Knockdown of Caveolin-1 by siRNA Inhibits the Transformation of Mouse Hepatoma H22 Cells *In Vitro* and *In Vivo*

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Caveolin-1 (Cav-1) is a main structural protein of caveolae and plays important roles in signal transduction and tumorigenesis. We previously showed that Cav-1 was highly expressed in mouse hepatoma cell lines and positively correlated with cell invasion capability. Thus, interfering with the expression and activity of Cav-1 might be a potential way to intervene with hepatoma progression. We used RNA interference to study the biological effects of silencing Cav-1 expression in hepatoma H22 cells, to validate its potential as a therapeutic target. Using small-interfering RNAs (siRNAs) targeting the mRNA region of Cav-1, we effectively suppressed Cav-1 mRNA and protein levels. This resulted in the decreased transformation ability of H22 cells *in vitro* and *in vivo*. In addition, downregulation of Cav-1 expression promoted the apoptosis of H22 cells *in vitro* and *in vivo*. These results suggest that the use of siRNA could be an efficient molecular therapeutic method for hepatoma with high expression of Cav-1.

Introduction

HEPATOCELLULAR CARCINOMA IS ONE of the most common malignant tumors in some areas of the world. Although advances have been made in its detection and treatment such as surgery, radiation, and chemotherapy, the mortality rate and prognosis have not been improved yet (Sell and Leffert, 2008). Therefore, an understanding of the molecular mechanisms involved in its formation and progression should be helpful in developing more effective treatments for hepatoma.

Caveolin-1 (Cav-1) is a major structural component of flask-shaped plasma membrane invaginations called caveolae (Anderson et al., 2002). Cav-1 is expressed in terminally differentiated cell types including adipose, endothelial, epidermal, and epithelial cells (Engelman et al., 1998). Cav-1 acts as a driving force for caveolae formation and plays a key role in signal transduction by interacting with some signaling molecules such as G-protein, eNOs, and EFG-R (Okamoto et al., 1998; Drab et al., 2001; Liu et al., 2002). In addition, Cav-1 has also been implicated in vesicular trafficking, cholesterol homeostasis, and lipid transport (Fielding and Fielding, 2001; Fielding et al., 2002). In the past decade, the functions of Cav-1 aforementioned have been studied considerably.

Currently, the exact functions of Cav-1 in tumor have been controversial. Downregulation of Cav-1 has been found

in most (but not all) cancer cells, and silencing of Cav-1 expression was sufficient to induce oncogenic transformation in NIH 3T3 cells (Koleske et al., 1995; Galbiati et al., 1998; Bender et al., 2000; Razani et al., 2001; Wiechen et al., 2001; Heighway et al., 2002; Williams et al., 2004). Exogenous expression of Cav-1 in certain human or mouse tumor-derived cell lines inhibited cellular transformation and survival capability (Lee et al., 1998; Fiucci et al., 2002). These results indicate that Cav-1 may act as a tumor suppressor. However, there is accumulating evidence that Cav-1 does not function similarly in all types of cancer cells. For example, Cav-1 was highly expressed in prostate cancer cell lines and the expression of Cav-1 is positively correlated with the tumor cell grade and its lymphatic progression stage (Pflug et al., 1999). Inhibition of c-myc-induced apoptosis by Cav-1 was proposed to promote progression of prostate cancer, and it may serve as a prognostic indicator for prostate cancer. The same conclusions have also been documented in bladder and esophageal carcinomas (Rajjayabun et al., 2001; Kato et al., 2002). Therefore, these results suggest that Cav-1 also acts as a tumor promoter in certain tumor cell types.

In previous study, we reported that Cav-1 was highly expressed in mouse hepatoma cells, and positively correlated with their invasive ability (Jia et al., 2006). Here, to explore the roles of Cav-1 and the possible utility of Cav-1 downregulation for hepatoma therapy, we used RNA interference

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(RNAi) technology to knockdown the expression of Cav-1 in H22 hepatoma cells. In this study, we examined the phenotypic changes resulting from the reduction in Cav-1 expression, including the mRNA and protein levels of Cav-1, and the transformation and apoptotic potential of transfected H22 cells both *in vitro* and *in vivo*.

Materials and Methods

Cell culture

Mouse hepatoma cell line H22 was obtained from Cell Center of Peking Union Medical University, Beijing and maintained in 90% Rosewell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin under a humidified atmosphere of 95% air and 5% CO₂.

Transfection of siRNA

The oligonucleotide pairs of Cav-1 small-interfering RNA-1 (siRNA-1), siRNA-2, and siRNA-3 are complementary to exon 3 (GenBank accession number NM_007616), and the sequences of the sense strands of Cav-1 siRNA-1, siRNA-2, and siRNA-3 are 5'-CCAUCAUUUGGAGACUAUtt-3', 5'-CCACUCAGCAACUGAAUGAtt-3' and 5'-GUACCU GAGUCUCCAGAAAtt-3', respectively. To check for specificity, two negative control siRNAs were used: scramble-siRNA in the Cav-1-siRNA sequence, and an unrelated siRNA (UR-siRNA), and the sequences of their sense strands are 5'-ACGACTAGCCTGAACTCAA-3' and 5'-CGCACGTATATGACAATCG-3', respectively. H22 cells were incubated in a six-well tissue culture dish without antibiotics, and 24 hours before transfection resulting in a 60%–80% confluency. Control siRNAs and specific Cav-1-siRNAs were mixed with lipofectamine TM2000 (Invitrogen) according to manufacturer's recommendation and added to the cells, respectively. After 6 hours at 37°C, the medium was changed, and the cells were cultivated in RPMI 1640 supplemented with 10% heat-inactivated FBS for different time.

Semiquantitative RT-PCR analysis for Cav-1 expression

Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using reverse transcriptase polymerase chain reaction (RT-PCR) kit (TaKaRa, Kyoto, Japan) according to the manufacturer's instruction. The sequences of the primers were as follows: 5'-CTCGAGATGTCTGGGGGCAAATACG-3' (F) and 5'-GAATTCTATCTTTTCT-GCGTGCTG-3' (R) for Cav-1; 5'-GGCCGTGAAGTCGTCAGAAC-3' (F) and 5'-GCCACGATGCCAGGAA-3' (R) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. Polymerase chain reaction analysis was performed under the following conditions: denaturation at 95°C for 3 minutes, and then 30 cycles of denaturation for 20 seconds at 94°C, annealing for 30 seconds at 55°C, and extension for 30 seconds at 72°C. The amplified products were analyzed by agarose gel electrophoresis using 1.0% gel, followed by ethidium bromide staining. DL2000 Marker was served as molecular

weight standard. Band intensities were analyzed using Bioluminescence imaging systems (UVP, Labworks, ver 4.6).

Western blot analysis

The cell lysates were mixed with 2×sodium dodecyl sulfate (SDS) sample buffer (0.5M Tris-HCL pH 6.8, 10% SDS, 5% glycerol, and 5% β-mercaptoethanol). Equal amounts of denatured proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis and then blotted onto polyvinylidene fluoride membranes (Pall Corporation, East Hills, NY). After blocking for 2 hours with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% powdered skim milk, the blots were incubated with rabbit anti-mouse Cav-1 (Santa Cruz Biotech, 1:500 dilution) overnight in 5% powdered skim milk buffer, washed thrice with PBS with 0.1% Tween 20, and then incubated with secondary antibody anti-rabbit–horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:2,000 dilution). GAPDH antibody (Santa Cruz Biotech; 1:500 dilution) was used as a control. All bands were detected using ECL Western blot kit (Amersham Biosciences, UK). The relative amount of protein on the blots was determined by densitometry using LabWorks software (UVP, Upland, CA).

Immunocytochemistry

Cells were grown on sterile glass coverslips, washed three times in ice PBS, and fixed for 5 minutes at –20°C with acetone. After fixation, cells were permeabilized with 0.1% TritonX-100 for 5 minutes, and blocked with 10% goat serum in PBS, then the cells were incubated with rabbit anti-mouse Cav-1 (1:500 dilution) for 1 hour, washed several times with PBS, and incubated for 30 minutes with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC). After being washed, the coverslips were mounted onto slides for photography, set for appropriate fluorophore excitation emission viewing and storage.

Colony-formation assay in soft agar

Soft agar–formation assay was used to determine the effect of Cav-1 downregulation on the H22 cells transformation capability. In brief, a bottom layer (0.6% low-melt agarose) was prepared with Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. A top layer (0.3% agar) was prepared with DMEM and the same media as described above but containing 1000 indicated cells. Plates were incubated at 37°C in 5% CO₂ in a humidified incubator for ~2 weeks. The plates were then scanned and photographed, and the number of colonies was quantified by Quantity one v.4.0.3 software (Bio-Rad, Hercules, CA).

Apoptosis assay

Terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Germany) according to the manufacturer's protocol. Briefly, formaldehyde fixed cells were rinsed in PBS and immersed in equilibration buffer (5 minutes, 37°C). Then the slides were incubated with a mixture of TdT and fluorescein-dUTP

(1:9) for 60 minutes at 37°C. After being washed in PBS, the slides were incubated with FITC-conjugated anti-fluorescein antibody-AP for 30 minutes at 37°C. 4',6-Diamidino-2-phenylindole (DAPI) stain for counting of total cell number. Slides were examined and recorded in pictures by a bright-field light microscope. Green fluorescence staining represented positive reaction.

For detection of phosphatidylserine externalization, trypsinized cells were double stained with FITC-conjugated Annexin-V (25 µg/mL) and propidium iodide (PI) (50 µg/mL) apoptosis detection kit (Beckman Coulter). Cells (1×10^4) were collected on a FACScan flow cytometer equipped with a 488-nm argon laser and analyzed using the CellQuest software (Becton-Dickinson).

In vivo animal experiment

Six hundred and fifteen mice (H22 tumor host) were obtained from Animal Facility of Dalian Medical University. Inbred 615 mice, 20 g–23 g weight, were used for this study. For measurement of tumor growth *in vivo*, H22/Cav-1-siRNA and H22/control cells (2×10^7 cells/mouse) were injected subcutaneously in the right flanks of mice, 8 mice/cell. After 2 weeks, tumors were isolated from mice, weighed, fixed in formalin, and stained with hematoxylin and eosin. Immunohistochemistry (IHC) analysis was used to detect Cav-1 protein expression. Rabbit anti-mouse Cav-1 polyclonal antibody (1:200 dilution; Santa Cruz Biotech, Inc., Santa Cruz, CA) was used in standard indirect immunoperoxidase procedures, as reported previously (Zhang et al., 2003). The expression of Cav-1 protein were calculated and analyzed with the Image-Pro Plus 4.5 software (Media Cybernetics, Inc., Bethesda, MD).

Statistical analysis

SPSS12.0 software was used. Each assay was performed at least three times. The data were expressed as mean $\pm$ SD and Student's *t*-test was used to determine the significance of differences in multiple comparisons; $*p < 0.05$ was considered to be statistically significant.

Results

Silencing of Cav-1 expression by siRNA in hepatoma cells

To investigate the functions of Cav-1 in hepatic oncogenesis and the possible utility of Cav-1 downregulation for hepatoma therapy, our approach was to use siRNA oligonucleotides to deplete Cav-1 expression in mouse hepatoma cells, and to study the biological effects of this suppression. Three Cav-1-specific siRNAs (Cav-1-siRNA1, Cav-1-siRNA2, and Cav-1-siRNA3) and their mixture (Cav-1-siRNA mix) were transfected into H22 cells (a tumorigenic cell line that overexpresses Cav-1). To exclude the nonspecific effect of oligonucleotides, we used lipofectamine TM2000 (lipid), scrambled siRNA (scramble-siRNA) and UR-siRNA as negative controls.

After transfection for 72 hours, the efficacy in extinguishing Cav-1 expression was firstly evaluated by immunocytochemistry assay. As shown in Figure 1A, the treatment with Cav-1-siRNA mix including three Cav-1-siRNAs was much

more effective than the treatment with Cav-1-siRNA alone in terms of downregulating the Cav-1 fluorescence level. In contrast, treatment of H22 cells with lipid, scramble-siRNA and UR-siRNA did not downregulate Cav-1 fluorescence level. We therefore chose to use Cav-1-siRNA mix for all subsequent experiments.

Transfection with Cav-1-siRNA mix caused a dose-dependent decrease in Cav-1 mRNA and protein levels 72 hours after transfection, with greatest suppression at 100 nM concentration in H22 cells. Treatment with 100 nM Cav-1-siRNA mix decreased Cav-1 mRNA expression in H22 cells by >80% compared with the cells transfected with control siRNAs, while the treatment of 30 nM Cav-1-siRNA mix resulted in less reduction (Fig. 1B). Cav-1 protein levels were suppressed by up to 76% in H22 cells by 100 nM Cav-1-siRNA mix (Fig. 1C). Further observation showed that Cav-1 mRNA content did not recover to basic levels until 15 days after transfection (Fig. 1D).

Taken together, Cav-1-siRNA mix could effectively downregulate Cav-1 mRNA as well as protein levels and might be used to target Cav-1 for hepatoma therapy.

Cav-1-siRNA inhibits the clonogenicity of H22 cells in vitro

Colony-formation assay is considered as the most stringent assay *in vitro* for measuring malignant transformation and tumorigenesis ability. To determine the effect of Cav-1-siRNA mix on the clonogenicity of H22 cells, we performed colony-formation assay *in vitro*. Our results demonstrated that colony numbers of H22 cells treated with Cav-1-siRNA mix (60 nM, 5.4 ± 0.7 ; 100 nM, 3.2 ± 0.5) were markedly lower than the numbers of nontreated (11.2 ± 1.1) and lipid-treated cells (10.9 ± 0.9) ($*p < 0.05$, Fig. 2). In contrast, treatment of H22 cells with scramble-siRNA and UR-siRNA (100 nM) did not reduce colony-formation numbers. Thus, Cav-1-siRNA significantly inhibited *in vitro* transformation capability of H22 cells, and might be a potential method for gene therapy.

Cav-1-siRNA suppresses the tumorigenesis of H22 cells in vivo

In vitro transformation assay described above suggest that tumorigenicity *in vivo* may also be inhibited by Cav-1 knockdown. To examine this possibility, we performed *in vivo* tumorigenesis assay in the 615 mice. H22, H22/Cav-1-siRNA, and control cells were injected subcutaneously on either side of the mice back, respectively. After cells inoculation in mice for 14 days, a significant reduction of mean tumor weight ($n = 8$) of H22/Cav-1-siRNA groups was observed, when compared with control groups ($*p < 0.05$, Fig. 3A). Thus, downregulation of Cav-1 could attenuate the ability of H22 cells to form tumors *in vivo*.

To provide evidence that the inhibition of tumor growth by Cav-1-siRNA was due to its ability to downregulate Cav-1 *in vivo*, a separate study was accomplished to analyze Cav-1 expression levels in tumor tissues by IHC analysis. As shown in Figure 3C and D, Cav-1 protein expression levels in H22/Cav-1-siRNA cells were less than those in control cells. However, histological examination did not reveal obvious morphological changes between the tumors generated from Cav-1-siRNA, control-siRNAs and its parental H22 cells (Fig. 3B).

FIG. 1. Downregulation of Cav-1 in H22 cells by small-interfering RNA (siRNA). H22 cells were transfected with Cav-1-specific siRNAs and control siRNAs, as described under “Materials and Methods”. (A) Efficiency of transfection of the oligonucleotides was evaluated by immunocytochemistry assay ($\times 200$). (B) Cav-1 mRNA content was analyzed by RT-PCR assay 72 hours after transfection. GAPDH was used as internal control. (C) Cell extracts were assessed by Western blot with anti-Cav-1 and anti-GAPDH antibodies. Each lane contains an equal amount of total protein. Relative signal intensities of Cav-1 mRNA and protein levels as compared with GAPDH were analyzed by LabWorks (TM ver4.6, UVP, BioImaging systems), respectively. (D) Cav-1 expression did not recover to original levels until 15 days after Cav-1-siRNA mix transfection.

Cav-1-siRNA increases the apoptosis numbers of H22 cells

To examine the contribution of Cav-1 knockdown to apoptosis of H22 cells *in vitro*, the TUNEL assay was used to measure DNA strand breakage, an apoptosis hallmark, in H22/Cav-1-siRNA and control cells 72 hours after

transfection. Apoptotic cells nuclei were stained by green fluorescence and total cells nuclei were stained by DAPI (Fig. 4A). The numbers of apoptotic cells were significantly increased in the Cav-1-siRNA transfected cells as compared to the control transfectants (Fig. 4B).

To test whether the Cav-1 knockdown also induced cell death in H22 cells *in vivo*, Annexin-V and PI staining were

FIG. 2. Effect of Cav-1-siRNA on colony formation of H22 cells. Cells were seeded at 1000/well density in 6-cm plates. After 14 days, cell colonies were photographed. (A) Representative field photograph by colony-forming assay of H22, H22/controls, and H22/Cav-1-siRNA cells ($\times 100$). (B) Number of colonies were compared between the H22/Cav-1-siRNA and control cells. Each value is the mean $\pm$ standard deviation (SD) of 10 different fields (* $p < 0.05$). Data indicated that downregulation of Cav-1 in H22 cells suppressed the colony-formation capability *in vitro*.

used. Cav-1-siRNA increased the apoptotic cells (AV+PI- and AV+PI+) compared with control groups 14 days after injection into mice (Fig. 4C and D). As a result, this indicated that Cav-1-siRNA induced tumor regression might be associated with apoptosis *in vivo*.

Taken together, Cav-1-siRNA transfection could result in an increased apoptosis of H22 cells *in vitro* and *in vivo*, might therefore be a potential method for gene therapy.

Discussion

RNA interference is a universal conserved phenomenon of post-transcriptional gene silencing in which gene expression is suppressed by the introduction of homologous double-stranded RNAs. Synthetic siRNA can trigger an RNA interference response in mammalian cells and cause strong inhibition of specific gene expression. Therefore, it is used widely as a powerful approach to silence mammalian gene expression for gene function studies (Kim and Rossi, 2007; Rana, 2007; Rossi, 2008). In this study, siRNA-1, siRNA-2, and siRNA-3 oligonucleotides directed at exon 3 of Cav-1 mRNA were synthesized to determine whether Cav-1-siRNA suppresses Cav-1 expression. Mouse malignant hepatoma H22 cells, which expressed the highest Cav-1 levels in mouse hepatoma cell lines (data not shown), were transfected with Cav-1-siRNA1, Cav-1-siRNA2, Cav-1-siRNA3, and their mixture (Cav-1-siRNA mix), respectively. Our results showed that Cav-1-siRNA mix was more effective than Cav-1-siRNA alone in reducing the Cav-1 expression levels. As a result of this finding, a pool of three Cav-1-siRNAs was chosen to use for all subsequent *in vitro* and *in vivo* experiments.

To study whether Cav-1-siRNA could significantly inhibit malignant phenotypes of H22 cells, we first analyzed the effect of Cav-1 silencing on transformation ability of H22 cells *in vitro* and *in vivo*. Cav-1 was first identified as a tyrosine-phosphorylated target when cells were transformed by the Rous sarcoma virus, suggesting its possible role in cellular transformation (Koleske et al., 1995). In previous study, Cav-1 was always considered as a negative regulator of cell transformation and the homologous mechanism was also elucidated (Galbiati et al., 2001). Therefore, in our research, H22/Cav-1-siRNA and control cells were examined for anchorage-independent growth in a soft agar medium. Our findings showed that downregulation of Cav-1 inhibited cell soft-agar colony-formation capability. The capacity of cancer cells for anchorage-independent growth in soft agar is well correlated with their tumorigenic potential *in vivo* (Cox and Der, 1994). To further confirm the results *in vitro*, tumorigenesis assay *in vivo* was executed; the result showed that H22/Cav-1-siRNA cells became less tumorigenic as reflected by reduced tumor weight than the H22/control cells. Therefore, these results suggested that Cav-1-siRNA inhibited the transformation of H22 cells *in vitro* and might have a strong antitumor effect *in vivo*. However, further studies will be necessary to provide a complete mechanistic understanding of exactly how Cav-1 regulates hepatoma cell transformation.

Cells, including tumor cells, constantly face the selection of whether to proliferate and survive or to undergo apoptosis. Apoptosis plays an important role in the inhibition of oncogenesis, tumor development, and growth. Currently, the role of Cav-1 in cell apoptosis still remains controversial. Cav-1 plays opposite role in different type tumor cells

FIG. 3. Effect of Cav-1-siRNA on tumorigenesis of H22 cells *in vivo*. **(A)** The right flanks of 615 mice were subcutaneously injected with 2×10^7 cells in 0.1 mL of PBS of H22/Cav-1-siRNA and H22/control ($n = 8$). After 2 weeks, tumors were excised and weighed. Note that H22/Cav-1-siRNA cells formed smaller tumors than H22/control cells. **(B)** Tumors were paraffin-embedded, sectioned, and stained with hematoxylin and eosin ($\times 400$). **(C)** Cav-1 protein expression was determined by immunohistochemistry staining in solid tumors derived from H22, H22/control, and H22/Cav-1-siRNA cells ($\times 400$). Differences in Cav-1 protein expression were shown **(D)**, $*p < 0.05$. The data were obtained from three independent experiments. Results suggested that downregulation of Cav-1 in H22 cells attenuated tumorigenesis capability *in vivo*.

(Li et al., 2001; Liu et al., 2001; Li et al., 2003). To observe the effect of Cav-1 downregulation on apoptosis of H22 cells, TUNEL, and Annexin V-PI analysis were used. Our results showed that the apoptotic numbers and proportions of H22/Cav-1-siRNA cells were increased when compared with those of control cells. This phenomenon is consistent with the prostate cancer cells which are more sensitive to apoptosis after Cav-1 silencing (Nasu et al., 1998). To understand the mechanisms of apoptosis induced by Cav-1-siRNA, we examined the changes of Bcl-2 family protein levels during the Cav-1-siRNA-induced apoptosis in H22 cells by Western blot. We found that the transfection of Cav-1-siRNA resulted in the downregulation of bcl-2 (antiapoptotic), and no apparent changes were obtained for other proteins such as Bid and bcl-X1 (data not shown). These results indicated that Cav-1-

siRNA increased the apoptosis of H22 cells, which might be attributed to the reduction of bcl-2 expression.

Off-target effects can occur when a siRNA is processed by the RNA-Induced silencing complex and downregulates unintended targets. As these changes in gene expression might lead to measurable phenotypes (i.e., false positives), it is of great importance to identify RNAi triggers that work at very low effective concentrations (Persengiev et al., 2004; Fedorov et al., 2006). In recent years, the applications of siRNA pool, chemical modifications, and bioinformatics means are increasing to minimize off-target interactions and improve the quality of RNAi experiments. In this study, we used a pool of three Cav-1-siRNAs and found that Cav-1-siRNA mix at a concentration of 100 nM could suppress the Cav-1 mRNA and protein levels to the greatest extent in H22 cells.

FIG. 4. Silencing of Cav-1 increases the apoptosis level of H22 cells. (A) Representative images of TUNEL assays. H22/Cav-1-siRNA and control cells were assayed for their survival capability by TUNEL apoptosis detection kit. Cells were stained with DAPI for counting of total cell number. Apoptotic cells with DNA strand breaks were labeled with TUNEL. The results are representative of 30 different fields ($\times 200$). (B) Mean numbers of apoptosis cells per high-powered microscopic field ($*p < 0.05$). (C and D) Flow cytometry analysis with Annexin V-PI staining was performed to evaluate the percentage of Annexin V-PI positive cells. Apoptotic cells detected by Annexin V-PI staining assay were obviously increased after RNAi. Values were averaged from three independent experiments ($*p < 0.05$).

However, to diminish the off-target effects, we used Cav-1-siRNA mix at low concentrations for *in vitro* experiments. The results showed that Cav-1-siRNA mix at a concentration of 30 nM (10 nM each) could slightly change cell phenotypes, and Cav-1-siRNA mix (20 nM each) could effectively inhibit cells transformation and promote the apoptosis. Cell phenotypic changes were positively correlated with the degree of Cav-1 downregulation. Usually, it is possible to knockdown endogenously expressed targets with siRNAs at a concentration of 20nM or below. Thus, more efficient siRNAs should be found in future experiments. For *in vivo* experiments, we used Cav-1-siRNA mix at a higher concentration (33 nM each) because of its relatively permanent RNAi effects. Therefore, it is very critical to correlate the phenotypic outcome with the effectiveness of suppression in RNAi experiments.

The aforementioned findings showed that Cav-1-siRNA could effectively inhibit the expression of Cav-1 in H22 cells,

and attenuate the transformation capability of H22 cells *in vitro* and *in vivo*. In addition, Cav-1 knockdown promoted the apoptosis of H22 cells. Thus, Cav-1 gene can be regarded as a very good target gene in genetic therapy for hepatoma and the use of Cav-1-siRNA deserves further investigations as a novel approach to cancer therapy.

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