Simvastatin exhibits antiproliferative effects on spheres derived from canine mammary carcinoma cells

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Abstract. Mammary cancer is the most frequent type of tumor in the female canine. Treatments are mainly limited to surgery and chemotherapy; however, these tumors may develop clinical recurrence, metastasis and chemoresistance. The existence of a subpopulation of cancer cells with stemness features called cancer stem-like cells, may explain in part these characteristics of tumor progression. The statins, potent blockers of cholesterol synthesis, have also shown antitumor effects on cancer mammary cells, changes mediated by a decrease in the isoprenylation of specific proteins. Few studies have shown that simvastatin, a lipophilic statin, sensitizes cancer stem-like cells eliminating drug resistance. The aim of the present study was to evaluate the effects of simvastatin on spheres derived from CF41.Mg canine mammary tumor cells, which were characterized by phenotypic and functional analyses. Spheres exhibited characteristics of stemness, primarily expressing a $CD44^{+}/CD24^{-/low}$ phenotype, displaying auto-renewal and relative chemoresistance. Exposure to simvastatin induced a decrease in the sphere-forming capacity and cell viability, accompanied by a concentration- and time-dependent increase in caspase-3/7 activity. In addition, modulation of β-catenin and p53 expression was observed. Simvastatin triggered a synergistic effect with doxorubicin, sensitizing the spheres to the cytotoxic effect exerted by the drug. Invasiveness of spheres was decreased in response to simvastatin and this effect was counteracted by the presence of geranylgeranyl pyrophosphate. Our results suggest that simvastatin targets canine mammary cancer stem-like cells, supporting its therapeutical application as a novel agent to treat canine mammary cancer.

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Introduction

Mammary cancer accounts for ~30% of all cancers observed in female dogs, displaying pathological and clinical heterogeneity (1) and exhibiting several similarities with breast cancer in humans (2,3). Approximately 50% of the tumors are malignant (4-6). To date, surgery is the treatment of choice for this disease; however, this therapeutic option is not feasible in the case of unresectable or extensive metastatic tumors (4,5). Currently, there is no effective therapy since some tumor cells may acquire resistance to commonly available drugs (7). A possible explanation for this situation is the presence of cancer stem-like cells (CSCs) within the tumor mass; CSCs exhibit self-renewal, resistance to several antitumor treatments such as chemotherapy and radiotherapy and possess tumor-initiating capacity (7,8). Several signaling pathways have been identified as relevant for maintaining the capacity of self-renewal and pluripotency of CSCs, including Wnt/β-catenin, hedgehog and Notch. The canonical Wnt/β-catenin pathway regulates several cell activities such as proliferation, migration and self-renewal, important features of CSCs (9). The chemoresistance appears to be related with the expression of ATP-binding transporters such as MDR, assuring efflux capability to CSCs (7,10). Mammary CSCs are characterized by a low expression of heat stable antigen CD24 and a high expression of hyaluronan receptor CD44 (7,9). The expression of other surface markers, such as CD133 and aldehyde dehydrogenase (ALDH), has been detected in these cells (9,11). Mammary CSCs exhibit the capacity to form spheres, structures that grow from disaggregated solid tumors cultured under harsh conditions in which only the more undifferentiated cells can survive and proliferate (7,8).

The statins are a group of commercially available therapeutic drugs used for the reduction in the circulating levels of cholesterol (12). These drugs exhibit additional effects, including an apparent prevention of the abnormal growth of tissues such as the prostate and mammary gland (12-15). In humans, several studies have suggested a link between statin use and a decrease in overall cancer incidence (15); however, other groups describe no association between the use of these drugs and a reduction in cancer risk (16). These controversial findings require further research to specify the role of statins in cancer. In veterinary medicine, no data on this topic have

been published making the generation of information vital. Statins reduce serum cholesterol levels by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) preventing the synthesis of cholesterol (13-15). The inhibition of HMG-CoAR causes a deficit in mevalonate, decreasing the formation of isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP); these participate in protein isoprenylation, a process that provides lipid attachment sites for some proteins, allowing them to participate in the regulation of cell survival and migration (17,18). In addition, it has been described that the effects of statins may occur via other mechanisms, such as reduction in the expression of CD44 via a transcriptional mechanism, non-related with HMG-CoAR (19). Thus, the precise mechanisms by which statins prevent mammary carcinogenesis are not completely understood; however, the participation of cell cycle-regulating proteins appears to be essential for this effect. Lipophilic statins such as simvastatin and lovastatin show antiproliferative, antimetastasic and pro-apoptotic effects in mammary tumor cells (20-22). These effects seem to be more powerful in estradiol receptor (ER)-negative breast cancer cells with permanently activated Ras or ErbB2 (mutated epidermal growth factor receptor) (23-25). Due to the high expression of CD44 in CSCs, we investigated the effect of statins on CSCs derived from canine mammary tumor cells. It should be mentioned that Gopalan *et al* recently demonstrated that simvastatin and γ-tocotrienol alone or in mixture decreased CSCs in drug-resistant human breast tumor cells (26); these observations appear to be related to the antiproliferative effect induced by simvastatin on karyotypically abnormal embryonic stem cells (27).

Based on the above-described data, it was imperative for us to explore new cytotoxic strategies to increase the efficacy of antitumor therapies on mammary CSCs, particularly in veterinary oncology. Therefore, in the present study, we characterized spheres derived from a canine mammary carcinoma cell line, analyzing the effects of simvastatin on sphere-forming capacity, cell viability, apoptosis and cell invasion. In addition, we explored various proteins (β-catenin and p53) associated with clonogenic ability and cell survival in response to simvastatin.

Materials and methods

Materials. Cell culture material was obtained from Nalge Nunc (Rochester, NY, USA). Ultra-low attachment plates were purchased from Corning (Corning, NY, USA). TGFβ, simvastatin, GGPP and paclitaxel were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Doxorubicin was purchased from Tocris Bioscience (Ellisville, MO, USA). CellTiter 96 Aqueous One Solution Cell Proliferation Assay and Apo-One Homogeneous Caspase-3/7 assay were purchased from Promega Corporation (Madison, WI, USA). ApopTag Peroxidase *In Situ* Apoptosis Detection kit was obtained from Millipore Co. (Billerica, MA, USA). BD BioCoat was obtained from BD Biosciences (Bedford, MA, USA). Most of the other biochemicals used were purchased from Sigma-Aldrich Inc. and Gibco by Life Technologies (Carlsbad, CA, USA).

Antibodies. APC rat anti-mouse CD44 clone IM7 (559250), PE rat anti-mouse CD24 clone M1/69 (553262) and mouse anti-β-catenin (610154) monoclonal antibodies were obtained from BD Pharmingen (San Jose, CA, USA). Anti-mouse CD133 monoclonal antibody clone 13A4 (14-1331) was obtained from eBioscience (San Diego, CA, USA). Polyclonal goat anti-p53 (sc-1311) and polyclonal rabbit anti-pp53 (sc-7997) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). FITC-conjugated goat anti-rat IgG (PA1-28775) was purchased from Thermo Scientific (Rockford, IL, USA). Mouse monoclonal anti-β-actin antibody (ab8226) was from Abcam (Cambridge, UK). Peroxidase-conjugated rabbit anti-goat IgG (A5420), peroxidase-conjugated goat anti-rabbit IgG (A6667) and peroxidase-conjugated goat anti-mouse IgG (A9917) were purchased from Sigma-Aldrich Inc.

Cell line. CF41.Mg epithelial cells from canine mammary cancer tissue (CRL-6232; ATCC, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. In each of the experiments, the cells were cultured at 37˚C in a humidified incubator, in a 5% CO_2 atmosphere.

Sphere formation assay. These experiments were performed as describes by Cocola *et al* (8). In brief, cultured CF41.Mg cells were detached and re-suspended in ultra-low attachment plates with serum-free DMEM/F12 culture medium containing 10 ng/ml bFGF, 10 ng/ml EGF, 5 μg/ml insulin, 4 μg/ml heparin, B27 and 20 μg/ml penicillin, 20 μg/ml streptomycin and 0.05 μg/ml amphotericin B (sphere medium). To assess self-renewal capacity, cells derived from spheres were disaggregated and reseeded in 6-well ultra-low attachment plates. The spheres that formed were observed and photographed at low magnification every other day up to 7 days of culture. The effect of TGFβ (10 ng/ml) on the sphere formation efficiency was studied by counting at 7 days of culture at low magnification using an Olympus phase contrast microscope (CKX41 model; Tokyo, Japan).

Flow cytometric analysis. Cells derived from the spheres were dissociated with 0.25% trypsin-EDTA, washed and resuspended with phosphate-buffered saline (PBS) plus 2% FBS, and then incubated with specific labelled antibodies against CD44, CD24 and CD133 at 4˚C for 45 min, as described by Michishita *et al* (7). For CD133 detection, secondary FITC-conjugated goat anti-rat antibody was used. The cell populations were analyzed within 30 min by flow cytometry using a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA).

Cell viability studies. CF41.Mg cells (~5,000/cm^2) were seeded in ultra-low attachment plates and incubated for 24 h; then, the cells were incubated with several concentrations of doxorubicin and paclitaxel. After that, incubations with different concentrations of simvastatin (0-20 μM), in the absence or presence of 30 μM GGPP and doxorubicin were performed. In parallel, similar experiments on parental cells (grown in DMEM-high glucose plus 10% FBS) were run. At

the completion of the incubation period, cell viability was estimated using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous One Solution); MTS is reduced to formazan by viable cells. The quantity of formazan was measured by absorbance on a BioTek Synergy MX microplate reader at 490 nm (Winooski, VT, USA). In addition, the effects of simvastatin on sphere formation efficiency were conducted. Each experiment was performed at least 3 times in triplicate.

Caspase activity. Apoptosis was analyzed by measuring the activity of caspase-3/7 (Apo-One Homogeneous Caspase-3/7 assay), following the manufacturer's instructions. Briefly, 5,000 cells/cm^2 were seeded in the sphere medium without or with different concentrations of simvastatin, for 3-6 days. Caspase-3/7 activity was measured with a BioTek Synergy Mx microplate reader at an excitation wavelength of 485 nm and emission wavelength of 520 nm (Winooski, VT, USA). Caspase-3/7 activity was expressed relative to the number of total living cells.

TUNEL assay. The *in situ* staining of DNA strand breaks, a typical characteristic of end apoptotic cells was detected by the TUNEL (ApopTag Peroxidase *In Situ* Apoptosis Detection kit) assay according to the manufacturer's instructions. Cells derived from the spheres were exposed to 10 μM simvastatin for 3 and 6 days, fixed in alcoholic glyoxal for 6 h and sedimented in warmed 1% agarose. After solidification, the cell blocks were dehydrated, embedded in paraffin, cut (4 μm) and rehydrated. The sections were digested with proteinase K and endogenous peroxidase quenched. After several washes, the slides were incubated with TdT enzyme for 60 min in a humidified chamber at 37˚C. The samples were then washed and incubated with anti-digoxigenin conjugate for 30 min at room temperature. After extensive washes, the samples were developed with 3,3'-diaminobenzidine, counterstained with Mayer's hematoxylin, dehydrated and mounted with Eukitt (Foster City, CA, USA). Finally, the samples were inspected with an Olympus light microscope (FSX100 model) fitted with a color CCD camera. In each experiment, the images were captured under fixed settings of illumination, exposure times and camera gain.

Immunocytochemistry assays. Spheres were exposed to 10 μM simvastatin for 1, 6 and 16 h, fixed in alcoholic glyoxal for 6 h and sedimented in warmed 1% agarose. After that, the cell blocks were dehydrated, embedded in paraffin, cut and rehydrated. The sections were subjected to heat antigen retrieval in a microwave with 0.01 M citrate buffer (pH 6.0) for 20 min. After cooling at room temperature for 20 min, blocking of endogenous peroxidase was performed with 10% H_20_2. After several washes, the slides were blocked with 5% bovine serum albumin for 30 min in a humidified chamber at room temperature. The samples were then washed and incubated with the anti-β-catenin antibody diluted 1:100 at 4˚C overnight. Then, the sections were incubated with Vector Universal reagent anti-mouse/rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature, following the manufacturer's instructions. After 3 washes, the samples were developed with vector 3,3'-diaminobenzidine, counterstained with Mayer's hematoxylin, dehydrated and mounted with Eukitt. Finally, the samples were inspected with an Olympus light microscope (FSX100 model) fitted with a color CCD camera. In each experiment, the images were obtained under fixed settings of illumination, exposure times and camera gain.

Invasion assays. These were carried out using BD BioCoat™ Matrigel™ invasion chambers (Transwell® 8-μm pore size, 24-wells; BD Biosciences, Bedford, MA, USA). Cells in the sphere medium containing 5 μM simvastatin and/or GGPP were incubated for 48 h against a gradient of 5% FBS. Non-invading cells were wiped from the upper side of the filter, and the nuclei of the invading cells were stained with DAPI. After fixation with cold methanol, the nuclei were inspected by epifluorescence (Olympus FSX100). For each condition, 3 Transwell units were used in the experiments; 5 microscopic fields were counted/insert.

Western blot analyses. For total cell protein extraction, statin-exposed and control cells were washed and harvested by scraping with RIPA lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupetin and protease inhibitors. Cellular lysates were centrifugated and the protein content was determined by the Micro BCA™ assay (Thermo Scientific, Rockford, IL, USA). For electrophoresis, 40 μg protein samples was boiled for 5 min and loaded on 10-15% polyacrylamide gels. Electrophoresis was run using Bio-Rad's Mini-PROTEAN chambers. Bands were electro-transferred onto PVDF membranes. Immunodetection using appropriate primary antibodies and peroxidase labelled secondary antibodies, and development with enhanced chemiluminescence were carried out. Relative levels of total protein in each sample were determined by stripping the phospho-specific antibodies from the membrane and re-probing with antibodies against the non-phosphorylated proteins. The bands were analyzed with NIH's ImageJ software.

Statistical analyses. Student's t-test and ANOVA followed by *post hoc* comparisons of means were used to evaluate differences between samples and the respective controls. $p<0.05$ was considered to indicate a statistically significant result. Data were analyzed with InfoStat for Windows Software, AR.

Results

The culture of the CF41.Mg (parental) cells under anchorage-independent conditions and the absence of FBS allowed the isolation of spheres. These cell formations showed some characteristics of stemness, such as the preference for expression of the CD44$^+$/CD24$^{-/low}$ phenotype; the cells, however, did not displayed CD133, a surface molecule frequently present in CSCs. The majority of parental CF41.Mg cells expressed high levels of CD44$^+$/CD24$^{-/low}$ phenotype (91.1%), nevertheless spheres expressed a higher amount of CD44 (Fig. 1).

Cells derived from the spheres were disaggregated and seeded; they showed self-renewal capacity, a classical property of CSCs (Fig. 2A-D). Epithelial-mesenchymal transition

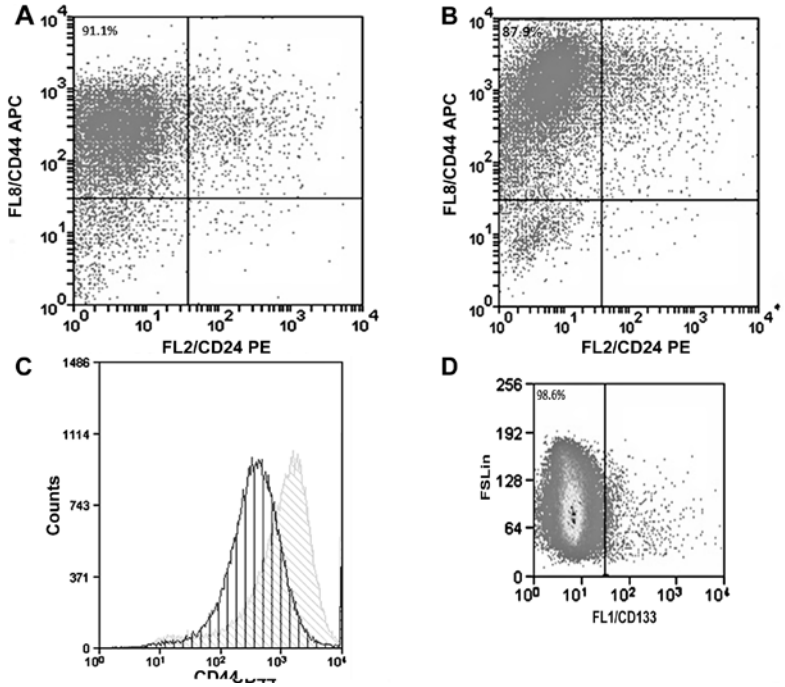

Figure 1. Flow cytometric analysis of the expression of CD44, CD24 and CD133 in CF41.Mg cells in culture. Expression of the $CD44^{+}/CD24^{-/low}$ phenotype in (A) CF41.Mg parental cells and (B) spheres derived from CF41.Mg cells. (C) Representative image that shows the overlay of CD44 expression in parental cells (gray curve) and spheres (light gray curve). (D) Expression of CD133 in the spheres. The mean percentage of cells expressing $CD44^{+}/CD24^{-/low}$ and $CD133^{-}$ is shown in each dot plot. Results are representative of 3 independent analyses.

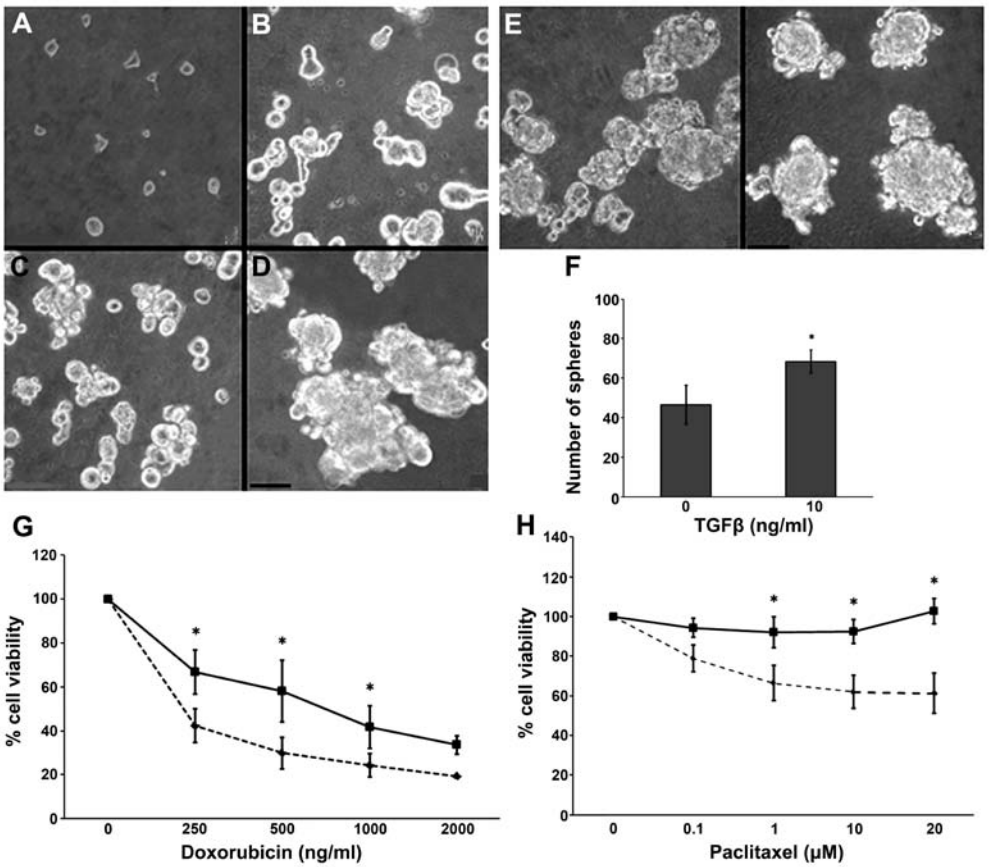

Figure 2. Functional assays of spheres derived from CF41.Mg cells in culture. (A-D) Sphere-forming capacity. Spheres were disaggregated in trypsin/EDTA and then cultured in ultra-low attachment plates for different times [(A) day 0, (B) day 1, (C) day 5, (D) day 7]. (E and F) Colony-forming ability in the presence of TGFβ. Representative image and histogram showing the number and morphology of the spheres in the presence of TGFβ after 7 days of culture. Values are mean ± SD of 3 independent experiments. $^{*}p<0.05$ compared with the non-stimulated control cells. Scale bar, 50 μm. (G and H) Cell viability assays in the presence of doxorubicin and paclitaxel. Spheres (solid line) and parental cells (dashed line) were treated with different concentrations of (G) doxorubicin and (H) paclitaxel for 72 h. Values are means ± SD of 3 individual experiments in triplicate. $^{*}p<0.05$ compared with the parental cells.

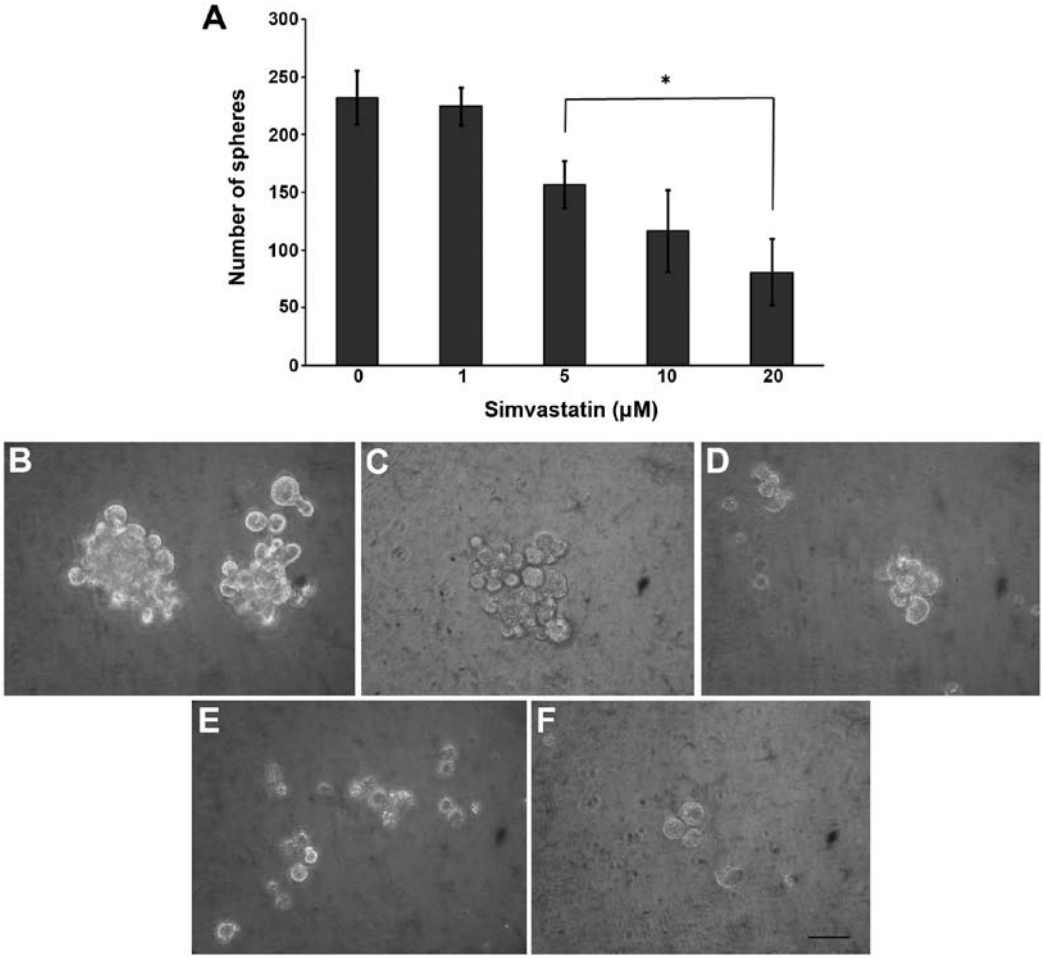

Figure 3. Sphere-forming capacity in response to simvastatin. CF41.Mg cells were cultured in ultra-low attachment plates. (A) Histogram and representative phase-contrast images showing the number and morphology of spheres in the presence of (B) 0, (C) 1, (D) 5, (E) 10 and (F) 20 μM simvastatin after 7 days of culture. Values are mean ± SD of 3 independent experiments. *p<0.05 for various between different concentrations of simvastatin and the control. Scale bar, 20 μm.

(EMT) is a process that can be elicited by TGFβ and increases the proportion of CSCs in certain tumors. We assessed this effect on the spheres by adding 10 ng/ml TGFβ to the culture medium. We obtained an increased number of spheres without any effect on their size (Fig. 2E and F). As compared to the parental CF41.Mg cells, the sphere-derived cells presented a relative drug resistance to doxorubicin and paclitaxel. The results from the resistance analyses are summarized in Fig. 2G and H.

Since simvastatin impairs tumor cells with high CD44 expression, we analyzed its potential antiproliferative effect on CSCs. The statin significantly decreased the sphere forming efficiency in a concentration-dependent manner (Fig. 3), reducing both the number and size. In response to simvastatin, we observed a reduction in β-catenin expression. It has been speculated that this protein complex is involved in the self-renewal ability of CSCs (Fig. 4).

Simvastatin decreased the cell viability in a concentration- and exposure time-dependent manner, reaching a maximum effect at 20 μM and after 6 days of contact with the drug. The spheres exhibited a relative resistance to simvastatin in comparison with the parental cells, which showed a high sensitivity to the statin. Representative graphs of these experiments are presented in Fig. 5A and B. The simultaneous addition of 30 μM GGPP efficiently blocked the effects of simvastatin on the spheres (Fig. 5C). To determine whether simvastatin exerts a synergistic effect with doxorubicin, we assessed the cell viability in spheres grown in the presence of both drugs. By adding 10 μM of simvastatin, the antiproliferative effect induced by doxorubicin was enhanced. This cytotoxicity was compared to the effects of statin alone at 6 days of culture (Fig. 5D).

Exposure to simvastatin for 72 and 144 h significantly induced the activity of caspase-3/7 in relation to the control (Fig. 6A). This increased activity was directly proportional to the concentration. In spite of no evident changes in the proportion of living cells in response to simvastatin during 3 days of exposure, an increase in caspase-3/7 activity was observed, suggesting the activation of apoptotic processes. Therefore, we evaluated apoptosis through the TUNEL method, based on the detection of DNA strand breaks characteristic of DNA fragmentation. For this purpose, spheres grown in presence of 10 μM simvastatin were included in cell blocks, and then cut and stained. The spheres exposed to simvastatin for 6 days exhibited a 45.8% increase in apoptotic cell death compared to the control condition in replicated experiments; the differences was statistically significant (Fig. 6B and C).

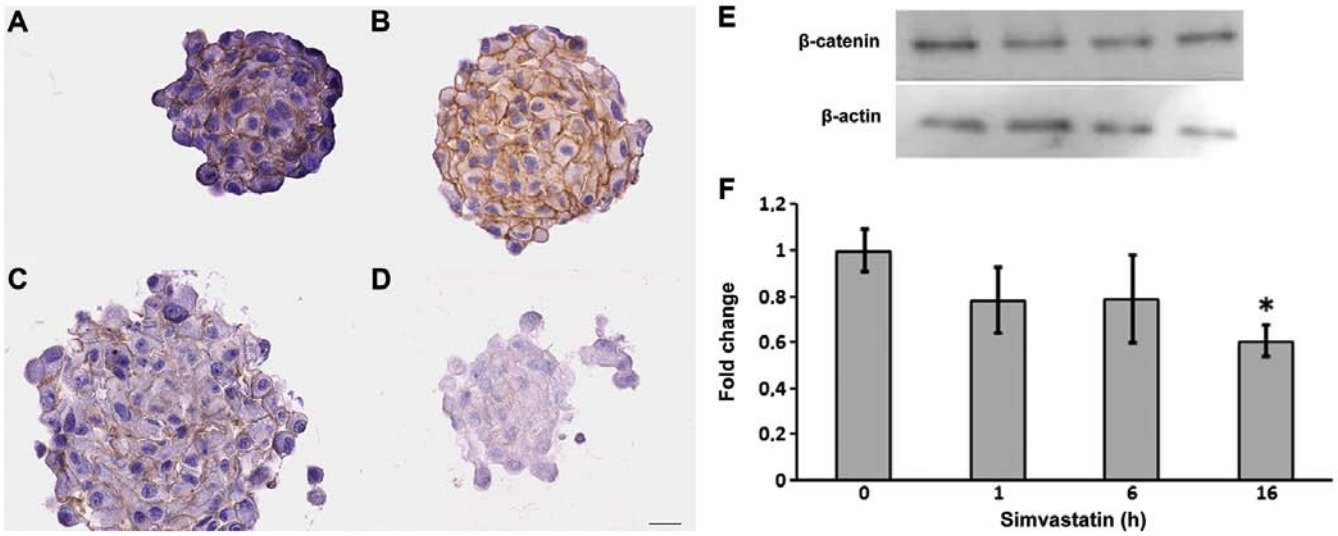

Figure 4. Effect of simvastatin on the β-catenin expression in spheres derived from CF41.Mg cells. (A-D) Representative immunocytochemistry images of β-catenin in spheres incubated with 10 μM simvastatin for (A) 0, (B) 1, (C) 6 and (D) 16 h. Scale bar, 16 μm. (E and F) Expression of β-catenin in the presence of 10 μM simvastatin. Representative western blot and histogram showing the expression. Values are mean ± SD of 3 independent analyses. *p<0.05 for the difference between various incubation times of simvastatin and the control.

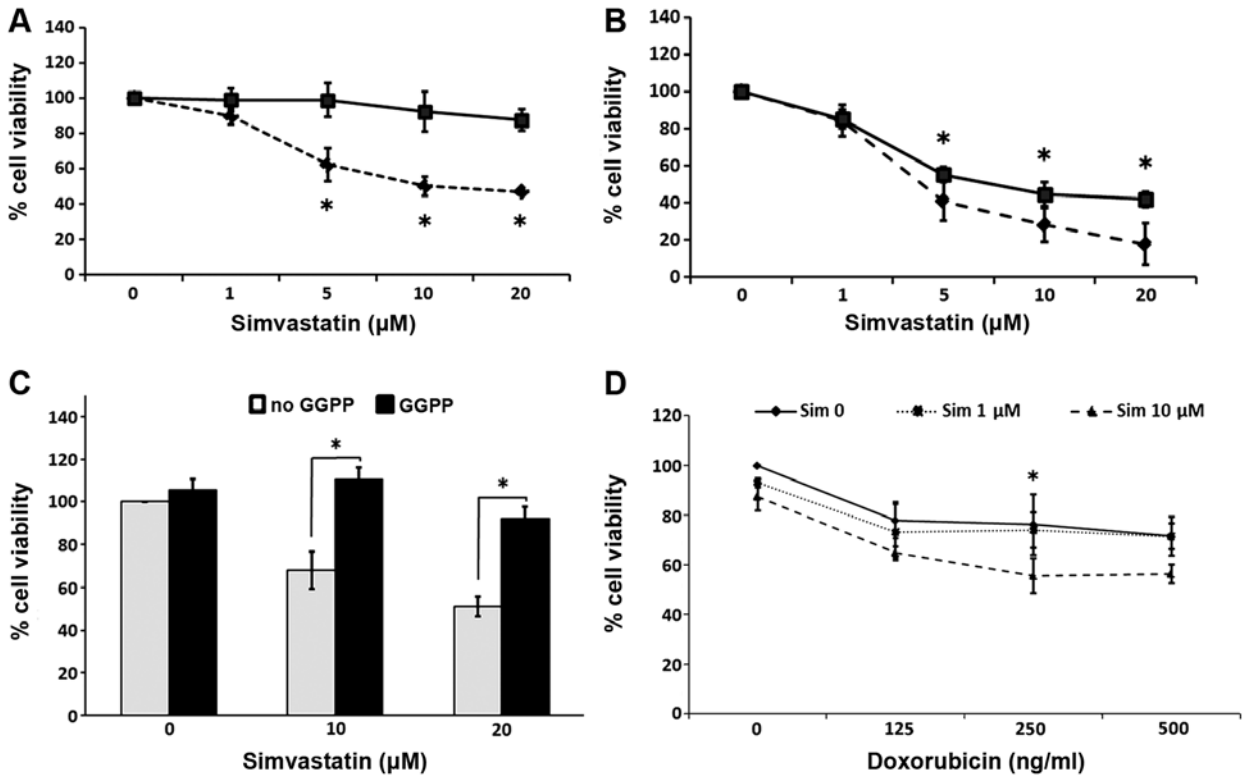

Figure 5. Cell viability of spheres derived from CF41.Mg cells after incubation with simvastatin, GGPP or doxorubicin. CF41.Mg cells and spheres were incubated for (A) 3 and (B) 6 days with simvastatin (0-20 μM). The percentage of viable cells was determined by an MTS assay. Values are mean ± SD of 3 independent experiments carried out in triplicate. *p<0.05 for differences between various concentrations of simvastatin and the control. (C) Cell viability of spheres incubated for 6 days with simvastatin (10 and 20 μM) and 30 μM GGPP. Values are mean ± SEM of 3 independent experiments in triplicate. *p<0.05 for differences between simvastatin alone or plus GGPP. (D) Spheres were incubated for 3 days with simvastatin (0 and 10 μM) in the presence of different concentrations of doxorubicin. Values are mean ± SD of 3 independent analyses in triplicate. *p<0.05 for differences between several concentrations of simvastatin in the presence of doxorubicin.

As shown in Fig. 7, a progressive increase in both p53 expression and its phosphorylation was detected in the spheres exposed to simvastatin for 16 h. This was consistent with the antiproliferative and apoptotic effects of the statin.

Since spheres have invasive potential, we performed Transwell invasion assays with 5 μM simvastatin in the presence or absence of GGPP. As shown in Fig. 8, the results indicated the inhibition of invasiveness in the presence of the

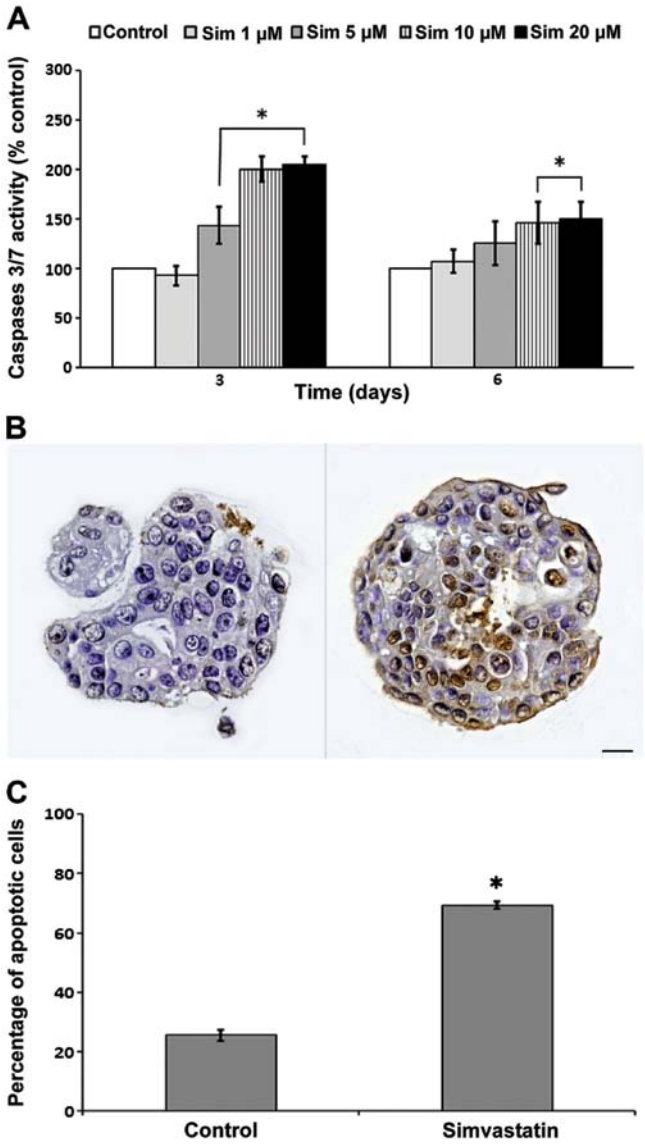

Figure 6. Simvastatin triggers apoptosis in spheres derived from CF41.Mg cells. (A) Caspase-3/7 activity induced by simvastatin (0-20 μM) for 3 and 6 days in spheres. This activity was determined per percentage of viable cells and is expressed as percentage of the control. (B and C) DNA fragmentation of the spheres was determined by TUNEL assay in response to 10 μM simvastatin for 6 days. (B) Representative immunocytochemistry image is displayed. Scale bar, 16 μm. Histogram shows the mean ± SD of the percentage of TUNEL-positive cells. *p<0.05 for differences between simvastatin and the control.

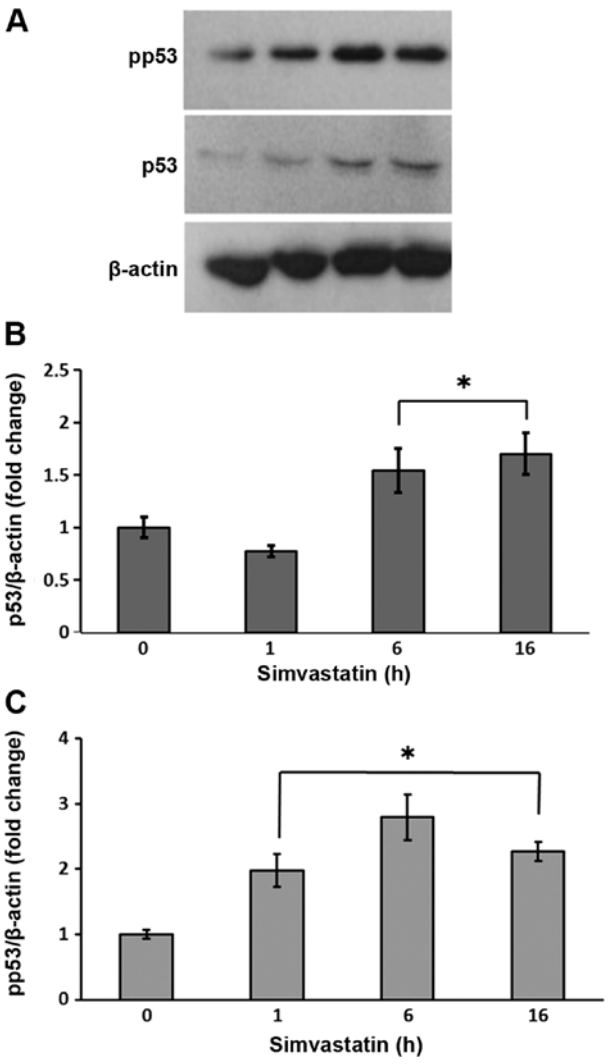

Figure 7. Analyses of p53 expression and its phosphorylation in spheres derived from CF41.Mg cells unexposed or exposed to simvastatin. (A-C) Expression and phosphorylation of p53 in the presence of 10 μM simvastatin at different times (0-16 h). Representative results from the assays of cell extracts as analyzed by western blotting. The histograms show the mean ± SD of 3 independent analyses. *p<0.05 for difference between various incubation times of simvastatin and the control.

statin. The effect was hindered by 30 μM GGPP, suggesting that the anti-invasive effect of simvastatin is dependent on the inhibition of protein prenylation as observed in the cell viability analysis.

Discussion

Similar to the mammary gland, solid tumors are heterogeneous, presenting different cell populations with varying rates of proliferation and differentiation, In addition, tumors exhibit variable metastatic capacity and response to treatments (28,29). According to the cancer stem cell model, also known as the 'tumor initiating cell model', a small subpopulation of tumorigenic cells located within the tumor exhibit capacities associated with stemness, that is, exhibit the ability for continuous self-renewal, differentiation, initiation and perpetuation of a tumor (30-32). To date, several groups have reported *in vitro* and *in vivo* data supporting the concept that CSCs are associated with malignancy (33,34). Yang *et al* found a high proportion of cancer stem-like cells in breast cancer tissues of poor prognosis (basal-like type and triple-negative form), suggesting that CSCs have prognostic value in this type of tumor (35). In canine mammary cancer, preliminary studies have shown that the presence of the $CD44^+/CD24^-$ phenotype is directly related to high histological grade carcinomas (36).

The tumor microenvironment is significant for CSC development since it regulates the stemness level (31), explaining in part the plasticity shown by these cells. In our experiments, spheres derived from CF41.Mg cells exhibited different features consistent with stemness (anchorage-independent growth, high expression of CD44, auto-renewal and chemoresistance). However, the cells did not express CD133, a biomarker usually

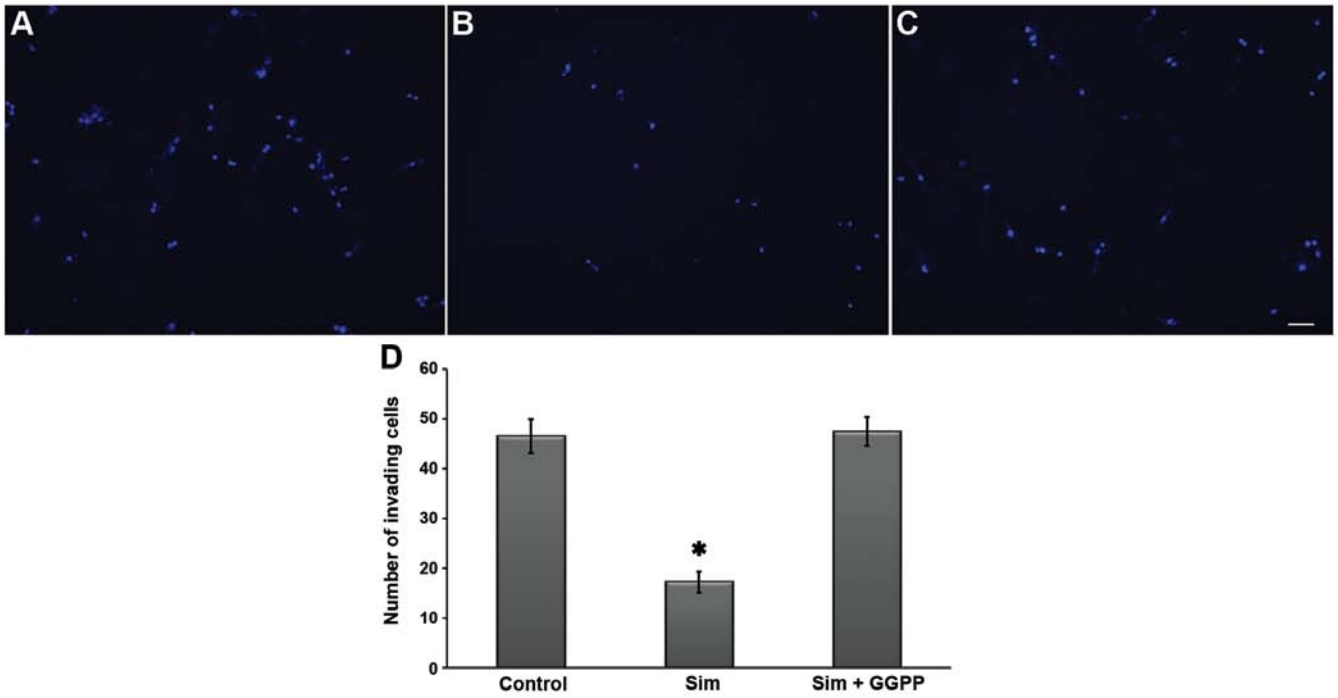

Figure 8. Effect of simvastatin on the invasiveness of spheres derived from CF41.Mg cells. (A-C) Invasiveness of cells derived from the spheres incubated with 5 μM simvastatin and 30 μM GGPP for 72 h. (D) The invading cells were stained with DAPI and counted using an epifluorescence microscope. Means ± SD of 3 independent Transwells. $^{*}p<0.05$ compared with the control cells. Scale bar, 20 μm.

present in CSCs. In veterinary literature, there are discordant data concerning the expression of CD133 in canine mammary CSCs derived from different cell lines (7,11), supporting the view of a high phenotypic variability for CSCs. Among others, the tumor microenvironment is the product of various cell types present, which promote a distinct pro-inflammatory and pro-tumorigenic niche. There is evidence that macrophages and adipocytes participate in this process, releasing molecules involved in CSC development, such as TNFα and TGFβ that modulate the Wnt/β-catenin pathway and EMT, respectively (37). In this context, we analyzed the effect of TGFα on the clonogenic capacity of spheres. The number of spheres was significantly increased by the treatment confirming that this growth factor is important to induce CSCs, as previously proposed (10).

In both canine and human mammary cancers, some CSCs become resistant to conventional antitumor treatments, including chemotherapy with doxorubicin, weakening the treatment of many mammary tumors (7,26,38). Concordant with the above, we observed that spheres showed chemoresistance to doxorubicin and paclitaxel in relation to the parental cells. In the presence of the highest concentrations of paclitaxel, the spheres tended to disaggregate, yet this did not mean greater cytotoxicity, suggesting this drug may impair sphere-forming capacity without changing their viability (data not shown).

The present study is the first report showing the antitumor properties of simvastatin on canine mammary carcinoma cell-derived CSCs. The statin impaired both the sphere-forming ability and cell viability, generating a maximum effect at 20 μM. The spheres exhibited resistance to simvastatin at 3 days of exposure, with almost no cytotoxicity as compared to the parental cells.

The canonical Wnt/β-catenin signaling pathway regulates critical processes related to cancer cells such as proliferation, migration and differentiation, among others (39). The self-renewal of cancer stem cells is also modulated by this pathway (40). Thus, the development of drugs that inhibit this signaling route is desirable. β-catenin is a protein that acts as a transcriptional co-regulator, and in conjunction with E-cadherin, is also involved in cell-to-cell adhesion (41). In the present study, β-catenin was expressed in the plasma membrane of cell spheres and the expression was reduced by simvastatin. These observations suggest that simvastatin partially impairs sphere-forming ability and cell viability through this pathway. Wang *et al* recently reported that β-catenin signaling is associated with drug resistance to upregulate ATP binding cassette subfamily G2 (ABCG2) proteins in high malignant breast cancer (42,43). On the other hand, activation of Akt and Wnt/β-catenin signaling confers chemoresistance to CSCs inducing a more efficient DNA repair mechanism (44). As shown in Fig. 5D, the addition of 10 μM of simvastatin exerted a synergistic effect with doxorubicin on spheres at 3 days of exposure, a time point at which they slightly respond to statin. The above described drug resistance mechanisms mediated by β-catenin may be inhibited by the statin through an unclear mechanism, which warrants further investigation explore in future studies.

According to of our results, simvastatin activate apoptosis, a process evaluated through caspase-3/7 activity and DNA fragmentation assays Despite the fact that simvastatin did not generate changes in cell viability at 3 days of incubation, the statin induced the activity of caspase-3/7 as a pro-apoptotic event. After 6 days of exposure to simvastatin, caspase activity remained and DNA fragmentation was increased, indicating a

late apoptotic event. In this context, these changes have been associated with an increase in the expression of cell cycle-negative regulatory proteins such as p53 (19) and p21 (45). We observed a higher expression and phosphorylation of p53 in response to simvastatin, confirming the results described in the literature (45,46). Apoptosis generated by simvastatin is probably related to the induction of intracellular radical oxygen species (45); the oxidative stress leads to an increase in p53 by inducing cell arrest and apoptosis (19). Thus, treatment with statin leads to an increase in pro-apoptotic Bax protein and activation of caspase-9 and -3 (46). In addition to the antiproliferative effects produced by simvastatin, this drug impaired invasiveness of spheres derived from CF41.Mg cells, promoting an antimetastatic potential effect. Mandal *et al* demonstrated that simvastatin decreased the invasive and metastatic potential of MDA-MB-231 CD44-positive cells (19), which is consistent with our observations.

Since the main mechanism of action of simvastatin is the disruption of this pathway through inhibition of HMGCoAR (14), we evaluated whether some of the effects of the drug were mediated by post-translational modifications associated with the inhibition of the mevalonate pathway. For this purpose, we conducted experiments adding the isoprenoid GGPP to the culture medium (14). The inhibitory effect induced by the statin on the cell viability and invasiveness was completely reverted, suggesting that these properties are dependent on protein isoprenylation. Several studies have shown that the effects of simvastatin and other statins are reversed in the presence of GGPP or mevalonate, but not by cholesterol or farnesyl pyrophosphate (46). This means that GGPP synthesis is relevant for the modulation of the activity of proteins involved in cell migration and survival, through an association with the plasma membrane. Our results suggest that simvastatin plays a role in the proliferative behavior of cancer stem-like cells. Therefore, statins represent valuable potential agents against CSCs by enhancing the effects of conventional chemotherapy in canines with mammary tumors.

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