primordium, it is critical for the maturation of the inter-renal organ (33). Malignant transformation is followed by up-regulation of *PROX1* in tumor cells reported for several types of cancer, such as gastric (34, 35), pancreatic (36), vascular (37) and renal (38) cancer. For all these cancer types, PROX1 overexpression is related to high aggressiveness and poor prognosis (35). PROX1 distribution and expression in BHK-21/C13 fibrosarcoma may be considered a promoter of aggressive behavior of these cells, clinically detected during the development of the tumor in both hamster and the chick embryo CAM model.

Weak and inconsistent expression of VEGF was related to lack of response to bevacizumab in the chick embryo CAM model. A previous report suggested that VEGF secreted by human fibrosarcoma cells promotes and sustains distant metastases after inhibition of primary tumor (39). We observed a similar phenomenon of development and persistence of distant metastases in the specimens treated with disodium cromolyn inhibition of primary tumor but not in those treated with bevacizumab. High EGFR expression of BHK-21/C13 fibrosarcoma is in concordance with previous data showing EGFR expression and modulation in soft-tissue sarcomas cell lines *in vitro* and *in vivo* by a combination of gefitinib and doxorubicin (40). These findings support the use of BHK-21/C13 fibroblasts in future research of tumor cell behavior after anti-EGFR agents.

Conclusion

Here we established the immunophenotype of BHK-21/C13 fibroblast-derived fibrosarcoma demonstrating that these fibroblasts represent a particular cell line with vimentin+/CD34-/CD117+/PROX1+/podoplanin-/EGFR+ phenotype, suggesting highly aggressive behavior based on several molecular peculiarities not previously described for this cell line. The heterogeneous response to disodium cromolyn, bevacizumab and anti-podoplanin support the use of these cells for the future evaluation of other new targeted therapies.

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Conflicts of Interest

The Authors have no conflict of interests to declare in regard to this study.

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