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Divergent expression and roles for caveolin-1 in mouse hepatocarcinoma cell lines with varying invasive ability

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Abstract

Caveolin-1 is the major component protein of caveolae and associated with a lot of cellular events such as endocytosis, cholesterol homeostasis, signal transduction, and tumorigenesis. The majority of results suggest that caveolin-1 might not only act as a tumor suppressor gene but also a promoting metastasis gene. In this study, the divergent expression and roles of caveolin-1 were investigated in mouse hepatocarcinoma cell lines Hca-F, Hca-P, and Hepa1–6, which have high, low, and no metastatic potential in the lymph nodes, as compared with normal mouse liver cell line IAR-20. The results showed that expression of caveolin-1 mRNA and protein along with the amount of caveolae number in Hca-F cells was higher than that in Hca-P cells, but was not detectable in Hepa1–6 cells. When caveolin-1 expression in Hca-F cells was down-regulated by RNAi approach, Hca-F cells proliferation rate in vitro declined and the expression of lymphangiogenic factor VEGFA in Hca-F decreased as well. Furthermore, in vivo implantation assay indicated that reduction of caveolin-1 expression in Hca-F prevented the lymphatic metastasis tumor burden of Hca-F cells in 615 mice. These results suggest that caveolin-1 facilities the lymphatic metastasis ability of mouse hepatocarcinoma cells via regulation tumor cell growth and VEGFA expression.

Keywords: Caveolin-1; Hepatocarcinoma; Lymphatic metastasis; VEGFA

Caveolae are 50–100 nm omega-shaped membranes that represent a subcompartment of the plasma membrane [1]. Caveolins (-1, -2, and -3) are known to be principal protein in caveolae [2]. Caveolin-1 (Cav-1) is responsible for the formation of caveolae along with cholesterol and sphingolipids [1]. Proofs reveal that caveolae and caveolins are involved in a variety of cellular processes including lipid transport, signal transduction, and oncogenesis [3–6].

Cav-1 is reported to interact directly with many key signaling molecules and keep them contact-inactive via a motif known as the scaffolding domain [5]. The high levels of Cav-1 are seen in terminally differentiated cell types, including adipocytes, epithelial, endothelial, smooth muscle cells, and type I pneumocytes [2]. These findings show that Cav-1 is associated with cell growth and proliferation.

Caveolin-2 (Cav-2) also exists in caveolae and forms a stable heterooligomeric complex with Cav-1, and it is proved that Cav-2 assists Cav-1 in fulfilling function but not necessary. Caveolin-3 (Cav-3) protein is expressed mainly in muscle cells, including smooth, skeletal, and cardiac myocytes. Cav-1 shares 31% identity with Cav-2 and 65% identity with Cav-3 at the amino acid level [7].

Evidence has accumulated suggesting that Cav-1 might act as a tumor suppressor. Down-regulation of Cav-1 has been found in various cancer types, including human breast [8,9], lung, colon [10], and ovarian carcinomas [11], as well as human sarcomas [12]. It seems that the loss of Cav-1 leads to a poorly controlled cell growth. But this idea has been controversial because Cav-1 over-expression is seen

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in some cancers such as prostate, lung, ovary, kidney, and esophageal cell carcinoma [13–17]. Cav-1 expression has even been associated with increased metastasis potential.

It has been proved that a large amount of molecules interact with Cav-1 [18]. Among these are vascular endothelial growth factors (VEGFs) [19], generally recognized as angiogenesis activators. Recently, some members from VEGF family are also proved to be lymphangiogenic factors, including VEGFA and VEGFC/VEGFD–VEGFR3 [20,21]. These cytokines have been reported to induce lymphatic metastasis. It is reported that endothelial-specific Cav-1 over-expression reduces VEGF-stimulated vascular permeability and decreases VEGF-mediated angiogenesis by 40% [22], suggesting that caveolin might be an intracellular target for angiogenesis activators. In another study, conversely, Cav-1 is shown to induce angiogenesis during the development of clear cell renal cell carcinoma [16]. Effects of Cav-1 on angiogenesis could explain partially the tumor suppressor and metastasis promoter functions of Cav-1. During different phases tumor cells face changed environment and need an adequate blood supply to survive and progress.

The mouse hepatocarcinoma cell lines Hca-F with a highly lymphatic metastasis rate over 70% and Hca-P with a low lymphatic metastasis rate less than 30% have been derived from hepatocarcinoma in mice [23]. Hca-F and Hca-P cells metastasize only to the lymph node, and do not disseminate to other organs [24]. However, the relationship between Cav-1 and lymphatic metastasis of mouse liver cancers is still not clear.

In this report, we demonstrated the divergent expression of Cav-1 in a series of mouse liver cancer cell lines. By the RNAi strategy, we found that partial loss of Cav-1 led to a significant decrease of VEGFA level in the Hca-F cells. A significant reduction of lymphatic metastasis tumor burden of Hca-F cells was seen followed by Cav-1 down-regulation. Taken together, our results suggest that Cav-1 down-regulation could decrease the lymphatic metastasis ability of mouse hepatocarcinoma cells possibly by inhibiting tumor growth and lymphangiogenesis.

Methods

Cell culture and animals. Mouse hepatocarcinoma cell lines, Hca-F and Hca-P, which have high and low metastatic potential in the lymph nodes (established and stored by Department of Pathology, Dalian Medical University) were maintained in 90% RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1× penicillin/streptomycin (Gibco) and 1 g/L sodium bicarbonate (Gibco). Mouse hepatocarcinoma cell line Hepa1–6 and mouse hepatocyte line IAR-20 (obtained from Cell Center of Peking Union Medical University, Beijing) were grown in required growth medium. Specifically, Hepa1–6 cells were grown in 90% Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Gibco), 1× penicillin/streptomycin and 1 g/L Hepes (hyclone), IAR-20 cells in 90% modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Gibco) and 1× penicillin/streptomycin. All cells were cultured in a humidified incubator at 37 °C with 5% CO_2. All animals were obtained from Animal Facility of Dalian Medical University.

In vivo lymphatic metastasis assay. Forty inbred 615-mice were equally divided into two groups. 2×10^6 of Hca-F and Hca-P cells were inoculated into 615 mice feet pad subcutaneously. 2×10^6 of Hepa1–6 cells were injected into 20 C57B1/6 mice feet pad subcutaneously. After RNA interference of Cav-1 in Hca-F cells, 24 615-mice were equally divided into three groups. 2×10^6 Hca-F cells treated with Cav-1 siRNA and control siRNA were inoculated into the feet pad of mice subcutaneously, 2×10^6 Hca-F cells without treating were injected into the feet pad of mice in control group. After 4 weeks, mice were sacrificed and their lymph nodes were isolated, weighed, sectioned, and stained with hematoxylin and eosin, then lymphatic metastasis rates were determined.

In vitro adhesion assay. Two hundred microliters of cell suspension (1×10^6 cells) was overlaid on the frozen sections of lymph nodes from 615 mice, then the sections were cultured at 37 °C for 16 h with 5% CO_2. After washing with cold normal saline, the sections were incubated at 4 °C for 30 min, fixed by 95% alcohol solution for 5 min after three washes with cold normal saline, and then stained with hematoxylin and eosin. Adherent cells were calculated.

cDNA microarray analysis. Total RNA was isolated from Hca-F and Hca-P cells, respectively, using Trizol (Invitrogen, Carlsbad, CA, USA) and mRNA was isolated from the total RNA using the mRNA midi Kit (Oligotex, Qiagen). Clonetech GeneChips were prepared, hybridized, and analyzed according to the manufacturer's protocol. Briefly, mRNA was labeled with [α^{33}P]dATP by reverse transcription. Hybridization was performed at 68 °C overnight. Hybridization signals were scanned using FLA-3000 microarray scanner (Fuji Photo Film). The data were analyzed using Clonetech® Microarray Suit Software 5.0.

Semi-quantitative RT-PCR. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using RT-PCR kit (TaKaRa, Japan) according to the manufacturer's protocol. For the semi-quantitative PCR, 1 μL cDNA-template was mixed with 3.0 μL of 10× PCR-buffer, 1.0 μL of 10 mM dNTPs, 1.0 μL of each primer (50 ng/μL), and 0.5 μL polymerase (Ampli-Taq., Gibco) in a total volume of 30 μL for each probe. The primer pairs for each gene were designed with Primer Premier 5.0 software, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for PCR. The Cav-1 primers were 5′-ACAGTTTCGACGGCATCTGG-3′ (forward) and 5′-CAAAGAGT GGATCGCAGAAG-3′ (reverse). Specific Cav-2 primers were 5′-GC GTCGACTATGCAGATCCT-3′ (forward) and 5′-GCAAGACCATT AGGCAGGTC-3′ (reverse). For Cav-3, the forward primer was 5′-GCTCGGATCATCAAGGACAT-3′ and the reverse primer was 5′-CT TCGCAGCACCACCTTAAT-3′. The primers of VEGFA were 5′-TG GCTTTACTGCTGTACCTC-3′ (forward) and 5′-ACTCCAGGGCT TCATCGTTA-3′ (reverse). The GAPDH primers were 5′-GGCCGT GAAGTCGTCAGAAC-3′ (forward) and 5′-GCCACGATGCCCAG GAA-3′ (reverse). The annealing temperature for amplification of Cav-1 and GAPDH was 58 °C, for Cav-2, 3, and VEGFA it was 60 °C. The number of amplification cycles was 30 for Cav-1, 35 for GAPDH, and 25 for Cav-2, 3, and VEGFA. The other conditions were the same, with initial denaturation for 5 min at 94 °C, cycle denaturation for 20 s at 94 °C, annealing for 1 min, and extension at 72 °C for 30 s. The PCR products were separated with electrophoresis on 1% agarose gel and photographed under ultraviolet radiation light. The size of the PCR-fragments was estimated using DL2000 (TaKaRa, Japan). Band intensities were measured using BioImaging systems (UVP, labworksTM, ver 4.6) and were normalized to those for GAPDH.

Western blot analysis. 1×10^6 cells were collected, then the cell lysates were prepared by adding of 1 ml of 2× concentrated electrophoresis sample buffer (125 mM Tris–HCl, pH 6.8, 5% glycerol, 2% sodium dodecyl sulfate, and 1% β-mercaptoethanol) to each Eppendorf tube. Utilizing a sterile toothpick to remove sticky DNA from lysates. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay kit assay. Twenty micrograms of total protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. After blocking for 2 h with phosphate-buffered saline containing 0.1% Tween 20 and 5% powdered skimmed milk, the blots were incubated with rabbit anti-mouse Cav-1 polyclonal antibody (Santa Cruz Biotech, Inc., 1:200 dilution) or rabbit anti-mouse

VEGFA polyclonal antibody (Santa Cruz Biotech, Inc., 1:200 dilution) overnight in 5% powdered skimmed milk buffer, and then incubated with secondary antibody anti-rabbit-HRP (Santa Cruz Biotech, Inc., 1:1000 dilution) after three washes with phosphate-buffered saline. GAPDH antibody (Santa Cruz Company 1:1000 dilution) was used as internal marker for control. All bands were evaluated using ECL Western blotting kit (Amersham Biosciences, UK). Protein band intensities were determined using BioImaging systems (UVP, labworksTM, ver 4.6).

Subcellular localization of Cav-1. 1×10^6 Hca-F cells in 1 ml sample buffer (125 mM Tris–HCl, pH 6.8, 5% glycerol, 1% β-mercaptoethanol, and 0.1 mM PMSF) were sonicated on ice at 200 W/cm^2 for 3 cycles with 10 s intervals, and then centrifuged at 36,000*g* for 30 min at 0 °C. The pellet that contained membrane fraction was harvested and washed twice with sample buffer, then resuspensed in 1 ml sample buffer. The supernatant was used as the cytosolic fraction. Twenty microliters of sample from the membrane, cytosol fractions, and the total lysate was subjected to SDS–PAGE and Western blotting analysis.

Quantitation of caveolae by electronic microscopy. Four kinds of cells (Hca-F, Hca-P, Hepa1–6, and IAR-20) were collected, sequentially washed in PBS, fixed with 2.5% glutaraldehyde for 2 h at 4 °C, and postfixed in 1% osmium tetroxide for 2 h at 4 °C after three washes in PBS. Specimens were dehydrated in a graded series of alcohol, then incubated at 37 °C for 3 h in a solution of Epon Polybed 812 and acetone (1:1). The solution was removed and the pellets were embedded in Epon Polybed 812, incubated at 65 °C for 72 h. The preparation was then sectioned and mounted on copper mesh grids, stained with uranyl acetate and lead nitrate, and examined with a transmission electron microscope (JEM-2000EX, Japan). Caveolae were identified by their characteristic flask shape and size (50–100 nm).

RNAi assay. Hca-F cells were incubated in a 6-well tissue culture dish to obtain a 60–80% confluency. Then the cell cultures were transfected with control siRNA and Cav-1-specific siRNA Transfection Reagent Complex (Santa Cruz Biotech, Inc., sc-29942), which was prepared according to the protocol. Transfer cells to 6-well tissue culture plate and incubate at 37 °C for 6 h, followed by incubation with complete medium for additional 24 h. After transient transfection was performed for 30 h, cells were harvested and followed by RT-PCR analysis, Western blot analysis, and lymphatic metastasis assay in vivo as previously described. Cell proliferation assay was used to determine the effect of Cav-1 down-regulation on the cells growth.

Cell proliferation assay. Hca-F cells were grown in 96-well plates and 400 μL MTS solutions were added to each well. After 4 h incubation, living cells converted MTS tetrazolium to a blued insoluble formazan product. One hundred microliters of 0.04 M NH_4Cl/isopropanol was added as a resolvent. The amount of formazan was estimated as absorbance at 490 nm in a spectrophotometer. Relative cell growth of cells treated with siRNA was expressed as fold increases of absorbance at 490 nm over cells control without being transfected. Data points in all figures are from three separate experiments [25].

Statistical analysis. Data were analyzed by SPSS10.0 statistical software. Student's *t* test was used to evaluate the significance of differences in multiple comparisons. $p < 0.05$ was considered to be statistically significant.

Results

Different invasive ability to regional lymph node of Hca-F, Hca-P, and Hepa1–6

First, the different invasive ability of Hca-F, Hca-P, and Hepa1–6 was determined by in vivo metastasis rate assay and in vitro adhesion assay. The metastasis rates to regional lymph nodes of Hca-F and Hca-P are 84% and 30% (Fig. 1A). No metastasis tumor was found in livers and lungs (data not shown). Hca-F cells also showed a 2.6-fold increase ($p < 0.05$) of adhesion ability to regional lymph node in vitro, when compared to Hca-P cells, as seen in Fig. 1B and C. No metastasis tumor was observed of Hepa1–6 cells in C57B1/6 mice (data not shown).

Higher Cav-1 expression in Hca-F cell line revealed by cDNA microarray analysis

To identify genes associated with the lymphatic metastasis, we analyzed the transcriptional profiles of 1185 mouse genes from highly lymphatic metastasis potential cell line Hca-F and low lymphatic metastasis potential cell line Hca-P using the Clonetech GeneChip® array method. Among those, 2.8-fold increase of *Cav-1* in Hca-F cells was observed when compared to Hca-P ($p < 0.05$), as shown in Fig. 2A–C, arrows point at *Cav-1* signal. We therefore focused on the changes of *Cav-1* gene and protein expression.

Higher CAV-1 expression in Hca-F cell line revealed by semi-quantitative RT-PCR

The relative levels of *Cav-1*, *2*, *3* transcripts in Hca-F, Hca-P, Hepa1–6, and IAR-20 cell lines were determined by RT-PCR. *Cav-1* and *Cav-2* were expressed in all cell types except in Hepa1–6 cell line, while *Cav-3* gene is absent in all cells (Fig. 3A). Up-regulation of *Cav-1*, *2* was seen in Hca-F ($p < 0.005$) and Hca-P ($p < 0.01$) when compared with IAR-20. Furthermore, *Cav-1*, *2* expressions appeared to be higher in Hca-F but reduced in Hca-P ($p < 0.01$) (Fig. 3B). These results were consistent with those of the cDNA microarrays. The small differences in the results may be attributable to the differences between these two methods. Nonetheless, these results supported the idea that *Cav-1* was not only a tumor suppressor gene but also a lymphatic metastasis-associated gene.

Up-regulation of Cav-1 protein expression in Hca-F cell line

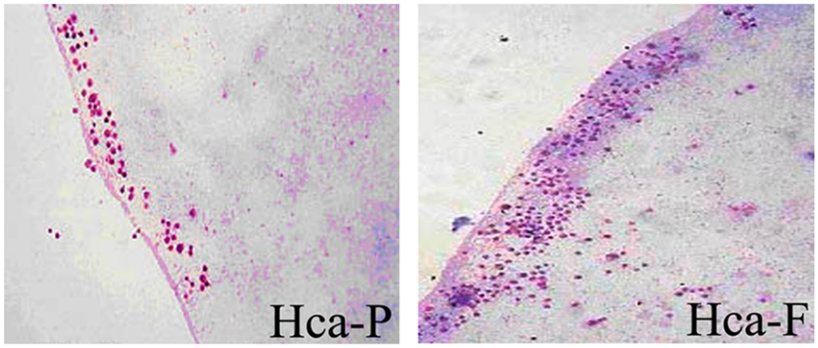
To confirm cDNA microarray and RT-PCR results further, Cav-1 protein expressions in Hca-F, Hca-P, Hepa1–6, and IAR-20 cells were investigated by Western blot analysis. Cav-1 expression was variable among the cell lines, with higher Cav-1 level detected in Hca-F cells and lower level in Hca-P ($p < 0.005$). Comparatively, Cav-1 level in IAR-20 cells was lower than that in Hca-F and Hca-P cell lines ($p < 0.001$, $p < 0.005$). In contrast, expression of Cav-1 protein in Hepa1–6 cells was undetectable, as shown in Fig. 4A and C. These results suggested that high Cav-1 expression might be associated with metastases, while loss of Cav-1 might be associated with primary tumor. In a separate experiment, we estimated the subcellular location of Cav-1 in Hca-F cell by Western blot analysis. The results indicated that Cav-1 mainly existed in the cell membrane fraction and less intensely in cytosol fractions (Fig. 4B), thus confirming the cell surface location of Cav-1.

A

Lymph Node Metastatic Incidence in 615 Mice

Cell type	Incidence(%)	N
Hca-P	30	20
Hca-F	84	19

B



C

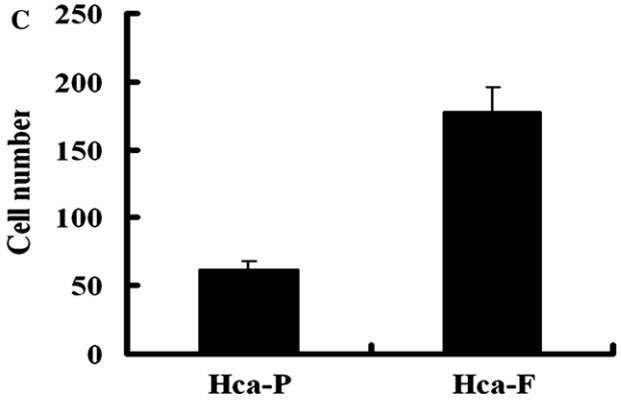


Fig. 1. Different invasive ability to regional lymph node of Hca-F, Hca-P. Hca-F and Hca-P cells were inoculated at 2×10^6 into 615 mice feet pad subcutaneously to assay lymphatic metastasis rate. The lymphatic metastasis incidences of Hca-F and Hca-P cells were 84% (16/19) and 30% (6/20) (A). Hca-F cells showed a high adhesion ability to regional lymph node in vitro, when compared with Hca-P cells ($p < 0.05$) (B,C).

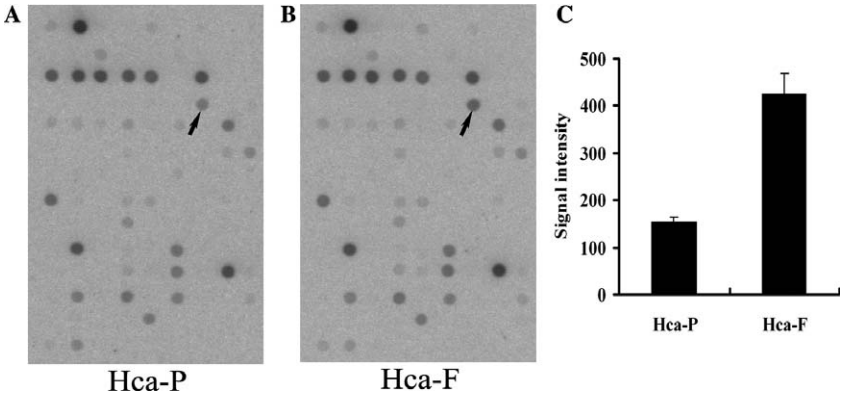


Fig. 2. Higher *Cav-1* expression in Hca-F cell line revealed by cDNA microarray analysis. The differential gene expressions in Hca-P (A) and Hca-F (B) cells were determined by cDNA microarray analysis. (A) and (B) are representative close-up view of gene chip images. The arrows indicated that Cav-1 was over-expressed in Hca-F cells. (C) Relative signal intensity of Cav-1 expression in Hca-F was significantly higher than that in Hca-P cell line ($p < 0.05$).

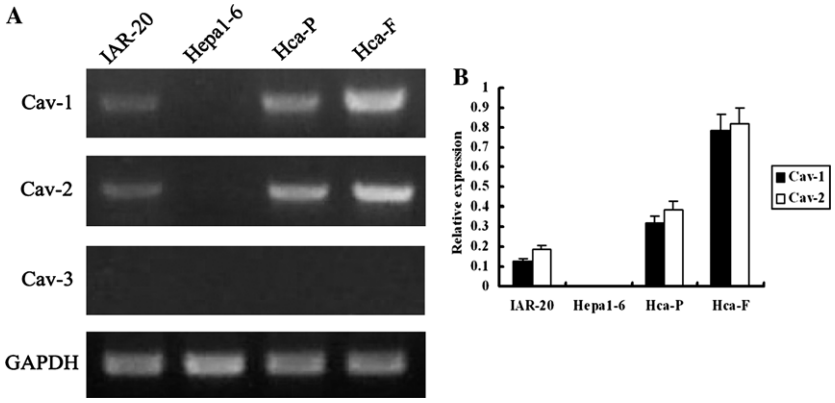


Fig. 3. Higher *Cav-1* expression in Hca-F cell line revealed by semi-quantitative RT-PCR. (A) The *Cav-1*, *2*, *3* genes were assayed by RT-PCR for their expression in Hca-F, Hca-P, Hepa1–6, and IAR-20 cell lines. (B) Relative mRNA levels were expressed as arbitrary units derived from signal intensities of the target cDNA normalized to those of GAPDH cDNA.

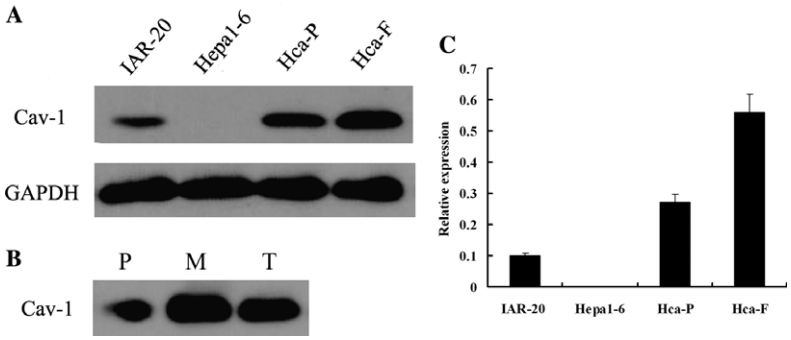


Fig. 4. Western blot analysis of the expression of Cav-1 protein in mouse hepatocarcinoma cell lines with varying invasive ability. (A) Cav-1 protein was in abundance in Hca-F cell line, less intensely in Hca-P cell line, lower level in IAR-20 cell line, and undetectable in Hepa1–6 cell line. (B) Cav-1 protein also was found in Hca-F subcellular fraction of (P, plasma; M, membrane; T, total cell lysates). GAPDH blotting was used as control. (C) Relative signal intensity of Cav-1 expression in Hca-F, Hca-P, Hepa1–6, and IAR-20 cell lines. The data were normalized against control samples.

Cav-1 expression is correlated with caveolae number in Hca-F

To determine whether Cav-1 expression was related to caveolae number, we scanned the plasma membranes of above four cell lines exhaustively for caveolae, which are defined as uniform 50–100-nm flask-shaped membrane invaginations. Caveolae number of 20 cells from each cell line was counted under transmission electron microscopy. We found that caveolae number in Hca-F (Fig. 5D) is larger than that in Hca-P (Fig. 5C) ($p < 0.05$). Caveolae were also present in IAR-20 (Fig. 5A), but fewer in number than caveolae in Hca-F ($p < 0.01$) and Hca-P ($p < 0.01$). No caveolae structure was observed in Hepa1–6, as shown in Fig. 5B. It seemed that both the loss of caveolin, which directly inhibited key signaling molecules, and the loss of caveolae, which regulated signal transduction, contributed to poorly controlled cell growth in Hepa1–6 cells. Changes of caveolae number in our four cells were consistent with different expression of Cav-1. These results showed that Cav-1 expression might influence caveolae formation.

Silencing of Cav-1 expression in Hca-F by RNAi approach

Next we sought to establish whether loss of Cav-1 was responsible for the decreased invasive capabilities of the Hca-F cells. We transfected Hca-F cells with Cav-1 siRNA and a control siRNA respectively, silencing the expression of target genes. As illustrated in Fig. 6A and B, RT-PCR and Western blot analysis confirmed a clear reduction of Cav-1 expression at both mRNA and protein levels after transfection, but no Cav-1 decrease was seen in Hca-F transfected with control siRNA. These results suggested that Cav-1 expression was indeed knockdown by Cav-1 siRNA.

Silencing of Cav-1 reduces VEGFA expression in Hca-F

A number of molecules have been shown to be associated with Cav-1 and some of them are involved in tumor lymphatic metastasis. We examined expression of lymphangiogenic factor VEGFA using Western blotting before and after Cav-1 silencing in Hca-F cells. Interestingly, we found that VEGFA expression was down-regulated in response to Cav-1 RNAi, as seen in Fig. 6A and B ($p < 0.05$). This result

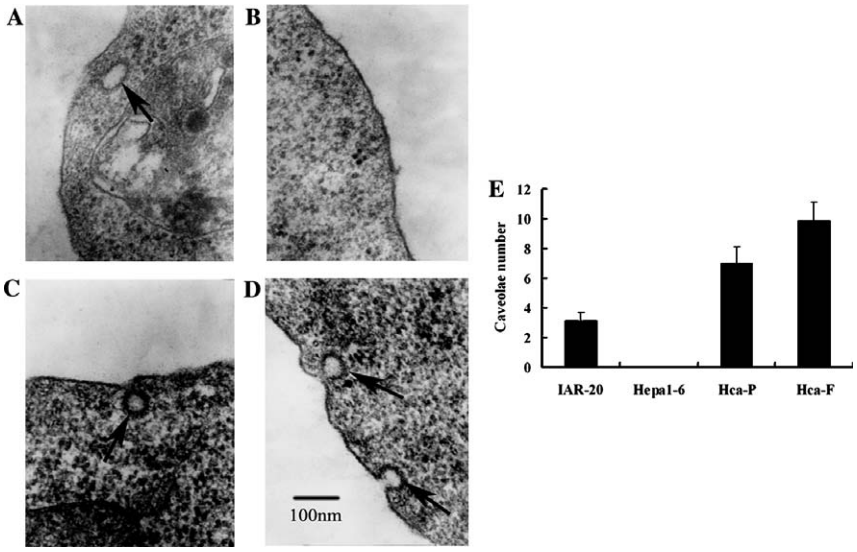


Fig. 5. Quantitation of caveolae by electronic microscopy. Representative transmission electron micrographs of caveolae structures in IAR-20 (A), Hca-P (C), and Hca-F (D) cell lines but not in Hepa1–6 cells (B). Average caveolae number per cell was determined by counting the number of plasmalemmal attached caveolae from 20 cells per cell line (E).

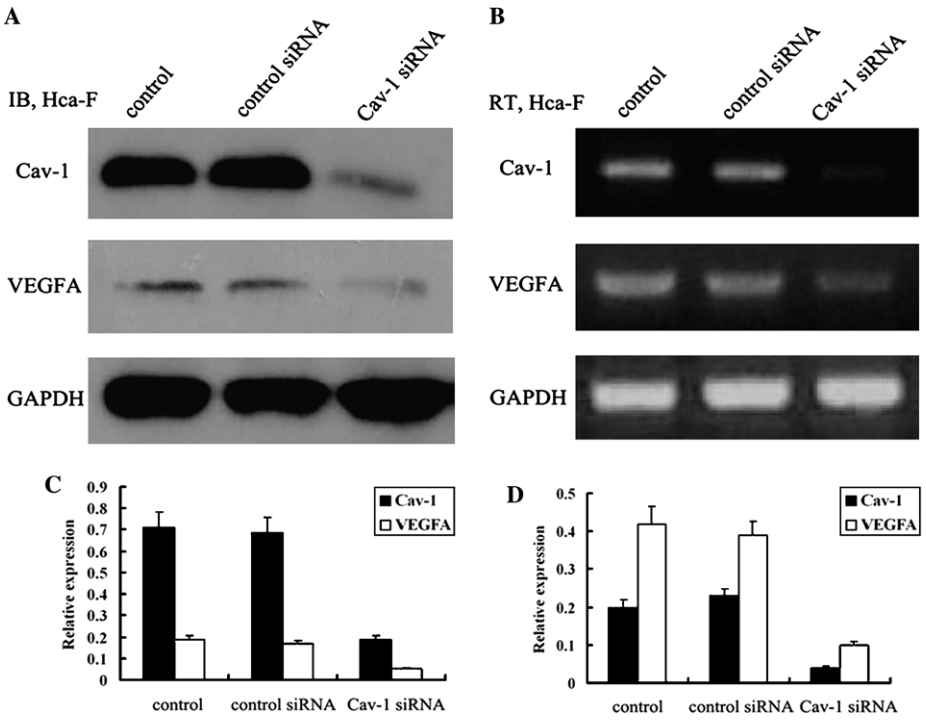


Fig. 6. Silencing of Cav-1 expression in Hca-F by RNAi approach. siRNA-mediated down-regulation of Cav-1 expression in Hca-F cell line. After Hca-F cells were transfected with Cav-1 siRNA and control siRNA for 30 h, Western blotting (A) and RT-PCR analysis (B) for Cav-1 and VEGFA were performed. GAPDH expression levels and gene levels were used as a control. Relative signal intensities of Cav-1 and VEGFA protein (C) and mRNA (D) levels were normalized against those of GAPDH, respectively.

suggested the potential involvement of VEGFA in the process of Cav-1 inducing lymphatic metastasis.

Partial loss of Cav-1 prevents Hca-F cells growth

To determine the influence of loss of Cav-1 on Hca-F cell growth, the MTS assay was used to measure cell growth of Hca-F cells transfected with the control siRNA or the targeted siRNA. Significant reductions in cell number were observed in Hca-F cells transfected with Cav-1-specific siRNA in 5 days experiments (Fig. 7) when compared to those untransfected, suggesting the Hca-F cells became less invasive with Cav-1 down-regulation. Meanwhile, growth of Hca-F cells transfected with control

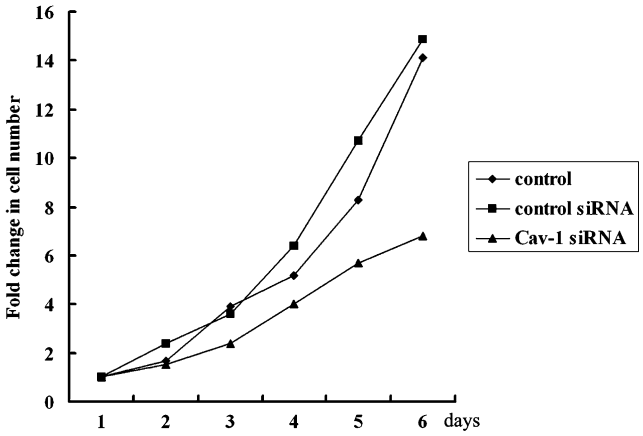


Fig. 7. siRNA-mediated down-regulation of Cav-1 expression prevented growth of the Hca-F cell line in vitro. Cells were harvested at 1, 2, 3, 4, and 5 days post-transfection and cell proliferation was measured by MTS assay, proliferation of transfected cells was expressed as a fold increase of proliferation of control cells. The data were obtained from three independent experiments.

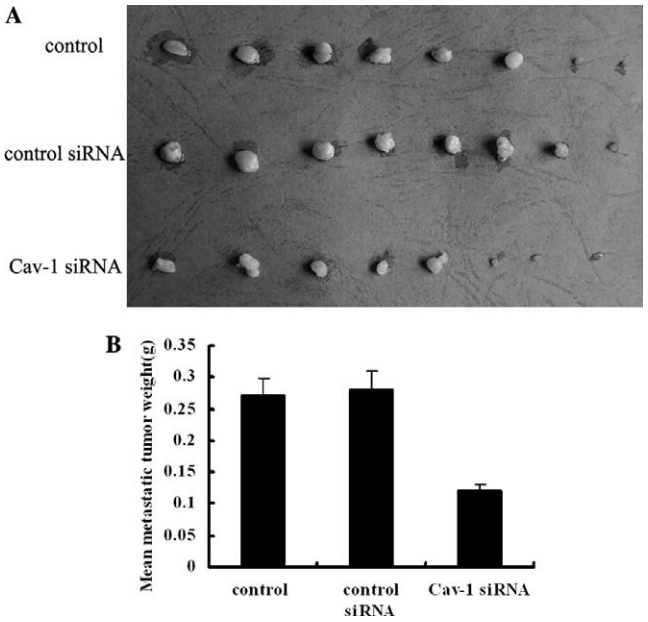


Fig. 8. Effects of Cav-1 silencing on in vivo lymphatic metastasis of Hca-F cells. (A) Three groups of 615 mice were injected subcutaneously with Hca-F, Hca-F/Cav-1 siRNA, and Hca-F/control siRNA cells. After 4 weeks, mice were sacrificed and their lymph nodes were isolated, weighed, and photographed. Standard histological analysis was used to determine the lymphatic metastasis rates. No significant reduction of lymphatic metastasis rate was seen after Cav-1 silencing. (B) Cav-1 siRNA group showed a significant decrease in mean metastatic tumor weight ($p < 0.05$), when compared to control and control siRNA group.

siRNA was not affected. Thus, forced low expression of Cav-1 did cause growth inhibition in Hca-F cells.

Cav-1 silencing delays the lymphatic metastasis of Hca-F cells

Next, we also examined the effect of Cav-1 down-regulation on lymph node metastasis of Hca-F cells in 615 mice. Interestingly, a significant decrease was seen in mean metastatic tumor burden after Cav-1 silencing though there is no significant reduction in lymphatic metastasis rate (Fig. 8A and B). This suggested that partial loss of Cav-1 was sufficient to attenuate the lymphatic metastasis of liver cancer and inhibit proliferation of tumor cells.

Discussion

A large amount of accumulated evidence reveals that Cav-1 expression is down-regulated in some forms of cancer, but up-regulated in many other cancer types. These data result in the implication that Cav-1 might act as both a tumor-suppressor gene and a metastasis-promoting gene. Concordant with this, we presented evidence that Cav-1 exhibited dual functions among series of mouse hepatocarcinoma cell lines including Hca-F, Hca-P, and Hepa1–6 with high, low, and no lymphatic metastasis potential. Using cDNA microarray approach we observed a 2.8-fold increase of *Cav-1* mRNA level in Hca-F cells compared to Hca-P cells. Similarly, Song et al. reported a 5.8-fold increase of *Cav-1* mRNA level in Hca-F cells versus Hca-P cells by gene chip assay [26]. We first demonstrate these results by RT-PCR and Western blot analysis, and that Cav-1 is expressed in normal mouse liver cell IAR-20 but absent in Hepa1–6 cells.

The molecular mechanism of controversial function of Cav-1 in tumors still remains a puzzle. One possibility is that tumor cells must constantly adapt to the changed environment. Cav-1 may exert its tumor-growth inhibition by contact inactivation of signaling molecules such as v-src, Ha-Ras, protein kinase A, PKC, and p42/44 MAP kinase within caveolae [27,28]. Down-regulation of Cav-1 could release and activate signaling molecules, promote cells transformation and proliferation during the first step of tumorigenesis. After that, tumor cells grow rapidly and might progress to an advanced metastasis. With the changed situation, Cav-1, as the center molecule of signal pathway, is over-expressed to meet the different requirements of tumor cells. Adjustment is necessary for cancer cells to survive in a new environment.

Some approaches have been taken to study the mechanisms of Cav-1 over-expression in various types of tumor cells with invasive ability. Ho et al. reported that up-regulated Cav-1 is necessary for mediating filopodia formation, which might enhance the invasive ability of lung adenocarcinoma cells [14]. Cav-1 can also be secreted. Cultivation of prostate cancer cells with conditioned medium from Cav-1-secreting cells increases their proliferation and protects against apoptosis [29]. More recently, it is shown that Cav-1 can suppress c-Myc-induced apoptosis in the development and progression of human prostate carcinoma, suggesting the potential for cooperation between c-Myc

and Cav-1 in malignant progression [30]. In many human tumor types, both increases of Cav-1 expression and caveolae number are involved in acquisition of multidrug resistance (MDR) and resistance to chemotherapy [31]. Therefore, Cav-1 expression has been associated with tumor-cell survival and metastatic potential.

Here, we first observed a reduced expression level of VEGFA, a putative lymphangiogenic factor, in response to down-regulation of Cav-1 by RNA interference in Hca-F. Lymphatic metastasis of cancers is one of the early steps of cancer aggravate [32]. Dissemination of cancer cells from the original locations to the lymphatic system occurs by invasion into lymphatic vessels [33]. VEGFA, a member of VEGF family, might function as joint switch for tumor lymphangiogenesis [21]. VEGFA also induces accumulation of inflammatory cells that might lead to lymphangiogenesis [34]. These studies suggest that VEGFA induce lymphatic metastasis. VEGF receptors have been found in caveolae subfractions and in complexes with Cav-1 [35,36], which show that there is relationship between VEGF family and Cav-1. In our study, down-regulation of Cav-1 in Hca-F directly reduced expression of VEGFA. An inhibition effect in Hca-F cell proliferation by Cav-1 RNA interference was also revealed by MTS assay. In vivo metastasis assay indicated that Cav-1 siRNA treatment decreased lymphatic metastasis tumor burden of Hca-F cells. These facts are consistent with the report by Williams et al. that inactivation of Cav-1 results in significant reductions in prostate tumor burden, as well as decreases in regional lymph node metastases in vivo [37]. Thus, as a metastasis-promoting gene in Hca-F, Cav-1 might promote tumor cell survival and be an intracellular target for lymphangiogenesis and lymphatic metastasis.

Cav-1 is proved to be an integral membrane protein. It is expressed mainly at the plasma membrane but also in the Golgi complex, the endoplasmic reticulum and at cytosolic locations [1]. In Hca-F cells, Cav-1 is recovered not only at a higher level from surface but also from intracellular fractions. In most cell types, Cav-2 is colocalized and coexpressed with Cav-1. Cav-3 is expressed mainly in muscle cells. Our results are consistent with those observations. Cav-2 is up-regulated in Hca-F and Hca-P cells but absent in Hepa1–6, just the same as Cav-1. No expression of Cav-3 has been detected in mouse hepatocarcinoma cells.

Cav-1 knock-out mice lacks visible caveolae on cells membranes [38], conversely, re-expression of Cav-1 in cells devoid of caveolae and caveolin expression led to the formation of caveolae [39], suggesting that Cav-1 plays an important role in caveolae biogenesis. We quantified caveolae number in our four kinds of cells and found significant differences. Caveolae were absent in Hepa1–6 and overexpressed in Hca-F and Hca-P. The presence of caveolae was fully correlated with the expression level of Cav-1 in cells. The result indicates that Cav-1 plays a central role in the formation of caveolae.

Taken together, our data suggest that Cav-1 has dual functions in mouse hepatocarcinoma cells with different invasive ability, both as a tumor suppressor protein and a lymphatic metastasis promoter protein. Furthermore, down-regulation of Cav-1 reduces lymphangiogenic factor VEGFA expression, which mechanism should be investigated deeply. These proofs will facilitate to understand the role of Cav-1 in lymphatic metastasis.

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