

## SHORT REPORTS

**Caveolin-1 inhibits breast cancer growth and metastasis**Erica K Sloan<sup>1,2</sup>, Kym L Stanley<sup>1</sup> and Robin L Anderson<sup>\*1</sup><sup>1</sup>Peter MacCallum Cancer Centre, Locked Bag #1, A'Beckett Street, Melbourne, Victoria 8006, Australia

**Caveolin-1 was identified in a screen for genes involved in breast cancer progression. Caveolin-1 is the major protein component of caveolae, flask-shaped invaginations found in a number of different cell types. Using an orthotopic model of spontaneous breast cancer metastasis, caveolin-1 was found to be expressed in low and non-metastatic primary tumors, but at much lower levels in highly metastatic 4T1.2 and 4T1.13 tumors. Exogenous expression of caveolin-1 at moderate levels in 4T1.2 cells was sufficient to suppress primary tumor growth after inoculation of cells into the mammary gland. Expression of high levels of caveolin-1 also inhibited subsequent metastasis to distant organs. Cells expressing high levels of caveolin-1 showed reduced capacity to invade Matrigel, diminished response to laminin-1 stimulation and decreased metastasis to lung and bone. This study provides the first functional evidence that caveolin-1 regulates primary breast tumor growth and spontaneous metastasis of breast cancer.**

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Breast cancer is one of the most common cancers of women in the Western world. While early detection is continuing to improve the survival rate, the prognosis is poor after the tumor has metastasized. Hence, there is an urgent need to identify the genes that drive breast cancer metastasis and, from this knowledge, to develop new therapies.

Caveolin-1 is the major protein component of caveolae, specialized lipid rafts that are recognized in electron micrographs as 50–100 nm invaginations of the plasma membrane. Caveolae are found primarily in terminally differentiated mesenchymal cells including adipocytes, endothelial cells and fibroblasts (Smart *et al.*, 1999). Caveolin-1 is involved in the processing and trafficking of cellular cholesterol through caveolae (Murata *et al.*, 1995) and is important in systemic lipid homeostasis *in vivo*. Mice deficient in caveolin-1 are

resistant to diet-induced obesity and have smaller fat deposits as a result of reduced lipid accumulation (Razani *et al.*, 2001). Many receptors and signaling molecules have been identified within caveolae, which are proposed to be able to integrate cellular signaling pathways (Garcia-Cardena *et al.*, 1997; Simons and Toomre, 2000).

There has been much recent interest in the role of caveolin-1 in breast cancer. Caveolin-1 was found to be downregulated in breast cancer samples and human breast cancer cell lines compared with matched normal tissue and normal epithelial cell lines (Lee *et al.*, 1998; Hurlstone *et al.*, 1999). Expression of caveolin-1 in MCF7 and T47D breast cancer lines slowed cell proliferation and growth in soft agar (Lee *et al.*, 1998; Fiucci *et al.*, 2002). The caveolin-1 gene is localized in a tumor suppressor locus at 7q31.1 (Zenklusen *et al.*, 1994; Engelman *et al.*, 1999). Since a number of other genes, including ST7, have also been implicated as the tumor suppressor gene at this locus, the importance of caveolin-1 remains unclear (Zenklusen *et al.*, 2001). Recently, a point mutation in codon 132 of caveolin-1 that induces cellular transformation and invasion has been reported in 16% of human breast cancers (Hayashi *et al.*, 2001). However, we were unable to reproduce this observation in a population of 83 largely Caucasian breast cancer patients (unpublished data). Until now, the role of caveolin-1 in breast cancer progression *in vivo* has not been investigated.

We identified caveolin-1 in a microarray screen for genes involved in breast cancer progression using an orthotopic model of spontaneous breast cancer metastasis (Lelekakis *et al.*, 1999). The model comprises cell lines that were derived from a spontaneous mammary carcinoma in a Balb/c/C3H mouse and exhibit different patterns of spontaneous metastasis following injection into the mammary fat pad (Aslakson and Miller, 1992; Lelekakis *et al.*, 1999). 4T1.2 and 4T1.13 tumors are highly metastatic to lymph nodes, bone, lungs and other organs. These lines provide a rare and valuable model that closely resembles the events that occur in metastatic breast cancer in humans, with spontaneous metastasis from the mammary gland to distal sites, including spine and femur. In contrast, tumors derived from the 66cl4 line show low levels of metastasis to lymph nodes and lung, while the 67NR line exhibits no metastatic capacity (Lelekakis *et al.*, 1999; Eckhardt *et al.*, submitted for publication).

Gene expression profiling using a cDNA microarray analysis of 5000 annotated human cDNA clones from

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the Research Genetics sequence verified set and with reduced-stringency cross-species hybridization revealed that caveolin-1 was highly upregulated in 66cl4 primary mammary gland tumors compared with 4T1.2 tumors (data not shown). Northern analysis was used to confirm the array data and to investigate the expression of caveolin-1 in other sublines of the mouse model. Highly metastatic 4T1.2 and 4T1.13 primary tumors expressed very low levels of caveolin-1 (Figure 1a). In contrast, caveolin-1 was expressed at a high level in low metastatic 66cl4 tumors and in nonmetastatic 67NR tumors.

To investigate whether caveolin-1 expression influences the metastatic phenotype, human caveolin-1 cDNA was stably expressed in the clonal cell line 4T1.2. Human caveolin-1 differs from mouse caveolin-1 at only two amino acids and is functionally indistinguishable from the mouse protein (Smart *et al.*, 1996). Caveolin-1 expression was demonstrated by Western analysis and four clones were characterized further. Clones 7 and 9 expressed high levels of caveolin-1, while clones 13 and 16 expressed moderate levels of caveolin-1

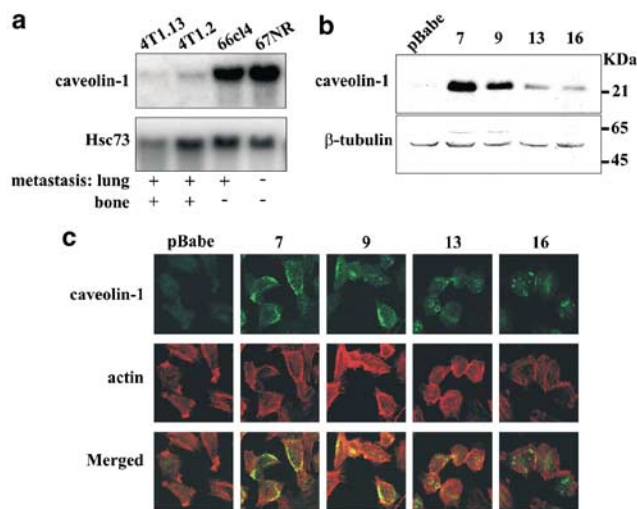
(Figure 1b). The clonal 4T1.2 cells transduced with empty vector were used as a control (4T1.2pBabe).

The distribution and internalization of caveolin-1 is influenced by actin organization. Cortical actin filaments confine caveolin-1 to the cell surface and depolymerization of the actin network results in a rapid movement of caveolin-1-containing structures towards the centrosome (Mundy *et al.*, 2002). In 3T3L1 cells, colocalization of caveolin-1 with F-actin increased during differentiation into mature adipocytes (Kanzaki and Pessin, 2002). In transduced 4T1.2 cells, caveolin-1 was partially localized at the plasma membrane, more so in the two higher expressing clones (7 and 9). Punctate staining of caveolin-1 was evident in the cytoplasm of the clones expressing lower levels of caveolin-1 (13 and 16) (Figure 1c). The same localization of caveolin-1 was observed with a polyclonal antibody raised against caveolin-1 (data not shown). The actin cytoskeleton, as revealed by phalloidin staining, showed partial colocalization with caveolin-1 in clones 7 and 9 (Figure 1c). Caveolin-1 did not colocalize with lipid droplets as assessed by Oil Red O staining (data not shown), in contrast to reports in other cell types (Ostermeyer *et al.*, 2001; Pol *et al.*, 2004).

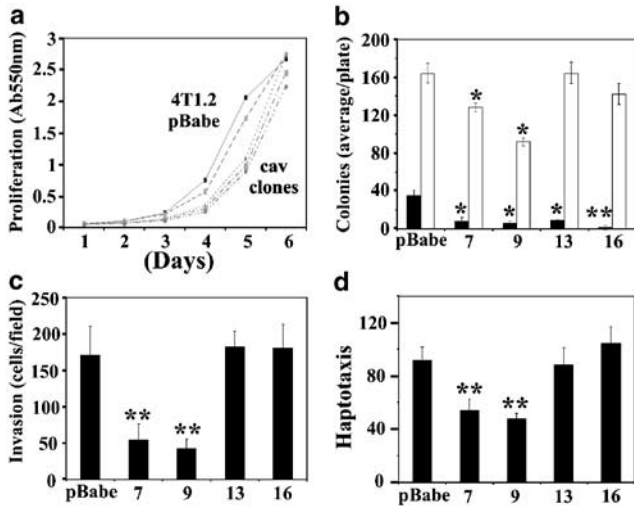
The effect of caveolin-1 on proliferation and anchorage-independent growth was investigated. Compared to 4T1.2pBabe cells, the 4T1.2 caveolin-1-expressing clones, with the exception of clone 7, showed an extended lag phase but then proliferated at a similar rate over a 6-day period (Figure 2a). Clone 7 grew at a similar rate to control cells. In soft agar cloning assays, all caveolin-1-expressing clones had reduced capacity for anchorage-independent growth as evidenced by fewer large colonies ( $>200\ \mu\text{m}$ ) (Figure 2b). Clones 7 and 9 also showed a significant reduction in the total number of colonies (Figure 2b). These results demonstrate that even modest levels of caveolin-1 are sufficient to slow anchorage-independent proliferation and are in agreement with previous data (Lee *et al.*, 1998; Fiucci *et al.*, 2002).

Invasion of the mammary basement membrane that separates the ducts from the surrounding stroma is an essential step in tumor progression. The effect of caveolin-1 on the capacity of tumor cells to invade Matrigel, a basement membrane substitute, was investigated. Invasion was significantly inhibited in clones 7 and 9, but not in clones 13 and 16 compared with 4T1.2pBabe ( $P<0.001$ ) (Figure 2c). Since Matrigel contains approximately 90% laminin-1, the effect of caveolin-1 expression on haptotactic migration toward laminin-1 was investigated. Migration was significantly reduced in clones 7 and 9 compared to 4T1.2pBabe, while clones 13 and 16 maintained high levels of laminin-stimulated migration (Figure 2d). Thus, cells expressing high levels of caveolin-1 exhibit reduced anchorage-independent viability, laminin-stimulated migration and basement membrane invasion, but no major changes in proliferative capacity.

Integrins mediate cell interactions with extracellular matrix molecules (Giancotti and Ruoslahti, 1999). Caveolin-1 has been shown previously to interact with



**Figure 1** Expression and localization of caveolin-1 in a mouse model of breast cancer metastasis. (a) Northern analysis of primary tumor total RNA using radiolabelled full-length human caveolin-1 or mouse Hsc73 (loading control) cDNA probes. (b) Western analysis of 4T1.2pBabe and 4T1.2 caveolin-1 clones using a rabbit polyclonal antibody against caveolin-1 (Transduction Laboratories) or anti- $\beta$ -tubulin (loading control) antibody (Santa Cruz) and enhanced chemiluminescence detection. Full-length human caveolin-1 cDNA, a kind gift from Dr Richard Anderson, University of Texas Southwestern Medical Center, TX, was subcloned into the pBabepuro retroviral vector and used to infect 4T1.2 cells via the Phoenix packaging line, a kind gift from Dr G Nolan, Stanford University, CA, USA. Stably infected cells were selected in  $2\ \mu\text{g}/\text{ml}$  puromycin and single cell cloned. (c) Immunofluorescence detection of caveolin-1 (green) and actin (red) and merged images of 4T1.2pBabe and caveolin-1 expressing 4T1.2 clones 7, 9, 13 and 16. Cells grown on coverslips were fixed, permeabilized and probed with anti-caveolin-1 monoclonal antibody (Transduction Laboratories) and an Alexa 488-conjugated secondary antibody. Cells were counter stained with rhodamine-phalloidin to detect actin and examined by confocal fluorescence. Cells were grown in alpha minimal essential medium/10% fetal calf serum/1% penicillin-streptomycin at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$

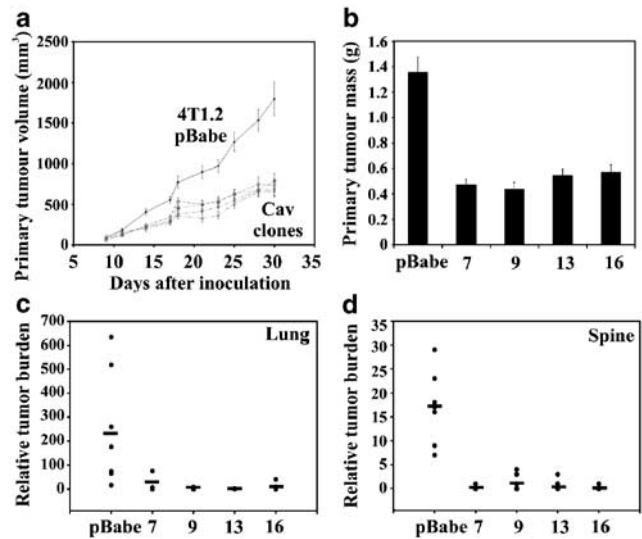


**Figure 2** *In vitro* effects of caveolin-1 expression in 4T1.2 cells. (a) Proliferation of 4T1.2pBabe (black line) and caveolin-1 clones (dashed lines) over 6 days in culture. A total of 300 cells/well were plated. On successive days, quadruplicate samples were fixed with trichloroacetic acid, stained with 0.4% sulforhodamine B and bound dye was quantitated by spectrophotometry. Representative of three separate experiments. Clone 7 proliferates at a similar rate to the parental cells, while the other clones (9, 13 and 16) had an extended lag phase followed by a similar growth rate to vector control cells. (b) Anchorage-independent growth in soft agar. Cells ( $10^5$ ) were grown in soft agar for 12 days. Colonies were stained with MTT and scored for size and number. Black bars: large colonies  $>200\mu\text{m}$ , open bars: total number of colonies. (c) Invasion through Matrigel towards a serum gradient. Cells ( $2 \times 10^5$ ) in 50% Matrigel/PBS were seeded in Transwell chambers. After 24h, cells that had invaded through to the lower side of the membrane insert were fixed in 10% buffered formalin, permeabilized in 0.1% Triton X-100 and visualized by staining with 1  $\mu\text{g}/\text{ml}$  DAPI (Sigma). The average number of cells in five randomly selected fields is shown. (d) Haptotactic migration towards 10  $\mu\text{g}/\text{ml}$  substrate-bound laminin-1 (Sigma) over 5h in the absence of serum, measured by the average number of migrated cells/field over five fields. For all experiments: \* $P < 0.01$ , \*\* $P < 0.001$

$\beta 1$  and  $\beta 3$  integrins and modulate their activity (Wary *et al.*, 1998; Wei *et al.*, 1999). However, consistent with previous findings (Fiucci *et al.*, 2002), expression of  $\beta 1$ ,  $\beta 3$ ,  $\alpha v$  and  $\alpha 6$  integrins as assessed by flow cytometry using specific antibodies was not altered by expression of caveolin-1 (data not shown).

The effect of caveolin-1 on spontaneous metastasis of breast cancer has not yet been investigated. To address this, we examined the ability of clones with modified caveolin-1 to grow as primary tumors and metastasize to distant sites. Primary tumors were palpable 7 days after injection of tumor cells into the mammary gland and mice were culled after 30 days. The growth rate of tumors was significantly reduced ( $P < 0.001$ ) in each caveolin-1 clone compared with 4T1.2pBabe (Figure 3a). This correlated with a threefold reduction in tumor weight in caveolin-1-expressing tumors (average weight  $\pm$  s.e.m. of all clones:  $0.45 \pm 0.03$  g) compared to 4T1.2pBabe ( $1.35 \pm 0.12$  g) ( $P < 0.001$ ) (Figure 3b).

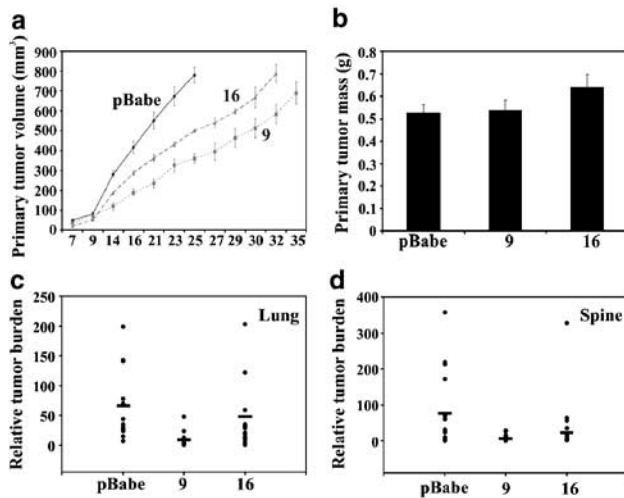
Real-time quantitative-polymerase chain reaction (RTQ-PCR) was used to determine the extent of lung and bone metastasis. RTQ-PCR is a sensitive method



**Figure 3** Caveolin-1 inhibits primary tumor growth and metastasis. (a) Average primary tumor size. Cells ( $10^5$  in  $20\mu\text{l}$  of 50% Matrigel) were injected into the fourth mammary gland of 6- to 8-week-old female Balb/c mice (10 mice/group; Animal Resources Centre, Western Australia). 4T1.2pBabe (black line), caveolin-1 clones (dashed lines). (b) Average primary tumor weights ( $g \pm$  s.e.m.) at the time of harvest (30 days after inoculation). Tumor burden in lungs (c) and spine (d) was measured by RTQ-PCR of genomic DNA using the ABI PRISM 7000. Whole organs were taken for DNA measurement. The fluorescence threshold was set within the range of linear amplification and the difference in cycle number (delta  $C_T$  value) was determined by subtracting  $C_{T(\text{Vim})}$  from  $C_{T(\text{LSXN})}$ . This number was corrected for copy number of LSXN in each clone and relative tumor burden (RTB) calculated according to the equation  $\text{RTB} = 2^{\text{Corrected}\Delta C_T} \times 10000$ . Each point represents the tumor burden for one mouse (not all points are distinguishable) and the average metastatic burden for each group is shown with a line

that directly quantifies metastatic burden (Tester *et al.*, 2002) and correlates closely with histological tumor analysis in our model (Parker *et al.*, 2004; Eckhardt *et al.*, submitted for publication). Briefly, a multiplex reaction was performed on genomic DNA from each intact organ to determine the ratio of vimentin DNA (present in all cells) to an LSXN sequence (a retroviral LTR sequence present only in tumor cells infected with the pBabepuro vector). Metastatic burden was reduced 53-fold in lungs ( $P < 0.001$ ) and 26-fold in spine ( $P < 0.001$ ) for 4T1.2 caveolin-1 expressing tumors (average of all clones) compared to 4T1.2pBabe tumors (Figure 3c and d). These results were replicated in a second experiment (data not shown) and demonstrate that even the moderate levels of caveolin-1 in clones 13 and 16 are sufficient to reduce tumor progression *in vivo*.

To establish whether metastasis was impacted directly by caveolin-1 expression or was secondary to the reduced size of the primary tumors, metastatic burden was assessed again after clone 9 (high caveolin-1 levels) and clone 16 (moderate caveolin-1 levels) tumors were grown for a longer time to achieve a similar average size as 4T1.2pBabe tumors (Figure 4a and b). Lungs and spines were assayed for metastatic tumor burden by RTQ-PCR. The average relative tumor burden



**Figure 4** Metastatic burden from primary tumors grown to similar size. (a) Cells ( $10^5$  in  $20 \mu\text{l}$  of 50% Matrigel) were injected into the fourth mammary gland (10 mice/group). Primary tumor volumes were measured three times per week. (b) Average primary tumor weights at time of harvest ( $\text{g} \pm \text{s.e.m.}$ ), which varied for each tumor line. Relative tumor burden in lungs (c) and spine (d) was measured by RTQ-PCR according to the protocol described in Figure 3. Each point represents the tumor burden for one mouse (not all points are distinguishable) and the average metastatic burden for each group is shown with a line

( $\pm \text{s.e.m.}$ ) in the lung from 4T1.2pBabe primary tumors was  $65 \pm 16$  compared to  $10 \pm 7$  ( $P=0.001$ ) for clone 9 expressing high levels of caveolin-1 and  $42 \pm 14$  ( $P=0.28$ ) for clone 16 expressing moderate levels of caveolin-1 (Figure 4c). In the spine, the average relative tumor burden ( $\pm \text{s.e.m.}$ ) was  $84 \pm 26$  from 4T1.2pBabe primary tumors,  $8 \pm 2$  ( $P=0.08$ ) from clone 9 and  $34 \pm 20$  ( $P=0.13$ ) for clone 16 (Figure 4d). Thus, even when primary tumors were grown to the same size, metastasis to lung remained significantly reduced in tumors expressing high levels of caveolin-1 (clone 9). A similar trend was observed for bone metastasis. These results demonstrate that high levels of caveolin-1 suppress both tumor growth and metastasis to distant organs.

The decreased capacity for anchorage-independent growth may contribute to the reduction in metastasis in mice bearing caveolin-1-expressing tumors. While non-transformed cells undergo anoikis in the absence of adhesion-mediated signals, transformed metastatic cells become refractory to the anchorage requirement for growth, allowing successful colonization of distant sites. Caveolin-1 may suppress anchorage-independent growth by sensitizing the cells to anoikis. Consistent with this, expression of caveolin-1 in T24 bladder carcinoma cells sensitizes them to caspase-3-mediated apoptosis (Liu *et al.*, 2001), although in MCF-7 cells,

caveolin-1 expression inhibits anoikis (Fiucci *et al.*, 2002). This may be related to the lack of caspase-3 in MCF-7 cells. Further, the reduced capacity for laminin-stimulated migration is likely to inhibit metastasis by preventing cells from crossing the basement membrane.

In normal breast development and function, caveolin-1 may serve as a regulator of growth and apoptosis, being expressed when growth must be restrained and switched off when tissue expansion is required. Consistent with this, caveolin-1 expression is suppressed by prolactin during late pregnancy when lobular-alveolar glands undergo proliferation (Park *et al.*, 2002). Further, caveolin-1-deficient mice develop hyperplasia by 6 weeks of age (Lee *et al.*, 2002), suggesting that caveolin-1 suppresses epithelial cell expansion within the developing breast.

The data presented here confirm and extend the *in vitro* findings of others and show for the first time that caveolin-1 suppresses breast cancer metastasis *in vivo*. We provide the first evidence that caveolin-1 reduces both primary tumor growth and spontaneous metastasis to lung and bone. The syngeneic metastasis model used here is one of very few spontaneous breast cancer metastasis models available to test the function of potential metastasis regulating genes.

Clinical studies reveal that the correlation of caveolin-1 expression with tumor progression varies with tumor type. In prostate, esophageal squamous cell carcinoma and lung carcinoma, expression of caveolin-1 correlates with increased metastasis and poor prognosis (Yang *et al.*, 1999; Li *et al.*, 2001; Ho *et al.*, 2002; Kato *et al.*, 2002). In contrast, caveolin-1 is expressed in normal and benign ovarian epithelial cells and in normal colon mucosa, but is lost in serous ovarian carcinomas (Wiechen *et al.*, 2001) and in colon carcinomas (Bender *et al.*, 2000). In one breast cancer study, caveolin-1 was upregulated in more aggressive tumors (Yang *et al.*, 1998), but, consistent with our data, the opposite result was found in three other studies (Sager *et al.*, 1994; Hurlstone *et al.*, 1999; Wiechen *et al.*, 2001). In future, it will be important to extend these observations to a larger number of tumors to clarify the impact of caveolin-1 in the progression of human breast cancer.

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