# Intratumoral Delivery and Suppression of Prostate Tumor Growth by Attenuated *Salmonella enterica* serovar *typhimurium* Carrying Plasmid-Based Small Interfering RNAs

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## Abstract

**The facultative anaerobic, invasive *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) has been shown to retard the growth of established tumors. We wondered if a more effective antitumor response could be achieved *in vivo* if these bacteria were used as tools for delivering specific molecular antitumor therapeutics. Constitutively activated transcription factor signal transducer and activator of transcription 3 (STAT3) promotes the survival of a number of human tumors. In this study, we investigated the relative efficacies of attenuated *S. typhimurium* alone or combined with Stat3-specific small interfering RNA (siRNA) in terms of tumor growth and metastasis. The bacteria preferentially homed into tumors over normal liver and spleen tissues *in vivo*. *S. typhimurium* expressing plasmid-based Stat3-specific siRNAs significantly inhibited tumor growth, reduced the number of metastastic organs, and extended the life time for C57BL6 mice bearing an implanted prostate tumor, versus bacterial treatment alone. These results suggest that attenuated *S. typhimurium* combined with an RNA interference approach might be more effective for the treatment of primary as well as metastatic cancer.** [Cancer Res 2007;67(12):5859–64]

## Introduction

RNA interference (RNAi) is an evolutionarily conserved, posttranscriptional gene-silencing mechanism wherein a small interfering double-stranded RNA (siRNA) directs a sequence-specific degradation of its target mRNA (1). Because of their unparalleled target specificity, there has been an intensive effort to use siRNAs as therapeutics for various diseases, especially for cancer therapy. Because synthetic siRNAs can only transiently decrease the target gene expression in proliferating cancer cells (2), a sustained, localized supply of anticancer siRNAs is critical for imparting a strong therapeutic benefit. Plasmid-based expression of gene-specific small hairpin RNAs (shRNA), under the control of RNA polymerase III–dependent promoters (e.g., U6 and H1), produces a sustainable and economical source of siRNAs for therapeutic purposes. The shRNAs are processed intracellularly by the enzyme Dicer into siRNAs. Some groups have reported successful application *in vivo* following systemic administration with shRNA-encoding plasmid DNA (3–5). Unfortunately, in most of these approaches, the therapeutics do not reach the tumors in effective doses, or distribution to unwanted sites and degradation by nucleases result in limited antitumor effect. The success of siRNAs as cancer therapeutics relies on the development of safe, economical, and efficacious *in vivo* delivery systems into tumor cells. Although siRNAs can be used as therapeutics *in vivo*, their intratumoral delivery, specifically across the plasma membrane of cells, is not achieved easily. Furthermore, they are ineffective at killing quiescent tumor cells that are distantly located from the vasculature and metastatic tumors because of their heterogeneous microenvironments. The ideal delivery system would be (*a*) nontoxic to normal cells and (*b*) able to deliver the therapeutic efficiently and specifically to the tumor.

The discovery that genes vectored by bacteria can be functionally transferred to mammalian cells has suggested the possible use of bacterial vectors as vehicles for gene therapy. Genetically modified, nonpathogenic bacteria have been used as potential antitumor agents, either to elicit direct tumoricidal effects or to deliver tumoricidal molecules (6–9). Bioengineered attenuated strains of *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) have been shown to accumulate preferentially >1,000-fold greater in tumors than in normal tissues and to disperse homogeneously in tumor tissues (10, 11). Preferential replication allows the bacteria to produce and deliver a variety of anticancer therapeutic agents at high concentrations directly within the tumor, while minimizing toxicity to normal tissues. These attenuated bacteria have been found to be safe in mice, pigs, and monkeys when administered i.v. (8, 9, 12, 13), and certain live attenuated *Salmonella* strains have been shown to be well tolerated after oral administration in human clinical trials (14–17). The *S. typhimurium phoP/phoQ* operon is a typical bacterial two-component regulatory system composed of a membrane-associated sensor kinase (PhoQ) and a cytoplasmic transcriptional regulator (PhoP; refs. 18, 19). *phoP/phoQ* is required for virulence, and its deletion results in poor survival of this bacterium in macrophages and a marked attenuation in mice and humans (18–21). *phoP/phoQ* deletion strains have been employed as effective vaccine delivery vehicles (20–22). More recently, attenuated salmonellae have been used for targeted delivery of tumoricidal proteins (7, 12). We report here the use of an attenuated *phoP/phoQ* null *S. typhimurium* as a delivery system for siRNA-based tumor therapy.

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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## Materials and Methods

**Construction of siRNA expression vectors.** A siRNA target located in the SH2 domain of human signal transducer and activator of transcription 3 (Stat3; nucleotides 2144–2162; Genbank accession no. NM00315) was chosen for use herein based upon our previous study (23). The sequence of Stat3-specific hairpin RNA is given as follows: GCAGCAGCTGAACAA-CATG*TTCAAGAGA*CATGTTGTTCAGCTGCTGCTTTTT. This oligonucleotide contains a sense strand of 20 nucleotides followed by a short spacer (loop sequence: *TTCAAGAGA*), the antisense strand, and five Ts (terminator). A scrambled siRNA (Ambion) was used as a negative control. Double-stranded DNA oligonucleotides were cloned into pGCsilencerU6/Neo/GFP, which also expresses a *green fluorescent protein* (GFP) gene (Jikai Chemical, Inc.), to generate plasmids pSi-Stat3 and pSi-Scramble (Fig. 1*A*).

**Bacteria, cell culture, and stable cell line establishment.** The attenuated *S. typhimurium phoP/phoQ* null strain LH430 was kindly provided by Dr. E.L. Hohmann (16). This strain was created from *S. typhimurium* strain SL1344 by deletion of the *phoP/phoQ* locus (24). Plasmids were electroporated into *Salmonella* before use. The mouse prostate cancer cell line RM-1 was obtained from the Shanghai Institute of Cellular Research. The cells were grown in Iscove's modified Dulbecco's medium (Invitrogen) with 10% fetal bovine serum. Cells were cocultured with recombinant bacteria ($1 \times 10^8$ cfu) at 37°C for 30 min. Cell lines were washed and treated first with 100 μg/mL gentamicin to kill all extracellular bacteria and then with 5 μg/mL of tetracycline to prevent intracellular bacterial multiplication. Stable RM-1 clones, containing integrated plasmids, were selected and maintained by treating the cells with 200 μg/mL G418.

**Tumorigenic assays.** C57BL6 mice ($n$ = 10 per group) were injected s.c. with the cell lines described above ($2 \times 10^6$ cfu) into the upper flank. Tumor development was followed for 60 days. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under assurance no. A3873-1.

**Northern and Western blotting.** Cell lysis, protein quantification, and Western blot analyses were carried out as described previously (23). Antibodies against Stat3, phosphorylated $Tyr^{705}$-Stat3 (p-Stat3), cyclin D1, c-Myc, VEGF, and antimouse were obtained from Santa Cruz Biotechnology. Antibody against Bcl-2 was obtained from DAKO Biotech. Antibody against Ki-67 was obtained from Biogenex. Protein bands were detected using enhanced chemiluminescence (Amersham). Total RNA (20 μg) and $^{32}$P-labeled cDNAs of Stat3 and actin were used as probes. mRNA level was quantified using a Molecular Dynamics PhosphorImager.

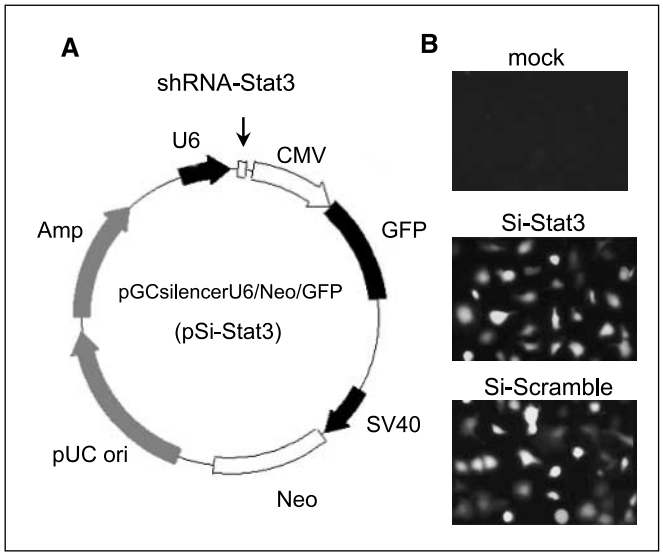


**Figure 1.** *A*, structure of pSi-Stat3 plasmid containing the sequence of Stat3-specific hairpin RNA (shRNA-Stat3; *arrow*). *B*, expression of GFP of pSi-Stat3 and pSi-Scramble in stable infected RM-1 cells versus mock uninfected cells. Magnification, ×400.

**Cell cycle, apoptosis, and proliferation assays.** Cell cycle phase distribution was determined by flow cytometry. An Annexin V-CY3 apoptosis detection kit (Sigma) was used for detecting apoptosis. Tumor tissue sections from animals were used for H&E staining and terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assays, as described previously (23). Cell proliferation was assayed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining kit (Sigma) as per manufacturer's protocol; the cell growth inhibition rate was calculated as follows: $A$ = (1 − absorbance of experimental group / absorbance of control group) × 100%.

**Antitumor activity of recombinant *S. typhimurium* on established prostate tumors.** RM-1 cells were transplanted into mice s.c. to generate a primary tumor. After the development of a palpable tumor at the site of inoculation, tumors were excised, and the primary tumor fragments (1.5 $mm^3$) were implanted by surgical orthotopic implantation in between two lobes of the prostatic gland in a recipient group of C57BL6 mice according to methods described previously (24, 25). Five days after implantation, mice were divided into three groups ($n$ = 10 per group) and injected i.v. with $1 \times 10^7$ cfu of attenuated *S. typhimurium* carrying different plasmids. One set of mice was sacrificed 18 days after administration of bacteria, and tumors were excised, weighed, and measured diameter. Tumor metastases were counted in the liver, lung, spleen, kidney, and lymph nodes. The remaining mice were followed over 70 days for survival after treatment with different plasmids.

**Analysis of bacterial distribution.** Tissue samples from the primary tumor, the liver, the spleen, and from other sets of tumor-bearing mice were used for bacterial distribution and clearance studies. Normal and tumor tissues were excised, weighed, minced thoroughly, and homogenized. The diluted tissue homogenates were plated onto Luria-Bertani agar containing ampicillin in triplicate, and the colony count was determined on the next day. The tissues were also observed under a fluorescence microscope to determine the extent of bacterial infection. A portion of the tissues was also prepared for histochemical analyses.

**Gelatin zymography assay.** The gelatinolytic activities of matrix metalloproteinase-2 (MMP-2) were examined according to the method described previously (26).

**Data analyses.** The significance of the *in vitro* and *in vivo* data was determined using the Student's two-tailed $t$ test. The significance of the differences between median data values was determined using the two-tailed Mann Test. $P < 0.05$ was deemed statistically significant. Data are presented as mean ± SD.

## Results and Discussion

**Construction of shRNA expression vectors and cell infection.** To show the utility of attenuated *S. typhimurium*–carried, Stat3-specific siRNA for tumor therapy, we first generated plasmid vectors that express a Stat3-specific siRNA (Si-Stat3) and a control scrambled siRNA (Si-Scramble). Because of its potent antitumor effects, the target for Si-Stat3 was chosen from the SH2 domain of human Stat3 based on our earlier study (23). Synthetic oligonucleotides (20 bp) capable of coding for the Si-Stat3 and Si-Scramble siRNAs were cloned into pGCsilencerU6/Neo/GFP, a plasmid containing the *GFP* gene. The resultant plasmids pSi-Stat3 and pSi-Scramble were transformed into *S. typhimurium* and used for transfection into RM-1 cells (Fig. 1*A*). Virtually identical transfection efficiencies were observed for each plasmid as determined by the expression of GFP in RM-1 cells (Fig. 1*B*).

In this study, the invasive recombinant *S. typhimurium* carrying either the pSi-Stat3 or pSi-Scramble plasmids were directly cocultured with a mouse prostate carcinoma cell line (RM-1), and stable cell lines RM-Si-Stat3 and RM-Si-Scramble were established after G418 selection. The continued expression of GFP, in the absence of bacteria in the cell lines, indicates that the siRNA expression vectors were stably integrated into the host cell genome.

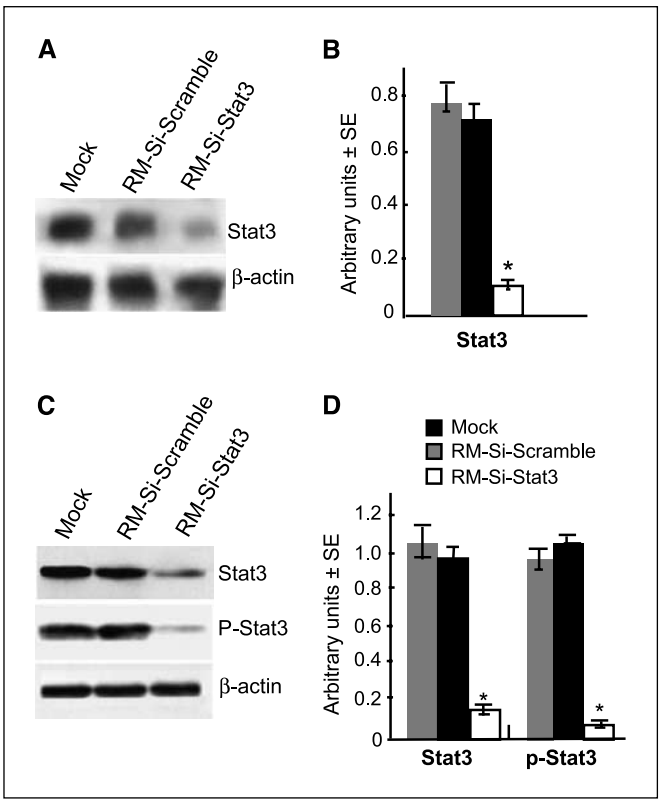


**Figure 2.** shRNA-mediated knockdown of STAT3 expression. Northern (*A*) and Western (*C*) blot analyses of Stat3 expression. Equal amounts of total RNA (20 μg) were used for Northern blot analysis. *B,* quantification of Stat3 mRNA from three separate experiments and normalized to that of β-actin. *, $P < 0.01$ versus mock and scrambled vector control. *D,* quantification of Stat3 protein levels.

**Effects of bacterially delivered shRNAs on cell growth and cycling.** The ability of these constructs to silence Stat3 was determined next using Western and Northern blot analyses. The Stat3 mRNA level in RM-Si-Stat3 was reduced to ~13% of that observed in RM-Si-Scramble (Fig. 2*A* and *B*). Western blot analyses with native Stat3 (Stat3)– and phosphorylated $Tyr^{705}$ Stat3 (p-Stat3)–specific antibodies also showed a strong inhibition of Stat3 or p-Stat3 proteins to ~18% or 10%, respectively, in RM-Si-Stat3 (Fig. 2*C* and *D*) compared with RM-Si-Scramble. The percentages of STAT3 knockdown observed in Northern versus Western blot analyses are similar and statistically significant. Thus, the bacterially introduced Si-Stat3 specifically knocks down the expression of Stat3. We also examined the effects of siRNAs on cell growth and cycling. Cells were stained with acridine orange and subjected to flow cytometry. Stat3-siRNA induced significant apoptosis (~23-fold) compared with the pSi-Scrambled control (Table 1). A further analysis of the flow cytometric data also showed that cells transfected with pSi-Stat3 accumulated significantly in $G_1$ phase compared with the control (Table 1). These findings indicate that inhibition of Stat3 promotes both cessation of cell growth and enhancement of cell death. Because the *Salmonella* have been eliminated from the stable cell line by treatment with antibiotics, the effects on cell growth and cycling from the Si-Scramble control was equivalent to the uninfected mock group. Cells transfected with pSi-Stat3 grew slower and showed strong apoptosis (Fig. 3*A*) compared with those transfected with pSi-Scramble. Cells transfected with pSi-Stat3 became confluent 6 days after seeding, in contrast to the control group, which reached confluence by 4 days. In a separate experiment, cellular metabolic activity (as an indicator of cell viability) was measured using MTT assays in RM-1 cells transfected with the various plasmids. MTT data, expressed as tumor cell viability, were significantly decreased in the cells treated with the pSi-Stat3 compared with the control groups at day 6 ($P < 0.05$, $n = 3$; Fig. 3*B*). Stat3 has been shown to play a key role in promoting the cell cycle, proliferation, differentiation, and inhibition of apoptosis (27). Persistently active Stat3 and its overexpression have been detected in a wide variety of human tumors (28), including prostate cancer (29). Constitutively active Stat3 promotes cell growth and survival via an overexpression of downstream targeted genes, such as the antiapoptotic *Bcl-2*, cell cycle regulators *cyclin D1* and *c-Myc*, and inducers of tumor angiogenesis *VEGF* and *MMP-2* (30–35). We, therefore, examined if the expression of these genes was altered by Si-Stat3. The expression of *Bcl-2, cyclin D1, c-Myc, VEGF*, and *MMP-2* was significantly knocked down in the presence of Si-Stat3 but not Si-Scramble (Fig. 3*C* and *D*). Thus, the Stat3-specific shRNA interferes with the expression of tumor growth–promoting factors and decreases tumor cell survival.

**Tumorigenic properties of RM-Si-Stat3 cells *in vivo*.** We next examined the tumorigenic properties of RM-Si-Stat3 cells *in vivo*. C57BL6 mice ($n = 10$) were injected with $2 \times 10^6$ cells via the s.c. route into the upper flank, and tumor growth was monitored for 60 days. Mice transplanted with RM-Si-Scramble cells developed tumors at the injection sites by 21 ± 3.6 days. In contrast, no tumors formed in the group injected with RM-Si-Stat3. Thus, the blockade of Stat3 reverses tumorigenicity of RM-1 prostate cancer cells.

**Inhibition of prostate tumor growth and metastasis *in vivo* by bacterially delivered shRNAs.** Although salmonellae have been effective in retarding the growth of established tumors, complete tumor regression has never been proven. We, therefore, first studied the effects of *S. typhimurium* alone or combined with Stat3-specific siRNA in terms of prostate tumor growth and metastasis. To this end, we employed a C57BL6 mouse tumor implant model. A primary tumor was first established with RM-1 prostate carcinoma cells. Upon development of palpable s.c. tumors at the sites of inoculation, the tumor was excised and used for initiating primary prostate tumor development via an orthotopic surgical implantation of tumor tissues into recipient naive mouse prostates. Five days after tumor implantation, mice were divided into four groups ($n = 10$ per group) and then injected with $1 \times 10^7$ cfu of attenuated *Salmonella* carrying different plasmids via the tail vein. Eighteen days after bacterial injection, mice were sacrificed, and the tumors were excised, weighed, and measured. As shown in Table 2, mice treated with buffer alone

**Table 1.** Effect of siRNAs on cell growth and apoptosis in RM-1 cells

| Group ($n = 10$) | Apoptotic cells, % (mean ± SD) | $G_0$-$G_1$, % (mean ± SD) | S, % (mean ± SD) |
|---|---|---|---|
| Mock | 0.4 ± 0.15 | 43.0 ± 2.02 | 45.7 ± 2.36 |
| pSi-Scramble | 1.3 ± 0.27* | 51.7 ± 2.65 | 36.2 ± 2.93 |
| pSi-Stat3 | 28.9 ± 3.14* | 71.2 ± 2.35* | 3.2 ± 0.35* |

*$P < 0.01$ versus pSi-Scramble.

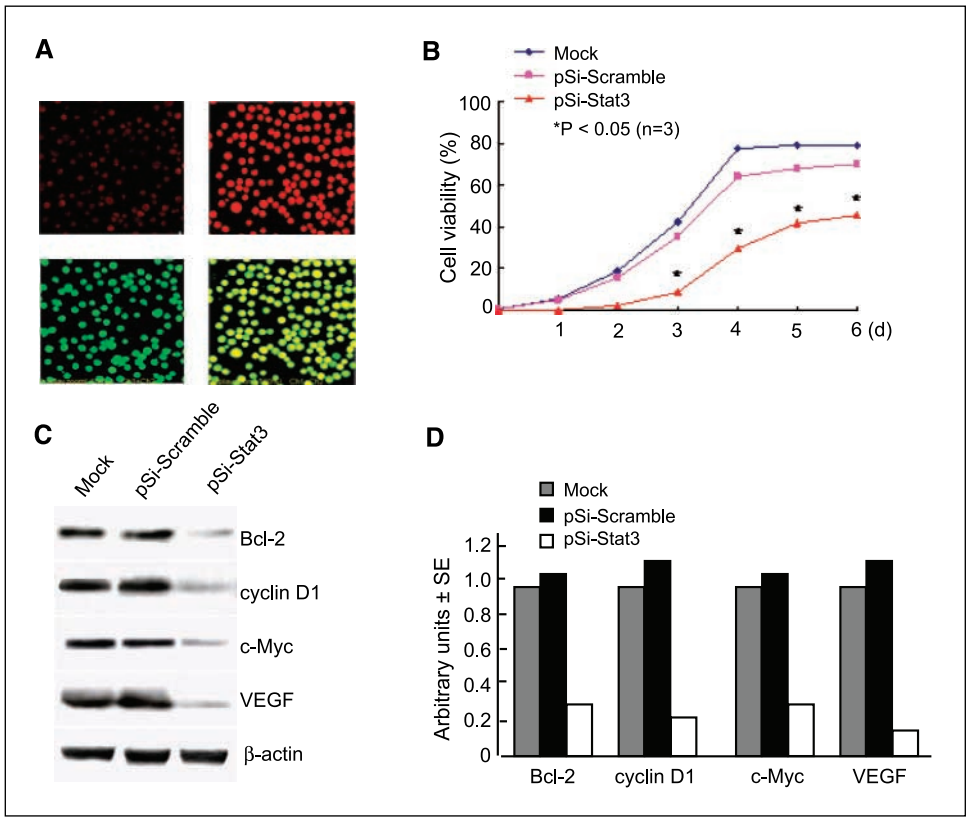


**Figure 3.** Si-Stat3 inhibits cell growth and induces apoptosis. *A,* cells were stained with AnnCy3 (*red*) and 6-CF (*green*) to visualize apoptotic cells using confocal microscopy. Live cells were labeled only with 6-CF (*green*); necrotic cells were labeled only with AnnCy3 (*red*); and cells undergoing apoptosis were double labeled yielding a yellow color in merged images. *B,* MTT assays. *Points,* mean of three separate experiments. *, $P < 0.01$ versus mock and pSi-Scramble. Tumor cell viability (*A* value) was significantly reduced by treatment with pSi-Stat3. $P < 0.05$ ($n = 3$). *C,* expression of Bcl-2, cyclin D1, c-Myc, and VEGF proteins as revealed by Western blot analyses. Mock, untreated cells. *D,* quantification of the images in (*C*).

(mock control) developed primary tumors with a mean volume of 2,458.51 ± 602.18 $mm^3$. In mice treated with *Salmonella*-Si-scramble, tumors grew to a volume of 589.22 ± 380.34 $mm^3$. In mice treated with *Salmonella* without any plasmid, the tumor grew to a comparable volume of 585.44 ± 220.21 $mm^3$. Thus, the bacteria carrying the scrambled-siRNA did not significantly affect tumor growth any differently compared with the *Salmonella* vector alone. However, mice treated with *Salmonella*-Si-Stat3 developed tumors with a median reduced volume of 216.42 ± 134.15 $mm^3$. Remarkably, tumors completely disappeared in one third of mice in this group over 18 days. The differences in tumor size between buffer control versus *Salmonella*-Si-scramble ($P < 0.05$) and buffer control versus the *Salmonella*-Si-Stat3 group ($P < 0.01$) were statistically very significant. The differences between *Salmonella*-Si-scramble or *Salmonella* alone versus *Salmonella*-Si-Stat3 group were also statistically significant ($P < 0.05$). In summary, ~3.9-fold higher tumor suppressive effect can be achieved with a single dose of bacteria transformed with a siRNA expression vector than those treated with *Salmonella* alone or *Salmonella* carrying Si-Scramble control, and ~11.4-fold higher than those treated with buffer control (Fig. 4*A, yellow arrowhead*; Table 2). Thus, attenuated *Salmonella* alone exert an antitumor effect, which can be further enhanced by genetically modifying these organisms in combination with Stat3-specific siRNA expression.

In addition to the primary tumor, metastases into liver, lung, spleen, kidney, and lymph nodes were examined in the recipient mice. A robust 84% reduction ($P < 0.01$) in the numbers of metastases in the *Salmonella*-Si-Stat3–treated mice (Supplementary Table S3) was observed. Tumor metastases occur primarily through tumor angiogenesis, aggressive growth of the primary tumor, and an extravasation of the tumor cells (36). Secretion of extracellular proteases by the tumor plays an important role in metastasis (36, 37). Among these, MMP-2/gelatinase A is believed to be essential for malignant behavior of cancer cells, such as rapid growth, tissue invasion, and metastasis (36, 37). Consistent with this observation, we found that the MMP-2 activity in RM-1 cells significantly decreased after treatment with pSi-Stat3 compared with mock or pSi-Scramble ($P < 0.05$; Supplementary Fig. S1). Furthermore, blockade of Stat3 correlated with a reduction of expression of the Ki-67 protein, a proliferation-associated antigen (38). Immunohistochemical analyses for Stat3 and Ki-67 expression in the RM-1 tumor cells after transfection with Si-Scramble and in untreated RM-1 tumor cells were highly positive for Stat3 and Ki-67. In contrast, RM-1 tumor cells treated with pSi-Stat3 stained weakly for Stat3 and Ki-67 (Fig. 4*B*).

**Table 2.** Antitumor effects of bacterially transferred Stat3-specific siRNAs

| Group ($n = 10$) | Mean weight (g) | | Mean tumor volume ($mm^3$) |
|---|---|---|---|
| | Mouse | Tumor | |
| Mock | 26.52 ± 3.06 | 3.43 ± 0.89 | 2,458.51 ± 602.18 |
| pSi-Scramble | 25.36 ± 2.58 | 1.45 ± 0.61* | 589.22 ± 380.34* |
| *Salmonella* alone | 25.00 ± 1.22 | 1.66 ± 0.23* | 585.44 ± 220.21* |
| pSi-Stat3 | 24.31 ± 2.36 | 0.38 ± 0.24† | 216.42 ± 134.15† |

*$P < 0.05$ versus mock.
† $P < 0.01$ versus mock.

Tumors from mice treated with pSi-Scramble or pSi-Stat3 were excised for H&E staining and analyzed with TUNEL assays (Fig. 4*C*). pSi-Stat3–treated tumors show massive apoptosis with sparsely dispersed chromatin, several TUNEL-positive cells, and some necrotic regions compared with the Si-scramble control, which showed a finely granular cytoplasm with evenly dispersed chromatin and no TUNEL-positive cells. These data show that the Stat3 siRNA carried by *Salmonella* exerts a strong apoptotic antitumor effect *in vivo*.

**The attenuated *S. typhimurium* expressing a Stat3-specific siRNA exerts a robust antitumor effect.** To further show the therapeutic utility of *Salmonella*-delivered siRNAs, tumor-bearing mice were injected with *Salmonella* carrying various plasmids or buffer. Mice were observed for 70 days. As shown in Supplementary Fig. S2, all mice ($n$ = 10) injected with buffer were dead before 30 days. In contrast, the mice injected with *Salmonella*-Si-Stat3 and *Salmonella*-Si-Scramble had nine and six surviving mice at 70 days, respectively. These data clearly show that the attenuated *Salmonella* expressing a Stat3-specific siRNA exerts a robust antitumor effect.

**Recombinant bacterial distribution in C57BL6 tumor-bearing mice.** To determine if the potent antitumor effects of *Salmonella* with shRNA vectors was due to a preferential homing of bacteria into tumor tissue, we monitored the kinetics of bacterial distribution in C57BL6 tumor-bearing mice at specified times after injection of bacteria (Supplementary Fig. S3*A*). Twenty-four hours after injection, similar numbers of bacteria were found in the liver, spleen, and tumors in tumor-bearing mice. The bacterial count (cfu) increased in tumors and decreased in the liver and spleen within 48 h after administration. By day 5, the number of bacteria in tumors increased significantly; the tumor to liver or tumor to spleen cfu ratio was 1,000:1 and 5,000:1, respectively, on average. By day 15, far more bacteria could be seen in the tumor compared with the liver, and no bacteria could be found in spleen tissues. On day 10, by using GFP expression as a marker, the bacterial distribution was also observed as markedly high in tumor tissue sections compared with those in spleen and liver tissue sections (Supplementary Fig. S3*B*). At present, it is not clear why or how *Salmonella* specifically home to the tumor. Both characteristics of *Salmonella* and the heterogeneous microenvironments in solid tumors may combine to allow these bacteria to deliver therapeutic molecules preferentially to tumors. These characteristics may include (*a*) bacterial motility leading to uniform penetration within tumors; (*b*) hypoxic regions, an environment to which facultative anaerobic salmonellae are well adapted and can multiply, and in which macrophages, neutrophils, and granulocytes, effectors of bacterial clearance, are reduced in number (39); (*c*) both antibodies and serum complement components, which together can be lytic to salmonellae, are greatly restricted from the tumor environment by the irregular vasculature and positive pressure that exist inside tumors (40); (*d*) nutrients, such as high availability of glucose in aggressively growing tumors, may promote locally increased bacterial growth (41); and (*e*) *Salmonella* may induce apoptosis in infected macrophages (42) at the tumor margins leading to increased antitumor inflammatory responses. An important recent advance in this field is the development of live, attenuated *Salmonella* vectors for DNA vaccine delivery (43). The mechanisms involved in *Salmonella* delivery of DNA vaccine plasmids to the cytosol of mammalian cells is yet unclear (44). However, several lines of evidence suggest that this bacterium can deliver nucleic acid vaccines *in vivo*, which elicit impressive levels of specific antibody response, T-cell proliferation, and CTL responses (45). Lastly, live *Salmonella* infection, but not *Escherichia coli*, induces the expression GRIM-19 (46), a protein

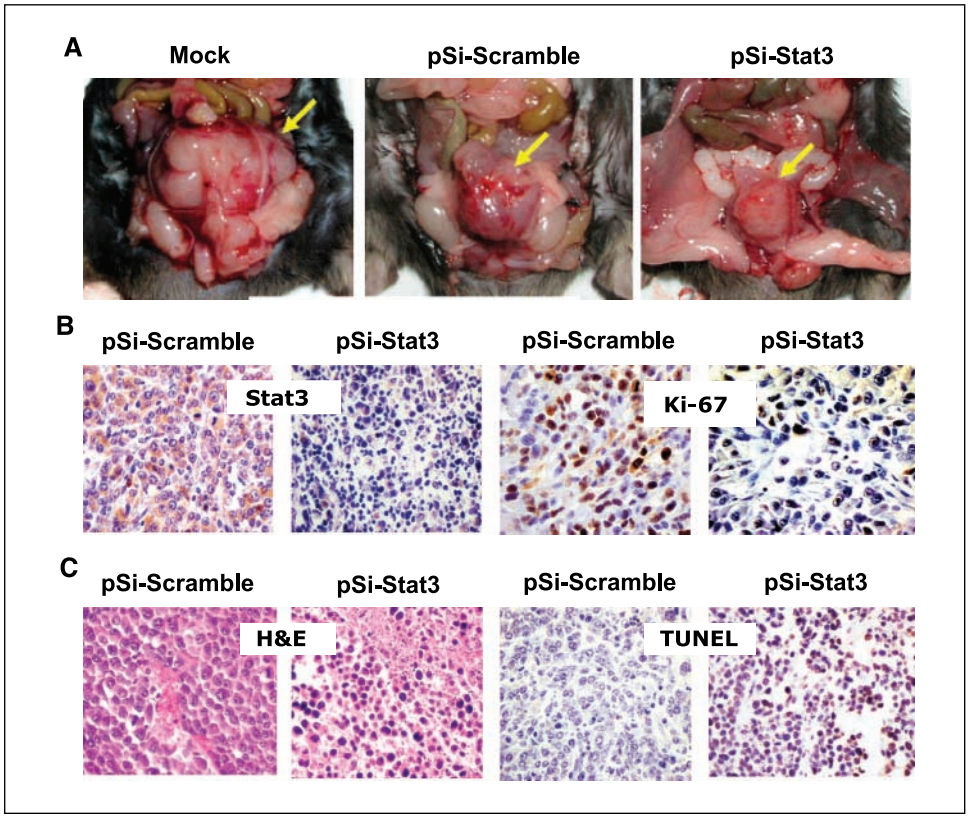


**Figure 4.** Effects of systemically administered recombinant *S. typhimurium* on prostate tumor growth *in vivo*. *A,* representative mice treated with recombinant bacteria carrying various plasmids after orthotopic implantation of prostate tumor. Note a significant loss of tumor volume in mice treated with *Salmonella*-pSi-Stat3 compared with the control. Tumor locations (*arrows*). *B,* immunohistochemical analyses of Stat3 and Ki-67 expression. Note a strong positive staining for Stat3 and Ki-67 in pSi-Scramble-treated tumor, in sharp contrast to those treated with Si-Stat3. Magnification, ×400. *C,* H&E staining and TUNEL (magnification, ×200) of tumors. TUNEL-positive cells (*brown*).

inhibitor of STAT3 (47, 48). Thus, the potent antitumor effect of *Salmonella* can, in part, be due to an inhibition of STAT3 activity by increased GRIM-19 in the tumor. When these bacteria are combined with Stat3-specific siRNAs, a double-edged inhibitory effect may be exerted on STAT3 *in vivo*.

Our results provide the first convincing evidence that *Salmonella* can be used for delivering plasmid-based siRNAs into tumors growing *in vivo*. The Stat3-siRNAs carried by an attenuated *S. typhimurium* exhibit tumor suppressive effects not only on the growth of the primary tumor but also on the development of metastases, suggesting that an appropriate attenuated *S. typhimurium* combined with the RNAi approach may offer a clinically feasible approach for cancer therapy. Ultimately, a live, attenuated *Salmonella* parenteral delivery system would likely be endotoxic in humans unless an *msbB* mutation was introduced, as reported previously (11).

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