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Different Roles for Caveolin-1 in the Development of Non-Small Cell Lung Cancer *versus* Small Cell Lung Cancer

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ABSTRACT

Caveolin-1 (CAV1), an essential structural constituent of caveolae that plays an important role in cellular processes such as transport and signaling, has been implicated in the development of human cancers. However, it is unclear whether CAV1 is acting like an oncogene or tumor suppressor gene. We found that CAV1 expression was reduced or absent in 95% of small cell lung cancers (SCLCs; $n = 21$ lines), whereas it was retained in 76% of non-small cell lung cancers (NSCLCs; $n = 25$ lines) compared with normal human lung epithelial cultures, where it was abundantly expressed. CAV1 expression was tightly linked to the ability to grow attached to the plastic cell culture surface, whereas CAV1-nonexpressing lung cancers of both SCLC and NSCLC type grew as suspension cultures. In addition, attached lung cancer cultures expressed phosphorylated focal adhesion kinase, whereas suspension cultures did not. Lack of CAV1 expression was tightly associated with *CAV1* promoter methylation ($P < 0.0001$) such that *CAV1* methylation was found in 93% of SCLCs ($n = 15$) and 9% of NSCLCs ($n = 11$), whereas 5-aza-2′deoxycytidine treatment restored CAV1 expression in SCLCs. Exogenous CAV1 expression in SCLCs significantly inhibited soft-agar colony formation but did not lead to attachment. By contrast, CAV1 knockdown in NSCLCs mediated by small interfering RNA against *CAV1* led to inhibition of cellular proliferation and soft-agar and liquid colony formation. Importantly, CAV1 knockdown led to reduced phospho-focal adhesion kinase and RalA, but not RalB, levels in NSCLC cells. These results suggest different roles for CAV1 in SCLC, where CAV1 acts like a tumor suppressor gene, and NSCLC, where it appears required for survival and growth.

INTRODUCTION

Caveolae are flask-shaped invaginations of the plasma membrane that play an important role in cellular processes, including molecule transport, cell adhesion, and signal transduction (1–3). Caveolin-1 (CAV1) is an essential structural component of caveolae and functionally regulates the activity of many signaling molecules, such as G-proteins, Src family kinases, H-Ras, protein kinase C, epidermal growth factor receptor, endothelial nitric oxide synthase, and integrins, which are potentially involved in the development of human cancer, by generating preassembled signaling complexes (4–7). Thus, CAV1 could be a key molecule for growth-related signaling and cancer development.

The *CAV1* gene has been considered as both a tumor suppressor gene and an oncogene. Down-regulation of CAV1 expression was observed in breast, lung, colon, and ovarian cancer, whereas oncogenic transformation of cells was associated with reduction of CAV1 expression, and antisense-mediated down-regulation of CAV1 expression was sufficient to drive oncogenic transformation in NIH 3T3 cells (8–15). Exogenous expression of CAV1 in oncogenically transformed cells and cancer cell lines inhibited cell growth and tumorigenesis (12, 16–18). This evidence indicates that CAV1 can act as a tumor suppressor. By contrast, other studies have reported that CAV1 expression was up-regulated in human cancers, including breast, lung, prostate, esophagus, colon, thyroid, and pancreas cancers, and that this up-regulation was associated with metastases and poor prognosis (19–25). These results suggest that CAV1 can function other than as a tumor suppressor. Thus, studies have implicated CAV1 in the development of several human cancers, but it appears to play quite different roles in the development of these cancers.

Human lung cancer is divided into two major histological types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC; Ref. 26). We evaluated endogenous expression of CAV1 protein in several lung cancer cell lines and found that CAV1 protein expression was reduced or absent in 95% of SCLCs ($n = 21$ lines), whereas it was retained in 76% of NSCLCs ($n = 25$ lines) compared with normal human lung epithelial cultures, in which expression was abundant. Methylation analysis revealed promoter hypermethylation of *CAV1* in 93% of SCLCs ($n = 15$) but only 9% of NSCLCs ($n = 11$). Furthermore, exogenous reexpression of CAV1 in SCLCs led to significant inhibition of colony formation, whereas knockdown of CAV1 in NSCLCs by RNA interference (RNAi) technology led to inhibition of cellular proliferation and colony formation in this histological type (27–31). These results suggest different roles for CAV1 in SCLC, where it has the properties of a tumor suppressor, whereas in NSCLC it appears required for growth.

MATERIALS AND METHODS

Cell Lines. All of the lung cancer cell lines were obtained from the Hamon Center collection (University of Texas Southwestern Medical Center) generated by the authors (32, 33). Most are also available from the American Type Culture Collection. Breast cancer cell line MCF7 was obtained from the American Type Culture Collection. Cells were cultured with RPMI 1640 supplemented with 5% fetal bovine serum. Normal human bronchial epithelial and small-airway epithelial cells were obtained from Clonetics (San Diego, CA). The immortalized human bronchial epithelial cell lines BEAS-2B, HBEC1, HBEC2, HBEC3, and HBEC4 were also used. We recently established the immortalized human bronchial epithelial cell lines HBEC1, HBEC2, HBEC3, and HBEC4, retrovirally transfected with CDK4 and the catalytic component of telomerase, which were obtained from bronchial biopsies.[6]

Preparation and Transfection of Synthetic Small Interfering RNA (siRNA). siRNAs targeting *CAV1* (CAV1-1 siRNA), *Tax* (the human leukemia virus gene), and *β-catenin* were designed and prepared as described previously (27, 34, 35). The siRNA sequences against *CAV1* (CAV1-1 siRNA) were 5′-AGACGAGCUGAGCGAGAAGCA-3′ (sense) and 5′-CUUCUCGCUCAGCUCGUCUGC-3′ (antisense) and (CAV1-2 siRNA) 5′-CAUCUACAAGCCCAACAACTT-3′ (sense) and 5′-GUUGUUGGGCUUGUAGAUGTT-3′ (antisense). The siRNA target sequences were tested in a BLAST search of GenBank (National Center for Biotechnology Information database)

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to ensure that only the corresponding gene is the target. RNA oligonucleotides were obtained from the core facility of the University of Texas Southwestern Medical Center (see the RNA Oligonucleotide Synthesis Core web site for details).[7] siRNAs were transfected into cells with use of Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA) as described (56). For siRNA transfection into NCI-H187 and NCI-H524 cells, Lipofectamine 2000 transfection reagent was used (Invitrogen). Cells were grown and harvested for further analysis.

Immunofluorescent Staining. After 24 h of siRNA transfection, cells were plated and grown on slides. After 48 h, the cells were washed with 60 mM PIPES–25 mM HEPES–10 mM EGTA–1 mM $MgCl_2$ (pH 7.4) and fixed for 10 min at 37°C in 3% paraformaldehyde in 60 mM PIPES–25 mM HEPES–10 mM EGTA–1 mM $MgCl_2$ (pH 7.4). After additional washes with PBS, the cells were permeabilized with 0.1% Triton in PBS for 10 min. After blocking with 3% gelatin–3% BSA–0.2% Tween-20 for 1 h at 37°C, the cells were incubated with mouse monoclonal anti-α-tubulin (Calbiochem, San Diego, CA) and the rabbit polyclonal anticaveolin (BD Transduction Laboratories, San Diego, CA) antibodies in gelatin/BSA blocking solution for 16 h at 4°C. The cells were incubated with Alexa Fluor 568 antimouse IgG (H + L) (Molecular Probes, Eugene, OR) and Alexa Fluor 488 antirabbit IgG (H+ L) (Molecular Probes) secondary antibodies for 1 h at 37°C. The stained cells were examined under a fluorescent microscope and photographed.

Western Blot Analysis. Cells were grown to 80–90% confluency and harvested, and cellular proteins were extracted with lysis buffer [40 mM HEPES-NaOH (pH 7.4), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl] containing Complete Mini, a cocktail of protease inhibitors (Roche, Indianapolis, IN). Total protein was separated on a SDS-polyacrylamide gel and electroblotted to nitrocellulose membranes (Schleicher & Schuell, Beverly, NH). After blocking with 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline, membranes were incubated at 4°C for 16 h with rabbit polyclonal anticaveolin (BD Transduction Laboratories), mouse monoclonal anti-β-catenin (BD Transduction Laboratories), rabbit polyclonal anti-phosphorylated focal adhesion kinase (FAK) pY^{397} (BIOSOURCE, Camarillo, CA), mouse monoclonal anti-FAK (BD Transduction Laboratories), mouse anti-RalA (BD Transduction Laboratories), rabbit anti-RalB (BD Transduction Laboratories), and mouse monoclonal antiactin (Sigma, Saint Louis, MO) antibodies. The membranes then were developed with peroxidase-labeled antibodies (Amersham Pharmacia, Piscataway, NJ) by Super Signal chemiluminescence substrate (Pierce, Rockford, IL). Actin protein levels were used as a control for adequacy of equal protein loading.

Bisulfite Genomic DNA Sequencing. One μg of genomic DNA was denatured by NaOH and modified by bisulfite (Sigma) as described previously (36). The modified DNA was purified with use of a Wizard DNA purification kit (Promega, Madison, WI), treated with NaOH to desulfonate the sample, precipitated with ethanol, and resuspended in water. Bisulfite-treated DNA was then used as template in PCR reactions for sequencing analysis. The sequences of the three primer pairs used are as follows: 5′-TGTGTATTTTGTAAATATGGTATAATTTG-3′ and 5′-CCATCTCTACCTTAAAACACAT-3′, 5′-GGGATTTATAAAGTTTAGATGTGA-3′ and 5′-CATTTTCCCTACTCTAAACCAC-3′, and 5′-ATAGGGTAGGATTGTGGATTGT-3′ and 5′-TAAACACATCCCCAAAATTCTAAC-3′. The PCR conditions were as follows: the reaction volume was 20 μl; initial denaturation was for 15 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, with final extension at 72°C for 10 min. PCR products were purified with exonuclease I and shrimp alkaline phosphatase (USB Corp., Cleveland, OH) and sequenced with an ABI 377 automatic DNA sequencer.

We evaluated the methylation status by measuring methylated CpG (mCpG) density as described previously (37). The bisulfite treatment converts unmethylated C to T but leaves methylated C as C. If only T is detected at this position in sequencing, the status of this CpG dinucleotide is defined as fully unmethylated (open square), which was counted as 0 in the calculation of mCpG density. If only C is detected at this position in sequencing, the status of this CpG dinucleotide is defined as fully methylated (filled square), which was counted as 1 in the calculation of mCpG density. Accordingly, a 50% filled square represents C and T detected at equal peak height in sequencing at this position; thus, the status of this CpG is defined as one-half methylated and one-half unmethylated and was counted as 0.5 in the calculation of mCpG density. A 75% filled square indicates that both C and T were detected in sequencing at this position but the height of the C peak was larger than that of the T peak; thus the status of this CpG is defined as 75% methylated and 25% unmethylated and was counted as 0.75 in the calculation of mCpG density. A 25% filled square indicates that both C and T were detected in sequencing at this position but the height of the T peak was larger than that of the C peak; therefore, the status of this CpG is defined as 75% unmethylated and 25% methylated and was counted as 0.25 in the calculation of mCpG density. The mCpG density per 100 bp was calculated by summing the scores of all methylated CpGs in the region from nucleotide (nt) 29442 to nt 29676, dividing by 235 bp, and multiplying by 100.

Treatment with 5-Aza-2′-deoxycytidine and Reverse Transcription-PCR Analysis. NCI-H146 and NCI-H2171 SCLC cell lines were incubated and grown in culture medium in the presence or absence of 4 μM 5-aza-2′-deoxycytidine (Sigma) for 6 days as described previously (36). Total RNA was extracted from the cells by TRIzol Reagent (Invitrogen) according to the manufacturer's instructions, and cDNA was synthesized using 2 μg of total RNA with the SuperScript II First-Strand Synthesis using oligo(dT) primer System (Invitrogen). Aliquots of the reaction mixture were used for the subsequent PCR amplification. The primer sequences for *CAV1* amplification were 5′-CGACCCTAAACACCTCAACGATG-3′ and 5′-GCAGACAGCAAGCGGTAAAACC-3′. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control to confirm the success of the reaction. The primer sequences for *GAPDH* amplification were 5′-CACTGGCGTCTTCACCACCATG-3′ and 5′-GCTTCACCACCTTCTTGATGTCA-3′. PCR conditions were as follows: the reaction volume was 20 μl; initial denaturation was for 15 min at 95°C, followed by 25 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, with a final extension at 72°C for 10 min. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

Methylation-Specific PCR. DNA methylation patterns in the CpG island of the *CAV1* gene were determined by the method of methylation-specific PCR (36). The primer sequences were 5′-TTATTTCGAAGCGTTTGGGAG-3′ and 5′-AACACTCGTTTACATCTAATCG-3′ for the methylated reaction, and 5′-TTATTTTGAAGTGTTTGGGAG-3′ and 5′-AACACTCATTTACATCTAATCA-3′ for the unmethylated reaction. PCR amplification was performed with bisulfite-treated DNA as a template, using specific primer sequences for the methylated and unmethylated forms of the gene. PCR conditions were as follows: the reaction volume was 20 μl; initial denaturation was for 15 min at 95°C, followed by 7 cycles of 30 s at 94°C, 55 s at 57°C, and 20 s at 72°C and 30 cycles of 20 s at 90°C, 30 s at 57°C, and 20 s at 72°C, with final extension at 72°C for 10 min. PCR products were analyzed on 2% agarose gels. We note that although CAV1 was not methylated in normal lung epithelium, it was methylated in B lymphocytes. Thus, caution must be used in applying this assay to samples containing lymphocytes.

Construction and Transfection of CAV1-Expressing Plasmid Vector. The full-length cDNA encoding human *CAV1* was amplified by reverse transcription-PCR with primers (5′-CGCGGATCCTCCTCACAGT-3′ and 5′-CCGGAATTCTATCCTTGAAATG-3′) and RNA isolated from BEAS-2B cells. The amplified DNA fragments were ligated into the *Bam*HI-*Eco*RI sites of the pcDNA3.1 (+) vector (Invitrogen) to construct the CAV1 expression plasmid pcDNA3.1/CAV1. The correct orientation and sequence of the insert were verified by DNA sequencing. The pcDNA3.1/CAV1 plasmid or the pcDNA3.1 plasmid was transfected into the cells with use of DMRIE-C reagent (Invitrogen) according to the manufacturers' protocol.

Colony Formation Assay. The *in vitro* growth characteristics were tested by colony formation assay. After 48 h of transfection with siRNA or CAV1 expression plasmid, cells were harvested, and the colony formation assay was performed as described previously (38) with some modifications. Briefly, 500 viable cells unstained with trypan blue were plated in each well of 6-well plates. Cells were cultured in RPMI 1640 supplemented with 5% serum, and surviving colonies were counted 14 days later after staining with methylene blue. For the anchorage-independent, soft-agar growth assays, 300 viable cells were suspended and plated in 0.33% agar in complete medium and layered over a 0.50% agar base containing complete medium in 12-well plates. After 4 weeks, the number of foci was counted.

Bromodeoxyuridine (BrdUrd) Incorporation Assay. Twenty-four h after transfection with siRNA, 3000 cells were replated and cultured in 96-well plates in replicates of 8. After 48 h, we examined DNA synthesis by quanti-

[7] http://cbi.swmed.edu/pages/oligonet_index.htm.

tating BrdUrd incorporated into the newly synthesized DNA during cell proliferation with use of the BrdUrd Labeling and Detection Kit III (Roche) according to the manufacturers' protocol. The absorbance ($A_{405\ nm}/A_{492\ nm}$) that was directly correlated to the level of BrdUrd incorporated into cellular DNA was determined with use of a microtiter plate reader.

PCR-Restriction Fragment Length Polymorphism Analysis. The *CAV1* missense mutation at codon 132 (P132L) was screened by PCR-restriction fragment length polymorphism analysis as described previously (39). The primer sequences for *CAV1* amplification were 5′-CTGTGCTCATGTTGTGTCAC-3′ and 5′-GTATTAGCAACTTGGAACTTG-3′. PCR conditions were as follows: 50 μg of genomic DNA was used in a reaction volume of 20 μl; initial denaturation was for 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C, with final extension at 72°C for 10 min. The amplified DNA fragments were digested with *Nla*III (New England Biolabs, Beverly, MA) at 37°C for 3 h. Complete digestion of the 460-bp PCR products produced 236- and 222-bp fragments for the wild-type allele, whereas digestion of the mutant allele produced a single, 460-bp fragment. The digested DNA was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining, and the genotypes were determined.

RESULTS

Endogenous Expression of CAV1 Protein Differs between SCLC and NSCLC. We first examined endogenous expression of CAV1 protein in 46 lung cancer cell lines, including 25 NSCLCs and 21 SCLCs (Fig. 1*A*). CAV1 protein expression was reduced or absent in 95% of SCLC cell lines (20 of 21), whereas 76% (19 of 25) of NSCLC cell lines retained CAV1 protein expression compared with the normal human lung epithelial cells. When we classified the cell lines into suspension and adherent cells according to their culture morphologies, CAV1 protein expression was absent in all cells growing as suspension cultures, including 19 SCLC cell lines and the NCI-H1155 NSCLC cell line. By contrast, all adherent cells, including 24 NSCLC cell lines and 2 SCLC cell lines (NCI-H1607 and NCI-H2227), that grew attached to the tissue culture dishes expressed CAV1 protein. We found a strong association between the loss of CAV1 protein expression and cell culture morphology ($P < 0.0001$, Fisher's exact test).

Because previous studies have reported that CAV1 is required for integrin-mediated FAK activation and cell adhesion (40), we asked whether CAV1 expression and attachment to the culture dish were correlated with FAK phosphorylation status. We therefore compared the level of phosphorylated FAK in CAV1-nonexpressing cell lines (four SCLCs and H1155 NSCLC), which grew as suspension cultures, and CAV1-expressing cell lines (two NSCLCs and H2227 SCLC). We found a perfect correlation between FAK phosphorylation and CAV1 expression (Fig. 1*B*).

CAV1 Promoter Methylation in SCLC. Previous studies reported promoter methylation of *CAV1* in breast, ovarian, and prostate cancers (9, 41, 42). We therefore examined whether *CAV1* is epigenetically inactivated through aberrant methylation of the promoter region in lung cancer cell lines that failed to express CAV1. We determined the methylation status of seven CpG dinucleotides (residing in the sequence between nt 29442 and nt 29676 of GenBank accession no. AC006159) in the 5′ region of *CAV1* by sequencing bisulfite-modified DNA in six SCLC cell lines (NCI-H82, NCI-H146, NCI-H345, NCI-H865, NCI-H1092, and NCI-H2171) and the MCF7 breast cancer cell line, all which lack CAV1 expression, as well as five CAV1-expressing lung cancer cell lines (NCI-H358, NCI-H460, NCI-H838, NCI-H1299, and NCI-H2227) and normal human bronchial epithelial cell lines HBEC1 and BEAS-2B (Fig. 2*A*). In MCF7 and the six SCLCs that did not express CAV1, the CpG dinucleotides were heavily or completely methylated, with a mCpG density range of 2.23–2.98/100 bp. The result was in agreement with a previous study by Engelman *et al.* (41), who reported methylation of the three CpG sites (nt 29487–29515 on the AC006159 sequence) in *CAV1* in two breast cancer cell lines, MCF7 and T-47D. In the NCI-H2227 SCLC cell line and three NSCLCs (NCI-H358, NCI-H460, and NCI-H838) that expressed CAV1, the CpG dinucleotides were only lightly methylated, with mCpG densities varying from 0.11 to 1.28, and these

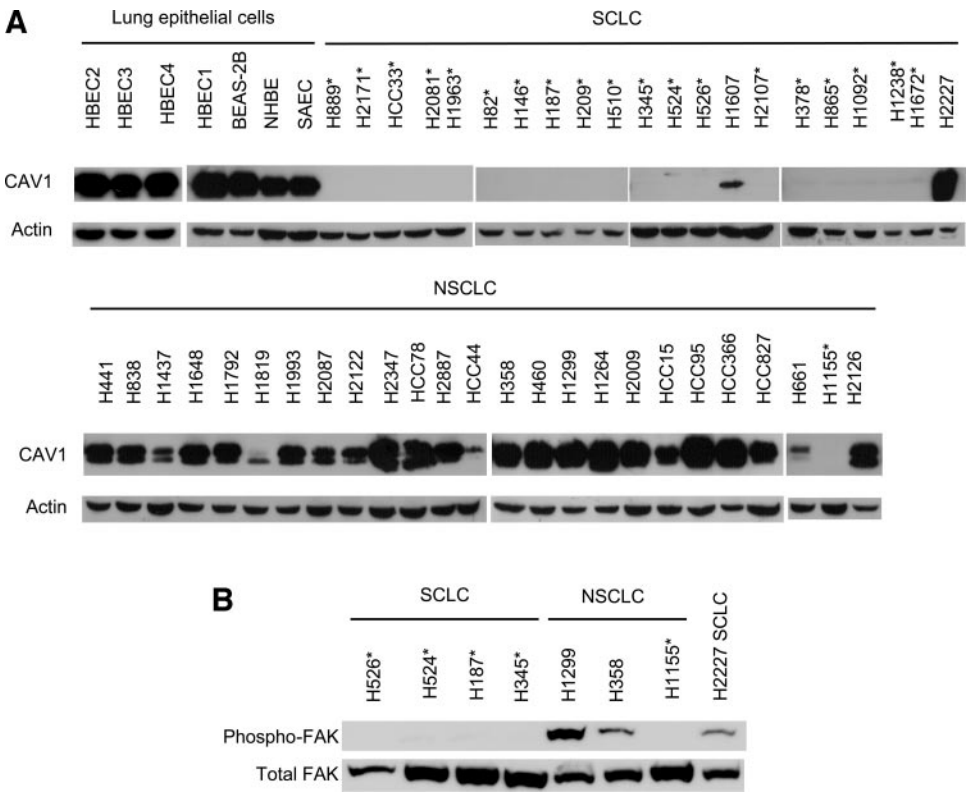

Fig. 1. *A*, endogenous expression of caveolin-1 (*CAV1*) protein in 46 human lung cancer cell lines, including 25 non-small cell lung cancers (*NSCLC*) and 21 small cell lung cancers (*SCLC*), and normal lung cells. Twenty μg of total protein were loaded in each lane, and Western blotting was performed. Actin expression levels were used as a control for equal protein loading. *B*, levels of total and phosphorylated focal adhesion kinase (*FAK*) in lung cancer cell lines. Conditions for Western blotting were the same as in *A*. Tumor cells that grew unattached as floating cultures are indicated by *.

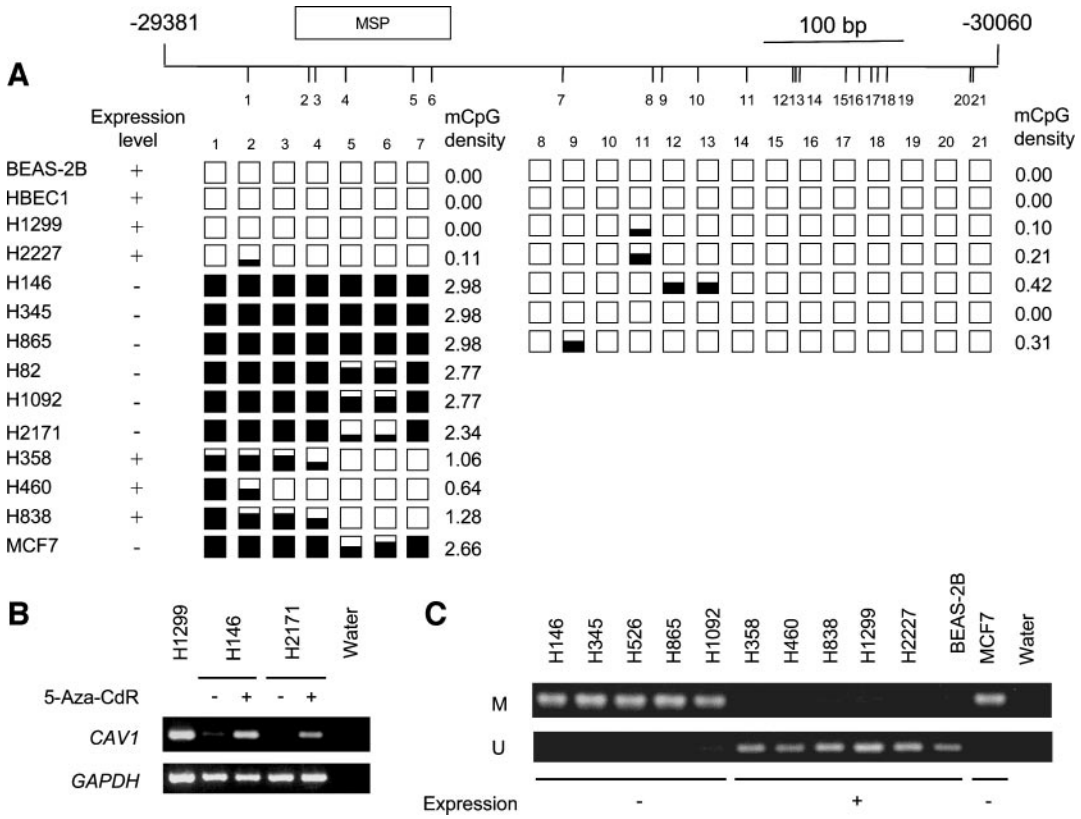

Fig. 2. Caveolin-1 (*CAV1*) promoter methylation in lung cancer cell lines. *A*, methylation status of each CpG dinucleotide in the promoter region of *CAV1* was determined by sequencing sodium bisulfite-treated genomic DNA. The *top panel* illustrates the region examined (GenBank accession no. AC006159; nucleotides 29381–30060), the *vertical bars* represent CpG sites (numbered 1–21), and the *open box* represents the amplicon for methylation-specific PCR (*MSP*) assay. The *bottom panel* illustrates the methylation status (*mCpG*) of individual CpG sites in the cell lines. +, normal protein expression level; −, undetectable level by Western blotting. *Open squares* indicate that CpG sites are fully unmethylated; *filled squares* indicate that CpG sites are fully methylated; *partially filled squares* indicate various degrees of CpG methylation (see "Materials and Methods"). *B*, expression of *CAV1* after 5-aza-2′-deoxycytidine (*5-Aza-CdR*) treatment in NCI-H146 and NCI-H2171 SCLC cell lines. The cells were grown in the presence (+) and absence (−) of 4 μM 5-aza-2′-deoxycytidine for 6 days. Total RNA was isolated, cDNA was prepared, and reverse-transcribed PCR was performed for *CAV1* with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the control gene. The cDNA obtained from NCI-H1299 cells was used as a positive control. *C*, methylation-specific PCR for the detection of methylated *CAV1* 5′-CpG sequences. Representative samples are shown. *M*, results with primers specific for methylated sequences; *U*, results with primers specific for unmethylated sequences. PCR products were visualized on 2% agarose gels stained with ethidium bromide. The cell lines expressing (+) and not expressing (−) *CAV1* are indicated.

CpGs were completely unmethylated in NCI-H1299 as well as in the immortalized bronchial epithelial cell lines BEAS2B and HBEC1. Methylation status of 14 CpG dinucleotides downstream (nt 29742–29981 of the AC006159 sequence) was further examined in four SCLC cell lines (NCI-H146, NCI-H345, NCI-H865, and NCI-H2227) and in NCI-H1299 and BEAS-2B. In all of these cell lines, the mCpG densities of the promoter region containing the 14 CpGs were <0.42/100 bp. Thus, we conclude that the 7 methylated CpG sites (nt 29442–29676 on the AC006159 sequence) but not the 14 downstream CpGs are possible targets for silencing of the *CAV1* gene.

To confirm that promoter methylation leads to loss of CAV1 expression in the lung cancer cells, we examined the effect of 5-aza-2′-deoxycytidine, a drug that inhibits DNA methylation, on CAV1 expression. Treatment of the CAV1-nonexpressing SCLC cell lines (NCI-H146 and NCI-H2171) restored CAV1 expression with no change in the expression of the housekeeping gene *GAPDH* (Fig. 2*B*), demonstrating that *CAV1* is functionally methylated in these SCLC cell lines.

Strong Association between *CAV1* Promoter Methylation and Loss of CAV1 Protein Expression. The methylation status of the *CAV1* promoter region was further determined by methylation-specific PCR in 25 lung cancer cell lines, including 14 SCLCs and 11 NSCLCs (Fig. 2*C*). Genomic DNA modified by sodium bisulfite was used as template to amplify a part (nt 29487–29579 of the AC006159 sequence) of the promoter region encompassing the five CpG sites that were heavily methylated in six SCLC cell lines and MCF7, which lacked CAV1 protein expression (Fig. 2*A*). The NCI-H1155 NSCLC cell line and 13 SCLC cell lines that lacked CAV1 protein expression exhibited the methylated band, whereas no methylated bands were detected in 10 NSCLC cell lines and the NCI-H2227 SCLC line, which expressed CAV1 protein. We found a significant correlation between *CAV1* methylation and loss of CAV1 protein expression ($P < 0.0001$ by Fisher's exact test).

Absence of Mutations in the *CAV1* Gene at Codon 132 (P132L) in Lung Cancer Cell Lines. A missense mutation in the *CAV1* gene at codon 132 (P132L) was found in 16% of primary breast cancers, as reported by Hayashi *et al.* (39). We therefore screened the mutation at codon 132 in 46 lung cancer cell lines by PCR-restriction fragment length polymorphism analysis. However, the *CAV1* P132L mutation was not detected in these cell lines, consistent with previous findings in lung cancer (10, 22).

Effect of Exogenous Expression of CAV1 in CAV1-Nonexpressing SCLC Cells. To assess the functional role of CAV1 in SCLCs, we examined the effect of exogenous reexpression of CAV1 on *in vitro* cell growth of the NCI-H187 and NCI-H524 SCLC cell lines, which lacked endogenous CAV1 expression, by colony formation assay in soft agar. Exogenous reexpression of CAV1 in the cells transfected with the pcDNA3.1/CAV1 vector was confirmed by Western blot analysis at 48 h post-transfection (Fig. 3*A*). The transfected cells were subjected to soft-agar growth assay, and colony formation was determined. We found a significant decrease in colony formation in cells expressing exogenous CAV1 compared with those transfected

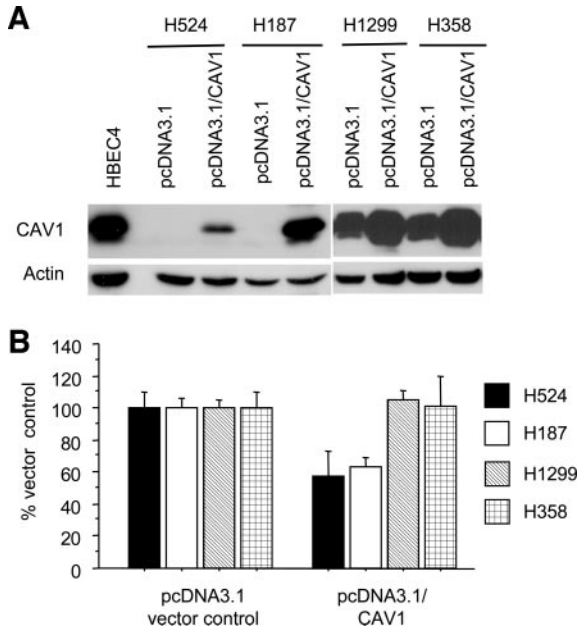

Fig. 3. Exogenous caveolin-1 (*CAV1*) expression inhibits colony formation by SCLC cells in soft agar. *A*, NCI-H524 and NCI-H187 small cell lung cancer and NCI-H1299 and NCI-H358 cell lines were transfected with pcDNA3.1/CAV1 or pcDNA 3.1. Cells were harvested at 48 h post-transfection, and Western blotting was performed. Fifteen μg of whole cell lysate were loaded in each lane. HBEC4, an immortalized bronchial epithelial cell line, was used as a positive control. *B*, cells were suspended in soft agar in complete medium containing 800 μg/ml G418 at 48 h post-transfection. Colonies were counted after 4 weeks. pcDNA3.1 vector transfection control was set at 100%. *Columns* represent the mean $\pm$ SD (*bars*).

with the pcDNA3.1 vector (Fig. 3*B*; NCI-H524, $P < 0.05$; NCI-H187, $P < 0.01$ by Student's *t* test). On the other hand, the forced expression of CAV1 did not affect colony formation of CAV1-expressing NSCLC cell lines NCI-H1299 and NCI-H358 (Fig. 3*B*). Thus, forced CAV1 expression inhibited soft-agar colony formation of the SCLCs lacking CAV1 expression but not of the NSCLC cell line expressing CAV1.

Growth Inhibition of RNAi-Mediated Down-Regulation of CAV1 Expression in NSCLC Cell Lines. In contrast to loss of CAV1 protein expression in SCLC cell lines, the majority of NSCLC cell lines expressed CAV1 protein, as did normal human bronchial epithelial cell cultures. To assess the role of CAV1 expression in NSCLC cells, we used RNAi technology. Two siRNAs targeting different sites of *CAV1* mRNA were used to verify that effect of CAV1 RNAi is specific. A siRNA targeting the human T-cell leukemia virus *Tax* gene was used as a negative control, as described previously (34). siRNAs against *CAV1* and *Tax* were transfected into NCI-H1299 cells, and the effect on gene silencing was monitored by immunofluorescent staining and Western blot analysis. As determined by immunofluorescent staining of NCI-H1299 cells 72 h after transfection with *CAV1* siRNA, a dramatic reduction in CAV1 expression was observed in ~90% of the transfected cells, showing the high transfection efficiencies (Fig. 4*A*). Western blot analysis showed that *CAV1* siRNAs, but not *Tax* siRNA, led to a marked down-regulation of CAV1 protein expression at 72 h post-transfection compared with the cells treated with Oligofectamine reagent alone (Fig. 4*B*). After 144 h, CAV1 reexpression began, and expression was completely restored after 9 days. On the other hand, siRNAs against *CAV1* and *Tax* did not affect the expression levels of α-tubulin, actin, and β-catenin (Fig. 4, *A* and *B*; Fig. 5, *A* and *B*). These results demonstrate that the effect of gene silencing mediated by siRNAs was dramatic and specific.

We then examined the effect of RNAi-mediated down-regulation of CAV1 expression on cellular proliferation and cell growth in NCI-H1299 NSCLC cells. siRNAs were transfected into the cells, and after 72 h, a BrdUrd incorporation assay was performed (Fig. 4*C*). CAV1 down-regulation mediated by CAV1 siRNAs resulted in a significant reduction in BrdUrd incorporation compared with Oligofectamine or *Tax* siRNA treatment ($P < 0.01$, Student's *t* test). At 48 h after the siRNA transfection, the cells were also harvested and plated for colony formation assay (Fig. 4*D*). siRNA-mediated down-regulation of CAV1 expression significantly inhibited colony formation in soft-agar and liquid culture ($P < 0.01$, Student's *t* test).

We further tested the effect of *CAV1* RNAi on the growth of other NSCLC cell lines, NCI-H358, NCI-H2009, and HCC15 by colony formation assay in liquid and soft-agar culture. In all of these cell lines, *CAV1* siRNA led to specific and dramatic down-regulation of CAV1 expression at 72 and 144 h post-transfection, but it did not affect β-catenin or actin expression (Fig. 5*A*). Again, CAV1 down-regulation mediated by *CAV1* siRNA inhibited colony formation significantly in these NSCLC cells compared with the Oligofectamine or *Tax* siRNA control (Fig. 5, *C* and *D*; $P < 0.05$, Student's *t* test). We also tested the effect of *CAV1* siRNA on soft-agar colony formation in CAV1-nonexpressing H187 and H524 SCLCs. Because CAV1 was not expressed in these cells, we used *β-catenin* siRNA as a control and verified that the transfection of siRNAs was successful (Fig. 5*B*). *CAV1* siRNA did not affect colony formation of these SCLCs (Fig. 5*D*). These results demonstrate that CAV1 expression is required for *in vitro* tumor growth of NSCLC cells that express CAV1.

RNAi-Mediated CAV1 Knockdown Led to Reduced Phosphorylation of FAK and RalA in NSCLC Cells. We wanted to determine how CAV1 RNAi inhibited anchorage-dependent and -independent growth of NSCLC cells. We were interested in the role of CAV1 in integrin-FAK signaling because evidence from previous studies indicated a role of CAV1 in integrin-mediated FAK activation, cell adhesion, and anchorage-dependent growth (40, 43). We were also interested in the role of CAV1 in RalA regulation because we recently showed that RalA is required for the anchorage-independent proliferation of transformed cells (44). Thus, we assessed the effect of RNAi-mediated CAV1 knockdown on FAK activation in H1299 and H358 NSCLCs expressing CAV1. In both NSCLC cell lines, CAV1 RNAi reduced the levels of phosphorylated FAK and RalA, whereas it did not affect the levels of RalB and phosphorylated extracellular signal-regulated kinase 1/2 (phospho-ERK1/2; Fig. 6). These results indicate that the growth-inhibitory effect mediated by CAV1 RNAi is associated with the loss of expression of phospho-FAK and RalA in NSCLC cells. By contrast, exogenous CAV1 expression in SCLCs, whereas associated with growth inhibition, was not associated with expression of phospho-FAK (data not shown).

DISCUSSION

Previous studies have reported that CAV1 is expressed in 21–36% of resected primary NSCLCs and that CAV1 expression is associated with poor prognosis of squamous cell lung cancer (8, 10, 11, 13, 25). In these studies, however, only two SCLC cancer cell lines were studied, no reasons for expression or nonexpression of CAV1 were provided, and no functional correlations were made. We therefore focused in the present study on comparing SCLC and NSCLC, the methylation status of *CAV1*, and the functional effects of altering CAV1 expression in these two major histological types of lung cancer. We examined endogenous expression of CAV1 protein in several lung cancer cell lines, nearly all of which came from metastatic (poor prognosis) lesions. CAV1 protein expression was reduced or absent in 95% of the SCLCs, whereas it was retained in 76% of NSCLCs compared with normal human lung epithelial cultures. We thus found

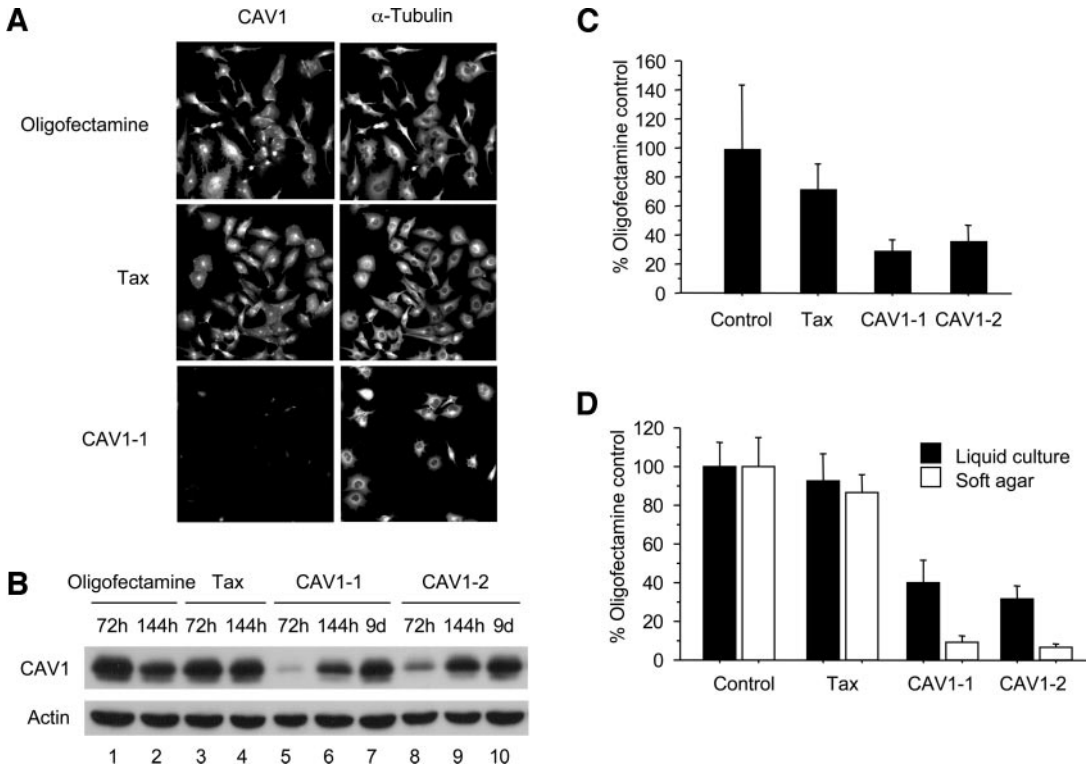

Fig. 4. Small interfering RNA (siRNA)-mediated down-regulation of caveolin-1 (*CAV1*) expression inhibited *in vitro* growth of the NCI-H1299 non-small cell lung cancer cell line. *A*, NCI-H1299 cells were transfected with 100 nM annealed sense and antisense 21-mer RNA oligonucleotides directed against either *CAV1* or *Tax*. Treatment with Oligofectamine reagent alone was used as a control. After 72 h, the cells were stained with anticaveolin or anti-α-tubulin primary antibodies and analyzed by fluorescent microscopy. *B*, cells were harvested at 72 h, 144 h, and 9 days post-transfection. Fifteen μg of total protein were loaded in each lane, and Western blotting was performed. Cells treated with Oligofectamine reagent only (*Lanes 1* and *2*) and *Tax* siRNA (*Lanes 3* and *4*) were used as controls. Two *CAV1* siRNAs, CAV1-1 (*Lanes 5–7*) and CAV1-2 (*Lanes 8–10*), which target different mRNA sites, were used. *C*, *CAV1* siRNAs inhibit cellular proliferation in the NCI-H1299 cell line. siRNAs were transfected into the cells, and the bromodeoxyuridine incorporation assay was performed in replicates of 8 at 72 h post-transfection. Oligofectamine control treatment was set at 100%. *Columns* represent the mean ± SD (*bars*). *D*, *CAV1* siRNAs inhibit colony formation by the NCI-H1299 non-small cell lung cancer cell line. Forty-eight h after siRNA transfection, cells were plated for colony formation assay in liquid culture and soft agar. Oligofectamine control treatment was set at 100%. *Columns* represent the mean ± SD (*bars*). The data were obtained from three independent experiments. Marked down-regulation of CAV1 protein expression in *CAV1* siRNA-transfected cells was confirmed by Western blot analysis for every experiment.

dramatic difference in endogenous expression level of CAV1 protein between SCLCs and NSCLCs.

Interestingly, when we classified the tumor cell lines as suspension or adherent cultures according to their culture morphologies, all cells growing as suspension cultures lacked CAV1 protein expression whereas all plastic adherent cells expressed CAV1 protein. We therefore found complete association between the CAV1 expression level and cell culture morphology and growth characteristics for lung cancer cells. It is known that integrins are caveolae-interacting molecules and that CAV1 is required for integrin-dependent FAK phosphorylation and adhesion (40, 43). In fact, we found a correlation between FAK phosphorylation, CAV1 expression, and culture morphology in lung cancer cell lines. The loss of CAV1 protein expression is therefore also related to the functional loss of integrin-dependent adhesion in lung cancer cell lines that leads to tumor cells growing in suspension. Although most SCLC cell lines grew as suspension cultures and most NSCLC cell lines grew attached to the culture dish, two SCLC cell lines expressing CAV1, H1607 and H2227, had the same culture morphology as the majority of NSCLCs, whereas NSCLC cell line H1155 grew as a cell suspension, similar to most SCLC cell lines. Obviously, these tumor cell lines represent interesting exceptions that provide further support to the CAV1 expression connections. In experiments not presented here, we performed oligonucleotide (Affimetrix HG-U133) expression profiles and clustering analysis, which showed that these tumor cells cluster with their assigned histological group but on the edges of these clusters. We therefore have expression profiling data placing these interesting tumor cells in the same histological classes (SCLC or NSCLC) as reported in their initial pathological diagnosis.

CAV1 has been implicated as a candidate tumor suppressor gene in several human tumors including lung cancer. However, to our knowledge, no evidence for mutations, deletions, or epigenetic alterations caused by promoter methylation in the *CAV1* gene has been reported in lung cancer (10, 22). Here we report aberrant methylation of CpG islands in the *CAV1* gene associated with transcriptional inactivation in SCLC cell lines. In six SCLC cell lines examined by bisulfite sequencing, heavy or complete methylation was found at seven CpG sites (nt 29442–29676 on the AC006159 sequence) in the promoter region of *CAV1*, whereas these CpGs were unmethylated or only partially methylated in CAV1-expressing lung cancer cell lines and in normal bronchial epithelial cell cultures. Furthermore, methylation-specific PCR analysis demonstrated that *CAV1* was methylated in 93% (14 of 15) of SCLC lines but only 9% (1 of 11) of NSCLC lines with a strong concordance between methylation status and CAV1 expression. The major mode of inactivation of many tumor suppressor genes in human cancers, including lung cancer, involves tumor-acquired promoter hypermethylation (45, 46). In fact, the promoter region of *CAV1* was methylated in breast and prostate cancers at the same CpG sites that were found to be methylated in the SCLC cells in this study (41, 42). Therefore, studies of breast, prostate, and now lung cancers have found that methylation of the same CpG sites is associated with loss of CAV1 expression. Although formal experiments documenting promoter activity need to be done, these results in

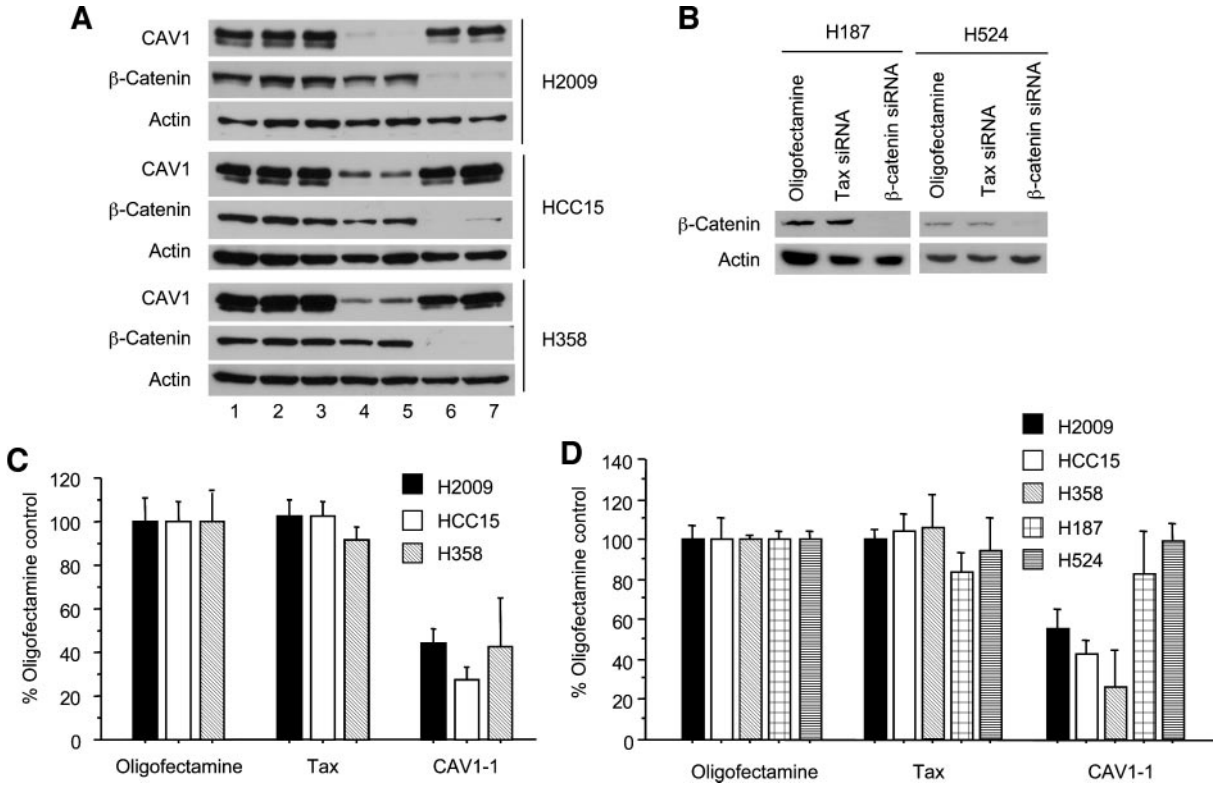

Fig. 5. *A*, specific small interfering RNA (siRNA)-mediated down-regulation of caveolin-1 (*CAV1*) protein expression in NCI-H2009, HCC15, and NCI-H358 NSCLC cell lines. siRNAs were transfected into NCI-H2009, HCC15, and NCI-H358 cells. Conditions for Western blotting were the same as Fig. 4*B*. *Lanes*: *1*, treatment with medium only; *2*, treatment with Oligofectamine only at 72 h post-transfection; *3*, *Tax* siRNA at 72 h post-transfection; *4*, *CAV1* siRNA (CAV1-1) at 72 h post-transfection; *5*, *CAV1* siRNA (CAV1-1) at 144 h post-transfection; *6*, *β-catenin* siRNA at 72 h post-transfection; *7*, *β-catenin* siRNA at 144 h post-transfection. *B*, *β-catenin* siRNA was used as a control to verify that siRNA transfection was successful because CAV1 was not expressed in NCI-H187 and NCI-H524 small cell lung cancers. After 72 h, cells were harvested, and Western blotting was performed. Conditions for Western blotting were the same as Fig. 4*B*. *C* and *D*, *CAV1* siRNA inhibits colony formation by NCI-H2009, HCC15, and NCI-H358 cells in liquid culture (*C*) and soft-agar colony formation of NCI-H2009, HCC15, and NCI-H358 cells (*D*) but not of NCI-H187 and NCI-H524 cells. The method was the same as in Fig. 4*D*. Oligofectamine control treatment was set at 100%. *Columns* represent the mean ± SD (*bars*). The data were obtained from three independent experiments.

diverse tumors highlight the importance of these CpG sites for CAV1 expression.

Recently, a missense mutation in *CAV1* at codon 132 (P132L) was detected in invasive scirrhous breast cancers, and recombinant expression of the *CAV1* P132L mutant could lead to mislocalization and intracellular retention of wild-type CAV1, cellular transformation, and promotion of invasive ability (39). Thus, the P132L mutant could behave in a dominant-negative manner (39, 47). However, we did not find the *CAV1* P132L mutation in the 46 lung cancer cell lines examined, consistent with previous studies describing no evidence of *CAV1* mutation in lung cancers (10, 22). It is therefore unlikely that the *CAV1* P132L mutation is important for the development of lung cancer.

We next assessed the growth-inhibitory effect of forced CAV1 expression in the SCLCs as was done in breast cancer cells in previous studies (16, 17). We found that exogenous CAV1 expression led to *in vitro* growth inhibition, as assessed by the soft-agar growth assay, in the NCI-H187 and NCI-H524 SCLC cell lines, which lacked CAV1 expression, but had no effect in tumor cells already expressing endogenous CAV1. These results suggest that CAV1 can act as a tumor suppressor in SCLCs, as it does in breast cancer.

Although *CAV1* has been implicated as a tumor suppressor gene, previous studies have reported CAV1 up-regulation in several human tumors, and the up-regulation was associated with tumor progression and poor prognosis (19–24). CAV1 plays a role in prostate cancer progression and protects prostate cancer cells from Myc-induced apoptosis (19, 23, 48). As for lung cancer, the up-regulation of CAV1 has been reported in lung adenocarcinoma with enhanced invasive/metastasis capacity (22). More recently, Yoo *et al.* (25) found that CAV1 was expressed in 32% of primary squamous cell lung cancers and that the CAV1 expression was significantly correlated with advanced pathological stage and poor prognosis. Two other studies using cDNA microarray analysis reported similar frequencies (21–

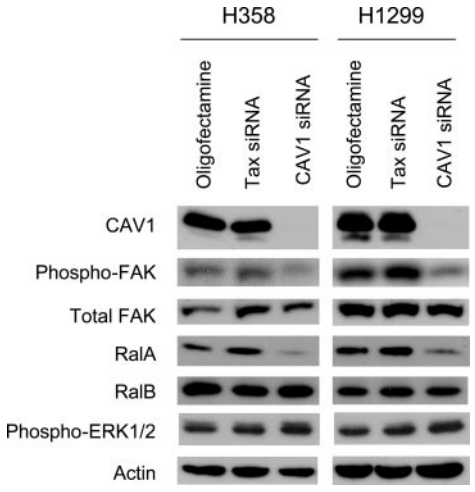

Fig. 6. Effect of RNA interference-mediated caveolin-1 (*CAV1*) knockdown on the expression of phosphorylated focal adhesion kinase (*Phospho-FAK*), focal adhesion kinase (*Total FAK*), RalA, RalB, phosphorylated extracellular-signal regulated kinase 1/2 (*Phospho-ERK1/2*), and actin. Twenty-five μg of total protein were loaded in each lane, and Western blotting was performed. *siRNA*, small interfering RNA.

36%) of *CAV1* mRNA expression in operable primary NSCLC tumors (10, 11). By contrast, in this study, we found that 76% of NSCLC cell lines retained CAV1 protein expression compared with normal lung cells. However, the NSCLC cell lines we studied were established from advanced lung tumors with metastasis and in most cases came from the metastatic lesions (32, 33). These tumor lines thus represent the bad prognostic lesions of Yoo *et al.* (25) and suggest that CAV1 expression may be required as a late event in the progression of NSCLC. It has been shown that that multidrug-resistant cancer cells, including NSCLC cells, express very high CAV1 levels and exhibit a high surface density of caveolae (49, 50). It was reported that phospholipase D, a constituent enzyme of caveolae, was up-regulated in human multidrug-resistant cancer cells (44) and that phospholipase D is a downstream target of RalA, which was down-regulated by CAV1 RNAi in this study (Fig. 6). Therefore, the high expression levels of CAV1 in NSCLC cells observed in this study may be associated with resistance to chemotherapy.

RNAi is a post-transcriptional gene-silencing mechanism initiated by double-stranded RNA homologous in sequence to the targeted gene (29, 30). Recently, RNAi technology has been developed as a method for generating specific and dramatic inhibition of gene expression in mammalian cells (27, 51). RNAi technology has great potential in studies of the function of the genes involved in the pathogenesis of human cancer (28, 52). To assess the role of CAV1 in NSCLC, we knocked down expression of CAV1 protein in CAV1-expressing NSCLC cell lines by use of the RNAi technology and found that knockdown of CAV1 mediated by RNAi led to inhibition of NSCLC proliferation and growth *in vitro*, demonstrating that CAV1 is required for tumor cell growth of NSCLC cells that retain CAV1 expression. CAV1 has multiple functions and can regulate many signal transduction molecules (5). It thus is likely that CAV1 may have opposite functions resulting in either growth inhibition or growth promotion, depending on the cellular context. Such opposite functions are also known for other proteins, such as the RAS protein, which induces either differentiation or transformation in distinct cell types by a similar signaling pathway (53). CAV1 also mediates integrin-dependent activation of ERK and FAK and promotes cell cycle progression (40, 43). In addition, the ERK pathway was frequently activated in our NSCLC cell lines, including NCI-H358, NCI-H1299, NCI-H2009, and HCC15 cells.[8] In this study, we found that RNAi-mediated CAV1 knockdown inactivated FAK in NSCLC cells but did not affect ERK phosphorylation. Importantly, we found that CAV1 RNAi down-regulated RalA expression in the NSCLC cells. RalA has been shown to be required for the transformed phenotype that induces Src and H-Ras, which interact with CAV1 (5, 54, 55). In addition, RalA is required for anchorage-independent proliferation of transformed cells (44). These results indicate that CAV1 is required for NSCLC growth through the activation of FAK and RalA signaling but not mitogen-activated protein kinase activation. By contrast, SCLC obviously does not use this mechanism because phospho-FAK was not affected by re-expression of CAV1.

In conclusion, the present study indicates quite different roles for CAV1 between SCLC and NSCLC. In SCLC, CAV1 has the properties of a tumor suppressor, whereas in NSCLC it appears to be required for tumor growth and is involved in FAK phosphorylation and RalA expression. This difference in the roles of CAV1 may reflect differences in the biological properties of SCLC and NSCLC. Further studies will be needed to elucidate the role of CAV1 in the development of lung cancer and to determine whether CAV1 expression and/or promoter methylation could be a diagnostic marker and therapeutic target for lung cancer.

[8] S. Muneer, J. Wilsbacher, M. Cobb, and J. D. Minna. Constitutive activation and abnormal cytoplasmic localization of ERK1/2 MAP kinases in breast and lung cancers, manuscript in preparation.

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REFERENCES

1. Anderson RG, Jacobson K. A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. Science (Wash DC) 2002;296:1821–5.
2. Severs NJ. Caveolae: static inpocketings of the plasma membrane, dynamic vesicles or plain artifact? J Cell Sci 1988;90(Pt 3):341–8.
3. Galbiati F, Razani B, Lisanti MP. Emerging themes in lipid rafts and caveolae. Cell 2001;106:403–11.
4. Okamoto T, Schlegel A, Scherer PE, Lisanti MP. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. J Biol Chem 1998;273:5419–22.
5. Liu P, Rudick M, Anderson RG. Multiple functions of caveolin-1. J Biol Chem 2002;277:41295–8.
6. Drab M, Verkade P, Elger M, et al. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. Science (Wash DC) 2001;293:2449–52.
7. Razani B, Engelman JA, Wang XB, et al. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. J Biol Chem 2001;276:38121–38.
8. Razani B, Altschuler Y, Zhu L, Pestell RG, Mostov KE, Lisanti MP. Caveolin-1 expression is down-regulated in cells transformed by the human papilloma virus in a p53-dependent manner. Replacement of caveolin-1 expression suppresses HPV-mediated cell transformation. Biochemistry 2000;39:13916–24.
9. Wiechen K, Diatchenko L, Agoulnik A, et al. Caveolin-1 is down-regulated in human ovarian carcinoma and acts as a candidate tumor suppressor gene. Am J Pathol 2001;159:1635–43.
10. Heighway J, Knapp T, Boyce L, et al. Expression profiling of primary non-small cell lung cancer for target identification. Oncogene 2002;21:7749–63.
11. Wikman H, Kettunen E, Seppanen JK, et al. Identification of differentially expressed genes in pulmonary adenocarcinoma by using cDNA array. Oncogene 2002;21:5804–13.
12. Bender FC, Reymond MA, Bron C, Quest AF. Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. Cancer Res 2000;60:5870–8.
13. Racine C, Belanger M, Hirabayashi H, Boucher M, Chakir J, Couet J. Reduction of caveolin 1 gene expression in lung carcinoma cell lines. Biochem Biophys Res Commun 1999;255:580–6.
14. Galbiati F, Volonte D, Engelman JA, et al. Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. EMBO J 1998;17:6633–48.
15. Koleske AJ, Baltimore D, Lisanti MP. Reduction of caveolin and caveolae in oncogenically transformed cells. Proc Natl Acad Sci USA 1995;92:1381–5.
16. Lee SW, Reimer CL, Oh P, Campbell DB, Schnitzer JE. Tumor cell growth inhibition by caveolin re-expression in human breast cancer cells. Oncogene 1998;16:1391–7.
17. Fiucci G, Ravid D, Reich R, Liscovitch M. Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. Oncogene 2002;21:2365–75.
18. Engelman JA, Wykoff CC, Yasuhara S, Song KS, Okamoto T, Lisanti MP. Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. J Biol Chem 1997;272:16374–81.
19. Mouraviev V, Li L, Tahir SA, et al. The role of caveolin-1 in androgen insensitive prostate cancer. J Urol 2002;168:1589–96.
20. Suzuoki M, Miyamoto M, Kato K, et al. Impact of caveolin-1 expression on prognosis of pancreatic ductal adenocarcinoma. Br J Cancer 2002;87:1140–4.
21. Kato K, Hida Y, Miyamoto M, et al. Overexpression of caveolin-1 in esophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. Cancer (Phila) 2002;94:929–33.
22. Ho CC, Huang PH, Huang HY, Chen YH, Yang PC, Hsu SM. Up-regulated caveolin-1 accentuates the metastasis capability of lung adenocarcinoma by inducing filopodia formation. Am J Pathol 2002;161:1647–56.
23. Yang G, Truong LD, Timme TL, et al. Elevated expression of caveolin is associated with prostate and breast cancer. Clin Cancer Res 1998;4:1873–80.
24. Nasu Y, Timme TL, Yang G, et al. Suppression of caveolin expression induces androgen sensitivity in metastatic androgen-insensitive mouse prostate cancer cells. Nat Med 1998;4:1062–4.
25. Yoo SH, Park YS, Kim HR, et al. Expression of caveolin-1 is associated with poor prognosis of patients with squamous cell carcinoma of the lung. Lung Cancer 2003;42:195–202.
26. Minna JD, Roth JA, Gazdar AF. Focus on lung cancer. Cancer Cell 2002;1:49–52.
27. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature (Lond) 2001;411:494–8.

28. Borkhardt A. Blocking oncogenes in malignant cells by RNA interference–new hope for a highly specific cancer treatment? Cancer Cell 2002;2:167–8.
29. Sharp PA. RNA interference—2001. Genes Dev 2001;15:485–90.
30. Hannon GJ. RNA interference. Nature (Lond) 2002;418:244–51.
31. Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc Natl Acad Sci USA 2001;98:9742–7.
32. Phelps RM, Johnson BE, Ihde DC, et al. NCI-Navy Medical Oncology Branch cell line data base. J Cell Biochem Suppl 1996;24:32–91.
33. Wistuba II, Bryant D, Behrens C, et al. Comparison of features of human lung cancer cell lines and their corresponding tumors. Clin Cancer Res 1999;5:991–1000.
34. Verma UN, Surabhi RM, Schmaltieg A, Becerra C, Gaynor RB. Small interfering RNAs directed against beta-catenin inhibit the in vitro and in vivo growth of colon cancer cells. Clin Cancer Res 2003;9:1291–300.
35. Braasch DA, Jensen S, Liu Y, et al. RNA interference in mammalian cells by chemically-modified RNA. Biochemistry 2003;42:7967–75.
36. Harada K, Toyooka S, Maitra A, et al. Aberrant promoter methylation and silencing of the RASSF1A gene in pediatric tumors and cell lines. Oncogene 2002;21:4345–9.
37. Xu XL, Wu LC, Du F, et al. Inactivation of human SRBC, located within the 11p15.5-p15.4 tumor suppressor region, in breast and lung cancers. Cancer Res 2001;61:7943–9.
38. Burbee DG, Forgacs E, Zochbauer-Muller S, et al. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst (Bethesda) 2001;93:691–9.
39. Hayashi K, Matsuda S, Machida K, et al. Invasion activating caveolin-1 mutation in human scirrhous breast cancers. Cancer Res 2001;61:2361–4.
40. Wei Y, Yang X, Liu Q, Wilkins JA, Chapman HA. A role for caveolin and the urokinase receptor in integrin-mediated adhesion and signaling. J Cell Biol 1999;144:1285–94.
41. Engelman JA, Zhang XL, Lisanti MP. Sequence and detailed organization of the human caveolin-1 and -2 genes located near the D7S522 locus (7q31.1). Methylation of a CpG island in the 5′ promoter region of the caveolin-1 gene in human breast cancer cell lines. FEBS Lett 1999;448:221–30.
42. Cui J, Rohr LR, Swanson G, Speights VO, Maxwell T, Brothman AR. Hypermethylation of the caveolin-1 gene promoter in prostate cancer. Prostate 2001;46:249–56.
43. Wary KK, Mariotti A, Zurzolo C, Giancotti FG. A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. Cell 1998;94:625–34.
44. Chien Y, White MA. RAL GTPases are linchpin modulators of human tumour-cell proliferation and survival. EMBO Rep 2003;4:800–6.
45. Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 2001;10:687–92.
46. Zochbauer-Muller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res 2001;61:249–55.
47. Lee H, Park DS, Razani B, Russell RG, Pestell RG, Lisanti MP. Caveolin-1 mutations (P132L and null) and the pathogenesis of breast cancer: caveolin-1 (P132L) behaves in a dominant-negative manner and caveolin-1 (−/−) null mice show mammary epithelial cell hyperplasia. Am J Pathol 2002;161:1357–69.
48. Timme TL, Goltsov A, Tahir S, et al. Caveolin-1 is regulated by c-myc and suppresses c-myc-induced apoptosis. Oncogene 2000;19:3256–65.
49. Yang CP, Galbiati F, Volonte D, Horwitz SB, Lisanti MP. Upregulation of caveolin-1 and caveolae organelles in Taxol-resistant A549 cells. FEBS Lett 1998;439:368–72.
50. Lavie Y, Fiucci G, Liscovitch M. Upregulation of caveolin in multidrug resistant cancer cells: functional implications. Adv Drug Deliv Rev 2001;49:317–23.
51. Brummelkamp TR, Bernards R, Agami R. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2002;2:243–7.
52. Harborth J, Elbashir SM, Bechert K, Tuschl T, Weber K. Identification of essential genes in cultured mammalian cells using small interfering RNAs. J Cell Sci 2001;114:4557–65.
53. Crespo P, Leon J. Ras proteins in the control of the cell cycle and cell differentiation. Cell Mol Life Sci 2000;57:1613–36.
54. Aguirre-Ghiso JA, Frankel P, Farias EF, et al. RalA requirement for v-Src- and v-Ras-induced tumorigenicity and overproduction of urokinase-type plasminogen activator: involvement of metalloproteases. Oncogene 1999;18:4718–25.
55. Xu L, Frankel P, Jackson D, et al. Elevated phospholipase D activity in H-Ras- but not K-Ras-transformed cells by the synergistic action of RalA and ARF6. Mol Cell Biol 2003;23:645–54.
56. Suzuki M. Sunaga N, Shames DS, Toyooka S, Gazdar AF, Minna JD. RNA interference-mediated knockdown of DNA methyltransferase 1 leads to promoter demethylation and gene re-expression in human lung and breast cancer cells. Cancer Res 2004;64:3137–43.