Regulation of cancer cell proliferation by caveolin-2 down-regulation and re-expression

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Abstract. We investigated whether altering caveolin-2 (cav-2) expression affects the proliferation of cancer cells. Cav-2 was not detected in HepG2, SH-SY5Y and LN-CaP cells, and the loss of cav-2 expression was not restored by 5-aza-2'-deoxycytidine treatment. In contrast, C6, HeLa, A549, MCF7 and PC3M cells expressed cav-2. Effects of re-expression of exogenous cav-2 in HepG2, SH-SY5Y and LN-CaP cells, and siRNA-mediated down-regulation of endogenous cav-2 in C6, HeLa, A549, MCF7 and PC3M cells on cancer proliferation were examined by MTT assay, colony formation assay and flow cytometric analysis. Cav-2 transfection in HepG2 hepatocellular carcinoma cells and knockdown in C6 glioma cells caused reduction in cell proliferation and growth with retarded entry into the S phase. Cav-2 re-expression in SH-SY5Y neuroblastoma cells and depletion in HeLa epithelial cervical cancer and A549 lung adenocarcinoma cells promoted cancer cell proliferation. Luciferase reporter assay showed that transcriptional activation of Elk-1 and STAT3 was significantly decreased in cav-2-transfected HepG2 hepatocellular carcinoma and down-regulated C6 glioma cells. Our data suggest that cav-2 acts as a modulator of cancer progression.

Introduction

Caveolins, caveolae coat proteins, have specific functional roles which can vary in different cell types. There are three members within the caveolin protein family: cav-1, cav-2, and cav-3. Cav-1 and -2 are co-expressed in most cell types, whereas cav-3 is primarily expressed in vascular smooth, cardiac and skeletal muscles (1,2). Caveolins have been found to be involved in diverse cellular processes ranging from cell migration, cell cycle and cell polarity to regulation of cell transformation and signal transduction (3-5). Cav-1 has been reported to inhibit cell cycle progression and cell migration by preventing EGFR-dependent MAP kinase cascade (6,7). In contrast, we recently demonstrated that cav-2 activates cellular mitogenesis by promoting insulin-induced ERK activation and nuclear targeting (8-10).

Cav-1 regulates multiple cancer-associated cellular processes, such as cell proliferation, growth, migration and invasion (4,11). The cav-1 gene is localized to locus D7S522 of human chromosome 7q31.1, which is often deleted in human cancers including ovarian adenocarcinomas (12), prostate and breast cancers (13), uterine leiomyomas (14), myeloid neoplasms (15), oral cancer (16), stomach adenocarcinoma (17) and renal carcinomas (18). Moreover, cav-1 P132L mutant, that disrupts the cav-1 scaffolding domain, exists in 16% of human breast cancers and acts as a dominant-negative for growth suppression (19). Thus, cav-1 is believed to act as a tumor-suppressor gene. However, cav-1 expression is often maintained or up-regulated in T-cell leukemia, esophagus squamous cell carcinoma, prostate cancer, thyroid papillary carcinoma, bladder cancers and multiple myeloma (20-25). Thus, although many studies have shown cav-1 as a potential prognostic marker for prediction of cancer, the prognostic significance of cav-1 varies between different types of human cancers.

The cav-2 gene is co-localized with cav-1 to the locus D7S522 of human chromosome 7q31.1 (26), and tissue distribution of cav-2 is very similar to cav-1 (2). Although it has been reported that cav-2 levels are not changed by oncogenic transformation (2,27), cav-2 expression is up-regulated in esophageal and urothelial carcinomas (21,28). Furthermore, cav-2 expression has been detected in various lung cancers and associated with shorter survival in stage I adenocarcinomas (29). Despite the fact that cav-2 is expressed in various types of cancers, the functional role of cav-2 is less well-defined in tumor growth and metastasis.

In the present study, modulation of cancer cell proliferation was investigated by alteration of cav-2 expression in various cancer cells using MTT assay, colony formation assay and flow cytometric analysis. Exogenous cav-2 expression in HepG2 hepatocellular carcinoma cells and endogenous cav-2 depletion in C6 glioma cells induced inhibition of cancer growth and

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proliferation with prevention of transcriptional activation of Elk-1 and STAT3. Our results showed that cav-2 plays a role as a potential regulator of cancer cell proliferation.

Materials and methods

Cell lines and culture. HepG2 (human hepatocellular carcinoma), HeLa (human epithelial cervical cancer), A549 (human lung adenocarcinoma) and C6 (rat glioma) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL) containing 5 mM D-glucose supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (Gibco/BRL) in a 5% CO_2 incubator at 37˚C. PC3M (human prostate carcinoma), MCF7 (human breast cancer) and LN-CaP (human prostate carcinoma) cells were cultured in RPMI-1640 medium (Gibco/BRL) containing 10 mM D-glucose supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin in a 5% CO_2 incubator at 37˚C. SH-SY5Y (human neuroblastoma) cells were grown in a 1:1 mixture of DMEM and Ham's F12-medium (Gibco/BRL) containing 17 mM D-glucose supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin in a 5% CO_2 incubator at 37˚C.

Plasmids and transfection. A full-length cav-2 cDNA (NM_131914) was subcloned into the pcDNA3 vector (Invitrogen Corp.) as described previously (9,10). The cDNA constructs were introduced into HepG2, SH-SY5Y and LN-CaP cells in culture medium, which was replaced with 50 μl of 2.5 M $CaCl_2$ and 2X HEPES-buffered saline and incubated for 24 h at 37˚C. The transfection medium was replaced with fresh culture medium, and incubation was carried out for another 24 h at 37˚C as previously described (8).

Reverse transcription (RT)-PCR analysis. Total RNA was extracted with TRIzol reagent (SolGent, Co. Ltd.) according to the manufacturer's instructions. cDNA was generated using a reverse transcription kit (Accupower RT PreMix kit; Bioneer Corp.). The cDNA was used as the template for subsequent PCR amplification. PCR primers were as follows: human cav-2, 5'-ACTCTTACGCAGCGGCAGG-3' and 5'-AGTAACTGCTGAGGTTGGTGTAGACC-3'; rat cav-2, 5'-ATGGGGCTGGAGACTGAGAAG-3' and 5'-TCAGTCATGGCTCAGTTGCATG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-ACCACCATGGAGAAGGCTGG-3' and 5'-CTCAGTGTAGCCCAGGATGCC-3'. PCR was performed using AccuPower PCR PreMix kit. The PCR fragments were separated by running on 1% agarose gels.

Protein isolation and immunoblot analysis. For the protein isolation, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA buffer [50 mM HEPES, 150 mM NaCl, 100 mM Tris-HCl (pH 8.0), 0.25% deoxycholic acid, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 5 mM 1,4-dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 20 μM leupeptin and 100 μM aprotinin]. The whole cell lysates (WCLs) were put on ice for 30 min and centrifuged at 12,000 rpm for 20 min at 4˚C. Aliquots from the clear supernatant were obtained for protein quantification as determined by the Bradford assay (BioRad Laboratories). Equal amounts of samples (50 μg) were separated on 15% (w/v) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked overnight at 4˚C with 5% (v/v) nonfat dry milk in TBS, 0.1% (v/v) Tween-20, and incubated for 2 h at room temperature (RT) in the primary antibody. The primary antibodies used were as follows: cav-2 (BD 610685; 1:500) and cav-1 (BD 610407; 1:500) antibodies from BD Transduction Laboratories; F-actin (sc-1616; 1:200) antibody from Santa Cruz Biotechnology. The membranes were washed with TBS, 0.1% (v/v) Tween-20 and incubated for 1 h at RT in horseradish peroxidase-conjugated anti-mouse (A4416, Sigma-Aldrich; 1:5000) or anti-goat (sc-2020, Santa Cruz Biotechnology; 1:5000) antibodies in 5% (v/v) nonfat dry milk in TBS, 0.1% (v/v) Tween-20. The immunoblots were developed using the ECL detection reagent (RPN2106, Amersham Biosciences) as described previously (30).

Treatment with 5-aza-2'-deoxycytidine. HepG2, C6 and LN-CaP cells were incubated in culture medium in the presence or absence of 4 μM 5-Aza-CdR (Sigma-Aldrich) for 6 days. Total RNA and proteins were extracted from the cells and subjected to RT-PCR and immunoblot analysis as described above.

Transfection of cav-2 small interfering (si)RNA. A549, C6, PC3M, MCF7 and HeLa cells were transfected for 48 h with either a SMARTpool of cav-2-specific siRNA or non-targeting siRNA (scramble control; Dharmacon, Lafayette, CO, USA) using DharmaFECT transfection reagents (Dharmacon) as described previously (9,31,32). The construct targeting cav-2 was comprised of the following 3' (sense) and 5' (antisense) primer pairs: 3'-GUAAAGACCUGCCUAAUGGUU and 5'-PCCAUUAGGCAGGUCUUUACUU, the non-targeting siRNA was 5'-GGAAAGACUGUUCCAAAAA-3'.

MTT assay. Cells (2 x10^4) were plated in 96-well plates and transfected with the pcDNA3 vector or pcDNA3-cav-2 for 18 h, or with scramble or cav-2 siRNA for 48 h. After 2 days of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M5655, Sigma-Aldrich) stock solution was added to each well at a final concentration of 0.5 mg/ml and incubated at 37˚C for 4 h, followed by lysis with 100 μl of dimethyl sulfoxide (DMSO) (BioShop, CA) to solubilize the final product of MTT metabolism, the formazan precipitate. After a 30-min incubation at 37˚C, the optical density at 540 nm was determined using a microplate reader (Model 550, BioRad Laboratories).

Colony formation in soft agar. The *in vitro* growth characteristics were tested by colony formation assay. After 48 h of transfection with the pcDNA3 vector, pcDNA3-cav-2, scramble or cav-2 siRNA, cells were prepared by trypsinization and homogenization. Cells were suspended in the culture medium at 2x10^5 cells/ml. The cells were plated onto each well of a 24-well plate at a density of 2x10^3 cells/well in culture medium containing 0.35% agarose (BioWhittaker Molecular Applications, Rockland, MD, USA) on a base layer of 0.5% agar (MP Biomedicals, France). The medium was

refreshed every 3 days. After 2 weeks of incubation at 37˚C, foci were stained with 0.02% crystal violet solution (212525, BD Biosciences, USA) for 1 h. The number of colonies was photographed using a dissecting microscope (Olympus, SZX12) and the number of large colonies (>0.042 mm in diameter) counted in each plate was scored using Image-ProPlus 6.1 (Media Cybernetics). The cells were tested in triplicate in three independent assays.

Analysis of the cell cycle by flow cytometry. Cells ($5x10^4$) were plated in a 24-well plate and transfected with pcDNA3 vector or pcDNA3-cav-2 for 18 h, or with scramble or cav-2 siRNA for 48 h. After 48 h of incubation, the cells were fixed with ice-cold ethanol and stained with 50 μg/ml propidium iodide (Sigma-Aldrich, USA), followed by analytic flow cytometry using FACS Calibur (BD Biosciences, USA). The numbers of cells in G_0/G_1, S and G_2/M phases were quantified with FCS Express software (De Novo Software). At least $2x10^4$ cells in each sample were analyzed to obtain a measurable signal.

Luciferase reporter assay. Elk-1 and STAT3 translucent reporter vectors (Elk-1-Luc and STAT3-Luc) to monitor the transcription factor binding activity of Elk-1 and STAT3 were purchased from Panomics (Redwood City, CA). HepG2 cells were transiently transfected using the Lipofectamine LTX reagent (Invitrogen Corp.) with 0.5 μg of plasmid DNA (Elk-1-Luc or STAT3-Luc) along with the pcDNA3 vector or pcDNA3-cav-2. C6 cells were transfected using the DharmaFECT transfection reagents with scramble or cav-2 siRNA for 24 h and then transiently transfected using the Lipofectamine LTX reagent with 0.5 μg of plasmid DNA (Elk-1-Luc or STAT3-Luc) in each well of a 24-well plate. The Renilla reporter construct pRL-TK (Promega Corp., Madison, WI, USA) was used to normalize the transfection efficiency. The cells were incubated for 48 h in culture medium and washed twice with ice-cold PBS, and lysed in 100 μl/well of passive lysis buffer (Promega Corp.). Luciferase activity was measured using a dual-luciferase reporter assay system (Promega Corp.).

Statistical analysis. Statistical significance of differences in MTT assay, colony formation assay, cell cycle analysis and luciferase reporter assay was analyzed using the Student's t-test. Results represent data from three experiments for each group, and a P-value of <0.05 was considered statistically significant.

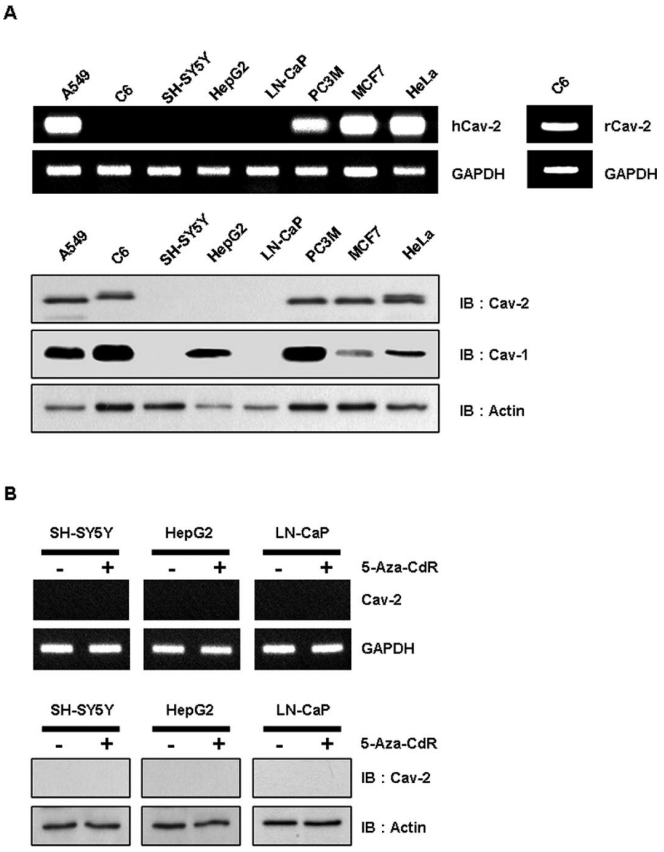

Figure 1. Cav-2 expression analysis in A549, C6, SH-SY5Y, HepG2, LN-CaP, PC3M, MCF7 and HeLa cells. (A) Cav-2 mRNA levels were analyzed by RT-PCR (hCav-2, human cav-2; rCav-2, rat cav-2) as described in Materials and methods. WCLs were separated by SDS-PAGE, and the protein levels of cav-2 and cav-1 were assessed by immunoblot analysis with anti-cav-2 and anti-cav-1 antibodies. Shown is a representative experiment that was repeated three times. (B) SH-SY5Y, HepG2 and LN-CaP cells were treated with or without 4 μM 5-Aza-CdR for 6 days. Total RNA was extracted, and the amount of cav-2 mRNAs was analyzed by RT-PCR. WCLs were subjected to immunoblot analysis with the anti-cav-2 antibody. Shown is a representative experiment that was repeated three times. GAPDH and actin expression levels were used as controls for equal mRNA and protein loading in A and B.

Results

Cav-2 expression in various cancer cells. We first evaluated mRNA levels and protein expression of endogenous cav-2 in A549, C6, SH-SY5Y, HepG2, LN-CaP, PC3M, MCF7 and HeLa cells. Both cav-2 mRNA and protein were detected in A549, C6, PC3M, MCF7 and HeLa cells, but not in SH-SY5Y, HepG2 and LN-CaP cells (Fig. 1A). Previous studies reported promoter methylation of cav-1 in human cancers including breast, prostate, ovarian and lung (33-36). To verify whether cav-2 is epigenetically inactivated by methylation of the promoter region in SH-SY5Y, HepG2 and LN-CaP cells expressing no cav-2, the cells were treated with 5-aza-2'-deoxycytidine (5-Aza-CdR), a drug that inhibits DNA methylation. As shown in Fig. 1B, cav-2 mRNA and protein were still not detected in the cells upon 5-Aza-CdR treatment. Endogenous cav-1 expression was observed in A549, C6, HepG2, PC3M, MCF7 and HeLa cells but not in SH-SY5Y and LN-CaP cells (Fig. 1A) as previously reported (37-42).

Effect of exogenous cav-2 expression on proliferation of cav-2-nonexpressing cancer cells. To assess the functional role of cav-2 in cav-2-nonexpressing HepG2, SH-SY5Y and LN-CaP cells (Fig. 1), we examined the effect of exogenous addition of cav-2 on cancer cell proliferation. Expression of exogenous cav-2 in the cells transfected with pcDNA3-cav-2 was confirmed by immunoblot analysis (Fig. 2A), and the cells were subjected to MTT assay (Fig. 2B). When HepG2 hepatocellular carcinoma cells were transfected with cav-2, cell proliferation was decreased by 22.7%, whereas cav-2-transfected SH-SY5Y neuroblastoma cells showed a 22.0% increase as compared to the vector control. Re-expression of cav-2 did not affect proliferation of LN-CaP prostate

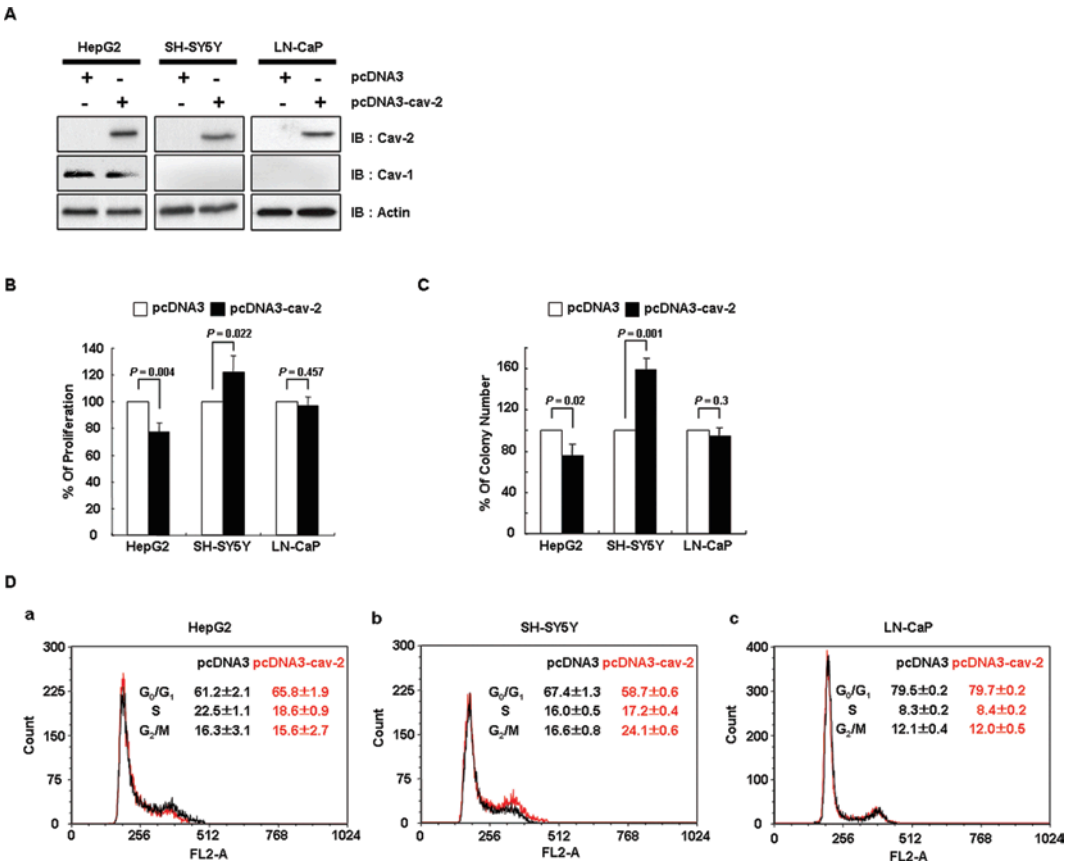

Figure 2. Mitogenic effects of exogenous cav-2 transfection in HepG2, SH-SY5Y and LN-CaP cells. (A) HepG2, SH-SY5Y and LN-CaP cells were transfected with the pcDNA3 vector or pcDNA3-cav-2 for 48 h. WCLs were subjected to immunoblot analysis with anti-cav-2, anti-cav-1 and anti-actin antibodies. Shown is a representative experiment that was repeated three times. (B) HepG2, SH-SY5Y and LN-CaP cells were seeded in a 96-well plate and transfected with the pcDNA3 vector or pcDNA3-cav-2 for 18 h. After 2 days of incubation, the cells were treated with 0.5 mg/ml MTT for 4 h and then dissolved in 100 μl of DMSO for 30 min. Absorbance was measured at 540 nm by a microplate reader as described in Materials and methods. The results represent the mean ± SE of three independent experiments. (C) HepG2, SH-SY5Y and LN-CaP cells were transfected with the pcDNA3 vector or pcDNA3-cav-2 for 48 h, and the cells were reseeded on the top layer in a 0.35% agarose medium on a base layer of 0.5% agar medium. After 2 weeks, colonies were stained with 0.02% crystal violet solution for 1 h and photographed using a dissecting microscope,and the number of large colonies (>0.042 mm in diameter) counted was scored using Image-ProPlus 6.1 as described in Materials and methods. The results represent the mean ± SE of three independent experiments. (D) HepG2, SH-SY5Y and LN-CaP cells were transfected with the pcDNA3 vector or pcDNA3-cav-2 for 48 h. The cells were harvested with trypsin, fixed in ethanol, stained with propidium iodide, and analyzed by flow cytometry as described in Materials and methods. Representative cell cycle profiles from one representative out of a total of three experiments are shown for the pcDNA3 vector- and pcDNA3-cav-2-transfected cells. The representative cell cycle profiles of the pcDNA3 vector- and pcDNA3-cav-2-transfected cells are indicated in black and red, respectively. The results represent the mean ± SE of three independent experiments. a, HepG2 hepatocellular carcinoma cells; b, SH-SY5Y neuroblastoma cells; c, LN-CaP prostate carcinoma cells.

carcinoma cells (Fig. 2B). We further investigated the effect of cav-2 on the *in vitro* growth of the cells by colony formation assay (Fig. 2C). Similar to the results of the MTT assay, exogenous cav-2 led to a 25.0% reduction and a 58.4% elevation in the growth of HepG2 and SH-SY5Y cells, respectively, as compared to the vector control. The growth of LN-CaP prostate carcinoma cells was again not influenced by cav-2 transfection (Fig. 2C). Regulation of the cell cycle by cav-2 re-expression was further investigated using flow cytometric analysis (Fig. 2D). In cav-2-transfected HepG2 hepatocellular carcinoma cells, the number of cell in the G_0/G_1 phase was increased from 61.2±2.1 to 65.8±1.9% (P=0.047), while the number of cells in the S phase was decreased from 22.5±1.1 to 18.6±0.9% (P=0.009) as compared to the cav-2-untransfected cells (Fig. 2D-a). In contrast, the number of cells in the G_0/G_1 phase was reduced from 67.4±1.3 to 58.7±0.6% (P<0.001) while the number of cells in the S and G_2/M phase was elevated from 16.0±0.5 to 17.2±0.4% (P=0.039) and from 16.6±0.8 to 24.1±0.6% (P<0.001), respectively, by cav-2 transfection in SH-SY5Y neuroblastoma cells (Fig. 2D-b). G0/G1 to S phase transition was not changed in cav-2-transfected LN-CaP prostate carcinoma cells. Thus, these results demonstrate that forced cav-2 expression inhibits the proliferation, cell cycle and growth of HepG2 hepatocellular carcinoma cells indicating that cav-2 acts as a negative regulator of the progression of cancer.

Effect of the down-regulation of cav-2 on proliferation of cav-2-expressing cancer cells. To verify whether endogenous cav-2 regulates cancer progression, we abrogated its expression in C6, HeLa, A549, MCF7 and PC3M cells using cav-2 siRNA. As shown in Fig. 3A, cav-2 siRNA duplexes effectively depleted cav-2 protein by >80.4-84.4% of levels observed in the scramble control siRNA-transfected cells. In C6 glioma cells, cav-2 down-regulation caused a 14.0 and 22.7% reduction in cell proliferation and colony formation,

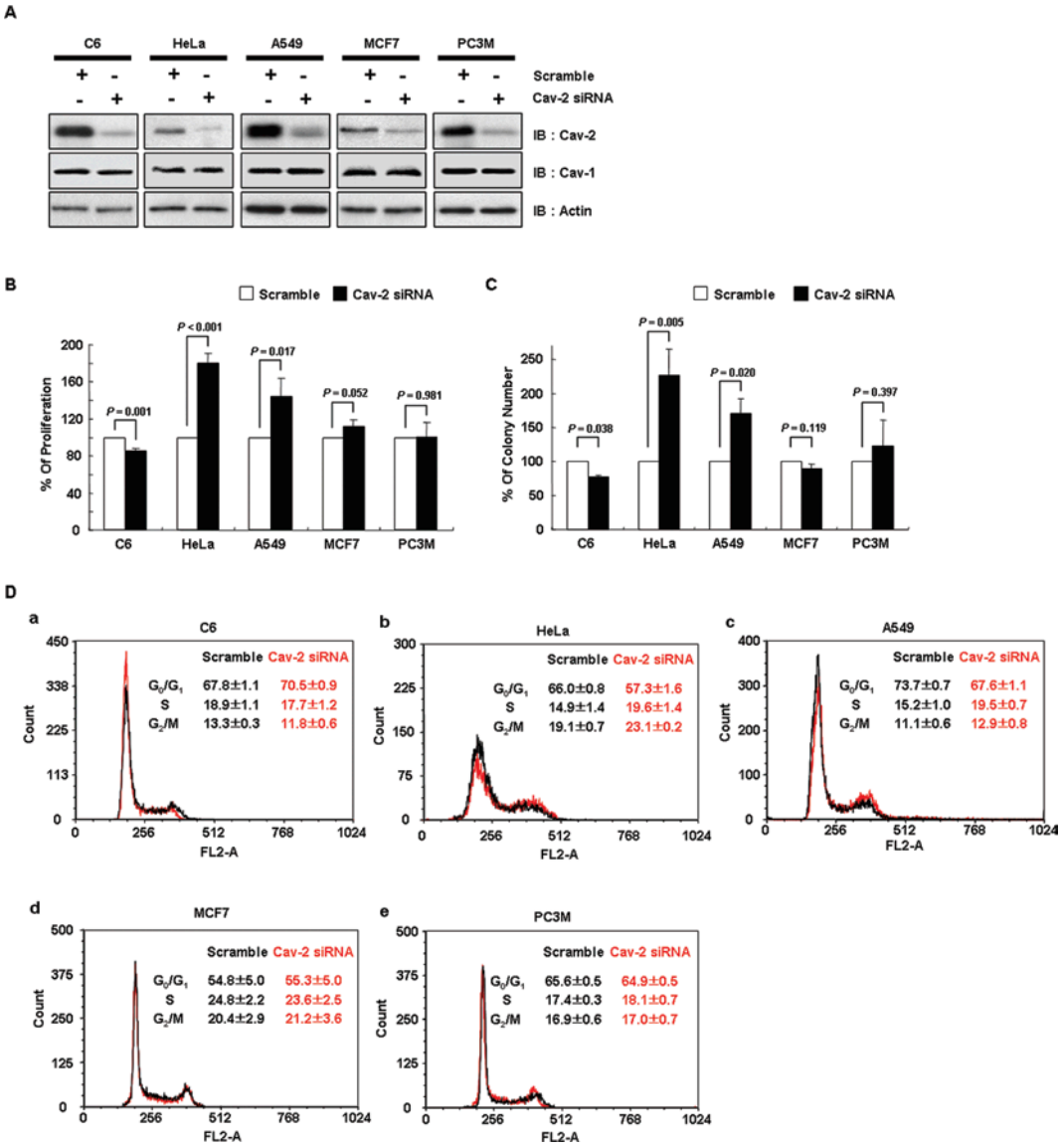

Figure 3. Mitogenic effects of cav-2 down-regulation in C6, HeLa, A549, MCF7 and PC3M cells. C6, HeLa, A549, MCF7 and PC3M cells were transfected with scramble or cav-2 siRNA for 48 h. (A) WCLs were subjected to immunoblot analysis with anti-cav-2, anti-cav-1 and anti-actin antibodies. Shown is a representative experiment that was repeated three times. (B) After 2 days of incubation, cells were treated with 0.5 mg/ml MTT for 4 h and then dissolved in 100 μl of DMSO for 30 min. Absorbance was measured at 540 nm by a microplate reader as described in Materials and methods. The results represent the mean ± SE of three independent experiments. (C) Cells were seeded on the top layer in a 0.35% agarose medium on a base layer of 0.5% agar medium. After 2 weeks, colonies were stained with 0.02% crystal violet solution for 1 h and photographed using a dissecting microscope and the number of large colonies (>0.042 mm in diameter) counted were scored using Image-ProPlus 6.1 as described in Materials and methods. The results represent the mean ± SE of three independent experiments. (D) Cells were harvested with trypsin, fixed in ethanol, stained with propidium iodide, and analyzed by flow cytometry as described in Materials and methods. Representative cell cycle profiles from one representative out of a total of three experiments are shown for scramble control and cav-2 siRNA-transfected cells. The representative cell cycle profiles of the scramble control and cav-2 siRNA-transfected cells are indicated in black and red, respectively. The results represent the mean ± SE of three independent experiments. a, C6 glioma cells; b, HeLa epithelial cervical cancer cells; c, A549 lung adenocarcinoma cells; d, MCF7 breast cancer cells; e, PC3M prostate carcinoma cells.

respectively, as compared to the scramble control (Fig. 3B and C). In contrast, HeLa epithelial cervical cancer and A549 lung adenocarcinoma cells showed a 79.5 and 44.2% increase in cell proliferation with a 126.6 and 69.5% elevation in colony formation, respectively, upon cav-2 siRNA transfection. In MCF7 breast cancer and PC3M prostate carcinoma cells, depletion of cav-2 did not affect cell proliferation and colony formation (Fig. 3B and C). To determine whether the modulation of cancer proliferation and growth by cav-2 down-regulation is reflected in the cancer cell cycle distribution, we performed flow cytometric analysis of the cav-2-depleted cancer cells (Fig. 3D). Cav-2 siRNA-transfected C6 glioma cells displayed an increase in number in the G_0/G_1 phase from 67.8±1.1 to 70.5±0.9% (P=0.027) and a decreases in cell number in the S and G_2/M phase from 13.3±0.3 to 11.8±0.6% (P=0.012) as compared to the scramble control (Fig. 3D-a). Upon cav-2 depletion, the number of cells in the G_0/G_1 phase was decreased from 66.0±0.8 to 57.3±1.6% (P=0.001) in the HeLa epithelial cervical cancer cells and from 73.7±0.7 to 67.6±1.1% (P=0.001) in the A549 lung adenocarcinoma cells, respectively, and the number of cells in the S and G_2/M phase was increased as compared to the scramble control (Fig. 3D-b

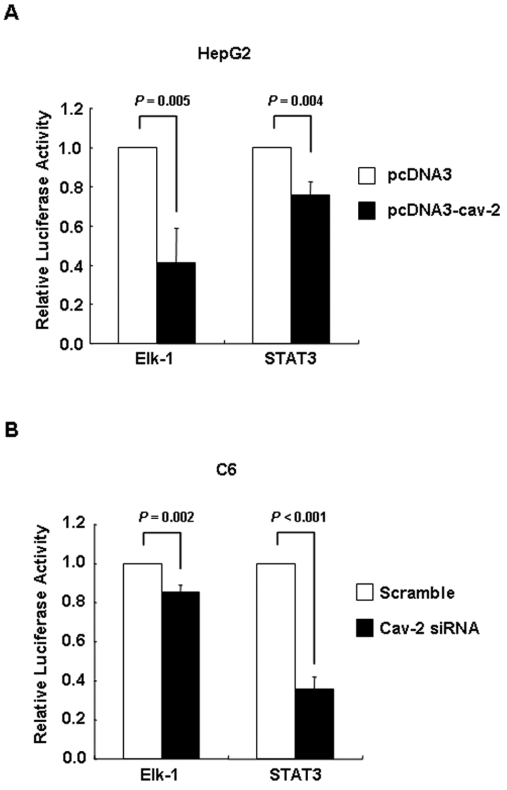

Figure 4. Effects of cav-2 re-expression in HepG2 hepatocellular carcinoma cells and cav-2 depletion in C6 glioma cells on transcriptional activation of Elk-1 and STAT3. (A) HepG2 hepatocellular carcinoma cells were transfected with plasmid DNA (Elk-1-Luc or STAT3-Luc) along with the pcDNA3 vector or pcDNA3-cav-2 with the pRL-TK vector for 48 h. Luciferase activity was measured with a dual luciferase assay system, and data are presented as the mean ± SE of three independent experiments performed. (B) C6 glioma cells were transfected with scramble or cav-2 siRNA for 24 h. The cells were transfected with plasmid DNA (Elk-1-Luc or STAT3-Luc) along with pRL-TK vectors for 48 h. Luciferase activity was measured with a dual luciferase assay system, and data are presented as the mean ± SE of three independent experiments performed.

and -c). As observed in Fig. 3B and C with no significant changes in proliferation and colony formation of MCF7 and PC3M cells, their cell cycle progression was also unaffected by cav-2 siRNA (Fig. 3D-d and -e). These data suggest that cav-2 siRNA serves as a modulator for the inhibition of proliferation, cell cycle and growth of C6 glioma cells.

Regulation of transcriptional activity by cav-2 in HepG2 hepatocellular carcinoma and C6 glioma cells. We previously showed that cav-2 regulates transcriptional activation of STAT3 by promoting mitogenic ERK signaling (9,31). As demonstrated in Figs. 2 and 3, cancer cell proliferation was reduced by re-expression of cav-2 in HepG2 hepatocellular carcinoma cells and by down-regulation of cav-2 in C6 glioma cells. To investigate whether the alteration of cav-2 expression inhibits transcriptional activation of Elk-1 and STAT3, which are ERK-regulated transcription factors (43,44), we performed a reporter gene assay using a luciferase reporter construct containing the Elk-1 or STAT3-binding site (Fig. 4). When HepG2 hepatocellular carcinoma cells were re-expressed with exogenous cav-2, transcriptional activation of Elk-1 and STAT3 was decreased by 2.4- and 1.3-fold, respectively as compared to the untransfected cells (Fig. 4A). In C6 glioma cells, down-regulation of cav-2 by cav-2 siRNA led to a 1.2- and 2.8-fold decrease in transcriptional activation of Elk-1 and STAT3, respectively, as compared to the scramble control (Fig. 4B). The data together with the results from Figs. 2 and 3 demonstrate that exogenous introduction of cav-2 and siRNA-mediated knockdown of cav-2 can be applied to delay cancer progression by retarding cancer cell proliferation and growth by reducing mitogenic transcriptional activation in HepG2 hepatocellular carcinoma and C6 glioma cells, respectively.

Discussion

Previous studies have reported that cav-1 regulates multiple cancer-related processes including cell growth, migration, metastasis and angiogenesis (4,11,45). Although cav-2 is widely present in tumor cells (2), the regulatory function of cav-2 in tumor progression has not been investigated. In the present study, we explored the effects of the alteration of cav-2 status in various types of cancer cells. We first examined cav-2 mRNA levels and protein expression in HepG2, SH-SY5Y, LN-CaP, C6, HeLa, A549, MCF7 and PC3M cells (Fig. 1). Cav-2 mRNA and protein expression was absent in HepG2, SH-SY5Y and LN-CaP cells, whereas it was present in C6, HeLa, A549, MCF7 and PC3M cells. Cav-2 was co-expressed with cav-1 in C6, HeLa and A549 cells.

Our results showed that cav-2 deficiency, produced by siRNA-mediated silencing, caused attenuation of cancer cell proliferation in C6 glioma cells and promotion in human HeLa epithelial cervical cancer and A549 lung adenocarcinoma cells with no change in cav-1 expression (Fig. 3A). When cav-2 was re-expressed in human HepG2 hepatocellular carcinoma cells which expressed cav-1 alone (Fig. 1), the cancer cell growth and proliferation were significantly reduced without a change in cav-1 expression (Fig. 2). In a recent study, we demonstrated that cav-2 regulates cellular mitogenesis of insulin signaling irrespectively of cav-1 expression (10). Thus, our present data support the regulatory role of cav-2 in cancer progression independently of cav-1.

Cav-1 expression is down-regulated or mutated in many types of human cancers, which suggests that cav-1 acts as a tumor suppressor (46). Of interest, we found that cav-2 was not detected while cav-1 was expressed in HepG2 hepatocellular carcinoma cells suggesting that cav-2 instead of cav-1 plays a role as a tumor suppressor in hepatocellular carcinoma (Fig. 1A). Down-regulation of cav-1 gene expression has been correlated with promoter methylation in human breast, ovarian, prostate and lung cancers (33-36). Accordingly, as the loss of cav-2 expression in HepG2 hepatocellular carcinoma cells by promoter methylation was examined using 5-Aza-CdR treatment, cav-2 expression was not restored indicating the absence of the cav-2 gene in the hepatocellular carcinoma cells (Fig. 1B). The present data demonstrated that exogenous addition of cav-2 markedly decreased cancer cell growth and proliferation of HepG2 hepatocellular carcinoma (Fig. 2). These results suggest that exogenous cav-2 can be used as an anticancer agent against human hepatocellular carcinoma.

ERK activation induces phosphorylation of Elk-1 (1) and STAT3 (44). In previous studies, we demonstrated cav-2-dependent promotion of ERK-mediated transcriptional

activation of STAT3 (9,31). In the present study, we investigated whether ERK-mediated transcriptional activation of Elk-1 and STAT3 is affected by re-expression of exogenous cav-2 in HepG2 hepatocellular carcinoma cells and down-regulation of cav-2 by siRNA in C6 glioma cells, which led to retardation of cancer cell growth and proliferation (Figs. 2 and 3). Changes in cav-2 status in the hepatocellular carcinoma and glioma cells induced a significant reduction in the transcriptional activation of Elk-1 and STAT3 (Fig. 4).

STAT3 is constitutively activated in many types of human cancers (47). Therefore, the cav-2 gene could be an appealing therapeutic target for inhibition of oncogenic STAT3 signaling. The regulatory role of cav-2 in human cancer progression warrants investigation in animal models by employing cav-2 gene transfer delivered by either direct injection into hepatocellular carcinomas or via hepatic artery and cav-2 antisense gene therapy for gliomas using viral or non-viral vectors. Further investigation of the therapeutic gene modulation of cav-2 may facilitate clinical application of cav-2-targeted cancer therapy.

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