

Original Article

Attenuated *Salmonella typhimurium* carrying shRNA-expressing vectors elicit RNA interference in murine bladder tumors

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Aim: To examine whether attenuated *Salmonella typhimurium* (*S typhimurium*) could be used as an anti-cancer agent or a tumor-targeting vehicle for delivering shRNA-expressing pDNA into cancer cells in a mouse tumor model.

Methods: Mouse bladder transitional cancer cell line (BTT-T739) expressing GFP was used, in which the GFP expression level served as an indicator of RNA interference (RNAi). BTT-T739-GFP tumor-bearing mice (4–6 weeks) were treated with *S typhimurium* carrying plasmids encoding shRNA against *gfp* or scrambled shRNA. The mRNA and protein expression levels of GFP were assessed 5 d after the bacteria administration, and the antitumor effects of *S typhimurium* were evaluated.

Results: In BTT-T739-GFP tumor-bearing mice, *S typhimurium* (1×10^9 cfu, *po*) preferentially accumulated within tumors for as long as 40 d, and formed a tumor-to-normal tissue ratio that exceeded 1000/1. *S typhimurium* carrying plasmids encoding shRNA against *gfp* inhibited the expression of GFP in tumor cells by 73.4%. Orally delivered *S typhimurium* significantly delayed tumor growth and prolonged the survival of tumor-bearing mice.

Conclusion: This study demonstrates that attenuated *S typhimurium* can be used for both delivering shRNA-expressing vectors into tumor cells and eliciting RNAi, thus exerting anti-tumor activity, which may represent a new strategy for the treatment of solid tumors.

Keywords: RNA interference; *Salmonella typhimurium*; shRNA-expressing pDNA; mouse bladder transitional cancer cell; tumor therapy

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Introduction

RNA interference (RNAi) is a naturally occurring gene silencing event in which small interfering RNA (siRNA) initiates the degradation of target mRNA that contains a sequence complementary to the siRNA. Since the original discovery of RNAi in *Caenorhabditis elegans*^[1], there has been an increasing interest in harnessing this endogenous mechanism to treat a variety of diseases in which aberrant expression of mRNA is problematic. The first clinical trial to test siRNA in the treatment of age-related macular degeneration (AMD) commenced in 2004^[2]. However, systemic siRNA therapy is hampered by barriers that prevent siRNA from reaching their intended targets in the cytoplasm and exerting their stable gene silencing activity. DNA vectors that transcribe short hairpin RNA (shRNA) provide another way to elicit RNAi. Previous studies have shown that the extent by which target gene expression

is repressed is nearly identical between siRNA and shRNA-expressing plasmid DNA (pDNA)^[3]. However, the gene-silencing effect induced by shRNA-expressing pDNA lasts significantly longer than gene silencing induced by siRNA^[4]. pDNA must be delivered into the nucleus of target cells to transcribe shRNA. The shRNA is then transported to the cytosol and processed into siRNA. As gene therapy studies have previously demonstrated, nuclear delivery is one of the biggest challenges in transfection, and limited transgene expression has largely hampered the clinical application of therapeutic DNA^[5]. By hijacking a variety of cellular mechanisms, viruses have evolved strategies to transport their genomic material into the nucleus^[6,7]. However, the safety concerns surrounding viral vectors have not been resolved.

In cancer research, efforts have been made to find a magic bullet that could selectively target and destroy malignant cells. More than 100 years ago, the German physician W Busch induced a bacterial infection in a woman with an inoperable sarcoma and observed a reduction in the size of the primary tumor^[8]. Recently, both the presence and the selective growth of anaerobic bacteria in human tumors have

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garnered attention. The conditions that permit anaerobic bacterial growth, such as impaired circulation and extensive necrosis, are found in many tumors. Therefore, bacteria may be a viable therapeutic option for a variety of malignancies. *Salmonella typhimurium* (*S typhimurium*), a facultative anaerobe, has been exploited as an anti-tumor agent that is capable of preferentially targeting tumors. The bacteria then amplify within the tumor and inhibit its growth^[9, 10]. Tumor targeting bacteria have been harnessed to deliver exogenous genes that facilitate anti-tumor effects^[11, 12]. To this end, our group has reported that *S typhimurium* can transfer plasmids expressing cytokines^[13] and prodrug converting enzymes^[14] across phylogenetic borders to mammalian cancer cells, thereby exerting anti-tumor effects. Given the tumor-targeting capacity of *S typhimurium*, we examined the ability of attenuated *Salmonella* to serve as an anti-cancer agent and a tumor-targeting vehicle that would deliver shRNA-expressing pDNA into tumor cells in a mouse model. The results indicated that attenuated *S typhimurium* SL3261 mediated the trans-kingdom gene transfer of shRNA-expressing pDNA into tumors to silence gene expression. This proof of principle study suggests that the combination of tumor-targeting and repressive actions by *S typhimurium*, as well as the therapeutic potential of RNA interference using *S typhimurium* carrying shRNA-expressing plasmids, can be exploited as a novel therapeutic approach for the treatment of cancers.

Materials and methods

Plasmids and bacteria

The plasmid pSuper (Oligoengine, Halo-Bio RNAi Therapeutics, Inc, USA) was used to express shRNA under the control of the H1 RNA polymerase gene promoter. The general strategy for constructing the RNAi plasmid involved subcloning an inverted repeat into pSuper. shRNA targeting green fluorescent protein (GFP) was designed using the online siRNA Selection Program (<http://jura.wi.mit.edu/bioc/siRNAext/reference.php>) according to Tuschl's siRNA user guide (<http://www.rockefeller.edu/labheads/tuschl/sirna.html>). Forward and reverse oligos for *gfp* were: (forward) 5'-GATCCC **GGAGCGCACCATCTTCTTC** TTCAAGAGA **GAA-GAAGATGGTGCGCTCC** TTTTGGAAA-3' and (reverse) 5'-AGCTTTTCCAAAA **GGAGCGCACCATCTTCTTC** TCTCTGAA **GAAGAAGATGGTGCGCTCC** GGG-3'. Oligos were annealed, cloned into pSUPER and designated as pGFPi. A similar method was used to construct the pscrGFP negative control that contained the same nucleotide composition as the *gfp* targeting shRNA but had no match to the *gfp* coding sequence. Two sequences were blasted against the mouse genome database and the EST database to exclude the possibility of off-target effects. Plasmids were transformed into *S typhimurium* LB5000 (LT2 Trp Met ErpsI flaA R⁻M⁺) and SL3261 (WARY hisG 46 aroA⁻ Del 407 Fusaricres R⁺M⁺) (provided by Bruce AD STOCKER, Stanford University) as previously described^[13].

Cell lines and mice

Mouse bladder transitional cancer cells (BTT-T739) were derived from BBN-(*N*-butyl-*N*-(4-hydroxybutyl) nitrosamine) exposed T739 mice^[15]. The GFP-expressing cell line BTT-T739-GFP was generously provided by Prof Qian WANG and Feng HUANG from the Central Experimental Laboratory of the First People's Hospital in Shanghai, China. Cells were cultured in RPMI-1640 medium (Gibco BRL) supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin and 10% FBS.

Four- to six-week-old female T739 mice were used for tumor growth models and infection studies. T739 adult mice were purchased from B&K Universal Ltd (Shanghai, China), and all animal experiments were performed according to the institutional animal care and use protocol.

Bacterial infection assay *in vitro*

BTT-T739-GFP cells were plated at a density of 5.0×10^5 cells in 12-well culture plates and incubated overnight without antibiotics. Bacteria in late log phase were washed and diluted in RPMI-1640 and then added to the BTT-T739-GFP cells (1.25×10^8 bacteria/well). After 3 h, the cells were washed and incubated in RPMI-1640 containing 100 units/mL of penicillin and 100 µg/mL of streptomycin until harvested.

Flow cytometry analysis

GFP expression was assessed using fluorescence activated cell sorting (FACS). Cultured cells were harvested, washed and resuspended in PBS. Ten thousand events per sample of cultured cells were captured. The geometric mean fluorescence was used as a measure of the relative intensity of green fluorescence.

Animal tumor model and *in vivo* bacterial tumor targeting assay

BTT-T739-GFP cells (5×10^5) were implanted subcutaneously in mice before bacterial administration. Treatments were initiated 15 d after tumor inoculation. Groups of 15–20 tumor-bearing mice were orally administrated with 1×10^9 cfu of *Salmonella* (SL3261/pGFPi or SL3261/pscrGFP) or saline. All mice were monitored for tumor growth and survival as previously described^[16, 17].

Groups of 3–5 mice were sacrificed to investigate bacteria distribution at various time points after bacteria treatment. Tumors, blood, livers and spleens from the mice were collected, weighed and homogenized in 2 mL of ice-cold sterile PBS. The homogenates were plated onto L broth (Sigma) agar plates containing ampicillin (100 µg/mL), and bacterial counts were performed. To assess GFP expression levels, tumor cells were filtered through 0.22 µm meshes, resuspended in PBS and processed as described above for cultured cells. Tumor samples from T739 mice were homogenized in liquid nitrogen and stored for future semi-qRT-PCR analysis.

Extraction of total RNA and DNA and semi-qRT-PCR assay

Total DNA and RNA from cultured cells and mouse tumors

were prepared using TRIzol reagent (Invitrogen), and cDNA was synthesized using oligo(dT) with the AccessQuick™ RT-PCR System (Promega). Total RNA (100 ng) was subjected to cDNA synthesis. The RT-PCR method was used to analyze GFP expression levels in different samples, and β -actin was used for normalization. Semi-qRT-PCR was performed as previously described^[18] using 100 ng of cDNA in a 25 μ L reaction volume. The expression level of each gene was determined by semi-qRT-PCR method. The primers for β -actin amplification were 5'-CCCAGAGCAAGAGAGGTATC-3' (forward) and 5'-GACCAGAGGCATACAGGGAC-3' (reverse). The primers used for GFP amplification were 5'-GAGCGACCA-TCTTCTC-3' (forward) and 5'-CGTCTAGATTACTTGTA-CAGCTCGTC-3' (reverse). β -actin and GFP were amplified using 27 and 35 cycles, respectively. The parameters for each cycle were 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. The amplified fragments were separated by 1% agarose gel electrophoresis. Images of the semi-qRT-PCR products stained with ethidium bromide were analyzed with Dolphin-1D software (WEALTEC Corp, Sparks, NV, USA). The band intensity of GFP was normalized to β -actin.

Statistical analysis

Statistical analysis was performed using the SPSS statistic software package (SPSS, Chicago, IL, USA). All results are expressed as means \pm SEM, and $P < 0.05$ was considered significant.

Results

Silencing of *gfp* expression by SL3261/pGFPi in tumor cells *in vitro*

To test whether shRNA-expressing pDNA can be delivered from *S typhimurium* to mammalian tumor cells and induce gene specific RNAi, BTT-T739-GFP cells were infected with SL3261/pGFPi, SL3261/pSUPER, and SL3261/pscrGFP. As shown in Figure 1A, different levels of green fluorescence protein (GFP) expression were detected in tumor cells infected with SL3261/pGFPi or SL3261/pscrGFP at 48 h after bacterial infection. GFP protein levels were analyzed by FACS at 72 or 96 h post-infection. At 96 h post-infection, GFP protein expression in the SL3261/pGFPi treated group was reduced by 40.2% and 33.9% compared to that of SL3261/pSUPER- and SL3261/pscrGFP-infected groups, respectively (Figure 1B). Collectively, these results demonstrate that eukaryotic shRNA-expressing plasmids can be transferred to, and expressed in, tumor cells using attenuated *S typhimurium* as a gene delivery vehicle.

Preferential accumulation of attenuated *S typhimurium* in tumors

To determine the localization of orally administered attenuated *S typhimurium* in BTT-739T-GFP tumor-bearing mice, we monitored bacterial burdens in the blood, liver, spleen and tumor at various time points after oral infection. As shown in Figure 2A, SL3261 (regardless of the plasmid it harbors) was detected in the blood, liver, spleen and tumor at 36 h after

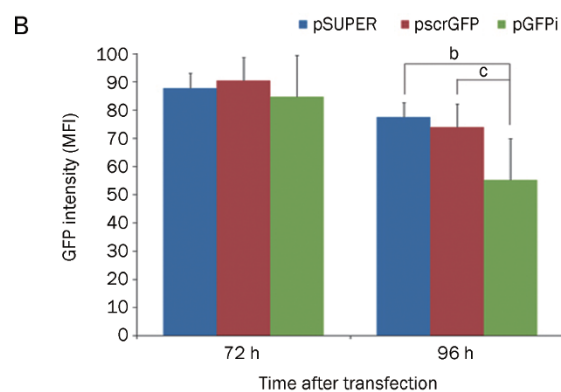
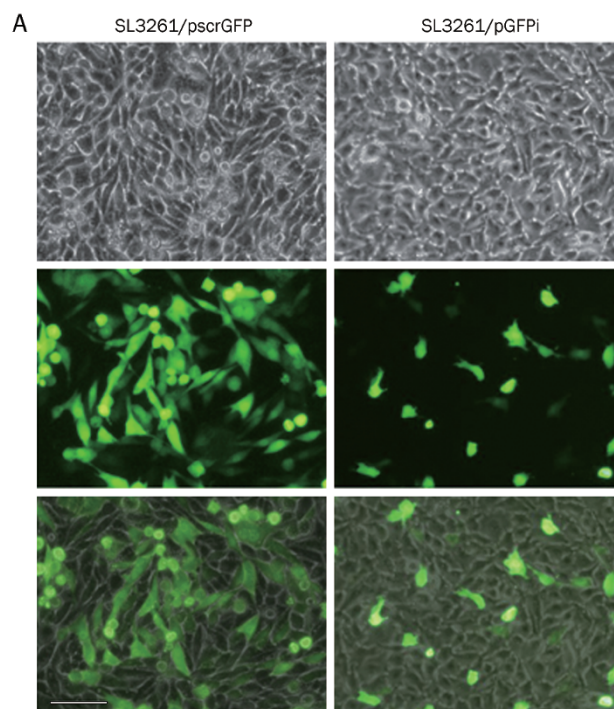


Figure 1. SL3261/pGFPi suppressed GFP expression in BTT-T739-GFP cells *in vitro*. (A) Different levels of GFP expression between SL3261/pGFPi and SL3261/pscrGFP treated groups were directly detected by fluorescence microscopy at 48 h following bacterial infection. The scale bar indicates 100 μ m. (B) Protein levels of GFP were analyzed by FACS at 72 and 96 h post-bacterial infection. The overall expression of GFP was reduced because the cultures deteriorated several days after bacterial infection. Notably, GFP expression was significantly decreased by SL3261/pGFPi compared with SL3261/pSUPER and SL3261/pscrGFP. ^b $P < 0.05$, ^c $P < 0.01$.

bacterial administration. By the 7th d, SL3261 had selectively accumulated within tumors, such that the tumor-to-normal tissue ratios were greater than 1000:1, and bacteria in the blood had been almost completely cleared. Notably, SL3261, while retained in the tumors, was undetectable in the spleens or livers of the mice by 21 d (Figure 2A, 2B). At 40 d after oral administration, bacteria were still present in tumors but were undetectable in the livers and spleens, as confirmed by

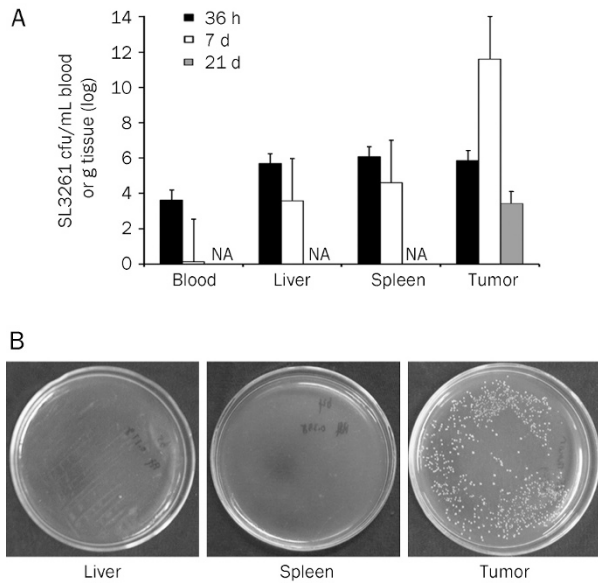


Figure 2. Preferential accumulation of *S typhimurium* in mouse bladder tumors. Tumor-bearing mice received the three different *Salmonella* treatments once tumors reached a predetermined size. (A) The amount of SL3261 in the tumors, livers and spleens was determined at 36 h, 7 d and 21 d post-infection. Data are shown as means±SEM. *n*=5. (B) Liver, spleen or tumor tissue (0.3 g each) was harvested from tumor-bearing mice on d 21 post-infection and homogenized and resuspended in 50 μ L PBS. The suspension was spread on LB agar plates containing ampicillin.

PCR using primer pairs specific to a region of *S typhimurium* genomic DNA (data not shown). Previous studies from our lab also investigated bacteria distribution in mouse lung, heart, brain and kidney. Bacteria was undetectable in the brain at 36 h after oral administration, and by 72 h the *Salmonella* were eliminated from all other organs (unpublished). Collectively, these results show that *S typhimurium*, when orally administered to mice bearing established tumors, preferentially accumulates in tumors and is retained for as long as 40 d.

Downregulation of *gfp* expression in mouse tumors treated with SL3261/pGFPi

We have shown that attenuated *S typhimurium* transferred shRNA-expressing plasmids to tumors and that shRNA was successfully expressed. Attenuated *S typhimurium* preferentially accumulated, and was retained in large amounts, within the tumors. Thus, we next examined *gfp* mRNA and protein levels in mice to determine whether attenuated *Salmonella* could deliver shRNA-expressing pDNA and induce RNAi in tumor cells *in vivo*. Compared to the SL3261/pscrGFP treated group, we observed considerably reduced *gfp* mRNA and protein expression in the tumors of SL3261/pGFPi treated mice at 5 d after bacteria administration. Gene silencing specificity was confirmed by the lack of change in β -actin expression. Representative results from this experiment are shown in Figure 3A, 3B. We next examined the presence of attenuated *S*

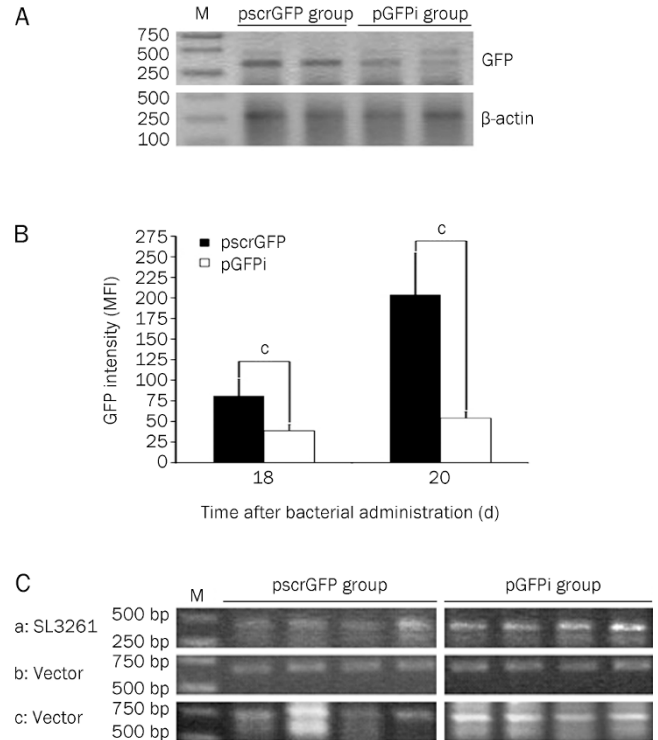


Figure 3. SL3261/pGFPi suppressed GFP expression in BTT-T739-GFP cells *in vivo*. Tumor cell suspensions were collected at 3 d and 5 d after bacterial treatment. GFP mRNA and protein levels were analyzed by semi-qRT-PCR (A) and FACS (B). Oral administration of SL3261/pGFPi significantly decreased GFP expression in tumor cells compared with SL3261/pscrGFP. (C) Dissociated tumor cells were treated with kanamycin to kill extracellular *Salmonella*, and the total DNA and RNA from tumor cells were extracted. Plasmid containing *Salmonella* was confirmed in tumor cells by PCR using *Salmonella* specific primers (a) and pSUPER specific primers (b). Tumor cell suspensions were dispersed onto ampicillin containing plates to confirm the presence of the different plasmids in the bacteria using colony PCR with pSUPER specific primers (c). $^{\circ}P<0.01$.

typhimurium and shRNA-expressing pDNA in tumor tissues. For this, tumors were dissected, and dissociated tumor cells were incubated with medium containing kanamycin to eliminate extracellular bacteria before extracting total DNA and performing PCR to detect SL3261 and pSUPER. As shown in Figure 3C (a, b), attenuated *S typhimurium* was localized in tumors, and shRNA-expressing pDNA was transferred into tumor cells 5 d after bacterial treatment. Previous studies have shown that plasmid instability may result in its loss from *Salmonella in vivo*. Therefore, we streaked the tumor cell suspension on LB agar plates and performed colony PCR using pSUPER specific primers. As shown in Figure 3C (c), *S typhimurium* in the tumor cells retained the shRNA-expressing plasmids. These results indicate that shRNA-expressing plasmids delivered by oral administration of attenuated *S typhimurium* can efficiently induce gene specific silencing in tumor cells.

Inhibition of tumor growth by *S typhimurium* oral administration

Despite its accumulation in diverse tumor subtypes, *Salmonella* has been shown to exhibit minimal, if any, impact on tumor growth^[19]. We next investigated whether SL3261 has therapeutic effects on bladder tumor progression in mice and whether the shRNA-expressing plasmids would interfere with its antitumor activity. Tumor volumes and survival time were examined in tumor-bearing mice treated with SL3261, SL3261/pGFPi, or SL3261/pscrGFP. SL3261 treatment significantly inhibited tumor growth compared to saline controls (Figure 4). SL3261/pGFPi and SL3261/pscrGFP exhibited antitumor effects similar to SL3261. This suggests that SL3261 can be used as both a shRNA-expressing pDNA delivery vehicle and an antitumor agent. The average tumor volume in the SL3261, SL3261/pGFPi, and SL3261/pscrGFP treated groups was significantly smaller than that in the saline control ($P < 0.001$) at d 30 and 35 post-tumor inoculation. We also recorded the survival rate of SL3261/pGFPi and SL3261/pscrGFP treated groups versus the saline treated group at different time points. SL3261/pGFPi and SL3261/pscrGFP treatment increased the life span by 35.7% ($P < 0.001$) compared to saline treatment, as determined by ED₅₀ (day at which 50% of the mice were dead). Gross adverse effects, such as loss of body weight, were observed during the experimental periods (data not shown). Different antitumor activities were not observed between SL3261, SL3261/pGFPi, and SL3261/pscrGFP treatments because the shRNA-expressing pDNA targets GFP rather than endogenous genes critical for tumor development. Collectively, these results suggest that oral administration of a shRNA-expressing plasmid containing *S typhimurium* can significantly suppress tumor growth and prolong the life span of tumor-bearing mice.

Discussion

We examined the potential of attenuated *S typhimurium* to be used as a gene transfer vehicle to mediate vector-based RNA interference in a mouse tumor model. We demonstrated that orally administered *S typhimurium* preferentially accumulated within tumors and mediated the trans-kingdom gene transfer of shRNA-expressing plasmids from bacteria to mammalian cells. The RNAi that was observed was presumably due to shRNAs produced from the plasmids.

In the past decade, there has been an increased interest in RNA interference, which is an evolutionarily ancient method of genome defense in many organisms. RNA interference is a widespread natural phenomenon that has the power to produce gene-specific inhibition. The sequence-mediated specificity and potency of RNAi make it a promising therapeutic strategy to treat diseases caused by aberrant gene expression. However, both siRNA and shRNA-expressing pDNA are negatively charged hydrophilic molecules that do not easily cross the cell membrane. Effective delivery of these molecules is perhaps the greatest challenge that needs to be overcome for RNAi to be translated to the clinic. Preclinical data have revealed multiple ways to deliver RNAi, including the injection of naked siRNAs into target organs, such as the lung

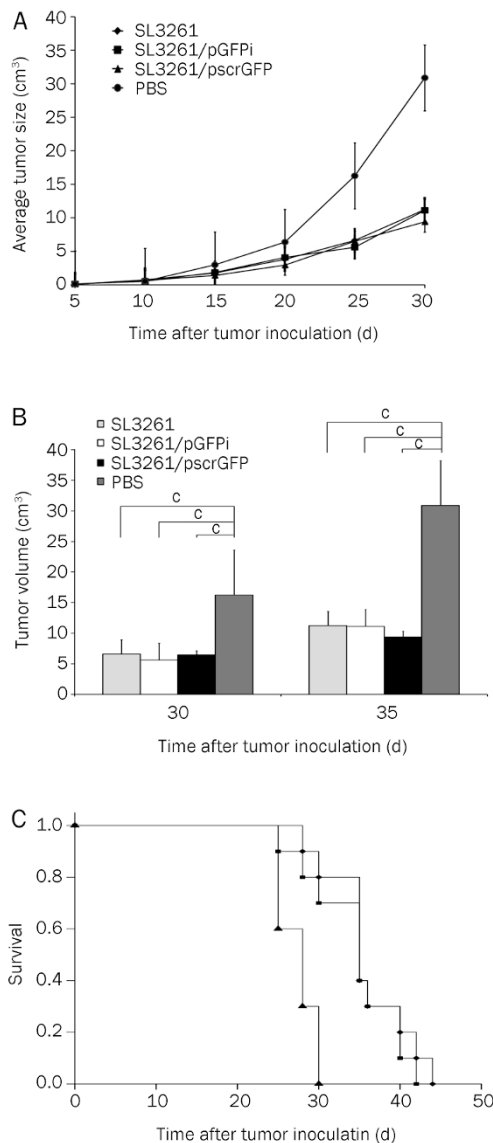


Figure 4. Antitumor effect of SL3261, SL3261/pGFPi, and SL3261/pscrGFP in the tumor-bearing mice. (A) Tumor growth curves of different treatment groups. On the 15th d after tumor inoculation, SL3261 (♦), SL3261/pGFPi (■), SL3261/pscrGFP (▲), or PBS (●) was orally administered. Tumor diameters were measured at a regular interval for more than 30 d, and the tumor volume was calculated. Results represent the mean \pm SEM ($n = 5-6$). (B) Comparison of tumor volumes on d 30 and 35. Compared to PBS, SL3261 alone, or SL3261 carrying either plasmid, exhibited an anti-tumor effect ($P < 0.01$). (C) Kaplan-Meier survival curves of tumor-bearing mice receiving the various treatments described in (A). SL3261/pGFPi or SL3261/pscrGFP treatment increased the life span by 35.7% ($P < 0.01$) compared to saline treatment, as evidenced by ED₅₀. ^c $P < 0.01$.

and eye, or the systemic delivery of siRNA in nanoparticles that are complexed to polycations, cholesterol groups or cell-surface receptors (reviewed by Daniela CASTANOTTO and John ROSSI^[20]). These methods are not suitable for delivering shRNA-expressing pDNA due to their size. The genes encod-

ing the hairpin structures are most commonly inserted into the backbones of viral vectors under the control of Pol II or Pol III promoters. Although some viruses are non-pathogenic, they are still potentially immunogenic and can cause insertional mutagenesis or trigger aberrant gene expression. A potential advantage of vector delivery is that a single treatment triggers long-term expression of the therapeutic RNAi. This is particularly appropriate for chronic viral diseases and makes it appealing to find a safer, yet efficient, way to deliver shRNA-expressing vectors to target cells.

The finding that genes of putative prokaryotic origins exist within the human genome strengthened the hypothesis that transfer of genomic information occurred during evolution. This suggested that bacteria could potentially be used to deliver DNA products into mammalian or human cells. The tumor may also provide an immunosuppressive environment that inhibits clearance of the bacteria. In addition to accumulating within tumors, attenuated *Salmonella* also exerts an inherent anti-tumor efficacy^[21-24]. The *Salmonella*-induced anti-tumor effect may be due to competition for nutrients between cancer cells and the bacteria and/or secretion of bacterial enzymes, such as proteases and other hydrolases. Additionally, apoptosis via the type III secretion system, as seen with macrophages *in vitro*, may also explain the anti-tumor efficacy caused by *Salmonella*^[25]. However, the precise mechanisms underlying the anti-tumor effects have not been fully elucidated.

Attenuated bacterial strains, such as *S typhimurium*^[26-28], *Clostridium*^[29], and *Bifidobacterium*^[30], preferentially accumulate in tumors because the hypoxic and necrotic areas in solid tumors are conducive to the proliferation of anaerobic bacteria. We previously used an attenuated *S typhimurium* SL3261 as a delivery vehicle to carry eukaryotic expression vectors encoding cytokines^[13] or pro-drug converting enzymes^[14] as anticancer agents. We have confirmed these findings and extended them to the use of *S typhimurium* as a tumor-targeted anticancer agent. Our results showed that *S typhimurium* delivered shRNA-expressing pDNA into tumor cells via systemic administration, and this resulted in effective induction of RNAi. Because *S typhimurium* preferentially colonized in tumors and survived for approximately 40 d after inoculation, a more sustained release of shRNA-expressing pDNA in tumor cells could potentially be achieved via *S typhimurium*-mediated gene transfer. Furthermore, *S typhimurium* has been genetically engineered to improve its ability to target tumors and to reduce toxicity^[24]. Because bacteria have served as a well-validated system in molecular biology and biotechnology and *Salmonella* grow well in an oxygen-rich environment, versatile RNAi vectors for therapeutic applications can be easily constructed, stored, and amplified in *S typhimurium*. Compared with synthetic siRNA, *S typhimurium*-mediated vector-based RNAi may result in a higher targeting specificity, a more sustained RNAi effect, and a more economical treatment method. Despite the fact that our shRNA was designed to target GFP, rather than an endogenous tumor-related gene, orally administered SL3261 carrying a pGFPi or pscrGFP plas-

mid showed antitumor effects. This indicates that synergistic antitumor effects may be achieved using *S typhimurium* to deliver shRNA-expressing pDNA targeting genes critical for tumor development. It has been 30 years since trans-kingdom gene transfer was first observed by Walter SCHAFFNER^[31], and extensive studies have examined the potential use of bacteria as a gene delivery method in tumor cells. However, little is known at the cellular level about the precise events involved in gene transfer between bacteria and target cells. Based on previous studies on the trans-kingdom gene transfer process (reviewed by Siegfried WEISS^[32,33], Christoph SCHOEN^[34] and John PAWELEK^[35]), it is known that *S typhimurium* containing shRNA-expressing vectors invade host cells, that the bacterial carriers are then lysed within the phagolysosomes, and that this results in the intraphagosomal release that transfers plasmid DNA into the cytosol. By a mechanism that is not fully understood^[36], these plasmids cross the vesicular membrane and reach the nucleus of the host cell where they are expressed. This process can occur with very high frequency.

In addition to intracellular bacteria, other researchers reported that *Escherichia coli* encoding siRNA can induce gene silencing in human colon cancer xenografts in mice^[37]. The plasmid used to make the siRNA was engineered to express the invasion gene *Inv* and the listeriolysin O gene *HlyA*, which are two bacterial factors that are essential for the transfer of siRNAs into mammalian cells. We had little problem with crosstalk between bacteria and mammalian cells because intracellular *S typhimurium* bacteria can circumvent host defense mechanisms. *S typhimurium* induce their own phagocytic uptake and avoid phagocyte-mediated death following internalization^[36]. The pDNA is released and can enter the nucleus following intracellular breakdown of bacterial carriers.

In conclusion, we demonstrated the potential of using *S typhimurium* to mediate the gene transfer of a shRNA-expressing vector for RNAi-based cancer therapy. The benefits of this system include tumor targeting and repression by *S typhimurium* and the therapeutic potential of RNA interference. Thus, *S typhimurium* that carry shRNA-expressing vectors that produce siRNA against genes critical in tumor development may be a promising approach for cancer treatment.

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Author contribution

Nan YANG, Sheng-hua LI, and Da-ming REN designed research; Nan YANG and Sheng-hua LI performed research; Li-shan CHEN and Yun-zhe LÜ contributed new analytical tools and reagents; Nan YANG and Sheng-hua LI analyzed data; and Nan YANG wrote the paper.

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