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# A *Salmonella* Typhimurium mutant strain capable of RNAi delivery

## Higher tumor-targeting and lower toxicity

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**Keywords:** *Salmonella* Typhimurium, RNA interference, delivering system, PhoP/PhoQ gene, anti-tumor, tumor-targeting, safety

**Abbreviations:** VNP20009, *S. Typhimurium* ATCC14028 (msbB<sup>-</sup>/purI<sup>-</sup>); VNP(PhoP/Q<sup>-</sup>), VNP20009 deleted phoP/phoQ gene; RNAi, RNA interference

Bacteria are highly versatile and useful tools that could deliver short interfering RNA. In this study, a phoP/phoQ double-deleted *Salmonella* Typhimurium named VNP(PhoP/Q<sup>-</sup>) based on the genetic background of VNP20009. The biological safety and function of VNP(PhoP/Q<sup>-</sup>) were also analyzed. Our study revealed the following results: (1) VNP(PhoP/Q<sup>-</sup>) exhibited lower titers in tumor-free livers and spleens than VNP20009, (2) The survival of VNP(PhoP/Q<sup>-</sup>) in macrophages and 4T1 tumor cells was significantly reduced compared with that of VNP20009, (3) The tumor-targeting ability of VNP(PhoP/Q<sup>-</sup>) was significantly enhanced compared with that of VNP20009, and the anticancer effects of VNP(pPhoP/Q<sup>-</sup>) and VNP20009 on tumor-bearing mice were similar, (4) VNP(PhoP/Q<sup>-</sup>) could release an shRNA-expressing plasmid and express the GFP reporter gene in tumor tissue. Therefore, VNP(PhoP/Q<sup>-</sup>) exhibited a better safety level in tumor-free mice and elicited an anti-tumor effect on tumor-bearing mice. Moreover, VNP(PhoP/Q<sup>-</sup>) could release an shRNA-expressing plasmid into the cytoplasm of host cells to silence targeted genes.

### Introduction

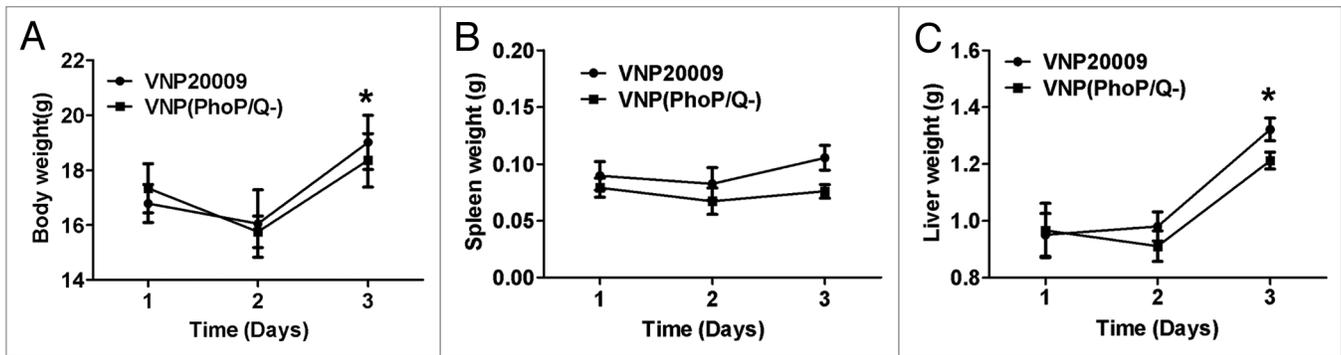
RNA interference (RNAi) has emerged as a powerful research tool that can silence particular target genes.<sup>1</sup> Moreover, the immense therapeutic ability of RNAi to treat cancers has been proposed.<sup>2</sup> Studies have demonstrated that RNAi can possibly target the degradation of a specific harmful RNA (point mutation, translocation, or overexpression); as a result, the proliferation or apoptosis of cancer cells can be inhibited.<sup>3-5</sup> However, advancements in RNAi technology for tumor therapy have been impeded by challenges involving the delivery of RNAi into the cytoplasm of cancer cells. Although viral vectors,<sup>6,7</sup> nanoparticles,<sup>8,9</sup> and liposomes<sup>10</sup> have been used to deliver RNAi, these delivery systems exhibit low security as well as target and silence the gene expressions of normal cells.

Facultative anaerobic *Salmonella* Typhimurium strains can preferentially accumulate in tumor tissues and elicit potential anti-tumor effects.<sup>11,12</sup> As a protein drug delivery system and shRNA-expressing plasmid carrier, *S. Typhimurium* significantly inhibits tumor growth and metastasis.<sup>13-15</sup> However, wild-type *S. Typhimurium* exhibits high toxicity. To reduce the possibility of lipopolysaccharide-induced septic shock in *Salmonella*-infected animals, researchers attenuated *S. Typhimurium* VNP20009 by genetic modification; as a result, purI and msbB genes are

deleted, thereby eliciting toxicity in mice and swine.<sup>16,17</sup> These mutants exhibit significantly reduced side effects, but tumor targeting, amplification, and growth suppression are still observed in mice. Clinical trials have also shown that VNP20009 can be used safely in humans.<sup>18</sup> As a drug delivery system, VNP20009 is used to express exogenous proteins (endostatin, TRAIL, and CCL21) to mediate anti-tumor effects because of its safety, replication, and high tumor specificity.<sup>19-21</sup> VNP20009 can survive and proliferate in host cells and it cannot or rarely release interference plasmids into the cytoplasm of host cells. So, VNP20009 is not suitable for use as a shRNA-expressing plasmid carrier for the purpose of gene silencing in mammalian cells because *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).<sup>22,23</sup> Preliminary studies showed that the phoP/phoQ locus modulates important virulence functions, such as the survival of bacteria in macrophages and resistance to endogenous antimicrobial peptides.<sup>24,25</sup> When deleted, the PhoP/PhoQ locus significantly reduces the survival of bacteria in macrophages and their virulence in BALB/c mice.<sup>26,27</sup> Strains with deleted PhoP/PhoQ have been used as an effective vaccine and shRNA-expressing plasmid delivery vehicles.<sup>28-31</sup> Considering the acceptable safety profile and anti-tumor effect of VNP20009,

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**Figure 1.** The toxicity effect of VNP(PhoP/Q<sup>-</sup>) infection in normal mice. Tumor-free mice were infected with VNP(PhoP/Q<sup>-</sup>) and VNP20009. Body weight, spleen weight and liver weight were determined at 1, 2 and 3 d post infection ( $n = 6$ ). (A) Body weight,  $*P < 0.05$ , body weight in mice of VNP(PhoP/Q<sup>-</sup>) or VNP20009 infection at 3 d post infection vs. 1 and 2 d post infection. (B) Spleen weight. (C) Liver weight,  $*P < 0.05$ , liver weight in mice of VNP(PhoP/Q<sup>-</sup>) or VNP20009 infection at 3 d post infection vs. 1 and 2 d post infection.

we aimed to delete PhoP/PhoQ based on the genetic background of VNP20009 to create a delivery carrier mediating shRNA plasmid expression with lower toxicity or better safety.

In our study, VNP(PhoP/Q<sup>-</sup>) was successfully engineered by red-mediated recombination mutagenesis and its biological characteristics were evaluated. Our results showed that VNP(PhoP/Q<sup>-</sup>) could be used as an RNAi delivery vector to target genes safely for cancer therapy.

## Results

### Toxic effect of VNP(PhoP/Q<sup>-</sup>) infection on normal mice

Body, liver, and spleen weights are major markers used to evaluate treatment-related toxicities in pre-clinical studies. Our results showed that the body, liver, and spleen weights of VNP(PhoP/Q<sup>-</sup>)- and VNP20009-infected mice at 2 d post-infection slightly decreased or remained unchanged compared with those at 1 d post-infection ( $P > 0.05$ ). The body and liver weights of VNP(PhoP/Q<sup>-</sup>)- and VNP20009-infected mice at 3 d post-infection were significantly enhanced compared with those at 1 d post-infection ( $P < 0.05$ ). However, VNP(PhoP/Q<sup>-</sup>) and VNP20009 infection did not result in any increase in spleen weight at 3 d post-infection ( $P > 0.05$ ). Compared with VNP20009 infection, VNP(PhoP/Q<sup>-</sup>) infection did not induce a significant change at 2 d post-infection ( $P > 0.05$ ). VNP(PhoP/Q<sup>-</sup>) infection significantly increased the liver and spleen weights compared with VNP20009 infection at 3 d post-infection ( $P < 0.05$ ) (Fig. 1A–C). The results suggested that phoP/phoQ deletion reduced the toxicity effect of VNP(PhoP/Q<sup>-</sup>) and further inhibited the swelling of the liver and the spleen.

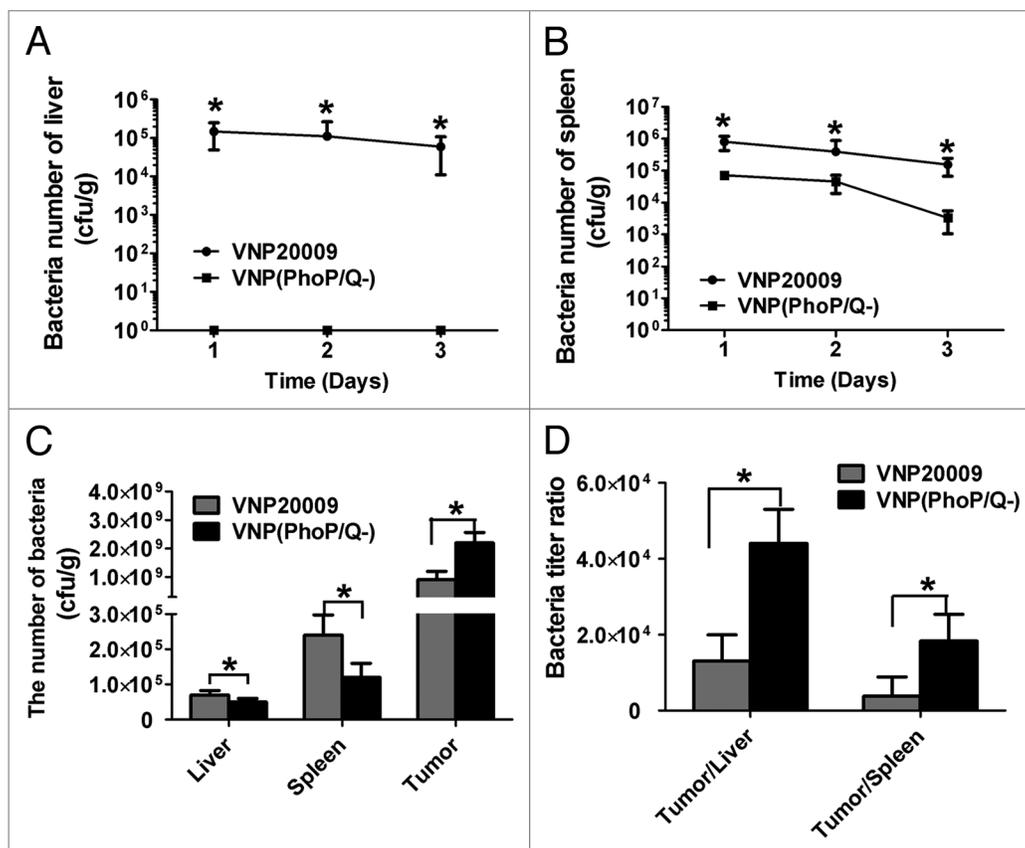
### VNP(PhoP/Q<sup>-</sup>) biodistribution in normal mice and tumor-bearing mice

To determine bacterial biodistribution, we inoculated VNP20009 and VNP(PhoP/Q<sup>-</sup>) in tumor-free and tumor-bearing mice. Tumor-free mice were sacrificed at 1, 2, and 3 d post-injection. The liver and the spleen were removed and then homogenized. VNP20009 and VNP(PhoP/Q<sup>-</sup>) were isolated. The results showed that the bacterial titers in the livers and the

spleens of mice infected with VNP20009 were significantly enhanced compared with those of mice infected with VNP(PhoP/Q<sup>-</sup>) at 1, 2, and 3 d post-injection ( $P < 0.05$ ) (Fig. 2A and B). Moreover, bacteria were observed in VNP(PhoP/Q<sup>-</sup>)-infected mice at 1, 2, and 3 d post-injection. In tumor-bearing mice, VNP20009 and VNP(PhoP/Q<sup>-</sup>) preferentially accumulated in tumors and reached  $1.0 \times 10^9$  cfu/g. Furthermore, the bacteria titers in the tumor tissues of mice infected with VNP(PhoP/Q<sup>-</sup>) were significantly enhanced compared with those of mice infected with VNP20009 ( $P < 0.05$ ) (Fig. 2C). In addition to tumor tissues, normal tissues, such as the spleen and the liver, were infiltrated with VNP20009 and VNP(PhoP/Q<sup>-</sup>) (Fig. 2C). However, the bacteria titers in the livers and the spleens of mice infected with VNP20009 were significantly lower than those of mice infected with VNP(PhoP/Q<sup>-</sup>) at 6 d post-injection ( $P < 0.05$ ) (Fig. 2C). The ratio of tumors in the liver to the tumors in the spleen of mice infected with VNP(PhoP/Q<sup>-</sup>) was significantly enhanced compared with that of tumor mice infected with VNP20009 ( $P < 0.05$ ) (Fig. 2D). In summary, VNP(PhoP/Q<sup>-</sup>) exhibited an excellent safety profile and a higher degree of tumor specificity than VNP20009.

### Immune cells of VNP(PhoP/Q<sup>-</sup>) infection in the liver and the spleen of normal mice

The above study showed that VNP20009 or VNP(PhoP/Q<sup>-</sup>) can multiply in the spleen and the liver of normal mice. To analyze the changes in immune cells during VNP20009 or VNP(PhoP/Q<sup>-</sup>) infection in the liver and the spleen of normal mice, we determined the percentage of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, macrophages, and granulocytes in the spleens and the livers at 1, 2, and 3 d post-injection by flow cytometry assay. The results showed that VNP20009 and VNP(PhoP/Q<sup>-</sup>) infection induced the infiltration of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, macrophages, and granulocytes; the peaks were reached at 2 d post-infection and the baseline levels were reduced at 3 d post-infection in the livers (Fig. 3A–D). The percentages of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, macrophages, and granulocytes in the livers of mice infected with VNP20009 or VNP(PhoP/Q<sup>-</sup>) did not statistically differ ( $P > 0.05$ ) (Fig. 3A–D). In the spleen, the percentages of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and macrophages



**Figure 2.** VNP(PhoP/Q<sup>-</sup>) biodistribution in normal mice and tumor-bearing mice. Tumor-free mice or tumor-bearing mice were inoculated with VNP20009 and VNP(PhoP/Q<sup>-</sup>) by intraperitoneal injection. Bacteria titer in liver and spleen of tumor-free mice were determined at 1, 2, and 3 d post-infection. Bacteria titer in liver, spleen, and tumor of tumor-free mice were determined at 6 d post-infection. (A and B) Bacteria titer in liver and spleen of tumor-free mice, \**P* < 0.05, VNP20009 vs. VNP(PhoP/Q<sup>-</sup>). (C) Bacteria titer of liver, spleen, and tumor in tumor-free mice with VNP20009 and VNP(PhoP/Q<sup>-</sup>) infection, \**P* < 0.05, VNP20009 vs. VNP(PhoP/Q<sup>-</sup>). (D) Bacteria titer ratio in tumor-free mice with VNP20009 and VNP(PhoP/Q<sup>-</sup>) infection, \**P* < 0.05, VNP20009 vs. VNP(PhoP/Q<sup>-</sup>).

increased with the duration of VNP20009 or VNP(phoP/Q<sup>-</sup>) infection in mice. Furthermore, the percentages of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and granulocytes in the spleens of mice infected with VNP20009 or VNP(PhoP/Q<sup>-</sup>) did not statistically differ at 1, 2, and 3 d post-injection (*P* > 0.05) (Fig. 3E, F, and H). The percentage of macrophages in the spleens of mice infected with VNP20009 or VNP(PhoP/Q<sup>-</sup>) did not statistically differ at 1 and 2 d post-injection (*P* > 0.05) (Fig. 3G). By contrast, the percentage of macrophages in the spleens of mice infected with VNP(PhoP/Q<sup>-</sup>) was significantly reduced than that of mice infected with VNP20009 at 3 d post-injection (*P* < 0.05) (Fig. 3G). In general, VNP20009 and VNP(PhoP/Q<sup>-</sup>) exhibited a similar level of biological safety, although the safety indexes of the latter were superior to those of the former.

#### Intracellular survival of VNP(PhoP/Q<sup>-</sup>) in vitro

PhoP/PhoQ participate in the intracellular survival of *S. Typhimurium*. We analyzed the intracellular survival ability of *S. Typhimurium* in vitro after PhoP and PhoQ genes were deleted. The results showed that the intracellular survival ability of VNP(PhoP/Q<sup>-</sup>) was significantly reduced than that of VNP20009 in RAW 264.7 macrophage and 4T1 tumor cells (*P* < 0.05) (Fig. 4A and B).

#### Antitumor effect of VNP(PhoP/Q<sup>-</sup>)

Our previous study showed that VNP20009 could significantly inhibit Lewis lung carcinoma model and B16F10 melanoma model by intraperitoneal injection. We further analyzed whether PhoP/PhoQ deletion could influence anti-tumor effect of VNP20009 in 4T1 breast cancer model. The results showed that VNP20009 or VNP(PhoP/Q<sup>-</sup>) significantly inhibited growth of 4T1 breast cancer than that of PBS (*P* < 0.05) (Fig. 5A). However, VNP20009 or VNP(PhoP/Q<sup>-</sup>) exhibited a similar anticancer effect (*P* > 0.05) (Fig. 5A). The tumor doubling time was significantly prolonged from 5.30 d (CI, 4.59 d to 6.25 d) in the PBS control group to 7.13 d (CI, 6.64 to 7.70 d) in VNP20009 group and 7.33 d (CI, 6.52 d to 8.38 d) in VNP(PhoP/Q<sup>-</sup>) group (*P* < 0.05) (Fig. 5B; Table 1). Tumor growth delay also significantly increased from 15.62 d (CI, 15.41 d to 15.91 d) in PBS control group to 22.07 d (CI, 21.51 d to 22.72 d) in VNP20009 group and 23.27 d (CI, 22.24 d to 24.60 d) in VNP(PhoP/Q<sup>-</sup>) group (*P* < 0.05) (Fig. 5C; Table 1).

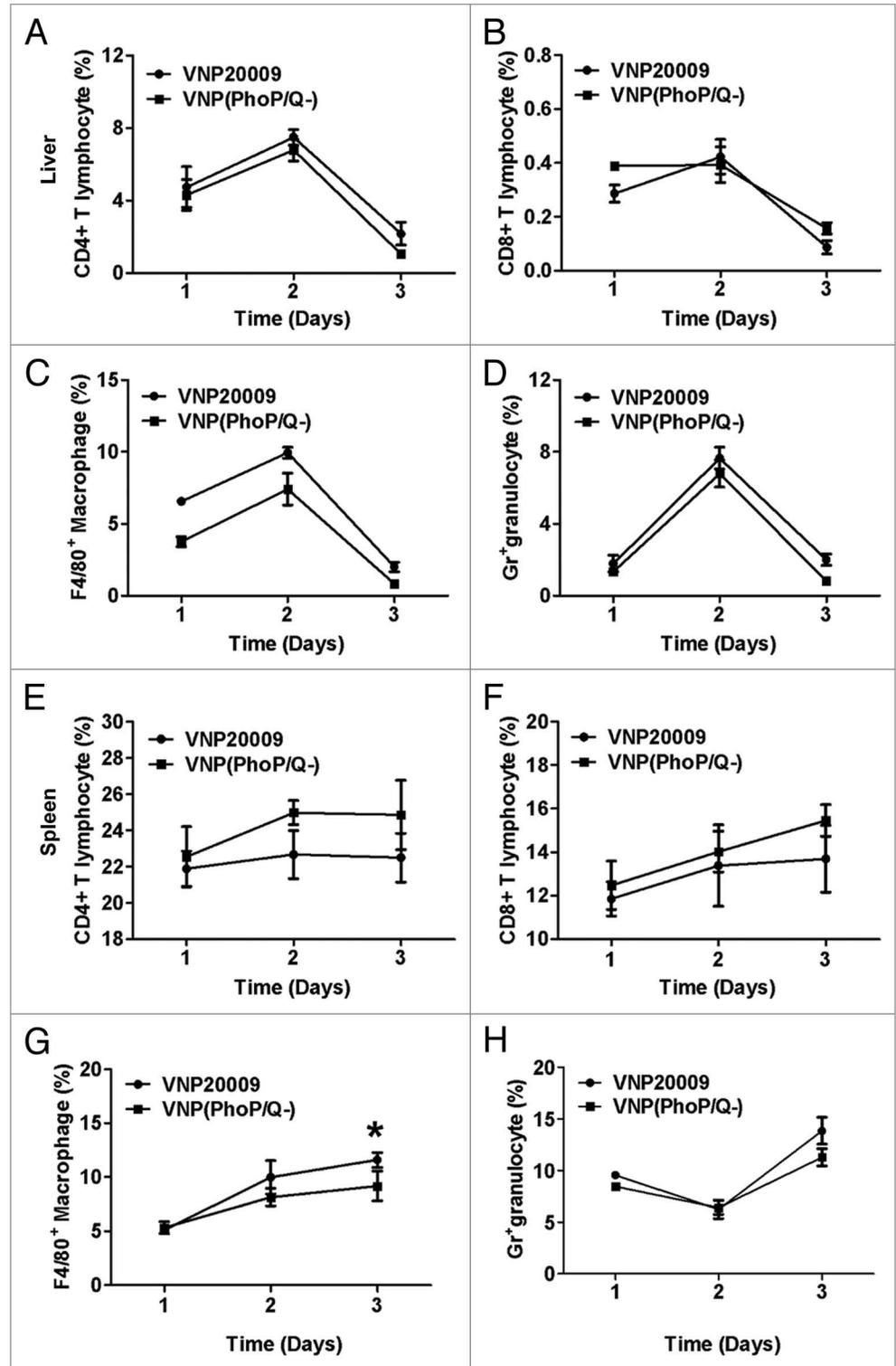
#### Effect of VNP(PhoP/Q<sup>-</sup>) on the released plasmid

We analyzed whether or not VNP(PhoP/Q<sup>-</sup>) can release a shRNA-expressing plasmid carrier into the cytoplasm of host cells to produce shRNA. The interference vector pRNA U6.1

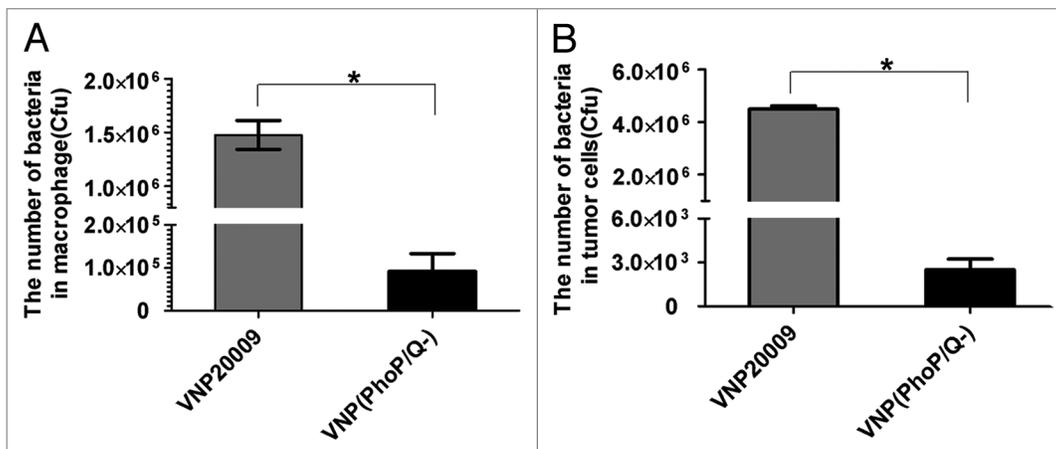
RNA (expresses EGFP) was targeted into the tumor tissue by VNP20009 and VNP(PhoP/Q<sup>-</sup>). EGFP expression indicated that the interference plasmid expression of shRNA was observed in the tumor cells. Immunofluorescence results at low-power lens also showed that the EGFP expression level in the tumor tissue of mice with VNP(phoP/Q<sup>-</sup>)-pRNA U6.1 RNA infection was significantly enhanced compared with that of VNP20009-pRNA U6.1 RNA infection (Fig. 6A). We further determined the cell that expresses the EGFP protein by co-localization. The results showed that EGFP protein was expressed by 4T1 tumor cells and macrophages (Fig. 6B and C). In summary, VNP(PhoP/Q<sup>-</sup>) could release shRNA-expressing plasmid into the cytoplasm of 4T1 tumor cells and macrophages to express shRNA.

## Discussion

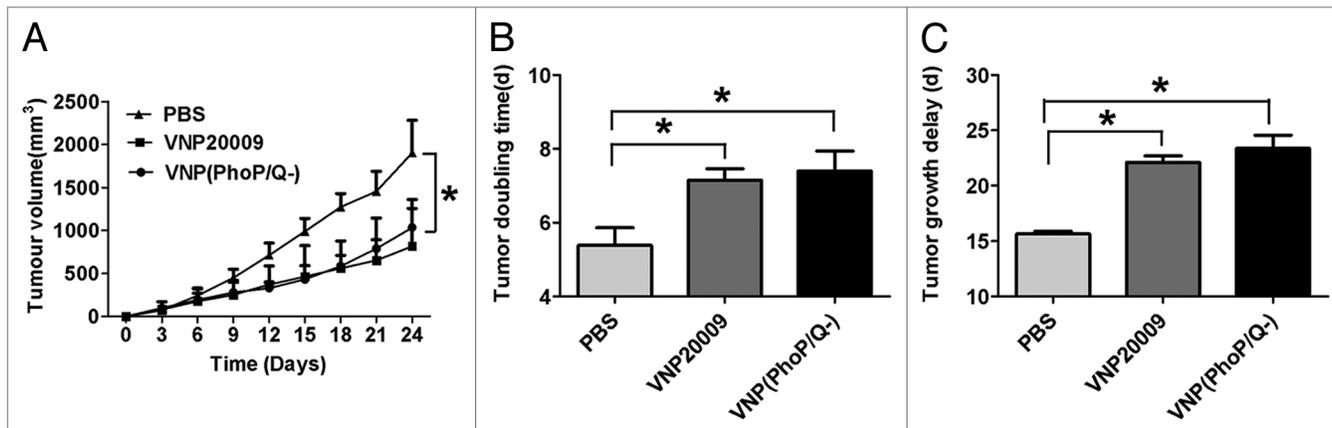
RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules.<sup>32,33</sup> As a therapeutic methods, RNAi has several advantages over traditional pharmaceutical approaches including high specificity and long gene silencing times (several days in vivo).<sup>34</sup> Although RNAi have enormous potential therapeutics function, to date, few clinical studies use this technology.<sup>35,36</sup> The reason is that the application of RNAi-based therapies in vivo has been hampered due to difficulties with delivery.<sup>37</sup> Recent, several strategies such as chemically modified siRNA, liposomes, nanoparticles and viral vectors were used to overcome delivery problem.<sup>38</sup> However, these methods were limited for expensive price and potential danger.



**Figure 3.** Subpopulation analysis of immune cells in liver and spleen of tumor-free mice. Tumor-free mice or tumor-bearing mice were inoculated with VNP20009 and VNP(PhoP/Q<sup>-</sup>) by intraperitoneal injection. Immune cells in liver and spleen of tumor free mice were determined at 1, 2, and 3 d post infection. (A) CD4<sup>+</sup> T cells in liver. (B) CD8<sup>+</sup> T cells in liver. (C) F4/80<sup>+</sup> macrophages in liver. (D) Gr<sup>+</sup> granulocytes in liver. (E) CD4<sup>+</sup> T cells in spleen. (F) CD8<sup>+</sup> T cells in spleen. (G) F4/80<sup>+</sup> macrophages in spleen. \**P* < 0.05, macrophage in mice of VNP20009 infection at 3 d post infection vs. VNP(phoP/Q<sup>-</sup>)infection at 3 d post infection. (H) Gr<sup>+</sup> granulocytes in spleen.



**Figure 4.** Intracellular survival of VNP(PhoP/Q<sup>-</sup>) in vitro. Raw 264.7 macrophages and tumor cells were incubated with VNP(PhoP/Q<sup>-</sup>) or VNP20009 for 1 h at 37 °C to permit phagocytosis. One hour after infection, gentamicin (25 μg/mL) was added to kill extracellular bacteria. After 3 h of culture at 37 °C, cells were lysed and the titer of bacteria was determined by counting colonies. **(A)** The number of bacteria in macrophage, \**P* < 0.05, VNP20009 vs. VNP(PhoP/Q<sup>-</sup>). **(B)** The number of bacteria in tumor, \**P* < 0.05, VNP20009 vs. VNP(PhoP/Q<sup>-</sup>).



**Figure 5.** Antitumor effect of VNP(PhoP/Q<sup>-</sup>). 4T1 breast cancer mice per group were injected i.p. with  $1 \times 10^4$  cfu of VNP20009 and VNP(PhoP/Q<sup>-</sup>), or with PBS at day 6 and 12. Tumor volumes among different groups were compared. Data are presented as mean  $\pm$  SD. **(A)** Tumor growth curves. \**P* < 0.05 for PBS vs. VNP20009 and VNP(PhoP/Q<sup>-</sup>). **(B)** Tumor doubling time, \**P* < 0.05 for PBS vs. VNP20009 and VNP(PhoP/Q<sup>-</sup>). **(C)** Growth delay time, \**P* < 0.05 for PBS vs. VNP20009 and VNP(PhoP/Q<sup>-</sup>).

**Table 1.** Regression analysis for treatment effects on tumor growth for treatment effects on survival

Group	Growth curves, $V(t)^a$	<i>r</i>	Tumor doubling time (d) <sup>b</sup>	Growth delay (d) <sup>c</sup>
PBS	$\ln(V) = 0.1309t + 4.8630$	0.8556	5.30 (4.59–6.25)	15.62 (15.41–15.91)
VNP(PhoP/Q <sup>-</sup> )	$\ln(V) = 0.0972t + 4.7626$	0.9627	7.13 (6.64–7.70)*	22.07 (21.51–22.72)*
VNP20009	$\ln(V) = 0.0945t + 4.7083$	0.8995	7.33 (6.52–8.38)*	23.27 (22.24–24.60)*

<sup>a</sup>Regression growth curves summarize volume (*V*, cm<sup>3</sup>) dependence on time (*t*, days) from initial treatment, with correlation coefficients indicated by *r*. <sup>b</sup>Tumor doubling time was derived from exponential growth curves. \**P* < 0.05 for PBS vs. VNP20009 and VNP(PhoP/Q<sup>-</sup>). <sup>c</sup>Growth delay was determined by assessing the time interval to 1000 mm<sup>3</sup>. \**P* < 0.05 for PBS vs. VNP20009 and VNP(PhoP/Q<sup>-</sup>).

In the study, we engineered VNP(PhoP/Q<sup>-</sup>), a genetically modified PhoP/PhoQ-deleted *S. Typhimurium* VNP20009, could release shRNA-expressing plasmid into the cytoplasm of host cells to produce shRNA. In contrast to viral vectors, VNP(PhoP/Q<sup>-</sup>) does not integrate the genetic material into the host genome and can be controlled by antibiotics.<sup>39,40</sup> Moreover, facultative anaerobic *S. Typhimurium* strains can preferentially accumulate and replicate in the hypoxic regions of solid

tumors.<sup>12,41,42</sup> This property provides the pre-conditions for VNP(PhoP/Q<sup>-</sup>) to deliver the fragment of RNAi to mediate anti-tumor effects.

As a RNAi delivery vector, VNP20009 should release RNAi plasmid into host cells to express shRNA. As an intracellular bacterial pathogen, VNP20009 can survive and proliferate in host cells. Therefore, VNP20009 cannot or rarely release interference plasmids into the cytoplasm of host cells. A previous

study showed that PhoP/PhoQ is required for virulence; if *phoP/phoQ* is deleted, *S. Typhimurium* poorly survives in macrophages.<sup>26,43</sup> Therefore, we deleted the *phoP/phoQ* genes of VNP20009 to construct VNP(*phoP/Q*<sup>-</sup>). Our study showed that VNP(*phoP/Q*<sup>-</sup>) strain not only has reduced survival rate in macrophages but also had decreased survival rate in tumor cells.

Studies have focused on the safety of VNP(*PhoP/Q*<sup>-</sup>). Although VNP20009 has shown efficient biological safety, whether or not *PhoP/PhoQ* knockout likely affects safety should be verified. Our study showed that VNP(*PhoP/Q*<sup>-</sup>) yielded lower titers in the livers and the spleens of tumor-free mice than VNP20009 at 1, 2, and 3 d post-injection. Moreover, the body weight, liver weight, spleen weight, and immune cell infiltration of mice infected with VNP(*PhoP/Q*<sup>-</sup>) and VNP20009 did not statistically differ at 1 and 2 d post-injection. Hence, the lysis of VNP(*PhoP/Q*<sup>-</sup>) in host cells induced inflammation and caused immune cell infiltration in the livers and the spleens. With the duration of infection time, inflammation in mice infected with VNP(*PhoP/Q*<sup>-</sup>) gradually decreased. In the spleen, immune cells, such as macrophages, were significantly reduced than those in mice infected with VNP20009.

In addition, our study showed that the bacterial titers in the tumor tissues of mice infected with VNP(*PhoP/Q*<sup>-</sup>) were significantly enhanced compared with those of mice infected with VNP20009. However, the study found that VNP20009 and VNP(*PhoP/Q*<sup>-</sup>) exhibited similar anti-cancer effects. This phenomenon was also observed in other gene knockout bacteria (unpublished data). Therefore, anti-tumor effect was possibly not correlated with bacterial tumor targeting. However, a higher tumor specificity of VNP(*PhoP/Q*<sup>-</sup>) possibly induced numerous bacteria to infect host cells and release interference plasmids into host cells.

VNP(*PhoP/Q*<sup>-</sup>) as RNAi delivery vehicle has several key advantages. First, VNP(*PhoP/Q*<sup>-</sup>) can be delivered by intravenous (iv), intraperitoneal (ip), or intratumoral (it) injection as VNP20009. Second, VNP(*PhoP/Q*<sup>-</sup>) preferentially accumulate to five times greater within the tumor as compared with VNP20009. This allows for the potential of delivering high levels of RNA interference plasmids and avoid systemic toxicity. Third, VNP(*PhoP/Q*<sup>-</sup>) as *Salmonella* mutation, it can target a broad range of solid tumors such as melanoma, breast and lung cancer. Fourth, VNP(*PhoP/Q*<sup>-</sup>) can continuously replicate and produce RNAi plasmids. Fifth, VNP(*PhoP/Q*<sup>-</sup>) exhibit better safety

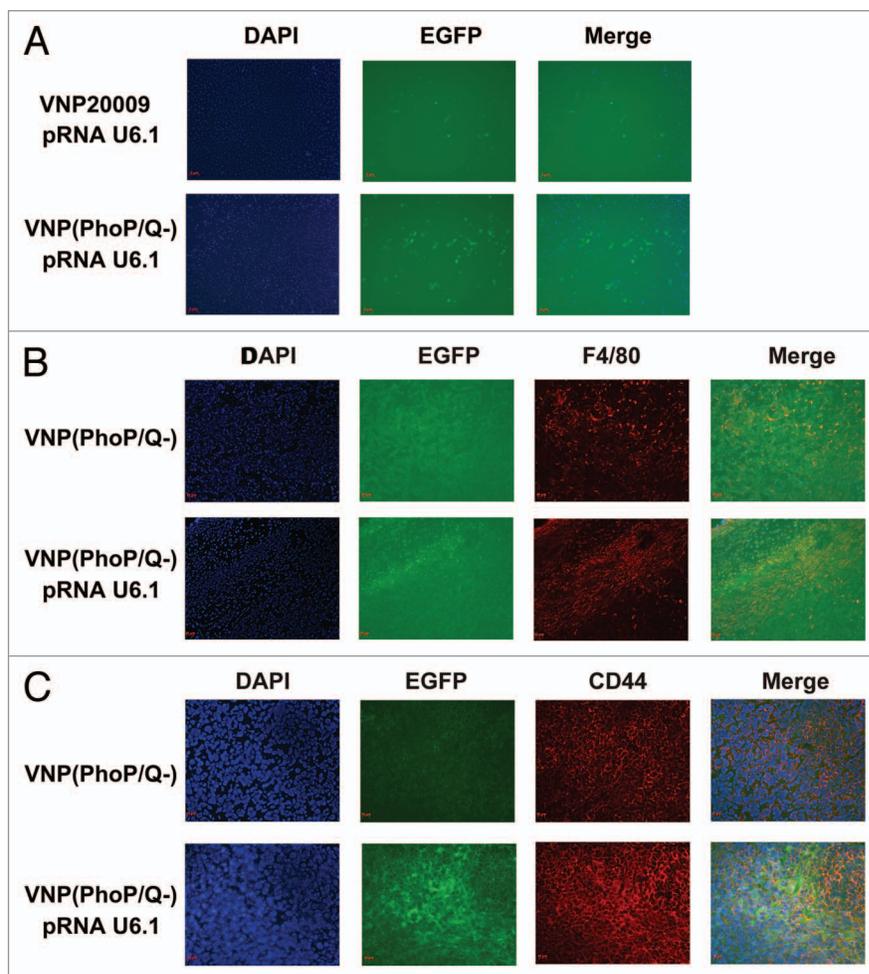
than VNP20009. Sixth, VNP(*PhoP/Q*<sup>-</sup>) does not integrate the genetic material into the host genome and can be allow cessation of treatment or post-treatment elimination by antibiotics.

In summary, VNP(*PhoP/Q*<sup>-</sup>) is a safe delivery system of RNA interference vector. Moreover, this delivery system could be used to target shRNA in tumor cells and TAMs to inhibit tumor directly or indirectly.

## Materials and Methods

### Animals, cell lines, gene, plasmids, and bacterial strains

Six-week-old male Balb/c mice were obtained from the Comparative Medicine Center of Yangzhou University and maintained in pathogen-free conditions for one week before the start of the experiment. Raw 264.7 macrophage cells were purchased from American Type Culture Collection (ATCC). Cell



**Figure 6.** Releasing plasmid effect of VNP(*PhoP/Q*<sup>-</sup>). 4T1 breast cancer mice per group were injected i.p. with  $1 \times 10^4$  cfu of VNP20009 and VNP(*PhoP/Q*<sup>-</sup>) bearing pRNAU6.1 RNA interference vector. Six days post-treatment, frozen tumor sections were prepared and EGFP expression in tumor tissues was analyzed. EGFP expression indicated the shRNA expression ability of interference plasmid in tumor cells. (A) EGFP expression in tumor tissue. (B) EGFP expression in macrophages of tumor tissues. (C) EGFP expression in 4T1 tumor cells of tumor tissues.

lines were cultured at 37 °C in 5% CO<sub>2</sub> in a humidified atmosphere in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS). 4T1-Luciferase murine breast cancer cells were purchased from Xenogen. Cell lines were cultured at 37 °C in 5% CO<sub>2</sub> in a humidified atmosphere in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS). pRNA U6.1 RNA interference vector were purchased from GenScript Corporation. *S. Typhimurium* VNP20009 (msbB<sup>-</sup>/purI<sup>-</sup>) (strain YS1646) was purchased from ATCC and cultured in modified Luria-Bertani (LB) media at 37 °C. Restriction enzyme and DNA polymerases were purchased TAKARA (China).

#### Construction of VNP(PhoP/Q<sup>-</sup>)

Construction of VNP(PhoP/Q<sup>-</sup>) were described by Datsenko et al.<sup>44</sup> Briefly, the phoP/phoQ genes on the *S. Typhimurium* chromosome were replaced with PCR generated kanamycin resistance cassettes. The antibiotic resistance cassette was amplified by PC-R from pkd4 plasmid with primers:

PhoP-F: 5'-CACCATAATC AACGCTAGAC  
TGTTCTTATT GTTAACACAA GGGAGAAGAG  
GTGTAGGCT GGAGCTGCTT C-3',  
phoQ-R: 5'-CGATTATAAC GGATGCTTAA  
CGAGATGCGT GGAAGAACGC ACAGAAATGT  
CATATGAATA TCCTCCTTAG-3'. At the 5' end of either

primer contains a 40–50 base-long extension with homology to the PhoP/PhoQ gene. The PCR products were used to replace the coding sequence of the PhoP/PhoQ genes on chromosome using the Lambda Red recombination system.

#### Construction of tumor model

4T1 breast cancer cells, which had been grown in RPMI medium 1640 plus 10% FBS, were collected and suspended in PBS (pH 7.4). To obtain primary tumor model, 2 × 10<sup>5</sup> 4T1 cells dispersed in 100 μL of PBS was injected subcutaneously into the hind flank region of the Balb/c mice. The tumors were allowed to grow. Animal studies designed to maintain a high standard of animal welfare were approved by the Nanjing University Animal Care and Use Committee.

#### Bacteria biodistribution in tumor free and tumor bearing mice

To determine bacterial biodistribution, tumor free mice or tumor bearing mice were inoculated with VNP20009 and VNP(PhoP/Q<sup>-</sup>) by intraperitoneal injection. Tumor free mice were sacrificed after 1, 2 and 3 d and tumor bearing mice were sacrificed after 6 d. The tissue samples (liver and spleen or tumor) were weighed and homogenized in a known volume of PBS and plated on LB agar (without salt) by serial dilution. After 16 h of culture at 37 °C, the titer of bacteria was determined by counting colonies and dividing them by the weight of the tissue (colony forming unit [cfu]/g tissue).

#### Flow cytometry analysis

Tumor-free mice were inoculated with VNP20009 and VNP(PhoP/Q<sup>-</sup>) by intraperitoneal injection. Mice were sacrificed after 1, 2, and 3 d. Liver and spleens were collected post-infection for subpopulation analysis of immune cells. Briefly, the livers were digested by collagenase IV and the spleens were ground. The single cell suspension were obtained using a 40-mL

cell strainer (BD Falcon), deleted red blood cells by Red Blood Cell Lysing Buffer (Sigma), and followed by washing twice in incomplete RPMI 1640 medium. To determine the percentage of immune cells in liver and spleen tissue, 10<sup>6</sup> cells resuspended in 100 μL phosphate-buffered saline (PBS) containing 1% FCS and stained for 30 min at 4 °C by using appropriate isotype controls and phycoerythrin-conjugated anti-CD4, anti-CD8, anti-F4/80, FITC-conjugated anti-Gr (BD Biosciences/PharMingen). Samples were incubated in dark for 45 min. After washing in PBS, the cells were analyzed on a FACSCalibur using CELLQUEST software (BD Biosciences).

#### Survival ability assay in macrophage and tumor cell

Intracellular survival of bacteria was measured by the method of Buchmeier and Heffron. Briefly, 1 × 10<sup>6</sup> Raw 264.7 macrophages and tumor cells in RPMI plus 5% FCS containing no antibiotics were allowed to adhere to each well of a 6-well plate. VNP20009 and VNP(PhoP/Q<sup>-</sup>) were added to each well at a ratio of 10 bacteria per macrophage and tumor cells. The cells were incubated for 1 h at 37 °C to permit phagocytosis, and the free bacteria were removed by four washes with PBS. RPMI plus 5% FCS plus gentamicin (25 μg/mL) (control extracellular bacteria) was added, and the cells were incubated at 37 °C. Wells were analyzed at 3 h after infection by removing the medium, lysing the macrophages and tumor cells with 0.5 mL PBS containing 0.1% Triton X-100, rinsing each well with 0.5 mL of PBS, and plated on LB agar (without salt) by serial dilution. After 16 h of culture at 37 °C, the titer of bacteria was determined by counting colonies.

#### Anti-tumor assay in vivo

4T1 breast cancer mice with a tumor volume of 150 mm<sup>3</sup> were randomly divided into three groups: PBS, VNP20009 and VNP(PhoP/Q<sup>-</sup>). The mice in the PBS group were injected intraperitoneally with 100 μL of PBS per mouse, whereas the mice in the VNP20009 and VNP(PhoP/Q<sup>-</sup>) were injected intraperitoneally with 1 × 10<sup>5</sup> cfu VNP20009 and VNP(PhoP/Q<sup>-</sup>) per mouse, respectively. After 6 d, the mice of the four groups were repeatedly treated with same treatment regime. The length and width of the tumor were measured every two days using a Vernier caliper (Mytutoyo Co.) across its two perpendicular diameters. Tumor volume was calculated using the following formula: tumor volume = length × width<sup>2</sup> × 0.52. The numbers and dates of death of mice were recorded to calculate the survival rate.

#### Immunofluorescence

4T1 breast cancer mice with a tumor volume of 150 mm<sup>3</sup> were randomly divided into two groups. One group mice were injected intraperitoneally with 100 μL of VNP20009 bearing pRNA U6.1 RNA interference vector. Other group mice were injected intraperitoneally with 100 μL of VNP(PhoP/Q<sup>-</sup>) bearing pRNA U6.1 RNA interference vector. After treatment for 6 d, mice in two groups were sacrificed. Frozen tumor sections were prepared using standardized procedure. To analyzed EGFP expression of 4T1 tumor cells, 4T1 tumor cells were stained with rat anti-mouse CD44 (BD Pharmingen<sup>TM</sup>). The secondary antibodies were goat anti-rat IgG labeled with Cy3 (Jackson ImmunoResearch). The nuclear of cells were stained by DAPI.

EGFP expression indicated interference plasmid expression shRNA ability in tumor cells.

### Statistical analysis

The results are presented as mean  $\pm$  SD. Data were assessed using the SPSS 13.0 software. Statistical significance for comparisons among two or three groups was analyzed using the Student *t* test or one way ANOVA, respectively. The level of significance was set at  $P < 0.05$ .

### Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflict of interest.

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