Caveolin-1 Levels Are Down-Regulated in Human Colon Tumors, and Ectopic Expression of Caveolin-1 in Colon Carcinoma Cell Lines Reduces Cell Tumorigenicity

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ABSTRACT

Caveolin-1 expression and function were investigated in human colon cancer. Low levels of caveolin-1 mRNA and protein were detected in several colon carcinoma cell lines. Moreover, caveolin-1 protein levels were significantly reduced in human tumor epithelial mucosa (3.6 ± 1.4-fold) when compared with normal colon mucosa for a majority (10 of 15) of the patients characterized. To directly assess the role of caveolin-1 in tumor development, caveolin-1 was reexpressed in the HT29 and DLD1 colon carcinoma cells, and the resulting HT29-cav-1 or DLD1-cav-1 cells were tested for tumorigenicity in nude mice. In most experiments, tumor formation was either blocked or retarded for HT29-cav-1 cells (10 of 13 mice) and DLD1-cav-1 cells (5 of 7 mice), as compared with both mock-transfected and parental HT29 or DLD1 cells. Interestingly, basal caveolin-1 levels were significantly reduced in HT29-cav-1 and DLD1-cav-1 cells isolated from tumors after injection into nude mice. Thus, reexpression of caveolin-1 in colon carcinoma lines reduced the probability of tumor formation in vivo, and when tumors did develop from either HT29-cav-1, DLD1-cav-1, or NIH-3T3 cells, lower basal levels of caveolin-1 were detected. Finally, evidence was obtained indicating that initial caveolin-1 down-regulation in colon cancer cells need not be an entirely irreversible process because cell survival on selection for either drug resistance or increased metastatic potential correlated with increased caveolin-1 expression levels.

INTRODUCTION

During progression from a normal epithelium to invasive or metastatic cancer, cells accumulate a combination of defects, including mutational activation of oncogenes such as Ras or Myc and inactivation of tumor suppressor genes like p53 (1). As a general consequence, several signal transduction pathways become constitutively activated, leading to enhanced cell proliferation, loss of adhesion, and a transformed phenotype coupled with insensitivity to apoptosis (2–4).

Since its identification (5, 6), caveolin-1 has been implicated in the process of cell transformation: caveolin-1 is a major substrate for phosphorylation on tyrosine upon cell transformation by the Rous sarcoma virus (6); caveolin-1 mRNA and protein levels are reduced in NIH-3T3 fibroblasts transformed by several oncogenes (7); and caveolin-1 levels are reduced in a variety of carcinoma cell lines, including human mammary carcinoma (8) and lung carcinoma cells (9). These results suggest that reduced caveolin-1 expression may represent a general characteristic or even a requirement of transformed cells and that caveolin-1 could play a central role as an inhibitor of tumor formation.

Caveolin-1 interacts directly with and inhibits or sequesters the inactive form of many key signaling molecules including heterotrimeric G proteins, Ha-Ras, c-Src, endothelial nitric oxide synthase, protein kinase Cα, MAPK, and tyrosine kinase receptors via a motif referred to as the scaffolding domain (10–15). Additionally, many of the aforementioned proteins contain a consensus motif for caveolin-1 binding (16, 17). Thus, caveolin-1 may reduce cell tumorigenicity by virtue of its ability to bind to and inhibit or sequester inactive forms of signaling proteins including oncogenes. Consistent with this hypothesis, caveolin-1 levels are reduced in oncogenically transformed cells (7), reexpression of caveolin-1 abrogates anchorage-independent growth in oncogene-transformed cells (18), and down-regulation of caveolin-1 by overexpression of caveolin-1 antisense RNA is sufficient to transform NIH-3T3 cells (19).

Despite such evidence favoring a role for caveolin-1 as a tumor suppressor gene in several cell systems, no mutations were found in human cancer cells. In addition, CpG islands associated with the caveolin-1 gene are not methylated in either primary tumors or tumor-derived cell lines in which caveolin-1 expression is low (20). Thus, although reduced caveolin-1 expression can clearly be linked to increased tumorigenicity in some cell systems, classification of caveolin-1 as a tumor suppressor remains controversial at this point.

Only a few studies have directly investigated caveolin-1 changes on tumor formation in humans and how reexpression of caveolin-1 modulates the tumor-forming ability of tumor-derived cells. In this study, these questions were addressed with a focus on the role of caveolin-1 in human colon cancer. The results presented show that: (a) caveolin-1 mRNA and protein levels were reduced in human colon carcinoma cell lines, as well as in human colon tumors; (b) reexpression of caveolin-1 in the colon carcinoma cell lines HT29 and DLD1 significantly reduced the tumorigenicity of these cells when they were injected in nude mice; and (c) tumor formation in vivo resulted in the selection of cells with lower basal caveolin-1 levels. To our knowledge, this is the first study showing that caveolin-1 reexpression in human carcinoma cells reduces their ability to form tumors in nude mice.

MATERIALS AND METHODS

Reagents and Antibodies. DMEM, RPMI 1640, trypsin/EDTA, and antibiotics (penicillin, streptomycin, and neomycin) were purchased from Life Technologies, Inc. (Paisley, United Kingdom). FCS was from Seromed-Biochrom KG (Berlin, Germany), IPTG was from Eurogentec (Seraing, Belgium), and hygromycin B was from Calbiochem (La Jolla, CA). The BCA protein determination kit was from Pierce (Rockford, ), prestained molecular weight protein markers were from New England Biolab Inc. (Beverly, MA), and the enhanced chemiluminescence kit was from Amersham International (Bucks, United Kingdom). The polyclonal anti-caveolin-1 antibody (C13630) 

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3 The abbreviations used are: MAPK, mitogen-activated protein kinase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ISREC, Swiss Institute for Cancer Research; MDCK, Madin-Darby canine kidney.
was purchased from Transduction Laboratories (Lexington, KY), and the monoclonal anti-actin antibody (010056) was from Bioscience (Seikagaku Corp., Tokyo, Japan). Goat antirabbit (170651) and goat antimouse (A4416) antibodies coupled with horseradish peroxidase were from Bio-Rad Laboratories (Hercules, CA) and Sigma (St. Louis, MO), respectively.

**Cell Culture.** The human colon carcinoma cell lines SW480, SW620, Co112, HT29, and its differentiated clones HT29-5 M12 and HT29-5 M21 (21), LoVo and the LoVo clones E2 and C5 (selected for higher metastatic potential; Ref. 22) were provided by Dr. Bernard Sordat (ISREC, Epalinges, Switzerland). Cell line DLD1 was provided by Dr. Emanuela Felley-Bosco (Institute of Pharmacology, University of Lausanne, Lausanne, Switzerland), and the cell lines Caco2 and MDCK strain II were provided by Dr. Walter Hunziker (Institute of Biochemistry, University of Lausanne, Lausanne, Switzerland). NIH-3T3 fibroblasts and NIH-3T3 EtTu, a population of NIH-3T3 cells isolated after tumor formation on nude mice (23), were provided by Dr Ernst Reichenbach (ISREC). HT29, HT29-5 M12, HT29-5 M21, Co112, Caco2, MDCK, NIH-3T3, and NIH-3T3 EtTu cells were cultured in DMEM supplemented with 10% FCS and penicillin, streptomycin, and neomycin. LoVo and LoVo clones were cultured in the same medium containing 0.1% Na2 CO3. SW480, SW620, and DLD1 cells were maintained in RPMI 1640 with 10% FCS and antibiotics as described above. All cells were cultured at 37°C under 5% CO2 and passaged every week using trypsin/EDTA.

**Isolation of Human Colon Crypts and Purification of Epithelial Cells.** Human colonic crypts and, subsequently, colonic epithelial cells or stroma were isolated as described previously (24, 25) after obtaining the informed consent of the patients. Operations were performed at the University Hospital of Geneva (Geneva, Switzerland) and at the Carl-Thiem Klinikum (Cottbus, Germany). Authorization was provided by the Ethics Committee. Epithelial cell viability after purification was >90% as determined by trypan blue staining. After cross-staining with a pan-anticytokinin antibody (CAM 5.12; Perkin-Elmer, Norwalk, CT), epithelial cell preparations were shown to be >95% pure by fluorescence-activated cell-sorting analysis.

**Plasmids.** Plasmid placOP-cav-1, which allows IPTG-inducible expression of caveolin-1 in transfected cells, was constructed as follows. The full-length cDNA encoding dog caveolin-1 was amplified by reverse transcription-PCR using caveolin-1-specific primers flanked by NotI restriction sites and RNA isolated from MDCK cells as a template. The resulting cDNA was purified and then cloned into the NotI site of placOP, which consists of vectors p3’SS and pOPRSV1 chloramphenicol acetyltransferase from Invitrogen (Carlsbad, CA) fused together as described previously (23). The sequence of the 5′ sense primer, which also included a Kozak motif (underlined) upstream of the initiation ATG codon, was 5′-CCAGGCCGGGCGGCGGTATCTGCTGGGGGAAATAC-3′, and the sequence of the antisense primer was 5′-TATCTGGCCGGCCTATGTTCTTCTTGATGTGTG-3′. NotI restriction sites are indicated in bold. The construct pGEM-cav-1 was used to produce caveolin-1-specific probes for Northern analysis and was obtained by amplifying a cDNA sequence conserved between dog and human (nucleotides 63–433 of the cDNA coding sequence) by reverse transcription-PCR using RNA isolated from MDCK cells as a template and appropriate primers to allow subsequent cloning of the amplified product into the XbaI/EcoRI sites of pGEM2 (Promega, Madison, WI). The sense primer (5′-GGGCAACATCTAGAAGCCCAACAAC-3′) was XbaI site (shown in bold). The antisense primer (5′-CTGATGCACTTATCAGAATGCAAG-3′) contained an EcoRI site (shown in bold). The pSP65m-β-actin plasmid (26) used for standardization of Northern blots was kindly provided by Markus Nahbolz (ISREC).

**Stable Transfection of HT29 and DLD1 Cells with a Plasmid Permitting Inducible Expression of Caveolin-1.** HT29 and DLD1 cells were stably transfected with placOP (mock) or placOP-cav-1 by calcium phosphate precipitation as described previously (27). Individual clones resistant to 500 μg/ml hygromycin B were screened for IPTG-induced expression of recombiant caveolin-1 by Western blot analysis. Induction of caveolin-1 was maximal after 24 h of stimulation with 1 mM IPTG.

**Northern Analysis.** Total cellular RNA was extracted with a purification kit in the presence of guanidinium thiocyanate (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Samples containing 15 μg of cytoplasmic RNA were fractionated on 1% agarose gels prepared in 10 mM sodium phosphate buffer (pH 7), transferred to a nylon membrane, and cross-linked to the membrane by UV irradiation as described previously (28, 29). Alternatively, multiple tissue or cell line Northern blots were purchased from Clontech Laboratories (Palo Alto, CA). After overnight preincubation at 55°C in hybridization buffer [50% formamide, 5% SSC, 1 mM EDTA, 0.2% SDS, 2× Denhardt’s solution, 0.5 mg/ml yeast RNA, and 0.25 mg/ml salmon sperm DNA in 50 mM sodium phosphate buffer (pH 6.5)], blots were further incubated for 24 h with 106 cpm/ml 13P-labeled RNA probes for caveolin-1 in hybridization buffer. Probes were synthesized as described previously (28, 29) from XbaI-linearized pGEM-cav-1. Blots were washed four times (for 15 min each time) at 65°C in 0.1× SSC/0.1% SDS solution and exposed to film (BioMax MR-1; Kodak, Rochester, NY). After caveolin-1 detection, blots were stripped (according to a protocol from Clontech Laboratories) and standardized to β-actin using a ribo-probe prepared from Mol-I-linearized pSP65m-β-actin.

**SDS-PAGE and Western Blotting.** Expression of caveolin-1 in carcinoma cell lines, transfected HT29 or DLD1 cells, human colon tissues, or NIH-3T3 cells was studied by Western blot analysis. Cells were grown until they reached 80% confluence. Culture medium was then removed, and the cells were washed twice with cold PBS and lysed in buffer containing 4% SDS, 125 mM Tris-HCl (pH 6.8), and protease inhibitors (10 μg/ml benzamidine, 2 μg/ml antipain, and 1 μg/ml leupeptin). Cell lysates were sonicated, and the protein concentration was determined with the BCA assay. Human colon tissues were lysed in a similar fashion, but homogenates were passed through a 25-gauge needle several times, sonicated, and cleared by centrifugation for 5 min at 10,000 × g in an Eppendorf centrifuge. The protein concentration of supernatants was determined by the BCA assay. All samples were adjusted to Laemmli buffer composition [Ref. 30; 2% SDS, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), 100 mM DTT, and 0.1% bromophenol blue], denatured by heating at 95°C for 5 min, and subsequently loaded on 10% gels. After separation, proteins were transferred onto nitrocellulose. Membranes were stained with Ponceau Red S (Sigma) to verify equal loading of samples and blocked overnight in PBS/3% milk/2 mM Na3B6H (the membranes were then incubated for 1 h at room temperature with either anti-caveolin-1 (1:10,000) or anti-β-actin (1:1,000) antibodies diluted in blocking solution. Membranes were then washed five times in PBS/0.1% Tween 20, incubated for 1 h with a second antibody (1:2,500) diluted in blocking solution (no azide), and washed again as described previously. Membrane-bound second antibodies were detected by enhanced chemiluminescence following the instructions of the manufacturer.

**Tumorigenicity Assays.** Cells (105) were suspended in 50 μl of DMEM and injected s.c. into 6–8-week-old nude mice. For each mouse, control cells (parental HT29 or DLD1 cells or mock-transfected cells) were injected on the left, and HT29 or DLD1 cells transfected with caveolin-1 [clones C13, C14, C16 (HT29 clones) or C2 or C4 (DLD1 clones)] were injected on the right. Large (D) and small (d) diameters of growing tumors were measured twice a week, and the corresponding volumes (V) were estimated using the equation $$V = \frac{d^2 \times D}{2 \times \pi}.$$ To reisolate tumor cells for further culture, the tumor tissue was excised, cut into small pieces under sterile conditions using scalpels, and washed with tetracycline/EDTA for 15 min at 37°C. Tumor cells were cultured until confluence in 10-cm Petri dishes, trypsinized, diluted 1:10 in fresh medium, and seeded again. After a second passage, when tissue debris and contaminating cells had been eliminated, ex-tumor cells were lysed at 80% confluence and processed for caveolin-1 detection as described previously.

**RESULTS**

**Caveolin-1 Expression in Normal Human Colon Tissue and Colon Carcinomas.** Caveolin-1 has been shown to suppress cell growth and tumorigenicity in several cell systems (7, 8, 18, 31). However, direct evidence showing that caveolin-1 can prevent tumor formation by human carcinoma cells is currently not available. To this end, we analyzed caveolin-1 mRNA and protein levels in a variety of human colon carcinoma cell lines, as well as in human tissues of normal or tumor origin. In initial experiments, caveolin-1 mRNA levels in human tissues (epithelium of the small intestine and colon) and the SW480 carcinoma cell line (Fig. 1, top panel) were compared by Northern blotting analysis. The 3-kb specific mRNA of caveolin-1 (22) was extremely abundant in the heart but undetectable in peripheral blood leukocytes, which served in these experiments as positive controls. In human normal and tumor tissue sections, caveolin-1 was detected in 20% of normal samples, and its expression in tumor tissues was significantly increased compared to normal tissue.
Caveolin-1 levels are reduced in human colon tumor samples as well as in colon carcinoma cell lines and that the decreased presence of the caveolin-1 protein may be attributed to reduced mRNA levels.

Caveolin-1 Down-Regulation Occurs during Tumor Formation. Although cell transformation by oncogenes (v-Abl, bcr-Abl, Ras, polyoma virus middle-sized tumor antigen, crkl, v-Src) led to caveolin-1 down-regulation (7), it was not clear at this point whether tumor formation itself is sufficient to reduce caveolin-1 expression. Because

![Image](https://cancerres.aacrjournals.org/doi/10.1158/0008-5472.CAN-00-0051)
levels were low in colon carcinoma lines, a different model system was required. NIH-3T3 fibroblast cells are ideal in this respect because they express caveolin-1 and are able to induce tumor formation in nude mice after extended periods of time (on the order of 50–60 days; Ref. 23). Comparison by Western and Northern blotting of parental NIH-3T3 cells with cells isolated after tumor formation in nude mice clearly revealed that the latter expressed lower levels of caveolin-1 protein (Fig. 4A) and mRNA (Fig. 4B). Thus, tumor formation in mice correlated with either reduction of caveolin-1 expression in NIH-3T3 cells or elimination of cells expressing caveolin-1.

Caveolin-1 Can Be Reexpressed in the Colon Carcinoma Cell Lines HT29 and DLD1. To assess whether the expression of caveolin-1 in colon carcinoma cells may represent a rate-limiting factor in tumor development of these cells, HT29 or DLD1 cells were transfected with a plasmid harboring a full-length dog caveolin-1 encoding cDNA under the control of an IPTG-inducible promoter (placIOP-cav-1). Several clones were isolated and checked for caveolin-1 expression levels and levels after IPTG induction were not identical in these clones, with clone C14 expressing the highest amount, and clone C16 expressing the lowest amount. Similarly, clones C2 and C4 expressed higher caveolin-1 levels than parental DLD1 and mock-transfected cells, but caveolin-1 expression levels were not increased by the addition of IPTG (Fig. 5B).

Reexpression of Caveolin-1 in HT29 and DLD1 Cells Reduced Tumor Formation in Nude Mice. The results obtained with NIH-3T3 cells in nude mice showed that caveolin-1 down-regulation occurred on tumor formation and suggested that the reintroduction of caveolin-1 into colon carcinoma cell lines like HT29 or DLD1 may block the tumor-forming ability of these cells. To test this hypothesis, nude mice were injected in each case with control cells (parental or mock-transfected cells) on the left side and HT29 (clones C13, C14, and C16) or DLD1 (clones C2 and C4) cells expressing caveolin-1 on the right side (total number of mice = n = 10, respectively; Figs. 6 and 7). All HT29 cells led to noticeable tumor formation 1 month after injection, but in 75% (n = 10) of the cases studied, tumors were either significantly smaller for caveolin-1-expressing HT29 clones (Fig. 6, A and B; n = 10) or almost undetectable (Fig. 6C; n = 3). Where tumors developed, the kinetics of tumor formation were different, with a lag time of 2–3 weeks before tumor formation was noticeable (Fig. 6, A and B). For 25% (n = 3) of the mice tested, however, no difference was detected in either the size of the tumors or the kinetics of tumor development (Fig. 6D).

Similarly, in 70% (n = 5) of the cases studied, tumor formation was reduced in DLD1 clones expressing caveolin-1 (Fig. 7, A–C). As for HT29-cav-1 clones, tumor formation was generally observed after an initial lag period of 2–3 weeks (Fig. 7, B and C). For 30% of the mice tested (n = 2), however, no difference in the size of the tumor was detected (Fig. 7D).

Caveolin-1 Expression Levels in HT29-cav-1 and DLD1-cav-1 Cells Were Reduced on Tumor Formation in Nude Mice. The experiments with NIH-3T3 fibroblasts (Fig. 4) revealed that tumor formation resulted in cell populations with reduced caveolin-1 levels. Thus, the possible explanations for why tumor formation had occurred in some cases with transfected HT29 and DLD1 cells were that this process may have led to either elimination of caveolin-1 expression, despite being under the control of an exogenous promoter, or to selection of cells with lower basal levels of caveolin-1 expression. To investigate these possibilities, cells were

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* Patient data were anonymized.

+ Tumors were characterized by staging criteria (tumor-node-metastasis system) describing local spread of the primary tumor (T), metastasis of regional lymph nodes (N), and distant metastasis (M). (Ref. 56).

+ Samples excised by surgery from colon cancer patients were analyzed by Western blotting as described in Fig. 3, and the relative levels of caveolin-1 expression were determined by scanning densitometry. For each patient, results are presented as a ratio between the levels of caveolin-1 measured in normal colon tissues (mucosa or stroma) and those detected in their tumor counterparts.

+ n.a. samples were not available.
isolated from excised tumors and put back in culture, and, after pure cell populations were available, cells were examined for caveolin-1 protein expression (Fig. 8, ExTumor). Directly after plating, cells derived from tumors were a mixture of host cells (mainly fibroblasts) and tumor cells, but only tumor cells underwent rapid proliferation. By contrast, host cells tended to detach and die rapidly (data not shown). When culture plates were confluent after two passages, homogenous tumor cell populations that were morphologically identical to parental HT29 or DLD1 cells but had the additional ability to grow in the presence of hygromycin B were obtained (data not shown). In these cells, basal levels of caveolin-1 expression were reduced when compared with those observed for HT29-cav-1 cells before injection into mice (Fig. 8, ExTumor and BI, respectively). Nevertheless, caveolin-1 expression could still be induced by the addition of IPTG (data not shown). Thus, selection for HT29 cells expressing lower levels of caveolin-1 occurred on tumor formation in nude mice. Similar results were obtained with DLD1 cells (data not shown).

Selection for Methotrexate Resistance and Metastatic Potential Enhanced Caveolin-1 Expression in Colon Carcinoma Cells. The previous experiments strongly favored the notion that tumor formation in humans and in nude mice correlates with reduced caveolin-1 expression levels. Alternatively, it became of interest to examine whether low caveolin-1 expression levels were an irreversible state in colon carcinoma cells. Given that more differentiated cells tend to express higher caveolin-1 levels (33), culture conditions promoting cell differentiation may be expected to enhance caveolin-1 expression in colon carcinoma cells. Indeed, the methotrexate-resistant, more differentiated HT29 clones HT29-5 M12 and HT29-5 M21 (21) expressed significantly higher levels of caveolin-1 than parental HT29 cells, with the difference being greatest for the enterocytic clone HT29-5 M12 and less apparent for the mucous-secreting HT29-5 M21 cells (Fig. 9A). However, caveolin-1 was not detectable in Caco2 cells cultured normally with frequent passaging (proliferative, undifferentiated cells; see Fig. 2) or in cells left for 5 weeks without passaging (differentiated cells; data not shown; Ref. 34). Taken together, these experiments suggest that variations in culture conditions favoring differentiation have little effect on caveolin-1 expression in colon carcinoma cells but that phenotypic changes correlate with increased caveolin-1 expression when observed in conjunction with drug resistance.

To test the possibility that caveolin-1 expression might vary with metastatic potential, as suggested from experiments with human prostate cancer cells (22), two clones isolated from the colon carcinoma cell line LoVo (E2 and C5) that display higher metastatic potential than the parental LoVo cells were characterized (35). Indeed, caveolin-1 expression levels in the LoVo E2 and C5 clones were significantly higher than that in the parental LoVo line (Fig. 9B), suggesting that up-regulation of caveolin-1 might occur during metastasis.
DISCUSSION

Several studies have recently assessed the potential role of caveolin-1 in tumor formation. When caveolin-1 was reexpressed in breast cancer cell lines, cell proliferation in culture and anchorage-independent growth in soft agar were reduced compared with those in parental cell lines, suggesting that caveolin-1 modulates growth parameters generally considered relevant to tumor formation in vivo (8). However, no direct evidence was provided showing that the presence of caveolin-1 prevented tumor formation. In a NIH-3T3 cell model system, reexpression of caveolin-1 in oncogenically transformed cells suppressed the transformed phenotype because anchorage-independent growth in soft agar was abrogated (18). In addition, downregulation of caveolin-1 by overexpression of an antisense caveolin-1 construct was sufficient to mediate cell transformation and promote tumor formation when cells were injected in nude mice (19). Taken together, these results show that caveolin-1 can reduce cell tumorigenicity in the NIH-3T3 mouse fibroblast cell line and suggest that it may do the same in human breast cancer cells.

Fig. 7. Tumor development in mice of DLD1 cells transfected with caveolin-1. Cells (1 × 10^6) were injected s.c. into 6–8-week-old nude mice. A total of seven mice were analyzed in the same fashion described in the Fig. 6 legend. Results from a representative series of experiments with four mice are presented.

Fig. 8. Immunoblot analysis of caveolin-1 expression in transfected HT29 cells after tumor formation in nude mice. Tumors that developed on injection of parental, mock-transfected, or caveolin-1-transfected HT29 cells were excised as described and cultured (ExTumor). When homogenous cell populations were obtained, cells were lysed, and proteins (50 μg) were analyzed by Western blot as described in the Fig. 2 legend. Caveolin-1 expression in the absence or presence of IPTG was compared in samples from cells before (Bl) and after (ExTumor) cell injection into mice. Results for cell populations obtained from two separate tumors (T1 and T2) are presented in each case.

Fig. 9. Caveolin-1 expression in colon carcinoma cells resistant to high doses of methotrexate or with high metastatic potential. A, expression of caveolin-1 in stably differentiated HT29 populations of the enterocytic (HT29-5 M12) or mucous-secreting (HT29-5 M21) phenotype obtained by exposure of HT29 cells to high concentrations of methotrexate was analyzed by Western blot analysis. Proteins from carcinoma (50 μg) or MDCK (5 μg) cells were analyzed as described in the legend for Fig. 2A. B, expression of caveolin-1 protein in the colon carcinoma line LoVo and in two derived clones selected for high metastatic potential (E2 and C5) was analyzed by Western blot analysis as described in A.

Fig. 10. A representative series of experiments with four mice are presented.
In complete agreement with this concept, caveolin-1 was identified as 1 of 26 candidate tumor suppressor genes in human mammary carcinomas using differential display and subtractive techniques (31). In addition, the caveolin-1 gene has been mapped to a tumor suppressor locus in both the human (locus D7S522) and mouse (locus 6-A2/731) genomes (36, 37). These regions are frequently deleted or contain breakpoint sites for chromosome translocation in a wide variety of tumors (36, 38). Furthermore, caveolin-1 was recently identified as a target protein for p53-dependent regulation (39). However, at the DNA level, there is virtually no evidence that caveolin-1 is a tumor suppressor gene because the caveolin-1 gene is neither mutated nor methylated in cancer cells (20), although methylation of the caveolin-1 promoter has been described in breast cancer cell lines (37).

Despite this wealth of information, few studies directly investigated how expression levels of caveolin-1 change on tumor formation in either mice or humans. In this study, we focused in particular on the role that caveolin-1 may play in human colon cancer. Our results showed that caveolin-1 mRNA and protein levels (Figs. 1 and 2) were reduced in colon carcinoma cell lines as compared with normal colon tissue. Thus, after lung (9) and possibly breast (8) carcinomas, colon carcinomas represent a third group of human carcinomas in which caveolin-1 levels are reduced as a consequence of what appears to be predominantly transcriptional regulation. Moreover, the comparison of samples from normal colon and colon tumor tissue revealed that caveolin-1 protein expression was reduced in tumor epithelium, thereby establishing a direct link between the reduced caveolin-1 expression levels observed in human colon carcinoma cell lines and a reduction of caveolin-1 expression observed in colonic epithelial cells on tumor formation.

Caveolin-1 down-regulation was not only observed in colon tumor mucosa but was also observed in the adjacent stroma, suggesting that carcinoma cells may be able to modulate expression levels of caveolin-1 in surrounding tissues, constituted predominantly of adipocytes and endothelial and muscle cells. In this context, it is interesting to note that angiogenesis activators such as vascular endothelial growth factor, basic fibroblast growth factor, and HGF down-regulate caveolin-1 in human endothelial cells (40).

To corroborate the notion that tumor formation correlates with reduction of caveolin-1 expression, caveolin-1 levels were compared in the parental NIH-3T3 cells and after tumors had developed in nude mice. Tumor formation in this experimental model system yielded a cell population with less caveolin-1 (Fig. 4), indicating that caveolin-1 may be rate-limiting for anchorage-independent growth and tumor formation in mice. Consistent with this idea, NIH-3T3 ex-tumor cells with lower caveolin-1 levels formed tumors more rapidly on reinjection into nude mice (data not shown).

Whereas our results are in perfect agreement with those obtained in mammary (8) and lung carcinoma cell lines (9), they disagree with results obtained by others for prostate and breast cancer (41, 42). There, elevated expression of caveolin-1 was associated with development of prostate and breast cancers in both the human and mouse systems (41). Alternatively, Hurlstone et al. (20) failed to detect any difference between the expression of caveolin-1 in tumor cells versus normal breast tissue. These results indicate that the role of caveolin-1 may vary considerably, depending on the tissue involved.

Interestingly, caveolin-1 levels were highest in metastases derived from primary prostate tumors, suggesting that accumulation of caveolin-1 relative to normal epithelium occurred with progression of prostate cancer (41). One major difference in that respect between prostate and colon tissue is that caveolin-1 mRNA and protein are present at high levels in normal colon epithelium (Figs. 1–3), whereas only minimal expression is observed in the corresponding prostate tissue samples (41). Thus, transformation and progression of malignancy in cells that normally express caveolin-1 may occur in two phases: (a) initially, down-regulation of caveolin-1 is required during primary tumor formation; and (b) subsequently, up-regulation of caveolin-1 may occur in methotrexate-resistant HT29 cells (Fig. 9A) as well as in multidrug-resistant human colon carcinoma HT29 cells and breast carcinoma MCF-7 cells (43). Alternatively, reexpression of caveolin-1 may be required during metastasis. In support of this idea, colon carcinoma clones selected from the LoVo cell line for higher metastatic potential (35) had elevated caveolin-1 protein levels when compared with parental cells (Fig. 9B).

Here, however, two additional points need to be considered. First, basal caveolin-1 levels are already higher in LoVo than in other colon carcinoma lines (see Fig. 9B); second, although the cell populations E2 and C5 were obtained by sequential injection into mice followed by isolation of cells from resulting lung metastases, this does not mean that metastases generally have higher levels of caveolin-1 expression than the original tumor. For instance, the primary colon tumor SW480 cells and matched metastatic colon cancer SW620 cells originating from the same patient both have equally low caveolin-1 levels (Fig. 2). Similarly low caveolin-1 levels were also observed (data not shown) for liver (Isrec02) and peritoneal (Isrec03) metastases derived from a primary ascending human colon cancer (Isrec01), cell lines characterized by Cajot et al. (44). Taken together, this would argue that control of caveolin-1 levels in colon carcinomas is complex and that no simple unifying hypothesis is currently available to explain all available observations. Clearly, more research is required to address these issues. In conclusion, down-regulation of caveolin-1 might be an early event that occurs in primary tumor formation of a limited set of epithelia that normally express high levels of caveolin-1, including colon (this study) and lung (9).

The precise mechanism by which reduced levels of caveolin-1 expression in epithelium would promote initial steps toward carcinoma formation is not clear. Several reports indicate that caveolin-1 possesses a specific motif, referred to as the scaffolding domain, that can bind to and inhibit the activity of a number of proteins involved in signal transduction, including heterotrimeric G proteins (11), Src family tyrosine kinases (10), endothelial nitric oxide synthase (45–48), Neu tyrosine kinase (49), epidermal growth factor receptor (50), and protein kinase C α (51). Thus, reduced levels of caveolin-1 would prolong cell stimulation linked to one of these numerous signal transduction pathways. Consistent with this notion, targeted down-regulation of caveolin-1 in NIH-3T3 cells leads to hyperactivation of the p42/p44 MAPK pathway and, as a consequence, cell transformation (19). However, overexpression of caveolin-1 inhibited both MAPK-dependent and -independent pathways in adipose cells, whereas in Cos-7 cells, caveolin-1 enhanced MAPK-dependent signaling (52). Thus, modulation of the MAPK signaling pathway as well as other signaling pathways by caveolin-1 may be differentially regulated, depending on the cell system studied. In addition, caveolin-1 levels are likely to be tightly controlled in cells because both up- and down-regulation alter cell signaling events.

More recently, caveolin-1 expression was reported to inhibit transcription of the cyclin D1 gene, suggesting that loss of caveolin-1 expression during tumorigenesis may lead to cellular transformation via the β-catenin/TCF/LEF signaling pathway (53, 54). Caveolin-1 is also involved in signal transduction events mediated by several integrins on binding to extracellular matrix proteins. There, caveolin-1 plays a key role by linking integrins to Fyn activation, which in turn is responsible for Shc recruitment, regulation of Ras–MAPK signaling, and cell cycle progression (55). Thus, anchorage-independent growth observed in transformed cells on down-regulation of caveolin-1 may be linked to this particular aspect of caveolin-1 function.
Finally, direct evidence for the importance of caveolin-1 in limiting the tumor-forming ability of colon carcinoma cells is provided here (Figs. 6 and 7). Expression of caveolin-1 in transfected HT29 and DLD1 clones generally reduced the size of tumors formed on injection into nude mice and delayed the onset of tumor formation in most cases (Figs. 6 and 7). When tumors were detectable, their presence correlated with a decrease in basal caveolin-1 expression in comparison with the levels detected before cell injection into mice (Fig. 8). These observations provide strong support for the notion that an initial period of selection exists. Those cells that have lower caveolin-1 levels and/or succeed in reducing caveolin-1 expression subsequently proliferate and are able to form tumors in nude mice.

The reduction of caveolin-1 mRNA levels observed in breast and lung tumor cell lines indicates that caveolin-1 down-regulation occurs primarily at the transcriptional level. The caveolin-1 gene is reportedly not methylated in either breast primary tumors or tumor-derived cell lines, indicating that the observed down-regulation of caveolin-1 mRNA expression in breast tumors does not result from transcriptional silencing or DNA methylation during tumor progression (20). However, those results are controversial because Engelman et al. (37) identified a CpG island within the caveolin-1 promoter region that was methylated in human breast cancer cell lines. Also, in our studies, reduction of the mRNA levels appeared to be an important mechanism by which caveolin-1 protein levels were regulated because both were dramatically reduced in colon carcinoma cell lines as compared with the levels observed in normal colon tissue (Figs. 1 and 2).

In summary, the experimental data presented here show that: (a) caveolin-1 protein levels were reduced in colon tumors from human patients; (b) colon carcinoma cells had low levels of caveolin-1 mRNA and protein; (c) expression of caveolin-1 in the colon carcinoma cell lines HT29 and DLD1 blocked or retarded tumor formation in nude mice; (d) the ability of HT29-cav-1 and DLD1-cav-1 (and also NIH-3T3 cells) to form tumors in nude mice despite the initial presence of caveolin-1 was linked to a selection process favoring the proliferation of those cells with reduced basal caveolin-1 levels; and (e) initial caveolin-1 down-regulation in colon carcinoma cells need not be an entirely irreversible event because cell survival on selection for either drug resistance or increased metastatic potential may require reexpression of caveolin-1.

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Caveolin-1 Levels Are Down-Regulated in Human Colon Tumors, and Ectopic Expression of Caveolin-1 in Colon Carcinoma Cell Lines Reduces Cell Tumorigenicity

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