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## Growth suppression of MCF-7 cancer cell-derived xenografts in nude mice by caveolin-1

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### ABSTRACT

Caveolin-1 is an essential structural constituent of caveolae membrane domains that has been implicated in mitogenic signaling and oncogenesis. However, the exact functional role of caveolin-1 still remains controversial. In this report, utilizing MCF-7 human breast adenocarcinoma cells stably transfected with caveolin-1 (MCF-7/cav-1 cells), we demonstrate that caveolin-1 expression dramatically inhibits invasion and migration of these cells. Importantly, *in vivo* experiments employing xenograft tumor models demonstrated that expression of caveolin-1 results in significant growth inhibition of breast tumors. Moreover, a dramatic delay in tumor progression was observed in MCF-7/cav-1 cells as compared with MCF-7 cells. Histological analysis of tumor sections demonstrated a marked decrease in the percentage of proliferating tumor cells (Ki-67 assay) along with an increase in apoptotic tumor cells (TUNEL assay) in MCF-7/cav-1-treated animals. Our current findings provide for the first time *in vivo* evidence that caveolin-1 can indeed function as a tumor suppressor in human breast adenocarcinoma derived from MCF-7 cells rather than as a tumor promoter.

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Caveolin-1, a 21–24 kDa integral membrane protein, is an essential protein within the caveolae coat structure [1,2]. Caveolin-1 has also been shown to be targeted in a cell type-specific manner to other organelles, such as endocytic vesicles, caveosomes, the Golgi apparatus, lipid droplets, and mitochondria [3]. Caveolin-1 modulates and interacts with multiple signaling pathways, suggesting that its expression profoundly affects cell function and cell fate [4]. Numerous experimental results have shown that caveolin-1 expression negatively regulates cell cycle progression and tumor cell growth [5,6]. These data, when taken together with the finding that the caveolin-1 gene is localized near a fragile site (7q31.1/D7S522) that is commonly deleted in a variety of human cancers [7], suggests that caveolin-1 is a tumor growth-inhibitory protein. However, other reported results revealed paradoxically higher caveolin-1 expression in advanced stages of specific malignancies, suggesting a role in cancer promotion [8,9]. Disparate results have also been obtained with respect to the regulation of apoptosis in cells and tissues, with caveolin capable of functioning either as a facilitator or suppressor [10–13], suggesting that caveolin-1 has distinct cell type-specific tumor-modulating functions, either

promoting or inhibiting tumor development depending upon the specific cellular context [14].

Globally, breast cancer is one of the most prevalent malignancies. Tissue invasiveness and metastatic spread of breast cancer cells are responsible for most of the morbidity and mortality associated with the disease [15]. Accumulating evidence has demonstrated that heterologous expression of caveolin-1 in breast MCF-7 cells attenuates cell proliferation rates and inhibits anchorage-independent growth (soft agar colony formation assay) and matrix invasion [4]. Surprisingly, MCF-7/cav-1 cells exhibited an opposite phenotype, i.e., the cells were found to be fully resistant to anoikis, suggesting that expression of caveolin-1 in MCF-7 breast cancer cells enhances matrix-independent cell survival by a mechanism involving up-regulation of IGF-I receptor expression and signaling [16,17]. Although the effects of caveolin-1 on proliferation in MCF-7/cav-1 cells are well documented *in vitro*, including soft agar colony formation and invasion activity through a filter membrane coated with Matrigel, the growth characteristics of MCF-7/cav-1 cells *in vivo* are currently unknown. Thus, we decided to conduct xenograft experiments in nude mice in order to shed light on the biological significance of caveolin-1 expression in MCF-7 cells. In the current study, we stably expressed caveolin-1 in MCF-7 cell lines lacking endogenous expression of caveolin-1 and found that MCF-7/cav-1 cells exhibited reduced migration *in vitro* as well as a decreased ability to form tumors following inoculation in nude mice.

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## Materials and methods

**Cell lines, plasmids, and reagents.** The human breast cancer cell line MCF-7 was obtained from the Cell Culture Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College. A monoclonal antibody (mAb) and a polyclonal antibody (pAb) against caveolin-1 were procured from Zymed Laboratories Inc. and Santa Cruz Biotechnology, respectively. Monoclonal anti-human Ki-67 antibody was obtained from DakoCytomation. Cell culture supplies (DMEM, FBS, L-glutamine, trypsin–EDTA, and penicillin/streptomycin) were purchased from Life Technologies Inc. The *in situ* Cell Death Detection Kit for carrying out terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL assay) was purchased from Roche Applied Science. Matrigel was procured from BD Biosciences and Sigma. The pCI-neo and pCI-neo-cav-1 plasmids were generous gifts provided by Dr. Eric J. Smart (Kentucky Medical School, USA). Hematoxylin and eosin (H&E) as well as other reagents were of the highest purity and obtained from commercial sources.

**Cell culture and transfections.** The parental human breast cancer cell line MCF-7 was cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The plasmid pCI-neo-cav-1 and its corresponding empty vector were transfected into MCF-7 cell lines using Lipofectamine 2000 according to the manufacturer's instructions [18,19]. Then we selected and propagated a clonal cell population [18]. Analysis of caveolin-1 expression in some monoclonal transfectants was subsequently confirmed by Western blotting. We then chose a pair of monoclonal cell lines, MCF-7/vector and MCF-7/cav-1, for future studies.

**Cell migration and invasion assays.** Cell migration and invasion were assayed in MilliCell chambers (12 mm diameter with 8 µm pores; Millipore) [20]. Briefly, exponentially growing cells were trypsinized using trypsin–EDTA and washed with PBS, and then 10<sup>5</sup> cells in 200 µl of serum-free DMEM were seeded into duplicate chambers, either coated with (for invasion assays) or without (for migration assays) 50 µl of Matrigel (diluted to 1 µg/µl in serum-free DMEM). Millicell units were placed into 24-well dishes containing 500 µl fresh DMEM growth medium (10% FBS). Cells were allowed to migrate or invade for 24 h at 37 °C. Chambers were fixed in methanol after removing the unpenetrated cells. The remaining cells were stained with hematoxylin for 10 min, rinsed in PBS, and examined under a bright-field microscope.

**Confocal microscopy images.** Cell samples were prepared and treated as previously described [18]. The cells were incubated for 2 h in diluted caveolin-1 (1:100) antibody in PBS. Immunostaining was performed by incubating the samples for 1 h in Texas Red-labeled goat anti-rabbit IgG diluted 1:200. After extensive washing in PBS, immunostained cells were examined with a Bio-Rad Radiant-2100 confocal microscope. Greater than 50 cells were inspected per experiment and typical results for each experiment are presented.

**Western blot analysis.** Cell extracts were prepared and analyzed by SDS–PAGE as described previously [19]. In brief, cell lysates were separated by SDS–PAGE employing standard reducing conditions. After electrophoresis, proteins were electroblotted onto a nitrocellulose membrane. The blots were blocked in Tris-buffered saline (10 mM Tris, pH 8.0, 135 mM NaCl) containing 5% nonfat dried milk and 0.05% Tween 20. The membranes were incubated with the designated primary antibodies at 4 °C overnight. Horseradish peroxidase-conjugated secondary antibodies (1:5000 dilutions, Pierce) were used to visualize bound primary antibodies, employing an enhanced chemiluminescence detection system (Amersham).

**Xenograft studies in nude mice.** All mice were handled according to the Guide for the Care and Use of Laboratory Animals. For

inoculation into nude mice, MCF-7/vector and MCF-7/cav-1 cells were washed with PBS, digested with trypsin, and resuspended in serum-free DMEM. After centrifugation, cells were resuspended in DMEM at a concentration of  $4 \times 10^6$  cells in 100 µl. The 100 µl cell suspension was injected subcutaneously (sc) into the left flank of 4-week-old female BABL/C athymic (nu/nu) mice. Tumor volumes were monitored based on caliper measurements of the length and width of the lesions and calculated using the following formula:  $0.5 \times \text{length} \times \text{width}^2$ . The mice were sacrificed approximately 32 days after inoculation, at which point the tumors were removed and fixed in 10% neutral buffered formalin for histologic and immunohistochemical analysis.

**Tumor immunohistochemical and histologic analysis.** Formalin-fixed tumors were embedded in paraffin and 4 µm tissue sections prepared. The sections were then deparaffinized, rehydrated, and subjected to either H&E, Ki-67, or TUNEL staining [21]. In brief, the tissue sections were mounted onto slides and stained routinely with H&E to confirm the presence of tumors. For Ki-67 staining, Ki-67 antigen was detected with a primary antibody and staining was carried out with the EliVision plus kit according to the manufacturer's instructions, using 3,3-diaminobenzidine (DAB) and hematoxylin. For assessing TUNEL-positive cells, the In Situ Cell Death Detection Kit was used. TUNEL staining was performed using TUNEL labeling mix, and Hoechst 33258 was used as the counterstain. Fluorescence images were captured using a Bio-Rad Radiant-2100 confocal microscope.

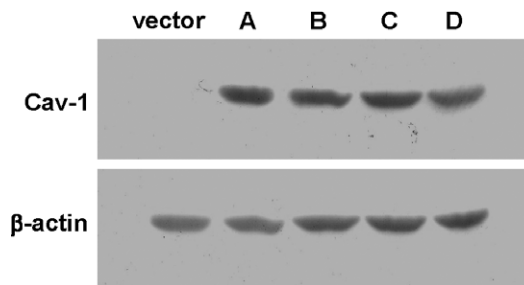
## Results

### The stable overexpression of caveolin-1 in MCF-7 cell lines

MCF-7 cells were transfected with pCI-neo-vector or pCI-neo-caveolin-1, and clonal cells selected and propagated. We carried out experiments with four stable cell lines expressing caveolin-1, with detection of caveolin-1 based on Western blot data. As shown in Fig. 1, a high level of caveolin expression was achieved in MCF-7/cav-1 cells (Fig. 1A–D), while no caveolin was detected in cells transfected with the pCI-neo-vector (i.e., MCF-7/vector cells) (Fig. 1, vector). The expression level of caveolin-1 in cells presented in Fig. 1A (i.e., A transfectants) is greater than in the other three cell lines. Thus, vector and A transfectants were selected for further studies and labeled as MCF-7/vector (as control) and MCF-7/cav-1, respectively.

### Caveolin-1 dramatically inhibits invasion and migration of MCF-7 cells

Tumor invasion of basement membranes is a crucial step in the complex multistage process that underlies the development of metastatic lesions [22]. To address the effect of caveolin-1 on MCF-7 cancer cell migration and invasion, we used a Boyden



**Fig. 1.** Caveolin-1 expression levels in MCF-7 transfectants by Western blot analysis. Vector, a stable cell line transfected with pCI-neo-vector, served as control. (A–D) Four independent stable cell lines transfected with pCI-neo-caveolin-1.  $\beta$ -Actin served as a loading control.

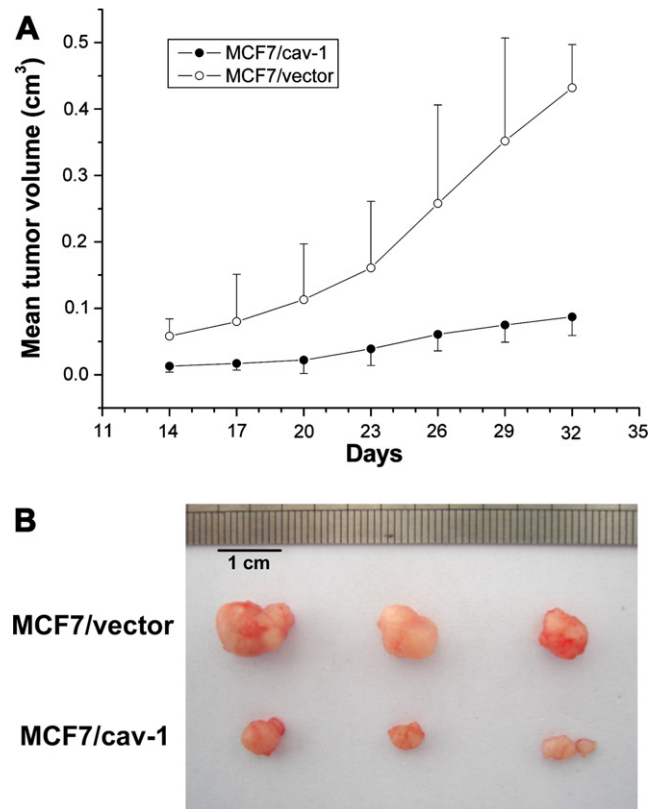
chamber assay employing porous filters containing or lacking a Matrigel barrier coating. Fig. 2 shows that the control MCF-7/vector cells display greater invasive and migration capacities as compared to MCF-7/cav-1 cells, which exhibit minimal penetration through the Matrigel barriers. Expression of caveolin-1 in MCF-7 cells results in a 70% and 42% inhibition of cell invasion and migration, respectively (Supplementary Fig. 1). Our findings demonstrate that caveolin-1 negatively affects two independent cellular processes, extracellular matrix dissolution and cell migration, suggesting that MCF-7/cav-1 cells should display a reduced metastatic capability relative to MCF-7/vector cells.

#### Caveolin-1 expression reduces growth of MCF-7 cell-derived xenograft

To assess whether our findings *in vitro* would extend to an animal model, as well as to investigate the important question pertaining to the biological significance of overexpression of caveolin-1 *in vivo*, MCF-7/vector and MCF-7/cav-1 cell lines were injected sc into nude mice ( $4 \times 10^6$  cells/mice). Tumors were palpable at day 10 following tumor cell injection in all mice except those implanted with the MCF-7/cav-1 cells, for which the earliest possible palpation of the tumors occurred at day 20–23 after cell injection. As shown in Fig. 3A, the growth measurements of the MCF-7/vector and MCF-7/cav-1 cell-derived tumors were plotted as log-growth curves for comparing the data. The results indicate that overexpression of caveolin-1 in MCF-7 cells produces a dramatic inhibition of tumor growth *in vivo*. Representative images of tumor samples removed from control and MCF-7/cav-1-derived xenograft tumors at day 32 after cell injection are presented in Fig. 3B. As can be seen, MCF-7/vector-derived tumors were larger, with an appearance similar to clusters of grapes, while MCF-7/cav-1 cells were smaller and paler.

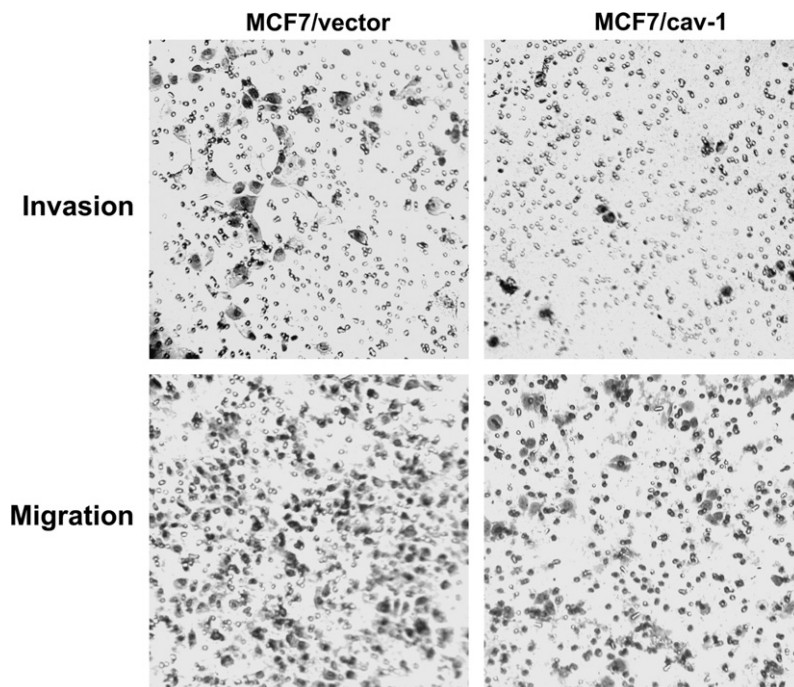
#### Caveolin-1 expression alters tumor histology and decreases proliferation but increases apoptosis of MCF-7 tumor cells *in vivo*

To investigate the effect of caveolin-1 on the histology of MCF-7 xenografts, tumor sections taken from mice were stained with



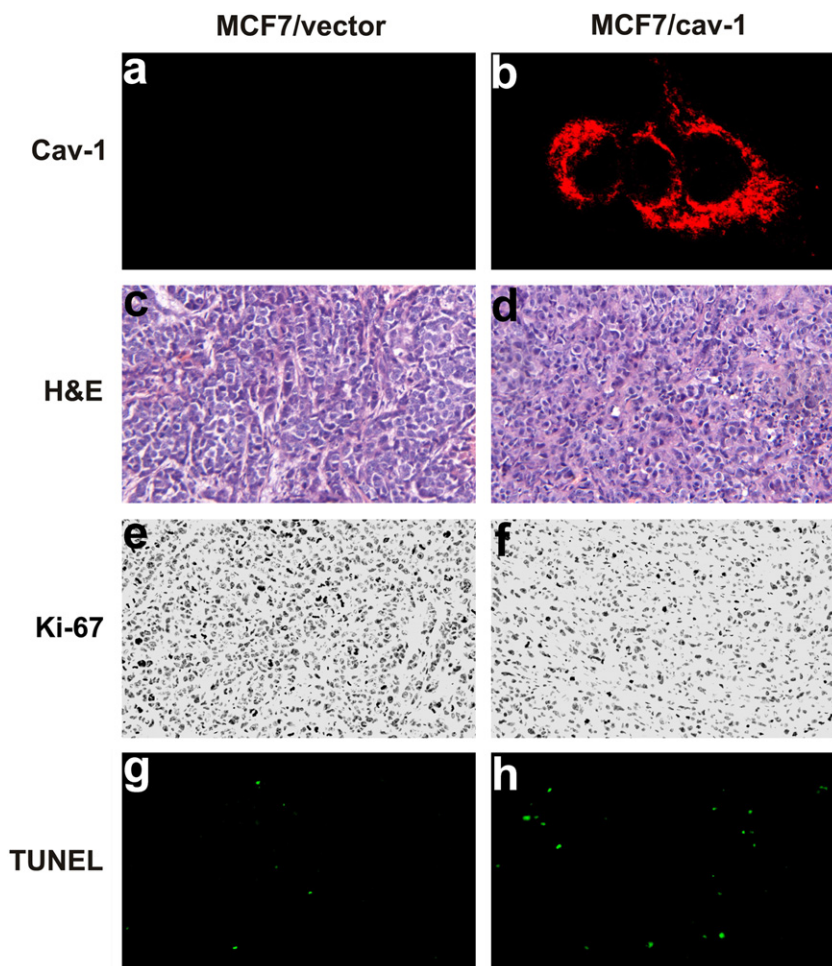
**Fig. 3.** Effects of caveolin-1 expression on MCF-7 cell xenograft growth. (A) Tumor volume was measured from xenograft appeared (at 14 day after injection). Bars represent average values  $\pm$  SD, which were counted in each set of nine nude mice from three independent experiments. (B) Isolated tumors from nude mice after 32 days.

H&E. As shown in Fig. 4, numerous cancer cells with higher nuclear fragmentation were apparent in H&E stained sections from MCF-7/



**Fig. 2.** Caveolin-1 expression inhibited invasion and migration of MCF-7 cells. Visual pictures of MCF-7 transfectant invasion and migration. The picture was representation of three independent experiments.





**Fig. 4.** Histological analysis of tumor sections from MCF-7 transfectant xenograft tumor model. Confocal analysis: Immunofluorescence staining of MCF-7 transfectant cells with anti-caveolin-1 was shown (a,b). Tumor samples from representative animals were fixed, embedded in paraffin, and sectioned at 4  $\mu$ m onto slides, and processed for H&E (c,d), anti-Ki-67 immunohistochemistry (e,f), and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) (g,h) analysis.

cav-1 cells as compared to MCF-7/vector cells (Fig. 4 c and d). All of the tumors are clearly carcinomas based on morphological characteristics. To quantitatively compare the proliferation and apoptotic index of the two transfectant cell populations, the tumor sections were stained for the proliferation marker Ki-67 and for DNA fragmentation by TUNEL analysis. A clear decrease in the level of proliferating cells was observed in MCF-7/cav-1 tumor cells (Fig. 4 e and f). The level of proliferating cells in these tumor sections was quantified based on 12 randomly selected microscopic fields, and the data show that overexpression of caveolin-1 in MCF-7 cells caused a 35% reduction in the percentage of proliferating cells (Supplementary Fig. 2). TUNEL staining detected greater apoptotic activity in MCF-7/cav-1-derived tumor cells (Fig. 4 g and h). A 2.8-fold greater number of apoptotic cells were found for the MCF-7/cav-1 cell population as compared to the control tumor cell samples (Supplementary Fig. 2). These findings suggest that as a result of caveolin-1 overexpression in MCF-7 cells, the promotion of apoptosis combined with a reduction in the proliferation rate of the tumor cells was responsible for the dramatic inhibition of tumor growth *in vivo*.

## Discussion

In the present study, we found that overexpression of caveolin-1 in MCF-7 breast adenocarcinoma cells lacking endogenous caveolin-1 synthesis caused suppression of cell invasion and migration

*in vitro*. Importantly, in order to confirm our *in vitro* observations, we performed *in vivo* experiments employing a nude mice xenograft model and demonstrated for the first time that expression of caveolin-1 in MCF-7 cells results in a dramatic suppression of tumor growth, with a concomitant increase in the degree of tumor cell apoptosis. It seems reasonable to conclude that the decreased cell proliferation and increased cell apoptosis contribute to the antitumor activity of caveolin-1 in MCF-7 cells not only *in vitro* but also *in vivo*.

Conflicting results have been reported in the literature concerning the role of caveolin-1 in regulating apoptosis. On the one hand, overexpression of caveolin-1 sensitizes fibroblasts to apoptotic stimuli, and caveolin-1 may act as a coupling or sensitizing factor in signaling apoptotic cell death in both fibroblastic and epithelial cells [12,23]. Similarly, Gargalovic and Dory reported that cell apoptosis is associated with increased caveolin-1 expression in macrophages [13]. On the other hand, caveolin-1 was shown to suppress c-myc-induced apoptosis in LNCaP cells [10], and caveolin-1 expression significantly reduced thapsigargin-stimulated apoptosis [11]. Caveolin-1 expression in small cell lung cancers significantly inhibited soft agar colony formation, whereas caveolin-1 appears to be required for the cell survival and growth of non-small cell lung cancers [24]. Interestingly, although both Hs578T and MCF-7 cancer cell lines represent breast adenocarcinoma cell lines, they respond quite differently when exposed to caveolin-1. Our previous findings demonstrated that caveolin-1 might play a

key role in regulating cell proliferation as a cell growth promoter rather than as a growth suppressor, mediated by inhibiting neutral-sphingomyelinase activity, reducing ceramide levels, and activating Akt signaling pathways in Hs578T cells [19]. However, up-regulation of caveolin-1 in MCF-7 cells decreased cancer growth *in vitro* and *in vivo*, with a concomitant increase in cancer cell apoptosis. It was hypothesized that at early stages of cancer progression, the expression of caveolin-1 is down-regulated to allow for enhanced cancer cell proliferation, but at later stages, expression of caveolin-1 is increased in order to support cancer cell survival [17]. This may be a reasonable explanation for how cancer cell growth proceeds through different stages, but does not explain the divergent roles of caveolin-1 as either a tumor promoter or suppressor, depending upon the type of cancer cell in which caveolin-1 is expressed. Although it is possible that the involvement of caveolin-1 in the regulation of cancer cell proliferation or apoptosis may be cell specific or depend on the cellular context, the molecular mechanisms that mediate the effects of caveolin-1 on cancer growth remain obscure.

We also wish to comment about tumor multidrug resistance (MDR), a phenomenon due to overexpression of the plasma membrane ATPase designated as P-glycoprotein (P-gp). The level of caveolin-1 expression is up-regulated in a number of human cell lines displaying an MDR phenotype and expressing P-gp, including colon, ovarian, breast, and lung carcinoma cells [25–27]. However, some reports also showed that expression of caveolin-1 and -2 were not detected in several MDR cell lines that expressed high levels of P-gp, i.e., caveolin-1 expression is not associated with P-gp synthesis or the MDR1 gene [28,29]. In addition, it is interesting to note in our previous experiments that parental and multidrug resistant (MDR) Hs578T cells were not tumorigenic in nude mice (data not published), in agreement with the data reported for the ATCC: Cell Biology Collection. However, heterologous overexpression of caveolin-1 in multidrug resistant Hs578T/Dox cells clearly led to tumorigenicity and metastasis *in vivo* [30]. Multidrug resistance is associated with reduced tumorigenicity, and P-gp expression in MDR cancer cells itself contributes to the loss of anchorage-independent growth and metastasis observed *in vitro* and *in vivo* [31–33]. Thus, the absence of P-gp in Hs578T/Dox cells due to overexpression of caveolin-1 [34] might facilitate tumor growth in nude mice. The physiological role of caveolin-1 remains elusive, and warrants further investigation.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.08.146.

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