Synergistic Suppression of Prostatic Cancer Cells by Coexpression of Both Murine Double Minute 2 Small Interfering RNA and Wild-Type p53 Gene In Vitro and In Vivo

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ABSTRACT

Our objective was to evaluate cell growth and death effects by inhibiting Murine Double Minute 2 (MDM2) expression in human prostate cancer cells overexpressing the wild-type (WT) p53 gene. Prostate PC-3 tumor cells were transfected with a plasmid containing either mdm2 small interfering (Si-mdm2) or the WT p53 gene (Pp53) alone, or both (Pmp53), using Lipofectamine in vitro and attenuated Salmonella enterica serovar Typhi vaccine strain Ty21a (Salmonella Typhi Ty21a) in vivo. Cell growth, apoptosis, and the expression of related genes and proteins were examined in vitro and in vivo by flow cytometry and Western blot assays. We demonstrated that human prostate tumors had increased expression of MDM2 and mutant p53 proteins. Transfection of the PC-3 cells with the Pmp53 plasmid in vitro offered significant inhibition of cell growth and an increase in apoptotic cell death compared with that of the Si-mdm2 or Pp53 group. These effects were associated with up-regulation of p21 and down-regulation of hypoxia-inducible factor 1α expression in Pmp53-transfected cells. To validate the in vitro findings, the nude mice implanted with PC-3 cells were treated with attenuated Salmonella Typhi Ty21a carrying the plasmids, which showed that the Pmp53 plasmid significantly inhibited the tumor growth rate in vivo compared with that of the Si-mdm2 or Pp53 plasmid alone. Tumor tissues from mice treated with the Pmp53 plasmid showed increased expression of p21 and decreased expression of hypoxia-inducible factor 1α proteins, with an increased apoptotic effect. These results suggest that knockdown of mdm2 expression by its specific small interfering RNA with overexpression of the WT p53 gene offers synergistic inhibition of prostate cancer cell growth in vitro and in vivo.

Introduction

Prostate cancer is the most common cancer in men (Kamangar et al., 2006), and an efficient therapy for this cancer remains an urgent need. Cell growth and death are determined usually by a balance between oncogenes and tumor-suppressor genes. p53 plays an essential regulatory role in the development and homeostasis of cells and tissues (Vosden and Lane, 2007). p53 inactivation caused by its negative regulators, such as Murine Double Minute 2 (MDM2), contributes to the development of a large number of human cancers. MDM2 is an E3 ubiquitin ligase that directly binds to p53 for its ubiquitylation (Tao and Levine, 1999). Tumors with overexpression of MDM2 were resistant to p53 gene

ABBREVIATIONS: MDM2, Murine Double Minute 2; WT, wild-type; MT, mutated; PI, propidium iodide; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA; PCNA, proliferating cell nuclear antigen; pRb, hypophosphorylated retinoblastoma; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; CFU, colony-forming unit; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; CDK, cyclin-dependent kinase; HIF-1α, hypoxia-inducible factor 1α; FCM, flow cytometry; Si-mdm2, plasmid containing mdm2 siRNA alone; Pp53, plasmid containing wild-type p53 gene alone; Pmp53, plasmid containing mdm2 siRNA and the wild-type p53 gene; Si-scramble, plasmid containing the scrambled small interfering RNA.
therapy (Wiman, 2006). Reportedly, prostate tumors with MDM2 knockdown were sensitive to radiation therapy both in vitro and in vivo (Mu et al., 2004a,b; 2008; Stoyanova et al., 2007). Therefore, an antitumor strategy interfering with the p53–MDM2 feedback loop holds promise to reactivate the p53 tumor-suppressing pathway (Wade and Wahl, 2009).

Several agents, such as Nutlin-3a, which disrupts the p53–MDM2 interaction, inhibit the growth of certain tumors but are less effective in other tumors (Patton et al., 2006; Wade et al., 2006). In addition, these agents also lack selectivity for tumor cells. Therefore, gene therapy selectively targeting tumor cells with little effect to normal tissue is an interesting and urgent tool to improve therapeutic efficacy (Lee et al., 2004). For instance, gastric cancer cell lines carrying the wild-type (WT) p53 gene were infected with adenovirus expressing RPL23 to stabilize WT p53 by inhibiting MDM2-mediated p53 degradation. The adenovirus delivery of RPL23 significantly inhibited proliferation of gastric cancer cells with WT p53 (Zhang et al., 2010).

In the above study, the successful restoration of p53 function needs the presence of endogenous WT p53 in the tumors. However, inactivation of p53 activity often occurs in the tumors due to its deletions, mutations, protein destruction, or protein sequestration (Bernal et al., 2010). Thus, reactivation of WT p53 function in tumors with endogenous mutated (MT) endogenous p53 is especially daunting (Bernal et al., 2010). A few studies have explored the forced expression of the WT p53 gene with MDM2 negative regulator genes, such as FHIT (Nishizaki et al., 2004) or FUS1 (Deng et al., 2007), to negatively inhibit MDM2, which in turn up-regulates exogenous WT p53 function in cancer cells. However, the expression of a single MDM2-negative regulator, either FHIT or FUS1, to down-regulate MDM2’s p53 suppression may not be ideal because which of these MDM2-negative regulators is the most effective in suppressing MDM2 function and whether these MDM2-negative regulators have other functions except for suppressing MDM2 function remain uncertain. Therefore, directly down-regulating MDM2 by a specific small interfering RNA (siRNA) in tumor cells with forced expression of exogenous WT p53 may be a better approach.

In addition, the transfection approaches (adenovirus and nanoparticle) used in the above studies (Nishizaki et al., 2004; Deng et al., 2007) are also not tumor cell selective. Reportedly, Salmonella would infect and preferentially accumulate within the tumor xenograft in vivo, with a tumor/normal tissue ratio of approximately 1000:1 (Pawelek et al., 2004). Attenuated Salmonella enterica serovar Typhi vaccine strain Ty21a (Salmonella Typhi Ty21a) is a facultative an aerobic bacterium and capable of replicating preferentially in tumor cells and has been explored for developing proteins therapeutic to tumors (Cryz et al., 1993; Kimura et al., 2010).

In the present study, therefore, we have made plasmids containing either mdm2 siRNA or human WT p53 alone, or both. The effects on tumor cell growth and apoptotic death by transfection with Pmp53 in prostate cancer cells in vitro and in vivo were compared with those by transfection with either mdm2-siRNA or WT p53 alone. The unique features of the present study are as follows: 1) we have cotransfected, for the first time, mdm2 siRNA to directly down-regulate MDM2 expression with the human WT p53 gene in prostate cancer cells in vitro and in vivo; 2) for the in vivo study, we have used, for the first time, Salmonella Typhi Ty21a to carry various plasmds predominantly transfected into prostate tumor xenografts in a nude tumor-bearing mouse model. This application of the Salmonella Typhi Ty21a bacteria carrying a plasmid containing both mdm2 siRNA and WT p53 will ensure that the transfected cells express WT p53 with efficient inactivation of the expression of its negative regulator MDM2 in tumor cells with or without the presence of WT p53.

**Materials and Methods**

**Construction of a Plasmid Vector Capable of Coexpressing mdm2 siRNA and WT p53.** By a method used in our previous studies (Zhang et al., 2007), we constructed a series of expression plasmids that contain siRNA specific to mdm2 and/or the WT p53 expression element. The pcDNA3.1-p53 plasmid containing the WT p53 coding region (Pp53) was generated as described previously (Shao et al., 2010). A siRNA with the sequence of 5'-GACACTTATACTTGAAG-3' (Genbank accession number NM002392) was selected to specifically target the fragment corresponding to nucleotides 152 to 171 of the MDM2 mRNA. The siRNA construct contains a sense strand of 19 nucleotides followed by a short spacer (loop sequence 5'-TTCAAGAGA-3'), an antisense strand, and a terminator containing five Ts. A scrambled siRNA was used as a negative control. Double-stranded DNA oligonucleotides were cloned into pcGsiencerU6Neo/GFP, which also expresses a green fluorescent protein gene (Jikai Chemical, Zhejiang, China), to generate plasmids Si-mdm2 and Si-scramble. A 585-base pair fragment, containing Si-mdm2 under the control of the U6 promoter, was amplified by polymerase chain reaction (PCR) from the plasmid Si-mdm2 using the following primers: forward, 5'-CTGGCGAAGGCGCACTTGAGACT- AATGACCC-3'%; reverse, 5'-GGAGATCACTTCTCGCCATCTGCAC- QGCC-3'. The underlined nucleotides in these primers correspond to the BglII and Nrul restriction sites, respectively. This fragment was subcloned into the BglII and Nrul sites of the Pmp53 vector to generate the plasmid pcDNA3.1-p53/U6 Si-mdm2, which can express both mdm2 siRNA and p53 protein from the same plasmid and was designated as Pmp53 (for short). All of the recombinant plasmids were verified by sequencing (Shenggong Bioengineering, Shanghai, China).

**Human Prostate Tissues.** We obtained 10 samples of normal prostate tissues from nontumor areas of prostatectomy specimens and 19 samples of cancer tissues from radical prostatectomy specimens of patients with organ-confined tumors without previous therapy. Both prostate normal and tumor tissues (Glascow score 9) were collected from the Inner Mongolia Forestry General Hospital (Yakeshi, China) and Prostate Disease Prevention and Treatment Research Center (Changchun, China), with the informed consent of the patients under an institutionally approved protocol.

**Cell Culture and Transfection.** PC-3 cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, penicillin-streptomycin, and l-glutamine, as described previously (Mu et al., 2005). Cells were transfected with various plasmids using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and 1% fetal bovine serum for various times before analysis of mRNA and protein levels, apoptosis, and cell proliferation. In combination treatments, plasmid-transfected cells were incubated for an additional 48 to 72 h before measurement.

**Cell Proliferation Assay.** Approximately 6 x 10^4 PC-3 cells per well were transfected with the plasmids of interest, and cell growth was evaluated after 48 and 72 h using the 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay following the instructions provided by the kit.

**Reverse Transcription PCR.** For reverse transcription PCR (RT-PCR) analysis at 48 h after transfection, tissues were collected and total RNA was extracted using the TRIzol reagent (Invitrogen). Approximately, 5 µg of total RNA (purified after DNase I treatment) from each sample was converted to complementary cDNA using the commercially available RT-PCR kit (Promega, Madison, WI). The resultant complementary DNAs were used in PCR with the gene-

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specific primers of interest (Table 1), and the optimal conditions for PCR, including cycles, annealing times, and primer concentrations for each gene, were obtained by performing a series of pre-experiments (data not shown). The PCR products at the optimal conditions for each gene of interest were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

**Flow Cytometry.** PC-3 cells were incubated with plasmids for 48 and 72 h, harvested, and counted, and 1 × 10⁶ cells were resuspended in 100 μl of phosphate-buffered saline (PBS). Then, 5 μl of propidium iodide (PI) (Beckman Coulter, Fullerton, CA) was added and incubated for 30 min at room temperature in the dark. Then, the cells were subjected to flow cytometry (FCM) to measure the apoptosis rate with an Epics-XL-MCL flow cytometer (Beckman Coulter).

**Xenograft Models.** The models of PC-3 xenografts were established using athymic nude male mice (nu/nu; 6–8 weeks) that were acquired from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China) and used in accordance with an Animal Care and Use protocol approved by Jilin University. Cultured cells were washed with and resuspended in serum-free media. The suspension (5 × 10⁶ cells in 150 μl per mouse) was inoculated subcutaneously into the right flanks of nude mice. The sizes of the tumors in these mice were measured starting from 10 days after cell injection until 21 days using calipers. These tumor-bearing mice then were divided randomly into five groups (six mice per group): 1) mock transfection alone; 2) scrambled siRNA vector alone (Si-scramble) as the vector control; 3) pcDNA3.1-p53 (Pp53) as WT p53 transfection alone; 4) U6-mdm2 (Si-mdm2) as mdm2 siRNA transfection alone; 5) pcDNA3.1-p53/U6 Si-mdm2 (Psi-mdm2-p53, Pp53) as the combined transfection. Live attenuated Typhi Ty21a was used for these studies. Plasmids [10⁸ colony-forming unit (CFU) per 50 μl of phosphate-buffered saline (PBS). Then, 5 μl of propidium iodide (PI) was added and incubated for 30 min at room temperature in the dark. Then, the cells were subjected to flow cytometry (FCM) to measure the apoptosis rate with an Epics-XL-MCL flow cytometer (Beckman Coulter).

**Immunohistochemical Staining.** Each paraffin-embedded 4-μm-thick tissue section was deparaffinized, hydrated, incubated in 3% H₂O₂, and microwaved for 10 min to block endogenous peroxidase activity. After formalin fixation and paraffin-embedding, the tissue sections were incubated with the primary antibodies [anti-MT p53, anti-WT p53, and anti-mdm2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-proliferating cell nuclear antigen (PCNA) (Beijing Biosynthesis Biotechnology, Beijing, China)] for 60 min, washed with PBS, and then incubated with the biotinylated secondary antibody. Finally, the sections were stained with 3,3′-diaminobenzidine and counterstained with hematoxylin. Serum blocking solution in place of the primary antibody was used as a negative control. Stained tissues were screened independently by two investigators and classified according to staining intensity (− or +). We considered the tissue to be positive (+) if the staining intensity was moderate to strong in >10% of cells examined. Weakly stained (<10% of total cells examined) or nonimmunoreactive cells were considered negative (−). Positive or negative reaction was determined after counting cells in five random high-power fields of each sample.

**Western Blot Analysis.** The protein levels of MDM2, WT p53, p21 (cyclin-dependent kinase (CDK) inhibitor 1A), CDK4, cyclin D1, hypophosphorylated retinoblastoma (pRb), E2F-1, hypoxia-inducible factor 1α (HIF-1α), and β-actin in cultured cells and xenografts were analyzed by Western blotting as described previously (Zhang et al., 2008). The monoclonal antibodies against MDM2, WT p53, p21, pRb, cyclin D1, and HIF-1α were obtained from Calbiochem (San Diego, CA). Anti-E2F-1 monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. . Anti-β-actin monoclonal antibody was obtained from Sigma-Aldrich (St Louis, MO).

**Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Staining of Apoptotic Cells.** The tissue sections of xenografts from the nude mice were dewaxed and hydrated and then were subjected to terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining following the instructions provided by the TUNEL kit (Promega). Four fields (400×) were chosen randomly and analyzed. The apoptotic index was defined as follows: apoptotic index (%) = 100 × apoptotic cells/total tumor cells.

<table>
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<th>Product Size</th>
<th>Annealing Temperature</th>
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<td>355 bp</td>
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</tr>
<tr>
<td>p21</td>
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<td>168 bp</td>
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<td>168 bp</td>
<td>55 °C</td>
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Transmission Electron Microscopy. Tissue blocks (−1 mm³) were fixed in 2.5% glutaraldehyde (in 0.1 M sodium cacodylate, pH 7.4) for 2 h and stained by 1% OsO₄ at 4°C for 2 h and 0.9% OsO₄ and 0.3% K₄Fe(CN)₆ at 4°C for 2 h. Eighty-nanometer-thick sections were deposited on carbon and Formvar-coated, 200-mesh, nickel grids (Electron Microscopy Sciences, Hatfield, PA) and were stained with 3% uranyl acetate and Reynolds lead citrate for visualization under a 120-kV JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan).

Statistical Analysis. For in vitro study, three separate experiments were done. For in vivo study, six animals were included. Data are expressed as the mean ± S.D. One-way analysis of variance was used to determine whether differences exist, and if so, a post hoc Tukey’s test performed with Origin 7.5 (OriginLab Corp., Northampton, MA) laboratory data analysis and graphing software was used for the difference analysis between groups. Statistical significance was considered at p < 0.05.

Results

MDM2 and MT p53 Are Overexpressed in Primary Human Prostate Cancers. Immunohistochemical analysis of MT p53 and MDM2 protein expression in human prostate tissues (Fig. 1A) showed that MDM2 expression mainly localized in the nucleus in the prostate tumors and was detectable in 68.4% of the primary prostate cancers (13/19), whereas MT p53 protein was detected in 52.6% of the primary prostate cancers (10/19). Semiquantitative analysis of each protein, as described under Materials and Methods, was presented in Fig. 1B. This study confirms the increased expression of MT p53 and MDM2 proteins in human prostate cancer tissues compared with that of normal prostate tissues.

Effects of Coexpression of mdm2 siRNA and WT p53 Gene in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α in Prostate Cancer Cells.

Effects on Gene and Protein Expression of MDM2 and WT p53 in Human PC-3 Prostate Cancer Cells by Transfection with Both mdm2 siRNA and WT 53. The expression of mdm2 and WT p53 genes and proteins in the PC-3 cells transfected with different plasmids (illustrations of the constructions of these plasmids are shown in Fig. 2A) was examined by RT-PCR and Western blot analysis, respectively. As expected, the mdm2 mRNA and protein expression decreased significantly in the PC-3 cells transfected with Si-mdm2 and Pmp53 compared with that in the cells transfected with mock and Si-scrumble (Fig. 2, B and C). In contrast, the WT p53 mRNA and protein expression increased significantly in PC-3 cells transfected with Pp53 and Pmp53 and increased slightly in PC-3 cells transfected with Si-mdm2 compared with that in cells transfected with mock and Si-scrumble (Fig. 2, B and C). The results suggest the effectiveness of the transfected plasmids on the target genes.

Effects on Cell Proliferation and Apoptotic Death by Coexpression of mdm2 siRNA and WT p53 Gene in Prostate Cancer Cells. The potential effects on cell proliferation and death by treatment with Pmp53 were compared with those with Pp53 and Si-mdm2 by two assays: MTT and apoptotic cell death. The MTT assay showed that treatments with Pp53, Si-mdm2, and Pmp53 resulted in significant inhibition of PC-3 cell proliferation at both 24 and 72 h after transfection, with maximal inhibition by Pmp53 treatment (Fig. 3A).

For apoptotic cell death, FCM was used to detect the fluorescence intensity after tumor cells were double-stained with Annexin V and PI at 72 h post-transfection. Representative raw FCM data are shown in Fig. 3B, in which the early (Annexin V positive only) and the late (Annexin V and PI positive) apoptotic cells are distributed in the Q4 and Q2 regions, respectively, and necrotic cells (PI positive only) are in the Q1 region. It is noted that there were a few necrotic cells and there was also no significant difference for the incidence of necrotic cells among the groups. Compared with necrotic cells, there was a significantly increased portion of apoptotic cells among the tumors cells of each group. Quantitative analysis based on FCM showed that the apoptotic cell death increased significantly in the cells transfected with Pp53 or Si-mdm2 plasmid compared with that of the cells transfected with mock or Si-scrumble but was most evident in the cells treated with Pmp53 plasmid among all of the groups (Fig. 3, B and C).

Effects of Coexpression of mdm2 siRNA and the WT p53 Gene on the Expression of CDK4, Cyclin D1, and HIF-1α in Prostate Cancer Cells. To explore the possible mechanisms underlying the induction of PC-3 cell death by coexpression of mdm2 siRNA and the WT p53 gene, gene and protein expression of p21, CDK4, cyclin D1, and HIF-1α were examined by RT-PCR and Western blot (Fig. 4B) assays. It was shown that p21 mRNA and protein levels were low in the mock and Si-scrumble groups but increased significantly in the cells transfected with Pp53 or Pmp53 plasmid (Fig. 4, A and B). In contrast, expression of CDK4, cyclin
D1, and HIF-1α were significantly evident in the cells treated with either mock or Si-scramble but decreased significantly in cells treated with Pp53, Si-mdm2, and Pmp53 plasmids (Fig. 4).

Preferable Transfection of the Salmonella Typhi Ty21a-Carrying Plasmids into Xenograft PC-3 Tumors and Efficient Expression of MDM2 and WT p53 Proteins. The models of PC-3 xenografts were established as described under Materials and Methods. Athymic nude male mice (nu/nu; 6–8 weeks) were implanted with the PC-3 tumor cell suspension at 5 × 10^6 cells per 150 μl per mouse at the upper backs of the mice. The sizes of the tumors were measured daily starting from 10 days after cell implantation. At 21 days postimplantation, the tumor-bearing mice were inoculated with bacteria carrying the specific plasmid for each group via intratumoral injection with 10^8 CFU per 50 μl per mouse.

To ensure that Salmonella Typhi Ty21a carrying these plasmids preferentially localized in the tumor tissue, we have monitored the kinetics of bacterium distribution in the xenograft tumor and different organs of the tumor-bearing mice with bacterium injection at specified postinjection times (Fig. 5A). At 24 h (1 day) after injection, bacteria could be found in the tumors and organs (data not shown). The bacteria significantly increased in the tumors and decreased in the liver and other tissues at 3 days after administration (Fig. 5A). At 10 days after bacterium injection, bacteria accumulation was observed predominantly in the tumor tissue, much less in the liver (Fig. 5A), and undetectable in other organs (data not shown). Quantitative analysis of the bacteria by CFUs, as described under Materials and Methods and presented in Fig. 5, B and C, confirms the predominant distribution of these bacteria in the tumor tissue.

To determine whether the transfected plasmids were expressed efficiently in tumor cells, the expression of MDM2 and WT p53 proteins was examined by Western blot analysis. As shown in Fig. 6A, MDM2 expression was significantly lower in the tumors of mice treated with Si-mdm2 and Pmp53 but not changed in the tumors of mice treated with Pp53, compared with that in the tumors of mice treated with Si-scramble or mock. This result suggests that the successful transfection of bacteria with mdm2 siRNA in vivo efficiently suppresses mdm2 expression. The expression of WT p53 increased significantly in the tumors of mice treated with Pmp53 and Pp53 and only slightly in the tumors of mice treated with Si-mdm2 compared with that in the tumors of mice treated with Si-scramble or mock (Fig. 6A). This confirms the successful transfection of the bacteria with the WT p53 gene to efficiently express the WT p53 protein. Furthermore, immunohistochemical staining for MDM2 and WT p53 is provided representatively in Fig. 6B, which confirmed that the expression of MDM2 decreased and the expression of WT p53 increased in tumor cells treated with Pmp53 compared with those of tumors cells treated with Si-scramble.

Suppression of Prostate Tumor Xenograft Growth by Coexpression of Mdm2-siRNA and WT p53. The potential therapeutic effect on prostate xenograft growth was examined in tumor-bearing mice treated with bacteria carrying plasmid containing both mdm2-siRNA and the WT p53 gene (Fig. 7). Dynamic tumor growth that was monitored from the 4th day until the 32nd day after bacteria treatment (Fig. 7A) showed that sizes of the tumor xenografts in the mice treated...
Effects of transfection with various plasmids on cell growth. A, growth inhibitory effects were evaluated with MTT assays for the PC-3 cells transfected with different plasmids, and the data were presented as the ratio to control (mock). Data shown are means ± S.D. of three separate experiments. **, p < 0.05 versus mock or Si-scramble group; †, p < 0.05 versus Pp53 group; ‡, p < 0.05 versus Si-mdm2 group. B, the raw data of FCM with Annexin V and PI staining for detecting cell death are shown representatively from each group. Q1, necrotic cells; Q2, late apoptotic cells; Q3, normal cells; Q4, early apoptotic cells. C, the average data of FCM are presented for the results at 72 h after treatment. **, p < 0.05 versus Si-scramble group; †, p < 0.05 versus Pp53 group; ‡, p < 0.05 versus Si-mdm2 group.

Figure 7B showed that the individual tumor xenografts from mice treated with the Pmp53 plasmid were significantly smaller than those from mice treated with Si-scramble. Figure 7C showed that tumor weights significantly decreased in tumor-bearing mice treated with Pp53, Si-mdm2, or Pmp53 compared with those of mice treated with mock or Si-scramble plasmid. However, the inhibitory effect on tumor growth was more evident in the mice treated with Pmp53 than that in the mice treated with either Pp53 or Si-mdm2 alone (Fig. 7, A and C).

Effects of Coexpression of mdm2 siRNA and WT p53 on Tumor Cell Death and Expression of the p53–MDM2 Downstream Signaling Targets in Prostate Tumor Xenografts. Histological examination with hematoxylin and eosin staining revealed a few focal areas of necrotic cells along with tissue disorganization in the Pp53, Si-mdm2, and Pmp53 treatment groups (Fig. 8A). With TUNEL staining (Fig. 8B), apoptotic cells around the necrotic area were observed. The apoptotic cells showed chromat condensation, chromatin crescent, and nucleus fragmentation under the examination with a transmission electron microscope (Fig. 8C). Although the incidence of apoptotic cells was increased in tumor tissues from all of the mice treated with Pp53, Si-mdm2, and Pmp53, the highest incidence of the apoptotic cells was seen in tumors from the mouse treated with Pmp53 (Fig. 8, B and C).

Tumor cell proliferation also was analyzed by immunohistochemical staining for PCNA, which acts a marker for cells in the early G1 and S phase of the cell cycle. Treatment with the Pmp53 plasmid caused maximal inhibition of cell proliferation, because there were significantly decreased numbers of PCNA-positive cells compared with those of other groups (Fig. 8D).

To determine the potential mechanism of tumor growth inhibition in vivo, p21 protein expression was examined by Western blot assay (Fig. 8E); p21 protein expression was very low in the Si-scramble group but increased significantly in the groups treated with Pp53 and Pmp53. In contrast, the protein expression of E2F-1, pRb, and HIF-1α decreased significantly in the groups treated with Pp53, Si-mdm2, and Pmp53 compared with that of the Si-scramble-treated group (Fig. 8E).

Discussion

Widespread use of prostate-specific antigen and digital rectal examination screenings has led to earlier diagnosis, but there remains a lack of a significantly efficient therapeutic approach (Derweesh et al., 2004). Gene therapy remains as a potentially efficient therapeutic approach (Freytag et al., 2007).

From human prostate cancer samples, we demonstrated significant increases in MT p53 and MDM2 expression compared with that of the normal prostate tissues (Fig. 1). These results indicated that increased expression of MDM2 and MT p53 may play important roles in the formation of prostate cancer. The tumor suppressor p53 is a powerful antitumor molecule that is often inactivated by overexpression of its negative mediators. Down-regulation of p53 results in the prevention of p53-mediated apoptosis and cell cycle arrest (Momand et al., 1992). MDM2 inhibits p53 transcriptional activity, favors its nuclear export, and stimulates its degradation. Therefore, inhibiting the p53–MDM2 interaction is a promising approach for activating p53 in tumor cells with the
presence of endogenous WT p53 (Gottifredi and Prives, 2001; Chène, 2003).

However, p53 also is frequently inactivated by mutations or deletions in cancer cells. Isaacs et al. (1991) reported for the first time p53 gene mutations in prostate cancer cells, and other studies also confirmed the high incidence of MT p53 in prostate cancer (Hainaut and Hollstein, 2000). The results from our own observation that approximately 52.6% of human prostate cancers showed the presence of MT p53 (Fig. 1B) also support these previous studies. Under such conditions, only inactivation of MDM2 would not be able to restore p53 function. To solve this issue, forced expression of both FHIT and WT p53 by an adenoviral vector in non–small-cell lung carcinoma cells was found to result in a synergistic inhibition of tumor cell proliferation in vitro and growth in nude mice (Nishizaki et al., 2004). This is because FHIT can inactivate MDM2 and interrupt the association of MDM2 with p53, leading to p53 stabilization. A similar study reported that the coexpression of FUS1 and p53 by nanoparticle-mediated gene transfer significantly and synergistically inhibited non–small-cell lung carcinoma cell growth and induced apoptosis (Deng et al., 2007). The observed synergistic tumor suppression by FUS1 and p53 concurred with the FUS1-mediated down-regulation of MDM2 expression. Although these findings showed that combinational expression of a gene to inhibit MDM2 function with exogenous WT p53 in tumor cells with MT p53 may be a gene therapeutic strategy, it maybe not ideal because which of these MDM2-negative regulators is the most effective in suppressing MDM2 function and whether these MDM2-negative regulators have other functions except for suppressing MDM2 function remain uncertain.

Thus, the novel finding in the present study is that we have demonstrated, for the first time, that the plasmid containing
both mdm2-siRNA to directly down-regulate mdm2 expression and function and WT p53 gene can synergistically inhibit the growth of prostate tumor cells in vitro and in vivo. The MTT assay and flow cytometric analysis of apoptotic cells showed maximal inhibition of PC-3 cell growth and induction of apoptotic cell death in the cells transfected with both mdm2-siRNA and WT p53 (Pmp53 group) compared with those of the groups treated with either mdm2-siRNA or WT p53 alone (Fig. 3). Treatment of PC-3 tumor-bearing mice with Pmp53 plasmid also provided maximal inhibition of the tumor xenograft growth, as shown by the smallest tumor volume and weight among the five groups (Fig. 6). Both in vitro and in vivo studies clearly showed the advantage of transfection of the cells or xenografts in the tumor-bearing mice with the plasmid containing both mdm2-siRNA and WT p53. In this case, forced expression of WT p53 can be optimized with efficient inactivation of mdm2 by its siRNA (Fig. 6).

In addition, MDM2 is known to interact with other regulatory proteins, such as pRb (Xiao et al., 1995) and E2F-1 (Martin et al., 1995) independent of p53. In the present study, we investigated the expression levels of these genes.
and proteins, such as p21, CDK4, cyclin D1, HIF-1α, pRb, and E2F-1. We demonstrated that treatment with Pmp53 plasmid increased the expression of p21 and inhibited E2F-1, pRb, and HIF-1α expression to a certain extent (Figs. 4 and 8).

Cell cycle arrest takes place when there is a block in...
cell-cycle division. It is known that p53 arrests the cell cycle by stimulating the expression of p21, which blocks DNA replication by inhibiting PCNA activity, and also mediates growth arrest by inhibiting the action of G1 CDKs. Inhibition of CDKs results in the inhibition of both G1-to-S and G2-to-M transitions by causing hypophosphorylation of retinoblastoma and preventing the release of E2F-1 (Zheng et al., 2006). Studies by Hsieh et al. (1997) and Kowalik et al. (1998) separately confirmed that overexpression of MDM2 limited native E2F-1’s ability to induce p53-dependent apoptosis. We also showed that restoration of WT p53 function resulted in the up-regulation of p21, which may be responsible for the down-regulation of its other downstream genes, such as pRb and E2F-1 expression (Fig. SE).

Yamakuchi et al. (2010) showed that p53 is able to inhibit HIF-1 by inducing microRNA-107, leading to suppression of tumor angiogenesis and tumor growth. The transcription factor HIF-1 is composed of the subunits HIF-1α and HIF-1β, which are basic helix-loop-helix DNA-binding proteins. The HIF-1α subunit is overexpressed in a variety of human cancers. To confirm this finding, we also demonstrated the inhibitory expression of HIF-1α by up-regulation of p53 in prostate tumor cells in vitro (Fig. 3) and in vivo (Fig. 8). On the basis of the above information, therefore, we assumed that the synergistically inhibitory effects of coexpression of mdm2-siRNA and WT p53 on prostate tumor cells observed in the present study are most likely due to two factors: 1) inhibition of HIF-1α activity by up-regulation of WT p53 expression would impair hypoxic signaling, resulting in inhibition of tumor growth, angiogenesis, and vessel maturation (Stoeltzing et al., 2004); 2) up-regulation of WT p53 in the tumor tissues enhances spontaneous apoptotic cell death (Deng et al., 2007).

Most human and rodent solid tumors contain a substantial fraction of cells that are hypoxic. Tumor hypoxia occurs because of the stochastic and slow development of the vasculature during tumor growth. Salmonella Typhi Ty21a has an excellent safety profile (Thamm et al., 2005) and a propensity for homing to tumors (Clairmont et al., 2000). In the present study, this process is advantageous. When the Salmonella Typhi Ty21a that carries the plasmids of interest infected the study, this process is advantageous. When the

References

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Authorship Contributions

Participants in research design: Ji, Shao, Zhao, Xu, Y. Li, and Cai. Conducted experiments: Ji, Wang, Liu, Shao, Xia, Li, and Xin Li. Contributed new reagents or analytic tools: Ji, Wang, Zhao, Zhang, and Hu. Performed data analysis: Ji, Wang, Xu, and Y. Li. Wrote or contributed to the writing of the manuscript: Ji, Wang, Zhang, Xu, Y. Li, and Cai.

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