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Silencing of hdm2 oncogene by siRNA inhibits p53-dependent human breast cancer

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RNA interference technology is a powerful tool for silencing endogenous or exogenous genes in mammalian cells. Here our results showed that hdm2-siRNA silenced its target mRNA specifically and effectively in human breast cancer cells, reduced tumor cell proliferation and induced apoptotic cell death. Other molecular features modified by hdm2-siRNA included decreased Bcl-2, NF-κB, survivin, Ras and Raf levels, elevated p53, p21, BRCA1, Bax, and caspase levels as well as altered expression of other genes. hdm2-siRNA also caused cell cycle arrest at G1 phases with reduction in cyclin and Cdk proteins. In addition, hdm2-siRNA displayed *in vivo* antitumor activity and increased therapeutic effectiveness of mitomycin in MCF-7 xenografts. Thus, hdm2-siRNA may be a promising gene-specific drug for the treatment of human breast cancer and other tumors.
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RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing (PTGS) directed by short interfering 21–23 nt double-stranded RNA (siRNA).[1,2] siRNAs act as a guide to activate the RNA-induced silencing complex (RISC), which cleaves homologous mRNA molecules.[3,4] A number of studies have demonstrated that the introduction of siRNAs into mammalian and human cells causes specific and effective suppression of the corresponding mRNA molecules.[1–5] Several lines of results that siRNA can inhibit *in vivo* expression of endogenous genes provide further support for the notion that oncogene-specific siRNAs may be a new alternative to gene-specific therapeutics of human cancers.[5–8]

hdm2 (Mdm2 in mouse) contains an N-terminal p53-binding domain, a central acidic domain, a zinc-binding motif, and a C-terminal *RING* finger motif. *RING* finger motifs are frequently found in proteins with the property of a ubiquitin-protein ligase E3. Through the *RING* finger E3 ligase, hdm2 can selectively recognize and catalyze substrate proteins of the ubiquitin-dependent proteolytic pathway. For example, hdm2 mediates ubiquitination of certain transcription factors, including the tumor suppressor p53 and the histone acetyltransferase Tip60.[9,10] Recent evidence revealed that the HIV-1 transactivator Tat interplayed with hdm2 and that hdm2 acted as an E3 ligase for Tat ubiquitination both *in vitro* and *in vivo*.[11] Genetic analyses on Mdm2-deficient mice indicated that Mdm2 was required to keep the growth-suppressive properties of p53 under control.

The hdm2 oncogene plays a pivotal role in tumorigenesis and especially in regulation of apoptosis and cell cycle progression.[12] Tumor suppressor p53 is an attractive therapeutic target in oncology because the stimulation of p53 activity can suppress the growth of tumour cells. hdm2 can form an autoregulatory loop with p53 by binding to its N-terminal domain, blocking p53 transcriptional function and targeting p53 for ubiquitin-mediated degradation. HDM2 also induces p53 nuclear export through a mechanism involving either the nuclear export signal (NES) of HDM2 or the RING finger of HDM2 and the NES of p53.[10,12] Thus, the suppression of p53–HDM2 interaction by the downregulation of HDM2 can lead to p53-mediated cell cycle arrest or apoptosis.[6] An hdm2 antisense approach to reduce HDM2 has been successfully applied on several carcinomas *in vitro* and *in vivo*.[13,14] However, the effect of hdm2 reduction on many p53-dependent and apoptosis-related genes remains unclear, as does the mechanism of hdm2-siRNA-induced apoptosis in human cancers. In this study, we attempted to investigate whether hdm2-siRNA could induce anticancer activity, cell cycle arrest and apoptosis, and what is the mechanisms underlying these cellular events.

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Materials and methods

Preparation of siRNA

In total, 21-nt siRNAs were preferably synthesized by Dharmacon Research (Lafayette, CO) using 2′-ACE protection chemistry. The siRNA sequence targeting hdm2 (GenBank: Accession No. NM_002392) corresponded to the coding regions 59–80 after the start codon, and the mock siRNA sequence was 5′-AAUA GUGUAUACGGCAUGCdTdT-3′.

Cell culture and transfection

Human breast cancer MCF-7 cells and other cancer cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in DMEM (Life Technologies) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. At 24 hours before transfection at 50–80% confluency, MCF-7 cells were trypsinized and diluted to appropriate concentration with fresh medium without antibiotics. Cells were treated with siRNA buffer alone for control group. Transfections of hdm2-siRNA or mock siRNA were performed with Lipofectamine 2000 (Life Technologies, New England) according to the manufacturer's instructions.

MTT assay

A total of 8×10^3 cells were seeded in each well of 96-well plate and allowed to attach for 18 hours. Cells were then treated with various concentrations of hdm2-siRNA or mock siRNA for three times in 15 hours intervals. At 48 hours after the first transfection, cells were assayed by MTT.[15] The optical density is determined with a microculture plate reader (Bio-Rad 3550) at 595 nm. Absorbance values were normalized to the values obtained for the vehicle-treated cells to determine the percentage of survival.

Hematoxylin and eosin staining

Cells were seeded in 24-well plates with glass slides on the bottom of wells, and treated with 300 nM siRNA for 36 hours. After the slides were washed with cold PBS gently, cells were fixed by 95% ethanol for 15 minutes. The resulting cells were stained with hematoxylin and eosin in standard protocol.[16] The morphological changes of cells were examined with Leica Imaging System.

TUNEL assay

At 36 hours after transfection for once, cells were stained by the DeadEnd™ Colorimetric TUNEL System (Promega) following the manufacturer's instruction. To quantify apoptosis, five fields with ~200 cells per field in each slide were examined. The experiments were repeated at least four times with duplicate slides for each condition.

Western blotting

Cells were washed twice in cold PBS and then extracted in 100 μl of RIPA lysis buffer (50 mM Tris-HCl pH 7.5; 1% NP-40; 150 mM NaCl; 1 mg/ml aprotinin; 1 mg/ml leupeptin; 1 mM Na_3VO_4; 1 mM NaF) at 4°C for 30 minutes. Cell debris was removed by centrifugation at 14000 *g* for 20 minutes at 4°C. Protein concentrations were determined by Bradford assay as described in standard protocol.[17] An amount of 40 μg total protein from whole cell lysates was resolved on 10% SDS-polyacrylamide gel electrophoresis and transfered to PVDF membranes (Amersham). The membranes were blocked with 5% nonfat milk TBS buffer overnight at room temperature, and incubated for 1 hour with primary antibodies. The expression of PCNA was used as loading control. The antibodies used included hdm2, p21, cyclin A, cyclin D, p53, Cdk2, CDC2, Bax and NF-κB (Santa Cruz Biotechnology), Bcl-2, Caspase 3 and PCNA (Oncogene Research) at 1:200 dilution. The membranes were then incubated for 1 hour with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Jackson Inc.) at 1:3000 dilution. Electrochemiluminescence was performed according to the manufacturer's instructions with ChemiImager 5500 imaging system (Alpha Innotech Co.).

Detection of cytochrome c *release*

Separation of mitochondrial and cytosolic fractions was performed with ApoAlert Cell Fractionation Kit (Clontech) according to the manufacturer's specifications. An antibody to COX4 (included in the kit, 1:200) was used to confirm the successful separation of mitochondrial fractions.[18] Amounts of 40 μg of supernatant were used for detection of cytochrome *c* by Western bloting.

RT-PCR analysis

Total RNAs were prepared from culture cells (3×10^6) transfected with 100 nM hdm2-siRNA for 24 hours. RT-PCR was carried out using a SuperScript™ One-Step RT-PCR kit (Invitrogen, Catalogue No.: 10928-034) as previously described.[19] Oligonucleotide primers used are as follows: HDM2 Pu1, 5′-TGGTGAGGAGCAGG CAAAT-3′; HDM2 Pu2, 5′-TGGCACGCCAAACAA ATC-3′; β-actin P1, 5′-CCCAGGCACCAGGGCGT GATGGT-3′; β-actin P2, 5′-GGACTCCATGCCCAG GAA GGAA-3′. β-Actin mRNA was analyzed as internal control. A measure of 1 μg of total RNA was reverse transcribed to synthesize cDNA at 50°C for 30 minutes, then the cDNA was subjected to PCR amplification with specific primers (0.06 μg each) in 25 μl mixtures. PCR comprised 35 cycles with denaturing at 94°C for 15 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 40 seconds in each cycle using an MJ PCR System. The PCR products were then subjected to 2% agarose gel electrophoresis.

Flow cytometric analysis

At each time point, cells attached to culture dishes were trypsinized and washed in PBS, and then fixed with 70% ethanol for 24 hours at −20°C. The cells were washed once with PBS followed by incubation in PBS containing 25 μg/ml propidum iodide and 200 μg/ml RNase A for 1 hour at room temperature in the dark. Stained nuclei were then analyzed using a Becton Dickinson FACScan with 10,000 events per determination.

Gene chips

Analyses were performed on a BioStarH-I cDNA chip (Biostar Genechip, Inc., Shanghai, China), which contained around 1024 individual genes. Transfected cells were used for RNA extraction and RT preparation of fluorescent cDNA probes labeled with Cy3- or Cy5-deoxy CTP (Amersham Pharmacia Biotech), respectively. Quantified probes were applied onto prehybridized BioStarH-I chips under a cover glass. After hybridization, the chips were scanned with a ScanArray 4000 (GSI Lumonics) at two wavelengths (532 and 653 nm for Cy3 and Cy5, respectively) to detect emission from both Cy5 (siRNA) and Cy3 (Non siRNA). The resulting images were analyzed using GenePix PRO 3 software (Axon Instruments, Inc.). Overall intensities were normalized using the corresponding GenePix default normalization factor.[20] We applied a cutoff intensity ratio of siRNA: non-siRNA at 1.2 and 0.8 for up- and downregulated genes, respectively. The changes of p53 in Western blotting and microarrays were taken as the standard to determine if changes in other genes tested in chips were significant. Experiments were performed two times, and two data sets were maintained in a Microsoft Excel spreadsheet.

In vivo *tumor model*

Female Balb-c nude mice (6–8 weeks old, 22–24 g) were kept under specific pathogen-free conditions. Tumor was raised by subcutaneous injection of 1×10^7 MCF-7 cells in 100 μl culture medium without FBS (fetal bovine serum) into the right flank. When the tumor nodules (4×4 mm) were established, mice were randomly divided into five groups, eight mice were for each group. From the seventh day after implantation on, intratumoral injection of siRNA (0.5 mg/kg) complexed with 50 μl Lipofectamine 2000 and 30 μl Opti-MEM medium (Life Technologies) were applied every other day for eight times. Mitomycin was administered by i.p. injection at a dose of 0.5 mg/kg on days 7, 10, 13, and 16. In the combinatory therapy, hdm2-siRNA and mitomycin were given at the same doses and schedule as above. The control group received physiological saline only. Growth inhibition of transplanted tumors was evaluated by measuring the tumor size every 2 days with the aid of microcallipers. Tumor volume was calculated using the formula $ab^2/2$, where a is the length and b is the width of the tumor.

Statistics

Data were described as means ± sem of the indicated number of separate experiments. A one-way analysis of variance was performed for multiple comparisons. If there was significant variation between treatment and control groups, the mean values were compared by using Student's t-test. P-values less than .05 were considered significant.

Results

hdm2-siRNA upregulates expression of tumor suppressor genes

To determine whether and what amount of siRNA inhibits the growth of cancer cells, we evaluated the effect of siRNA duplexes directed against hdm2 mRNA molecule on three different cancers. We transfected siRNA duplexes into various human cancer cells using cationic liposome and then measured cell survival 48 hours after transfection by MTT assay. The results showed that transfected hdm2 siRNA markedly inhibited the growth of MCF-7 cells while mock siRNA had some toxicity to MCF-7 cells (Fig 1). Thus, to suppress the growth of MCF-7 cells effectively and decrease the toxicity of siRNAs, we used 300 nM of hdm2-siRNA for following experiments unless the special amount was indicated.

To ascertain whether the effect is p53 dependent, we employed three kinds of cancers with different p53 status. MCF-7 and BEL-7402 cancer cells are characterized by wild-type p53 while the HT-29 cells are in p53 mutant status. Figure 1 showed that tumors with a p53 mutation were resistant to hdm2-siRNA attack in contrast to

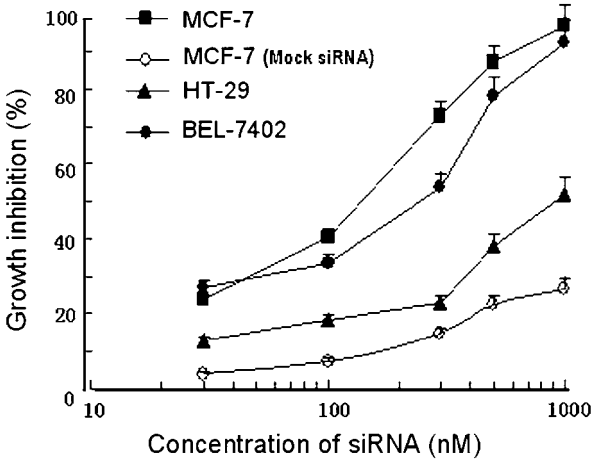

Figure 1 hdm2-siRNA inhibits different human cancer cells. BEL-7402 and HT-29 cells were transfected with hdm2-siRNA three times at the interval of 15 hours at different concentrations. MCF-7 cells were transfected with hdm2-siRNA and mock siRNA in the same manner as above, respectively. The inhibitory rate of cell growth was determined by MTT analysis. Mean ± SD, of triplicate wells for all data points, representative of three independent experiments are given.

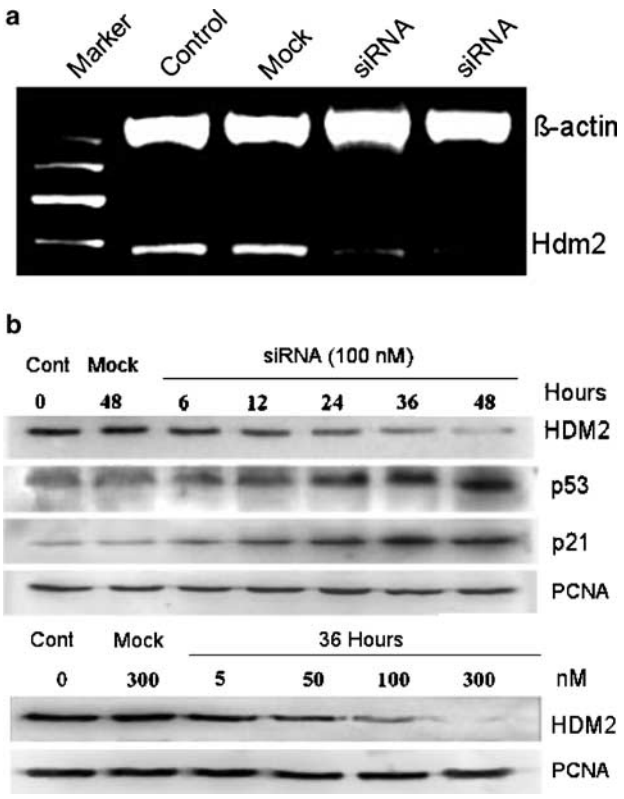

Figure 2 hdm2-siRNA reduces expression of *hdm2* gene in MCF-7 cells. (**a**) RT-PCR analysis of hdm2 mRNA levels in MCF-7 cells treated with hdm2-siRNA. β-Actin was employed as a loading control. Cycle numbers were optimized in several experiments with determination of linear phase PCR reaction. The results shown are representative of three independent experiments. (**b**) HDM2, p53 and p21 proteins were analyzed by Western blotting. PCNA protein was used as a loading control. Cells were treated with hdm2-siRNA for 6, 12, 24, 36, 48 hours, or transfected with different concentrations of hdm2-siRNA for 36 hours.

tumors with wild-type p53 that tended to undergo apoptosis readily.

To explore the mechanism of anticancer activity of hdm2-siRNA, MCF-7 cells were transfected by hdm2-siRNA. We assessed the silencing effect of hdm2-siRNA on its target mRNA by RT-PCR and Western blotting. RT-PCR data (Fig 2a) showed that hdm2 mRNA level was markedly downregulated in MCF-7 cells transfected with hdm2 siRNA for 24 hours, while mock siRNA was ineffective in hdm2 mRNA, confirming that HDM2 silencing occurred by degrading mRNA target. Western blot analysis (Fig 2b) indicated that the transfection of siRNAs directed against hdm2 mRNA resulted in a decrease in HDM2 levels in a dose-dependent manner, whereas p53 levels increased as the same mode. Densitometric analysis of the p53 Western blot showed that p53 levels were increased by 4–5-fold. To determine whether the inhibitory effect of siRNA on its cognate gene is time-dependent, we assayed its time course for the best effectiveness. The result indicated that 100 and 300 nM of siRNA significantly resulted in the degradation of its target mRNA 48 and 36 hours after transfection, respectively (Fig 2b). In addition, the suppression of hdm2 expression caused increased p21 levels in the same manner as p53 did (Fig 2b). This suggested that hdm2-siRNA could mediate p53-induced cell cycle arrest through the upregulation of p21, an inhibitor of cyclin-dependent kinases.[21]

hdm2-siRNA induces apoptosis of MCF-7 cancer cells

We next investigated whether hdm2-siRNA induced apoptosis in human breast cancer MCF-7 cells. At 36 hours after hdm2-siRNA treatment, the growth inhibition and chromatin condensation were observed (Fig 3a) in MCF-7 cells by staining with hematoxylin and eosin,[22] indicating that apoptotic cell death was induced by siRNA. Moreover, the terminal deoxytransferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay was used to monitor the extent of DNA fragmentation due to apoptosis (Fig 3a). At 36 hours of culture with hdm2-siRNA, the percentage of apoptotic cells was 0, 7 and 37.5% for control, mock and experimental MCF-7 cells, respectively, with $>90\%$ cell death at Day 3 (data not shown).

Cytochrome *c* release, a hallmark of outer membrane damage in mitochondria, is a critical step of the apoptotic process. It occurs upstream of caspase activation, and is inhibited by bcl-2 overexpression and promoted by bax activation. To illuminate the mechanism of apoptosis mediated by hdm2-siRNA, we examined the release of cytochrome *c* from the mitochondria after siRNA treatment. Results showed that without hdm2-siRNA treatment, cytochrome *c* was detected exclusively in the mitochondrial fraction, while the cytochrome *c* was released from the mitochondria into the cytosol 36 hours after transfection with hdm2-siRNA (Fig 3b), suggesting that the MCF-7 cells undergo apoptosis mediated by mitochondria pathway.

The apoptotic machinery in human cells is composed of a molecular network of many proteins. These proteins manipulate a molecular flow from signaling, commitment to execution stages of apoptosis through multiple parallel pathways. Bcl-2, Bak, Bax, and p53 are regulators of apoptosis.[23–25] Caspase family members can promote apoptosis in most cell lines. However, caspase 3 was not expressed in MCF-7 cells (Fig 3c), and the expression of Bcl-2 was stimulated by nuclear factor NF-κB. To understand the molecular mechanisms of apoptosis evoked by hdm2-siRNA, we examined the expression profiles of related genes such as Bcl-2, Bak, Bax and p53 following siRNA treatment. Westernblot analysis (Fig 3d) revealed that Bax protein level was increased while Bcl-2 and NF-κB protein levels were decreased 36 hours after transfection. In contrast, there was no difference in Bak protein level in apoptotic MCF-7 cells. At the same case, the p53 and p21 protein levels were increased remarkably after treatment with hdm2-siRNA (Fig 2b). Moreover, cDNA microarray measurement of other related gene expression (Table 1) indicated that caspases 6 and 9 and caspase-activated DNase remarkably increased,

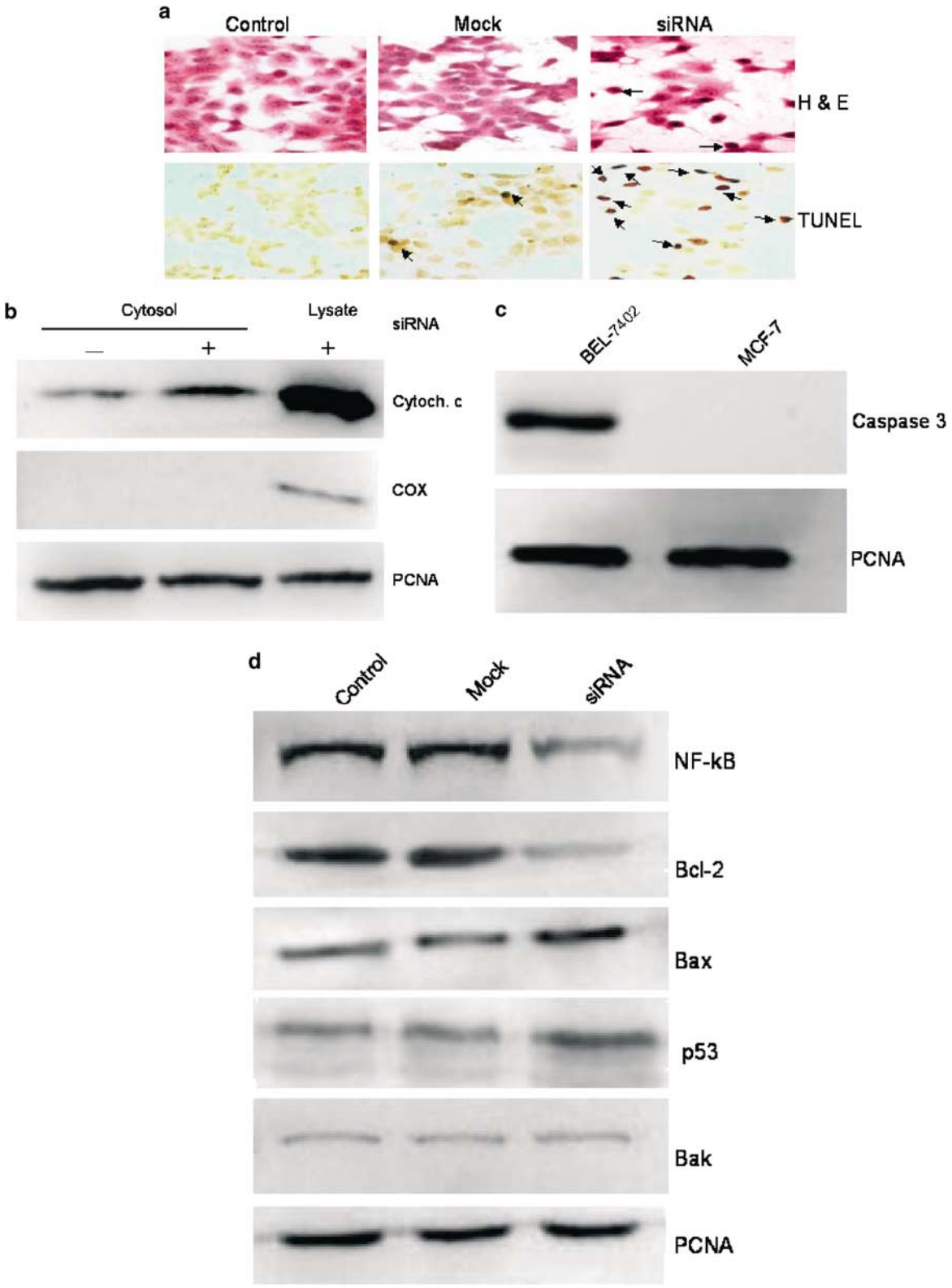

Figure 3 hdm2-siRNA induces growth inhibition, chromatin condensation and fragmentation in MCF-7 cells. (**a**) Cells were stained by hematoxylin and eosin and TUNEL. The arrows indicated apoptotic cells. The magnification is $\times$ 400. (**b**) hdm2-siRNA induces cytochrome *c* release in treated MCF-7 cells. The cytochrome *c* (Cytoch. c) levels were analyzed by Western blotting. Proliferating cell nuclear antigen (PCNA) was used as a loading control. COX is the abbreviation of cytochrome *c* oxidase subunit IV. (**c**) Caspase 3 protein was expressed in BEL-7402, compared with the absence in MCF-7 cells. (**d**) Effects of hdm2-siRNA treatment on Bcl2, Bax, Bak, p53 and NF-κB protein levels. Cells were transfected with 100 nM siRNA for 36 hours. The protein levels were detected by Western blotting.

Table 1 Expression profiles of some interested genes in MCF-7 cells treated with hdm2-siRNA

Genbank_ID	Definition of mRNA (*Homo sapiens*)	Ratio
Apoptosis-related		
NM_003789	TNFRSF1A-associated via death domain (TRADD)	2.144
NM_003844	Tumor necrosis factor receptor superfamily, member 10a (TNFRSF10A)	1.344
NM_003810	Tumor necrosis factor (ligand) superfamily, member 10 (TNFS10)	1.557
NM_001066	Tumor necrosis factor receptor superfamily, member 1B (TNFRSF1B)	1.315
NM_001229	Caspase 9, apoptosis-related cysteine protease (CASP9)	1.467
NM_004402	DNA fragmentation factor, beta polupeptide (caspase-activated Dnase)	1.368
NM_001226	Caspase 6, apoptosis-related cysteine protease (CASP6)	1.375
Cell growth signal-related		
NM_002880	v-raf-1 murine leukemia viral oncogene homolog 1(RAF1)	0.781
NM_006609	Mitogen-activated protein kinase kinase kinase 2 (MAP3K2)	0.740
NM_004972	Janus kinase 2 (a protein tyrosine kinase) (JAK2)	0.801
Matrix metalloproteinases		
NM_032950	Matrix metalloproteinase 28 (MMP28)	0.671
NM_005941	Matrix metalloproteinase 16 (MMP16)	0.750
ATPase		
NM_005765	ATPase, H^+ transporting, lysosomal (vacuolar proton pump) membrane sector associated protein M8-9 (APT6M8-9)	0.629
NM_002805	Proteasome 26S submit, ATPase, 5 (PSMC5)	0.793
Others		
NM_007295	Breast cancer 1, early onset (BRCA1)	1.634
NM_001168	Baculoviral IAP repeat-containing 5 (surviving)	0.753
NM_000546	Tumor protein p53 (Li–Fraumeni syndrome) (TP53)	1.519

Values in the ratio column are the mean of two independent experiments.

demonstrating that the apoptosis of MCF-7 cells evoked by hdm2-siRNA might go through p53-dependent pathway.

hdm2-siRNA alters cell cycle progression and gene expression

To investigate whether hdm2-siRNA regulated cellular proliferation and cell cycle progression, we analyzed the alteration of cell cycle distribution and cell cycle-related protein levels in the transfected cells. Flow cytometry results revealed that hdm2-siRNA induced cell arrest at G1 phase in a time-dependent manner (Fig 4a), and that the proportion of G1 phase was elevated from 49.8 to 83.2% compared with control, while mock siRNA was ineffective in cell cycle distribution. Furthermore, the results showed that apoptotic cell death was partially induced 36 hours after treatment. At the same condition, Western blotting analyses showed decreased cyclin D3, cyclin A, CDC2 and Cdk2 protein levels, coincided with G1 phase arrest (Fig 4b). On the other hand, decreased Cdk2 activity and declined Survivin expression (Table 1) accompanied by elevated p21 level (Fig 2b) would favor S phase arrest. These results suggested that hdm2-siRNA induced cell cycle arrest in a binary way.

To identify genes generally involved in carcinogenesis and apoptosis initiated by silencing hdm2, we compared expression profiles between MCF-7 cells with and without hdm2-siRNA treatment by means of cDNA microarray. We found that hdm2-siRNA induced diverse expression of many genes relating to proliferation, apoptosis, and metastasis of cancer cells (Table 1). Based on the changes of p53 in Western blotting and microarray, relative differences in expression of other genes could be evaluated. Among these genes, eukaryotic translation elongation factor 2 was upregulated the most dramatically, while glutaredoxin was mostly downregulated in MCF-7 cells 24 hours after treatment with hdm2-siRNA. Intriguingly, expression of several genes associated with cell death, including p53, BRAC1, TRADD, TNFR, TNF and some CASP members were commonly enhanced after transfection. As modulators of cell proliferation, raf, ras, map2, jak2, survivin, and proteasome were reduced following the introduction of hdm2-siRNA into MCF-7 cells. In addition, some altered genes were involved in transcription, signal transduction pathways and other functions, implying that hdm2-siRNA could suppress cancer growth by fine-tuning up a signal network for cell proliferation and apoptosis.

hdm2-siRNA displays in vivo *antitumor activity*

To investigate whether hdm2-siRNA suppressed tumor proliferation *in vivo*, we evaluated antitumor activity of hdm2-siRNA in a human breast cancer MCF-7 model. The inhibition of tumor growth by hdm2-siRNA was

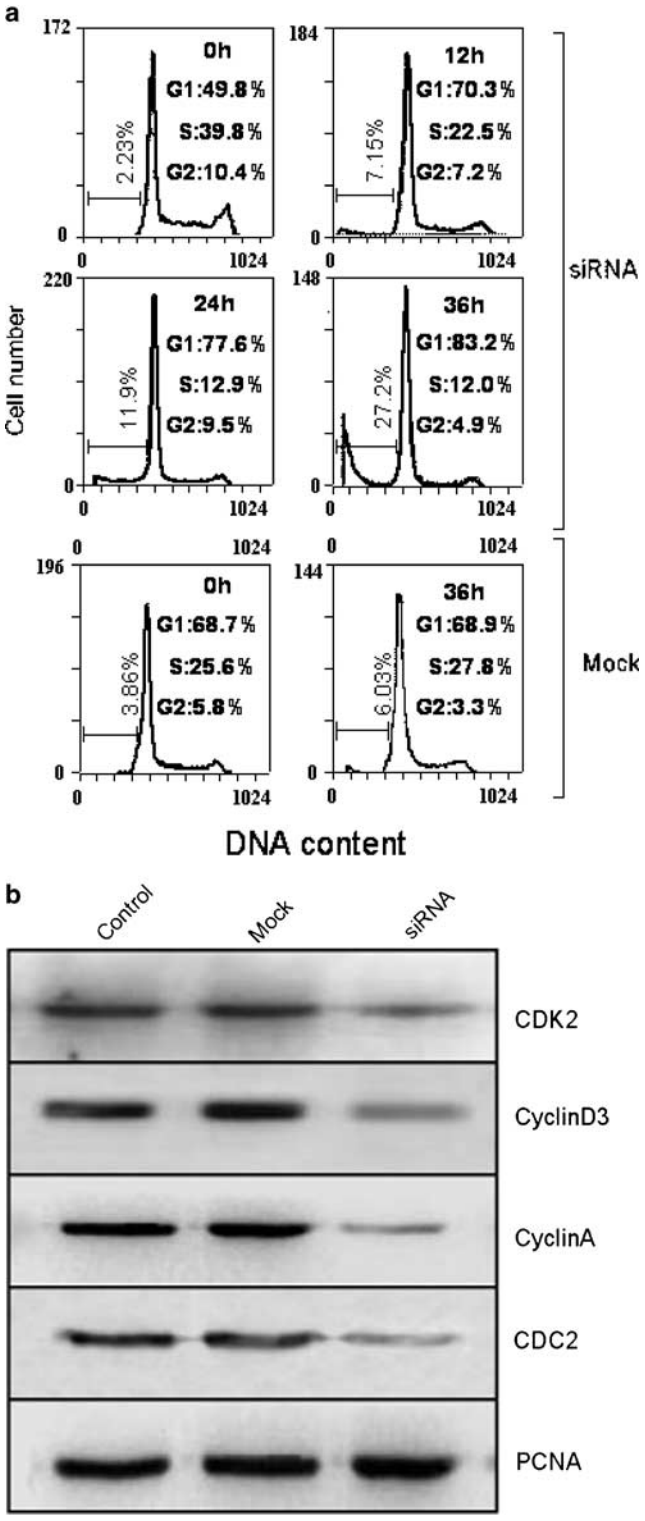

Figure 4 Cell cycle arrest and apoptosis induced by hdm2-siRNA in MCF-7 cells. (**a**) Cells were treated with hdm2-siRNA and mock siRNA for different times, then the cells were harvested and analysed by flow cytometry. Cell cycle distributions displayed here were representative of three experiments. (**b**) Effects of hdm2-siRNA on cyclin D3, cyclin A, CDC2 and Cdk2 protein levels. Cells were transfected with 300 nM siRNA for 36 hours, then proteins levels were detected by Western blotting. The expression of PCNA was used as a loading control.

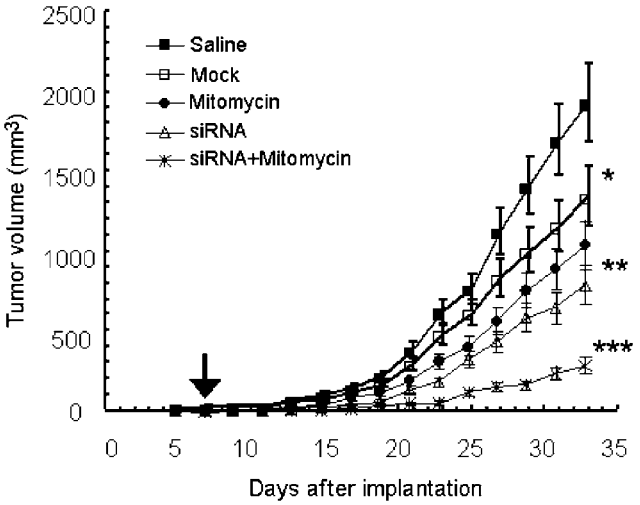

Figure 5 *In vivo* antitumor activity of hdm2-siRNA in nude mice bearing MCF-7 human breast cancer xenografts. At 7 days after implantation shown by arrow, intratumoral injection of siRNA (0.5 mg/kg) complexed with 50 μl Lipofectamine 2000 will be delivered every other day for eight times. Each point represents the mean $\pm$ SE of tumor mass from seven mice. *$P<.103$ (mock siRNA), **$P<.05$ (mitomycin or hdm2-siRNA), and ***$P<.01$ (mitomycin and hdm2-siRNA), compared with the saline control group.

observed with administration of intratumoral injection at a dose of 0.5 mg/kg hdm2-siRNA every other day for eight times. On day 33, average body weights of mice with and without treatment of hdm2-siRNA were almost the same, accompanied by no any died mice in the groups exposed to hdm2-siRNA. The tumors in the control group averaged 1926 mm,^{3} compared with 1344.8 mm^3 in the mock siRNA group, 809 mm^3 in the hdm2-siRNA group and 303 mm^3 in the group treated with hdm2-siRNA and mitomycin. These results demonstrated that hdm2-siRNA had significantly inhibitory effect on tumor growth without apparent toxicity to mice (Fig 5).

Importantly, the data showed significant synergic effect played by hdm2-siRNA and mitomycin (Fig 5). On day 23, the tumor size treated with the combination of hdm2-siRNA and mitomycin was 11.2% of that in the control, whereas the tumor sizes treated with hdm2-siRNA or mitomycin alone were 32.2 and 53.2%, respectively. The effect of combinatory therapy on tumor growth was remained after cessation of administration. The transfection reagent of Lipofectamine 2000 had slightly inhibitory effect on tumor growth (data not shown). These data suggest that hdm2 siRNA might be a new and effective antitumor agent for the treatment of human breast cancer.

Discussion

In this report, we investigated the effect of p53 pathway activation by blocking the p53-hdm2 interaction through the specific degradation of hdm2 mRNA molecules in a dose- and time-dependent manner. The silencing of hdm2

mRNA resulted directly in enhanced MCF-7 cell apoptosis and decreased cell proliferation. In addition, hdm2-siRNA transfection not only specifically reduced target gene expression, but also induced changes in expression of many other genes. These results provide clearcut evidence that siRNA technology can be an effective method to inhibit oncogene expression and activate apoptotic and tumor suppressor genes.

MCF-7, BEL-7402 and HT-29 cancer cells had different sensitivities to hdm2-siRNA, regardless of p53 status. What are the mechanisms underlying different sensitivities? It may be different transfection rate, diverse amounts of RISC and miscellaneous anti-siRNA components in different cancer cells.[26] Of these three kinds of cancer cells, MCF-7 cells are the most sensitive to siRNA. It is the reason why we took MCF-7 cells for our experiments. Our data indicated that hdm2-siRNA induced the increase of p53, p21 and BRCA-1 levels in MCF-7 cells. This effect may be explained by the fact that accumulated p53 protein stimulates large expression of the p21 and regulates Brca1 transcription.[27–30] These findings provide a molecular mechanism for hdm2-siRNA-based therapy for breast cancer and other cancers.

Our present study demonstrated that MCF-7 cells underwent apoptosis after treatment with hdm2-siRNA. Transfected MCF-7 cells exhibited typical apoptotic characteristics indicated in our results. Changes in the Bcl-2/Bax ratio after transfection may facilitate apoptotic process,[23,24,31] in which the mitochondrial membrane PT pore opens and cytochrome *c* is released. Despite the absence of caspase 3, MCF-7 cells still underwent apoptosis following hdm2-siRNA treatment. It is possible that cytochrome *c* activates caspase 9 that in turn induces sequential activation of caspase 7 and caspase 6.[25,32] Moreover, hdm2-siRNA also induced cell growth arrest at G1 phase with upregulation of p21. On the other hand, decreased cyclinD3 protein level and CyclinD3–CDK complex activity may play a critical role in G1 arrest induced by hdm2-siRNA according to the results of flow cytometry.[33]

It has been shown that tumor necrosis factor alpha (TNF-α) can induce apoptotic plasma membrane disruption. TNF-α-induced necrosis and apoptosis share some common signaling events downstreaming the TNF-α receptor such as the recruitment of TRADD, FADD, the cytosolic domains of TNF receptors, and other cytosolic effector proteins.[34] Our data shown in Table 1 demonstrated that hdm2-siRNA could elevate the expression of TNF-α, TNF receptor and TRADD, suggesting that death factor pathway may play some roles in MCF-7 cell death mediated by hdm2-siRNA. This notion was further supported by our microarray analysis that hdm2-siRNA could result in reduction in lysosome-related ATPase.[35] Subsequently, lysosome rupture could trigger the mitochondrial apoptotic pathway by releasing proteases that activate Bid.[36] In addition, hdm2-siRNA might block cancer metastasis through the downregulation of matrix metalloproteinase expression (Table 1).[37]

The comparison of relative efficacy of siRNA with antisense oligonucleotides (AO)[13,14] to inhibit tumor growth demonstrated that hdm2-siRNA suppressed tumor growth more efficiently than AO did. More importantly, we found that hdm2-siRNA could not only have better effects in suppressing the MCF-7 growth *in vivo*, but also synergize with mitomycin in effectively killing breast cancer cells, suggesting that hdm2-siRNA could significantly augment the sensitivity of caspase-3-deficient MCF-7 cells to undergo apoptosis in response to mitomycin. It can be predicted that cancer may be overcome when siRNA can be effectively delivered into each cancer cell. This study is the first report that siRNA technology can be taken as a new strategic approach for the treatment of human breast cancer. The stable expression of siRNA-vector[5,38] or chemically modified siRNA molecule may create a more promising and efficacious siRNA-based gene drugs against tumors,[8] viruses[39] and other diseases.

Acknowledgments

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