

## Priority Report

Genetically Engineered *Salmonella typhimurium* as an Imageable Therapeutic Probe for CancerVu H. Nguyen<sup>1</sup>, Hyung-Seok Kim<sup>2</sup>, Jung-Min Ha<sup>1</sup>, Yeongjin Hong<sup>3</sup>, Hyon E. Choy<sup>3</sup>, and Jung-Joon Min<sup>1,3,4</sup>

## Abstract

Tumor-targeting bacteria have been studied in terms of their ability to visualize the infection pathway (through imaging probes) or to carry therapeutic molecules to tumors. To integrate these monitoring and therapeutic functions, we engineered attenuated *Salmonella typhimurium* defective in guanosine 5'-diphosphate-3'-diphosphate synthesis to carry cytotoxic proteins (cytolysin A) and express reporter genes. We successfully visualized the therapeutic process with these engineered bacteria in mice and found that they often mediated complete tumor (CT-26) eradication on cytotoxic gene induction. Furthermore, treatment with the engineered bacteria markedly suppressed metastatic tumor growth. *Cancer Res*; 70(1): 18–23. ©2010 AACR.

## Introduction

Attenuated *Salmonella typhimurium* reportedly has a propensity to naturally accumulate and replicate in a wide variety of solid tumors (1). For cancer therapy, attenuated *S. typhimurium* has been applied alone (2) or in combination with conventional therapeutics (3) and has also been manipulated to deliver therapeutic molecules (4). *S. typhimurium* bioengineered to generate bioluminescence (5, 6) or fluorescence (7) signals has been used to monitor bacterial migration to tumors in living small animal models, which may facilitate the prediction of the therapeutic efficacy associated with bacteriotherapy, through visualization of bacterial accumulation and replication in specific organs.

Here, we engineered attenuated *S. typhimurium* defective in guanosine 5'-diphosphate-3'-diphosphate (ppGpp) synthesis ( $\Delta$ ppGpp strain) to express both the cytotoxic protein cytolysin A (ClyA) to kill tumor cells and the bacterial luciferase gene *lux* to generate imaging signals. ClyA is a 34-kDa pore-forming hemolytic protein found in *E. coli* and *Salmonella enterica* serovars Typhi and Paratyphi A (8). A major problem for the full exploitation of cytotoxin-expressing *Salmonella* in cancer treatment is its toxicity in nontumoral reticuloendothelial organs, mainly the liver and spleen, due to their initial localization in these organs after tail vein injection (6, 9). Constitutive cytotoxin expression would thus inevitably re-

sult in hepatic or splenic injury. Therefore, for tumor-specific therapy with reduced toxicity in normal organs, it is necessary to use inducible promoter systems. Here, we used the  $P_{BAD}$  promoter from the *E. coli* arabinose operon, which can be activated by the sugar L-arabinose.

## Materials and Methods

**Bacterial strains.**  $\Delta$ ppGpp *S. typhimurium*, SHJ2037 (*relA::cat*, *spoT::kan*), has been previously described (10). Bacterial doses were  $4.5 \times 10^7$  colony-forming units per mouse suspended in 100  $\mu$ L of PBS.

**Cell lines.** The murine CT-26 colon carcinoma and Hep3B2.1-7 human hepatocellular carcinoma cell lines were obtained from the American Type Culture Collection (CRL-2638, HB-8064). The cells were grown in high-glucose DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin.

**Plasmids.** The expression plasmid pBAD-RLuc8 has been previously described (11). The *clyA* gene was amplified with 5'-AGTCCATGGTTATGACCGGAATATTTGC-3' (forward primer) and 5'-GATGTTTAACTCAGACGTCAGGAACCTC-3' (reverse primer) using the *S. typhi* genomic DNA as a template (12). Amplified DNA was cut by *NcoI* and *PmeI* and used to directly replace RLuc8 at the same site in pBAD-RLuc8.

**Optical bioluminescence imaging.** Bioluminescence imaging was done as previously described (13) using IVIS 100 (Caliper).

**Animal models.** Five- to six-week-old male BALB/c and BALB/c athymic nu<sup>-</sup>/nu<sup>-</sup> mice (20–30 g body weight) were purchased from the Orient Company (Korea). All animal care, experiments, and euthanasia were done in accordance with protocols approved by the Chonnam National University Animal Research Committee. Cells ( $10^6$ ) were harvested and suspended in 100  $\mu$ L of PBS and injected s.c. into the right thigh of each mouse. Tumor volumes (mm<sup>3</sup>) were estimated with calipers every 4 d after tumor cell injection (6).

**Western blot analysis.** Homogenized samples, standardized for protein content at 180  $\mu$ g, were subjected to SDS-PAGE on (12%) linear gradient gels and subsequently

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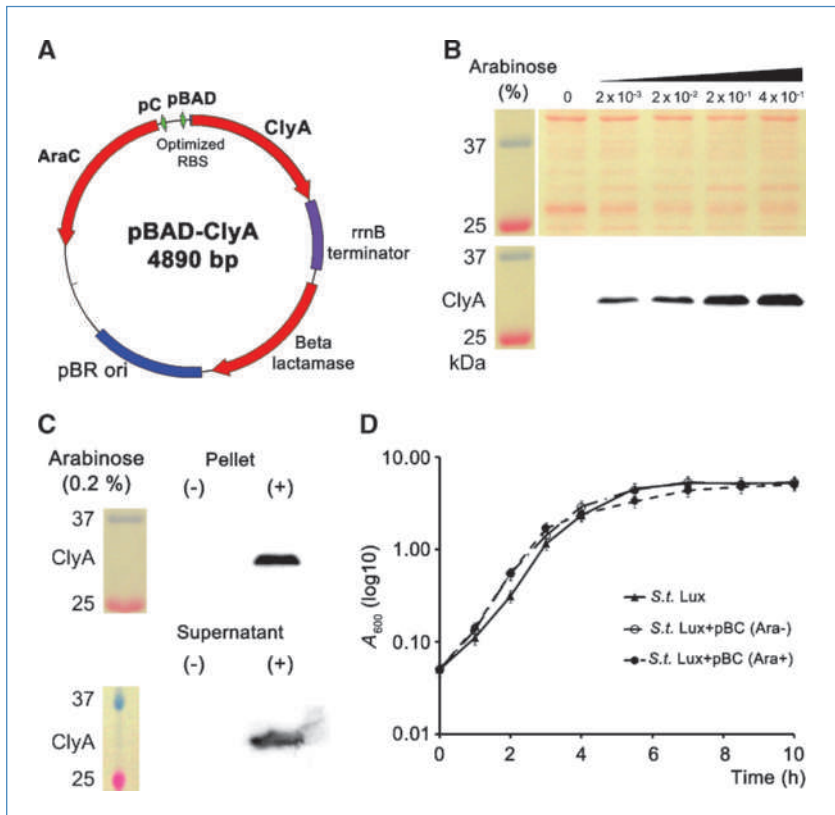
**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-09-3453

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**Figure 1.** L-Arabinose–induced ClyA expression by ppGpp-defective *S. typhimurium*. **A**, map of the bacterial expression plasmid containing ClyA (pBAD-ClyA). **B**, the ppGpp-defective *S. typhimurium* strain was transformed with pBAD-ClyA. Various concentrations (0–0.4%) of L-arabinose were added to fresh bacterial cultures (1 h) at  $A_{600}$  of 0.5 to 0.7. After 3 h of incubation,  $\sim 4 \times 10^7$  colony-forming units of bacteria were collected for immunoblot analysis with an anti-ClyA antibody. Coomassie staining indicated equal loading of bacteria in each well. **C**, expression and secretion of ClyA protein (34 kDa) was verified by Western blot analysis with an anti-ClyA antibody. Bacterial pellet (top) and culture medium (bottom) were collected 3 h after induction with or without L-arabinose (0.2%). **D**, Salmonella growth was assessed in untransformed *S. typhimurium* (*S.t.Lux*) or in transformed *S. typhimurium* without [*S.t.Lux*+pBC (*Ara*-)] or with [*S.t.Lux*+pBC (*Ara*+)] induction of ClyA expression. To induce the pBAD system in cultured bacteria, 0.2% L-arabinose was added to the cultures at mid-log phase (2 h after starting culture). The  $A_{600}$  was determined every 1 or 1.5 h for 10 h.



transferred to nitrocellulose membranes (Bio-Rad). To analyze ClyA, the membrane was probed with rabbit anti-ClyA antibody (1:250 dilution), horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000 dilution), and luminol reagent (Santa Cruz).

**Statistical analyses.** Two-tailed Student's *t* tests were used to determine the statistical significance of the primary tumor growth difference between the control and treatment groups. In the case of metastasis scores, the statistical significance was determined by Mann-Whitney *U* tests. The survival analysis was done using the Kaplan-Meier curve and log-rank test.  $P < 0.05$  was considered significant for all analyses. All data are expressed as mean  $\pm$  SD.

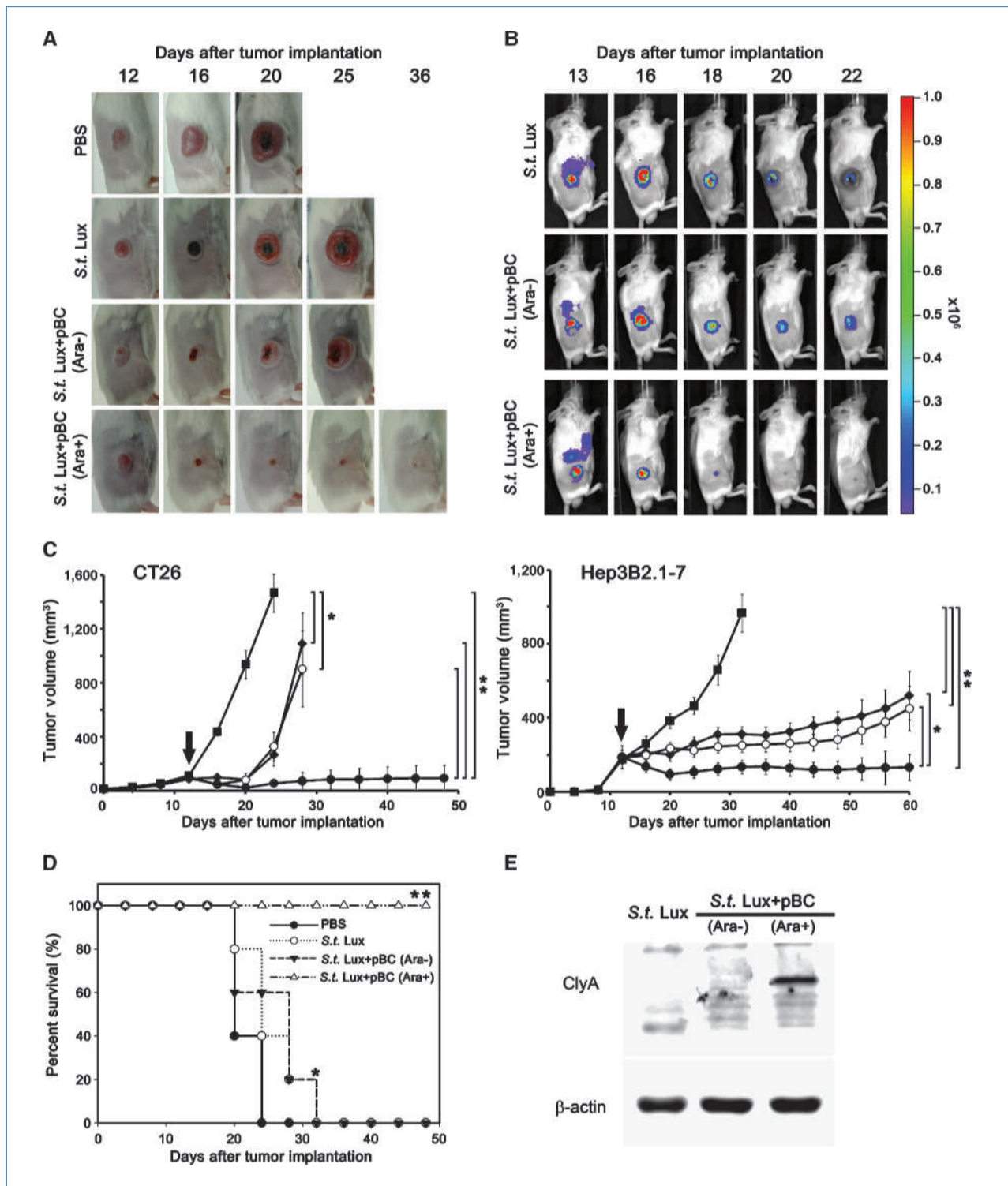
## Results and Discussion

To verify that remote control of the bacterial gene expression is possible, a bacterial expression plasmid was constructed in which a *Renilla* luciferase variant (RLuc8) was placed under the control of the  $P_{BAD}$  promoter (pBAD-RLuc8; Supplementary Fig. S1A). The mutant *S. typhimurium*  $\Delta$ ppGpp strain was electrotransformed with the pBAD-RLuc8 vector. Western blot analysis revealed that the intensity of RLuc8 expression (36.9 kDa) was directly proportional to the concentration of inducer, L-arabinose (0–0.4%), added to the cultured bacterial cells. RLuc8 was not detected in the absence of L-arabinose (Supplementary Fig. S1B and C). We i.v. adminis-

tered the *S. typhimurium*  $\Delta$ ppGpp strain carrying pBAD-RLuc8 into immunocompetent BALB/c mice bearing CT-26 ( $n = 5$ ). Because our previous observation (9) revealed that bacteria are cleared from the liver and spleen, and that they proliferate in tumors at 4 days post-inoculum (dpi), we induced RLuc8 expression with L-arabinose (60 mg) at 4 dpi. Bioluminescence was detected in the tumors after L-arabinose administration, but not in its absence (Supplementary Fig. S1D). These results strongly support the feasibility of using remote control of gene expression in tumor-targeting bacteria.

To control the therapeutic gene expression, the *clyA* gene was substituted for the *RLuc8* gene in the pBAD-RLuc8 plasmid, generating the pBAD-ClyA plasmid (Fig. 1A). To noninvasively monitor the bacterial migration in mice, the bacterial luciferase (*lux*) operon from *S. typhimurium*-Xen26 (Xenogen-Caliper) was transduced into the SHJ2037 strain by P22HT *int* transduction (14). ClyA expression was proportional to the L-arabinose concentration ( $\sim 0.4\%$ ), and ClyA was not detected in its absence (Fig. 1B). Western blot analysis revealed the presence of ClyA protein (34 kDa) in both the pellet and the culture medium of the strain carrying pBAD-ClyA only after L-arabinose administration (Fig. 1C). This result indicates that ClyA protein expression was specifically induced and that the protein was secreted from the bacteria. The growth of salmonellae was not affected by ClyA induction (Fig. 1D).

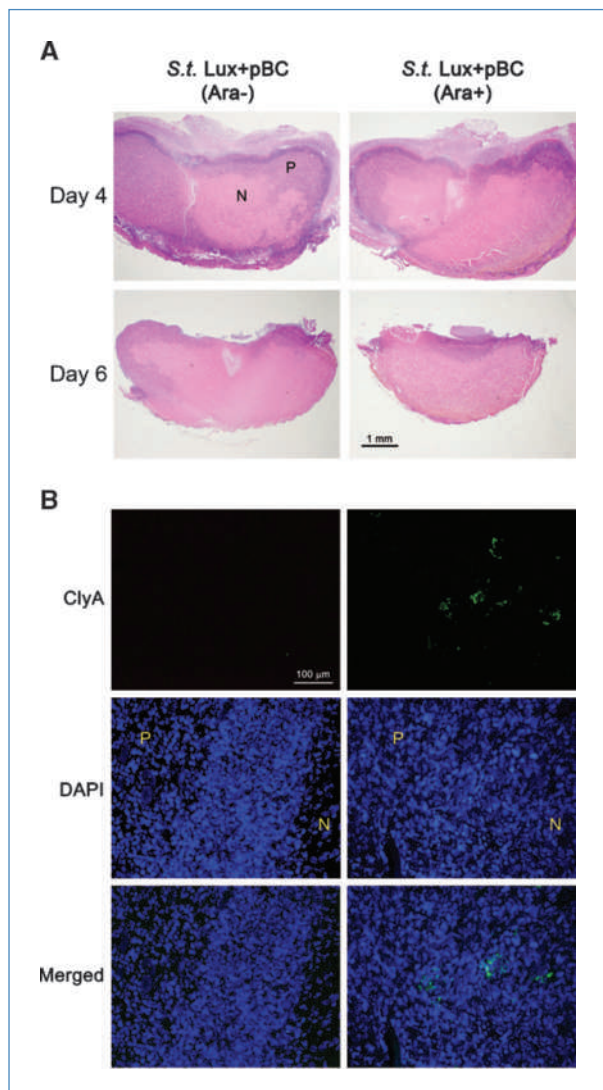
To explore the *in vivo* antitumor activity of our engineered *S. typhimurium*, CT-26- or Hep3B2.1-7-bearing mice were



**Figure 2.** Imaging and therapeutic effect of ClyA-expressing *S. typhimurium* in tumor-bearing mice. The mice ( $n = 5$  in each group) were injected s.c. with CT-26 or Hep3B2.1-7 cells. After tumors reached 130 mm<sup>3</sup> in volume (C, arrows), tumor-bearing mice were treated with PBS, untransformed *S. typhimurium* (S.t. Lux), or transformed *S. typhimurium* [S.t. Lux+pBC (Ara-)]. In a separate group of tumor-bearing mice ( $n = 5$ ), 60 mg of L-arabinose were i.v. injected everyday after day 4 of injection with transformed *S. typhimurium* [S.t. Lux+pBC (Ara+)]. A, photographs of subcutaneous tumors in representative mice. B, noninvasive *in vivo* imaging of bacterial bioluminescence in the representative mice presented in A. C, changes in tumor sizes. ■, PBS; ◆, S.t. Lux; ○, S.t. Lux+pBC (Ara-); ●, S.t. Lux+pBC (Ara+). D, Kaplan-Meier survival curves in CT-26-bearing mice ( $n = 5$  each group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . E, expression of the 34-kDa ClyA protein was verified by Western blot analysis of CT-26 tumor tissue from mice injected with S.t. Lux, S.t. Lux+pBC (Ara-), or S.t. Lux+pBC (Ara+).



treated with PBS and with untransformed or pBAD-ClyA-transformed *S. typhimurium*. Treatment of mice with transformed *S. typhimurium* in the presence of L-arabinose led to significant tumor shrinkage and, in some cases, tumor eradication (Fig. 2A and C). Survival was significantly prolonged in the group that received transformed *S. typhimurium* expressing ClyA by L-arabinose addition than in the other groups (Fig. 2D). Tumor shrinkage or the survival time following treatment with untransformed *S. typhimurium* or transformed *S. typhimurium* without induction was significantly better than that with PBS (control), but these tumors had a tendency to regrow. These results indicate that salmonellae alone are insufficient for significant tumor suppression.



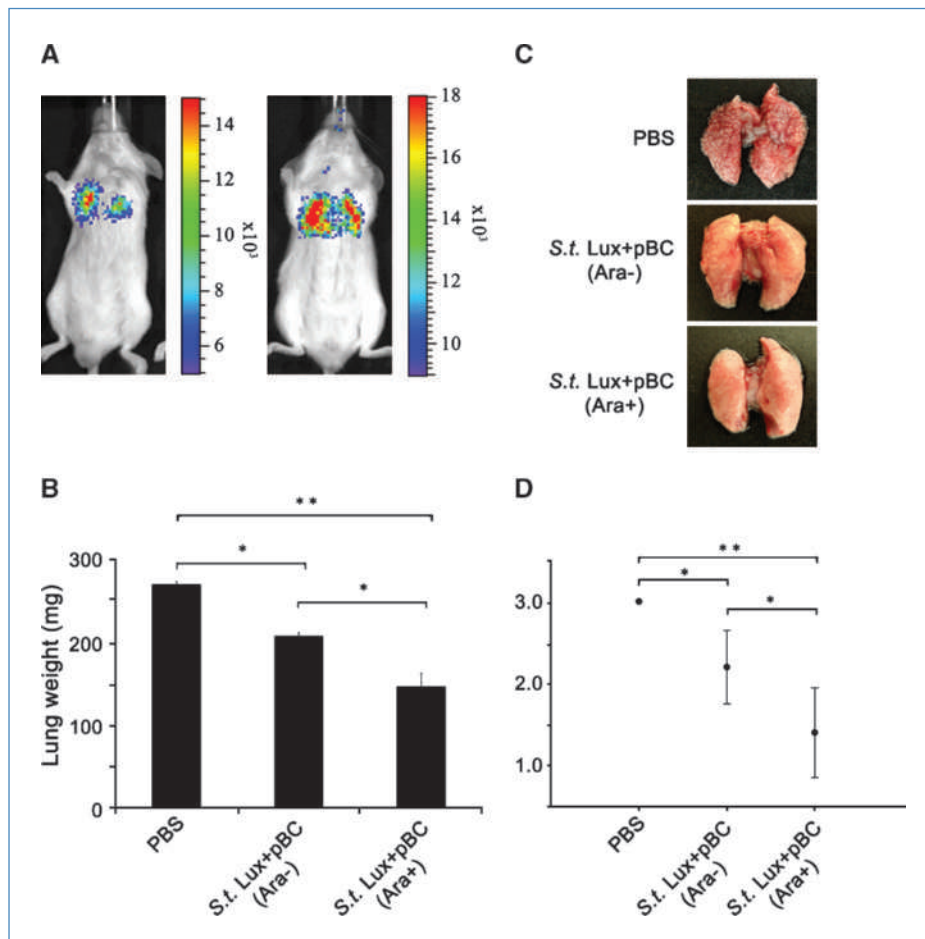
**Figure 3.** Histologic examination of CT-26 allografts after treatment with transformed *S. typhimurium*. A, H&E staining of a section of allografts at 4 d (top) or 6 d (bottom) after treatment with transformed *S. typhimurium*. B, immunofluorescence of a tumor 4 d after i.v. injection of transformed *S. typhimurium* with induction of ClyA expression [right; *S.t.Lux+pBC (Ara+)*]. N, necrosis; P, proliferative area.

Strong bioluminescence signals from the chromosomal *lux* operon of salmonellae were detected specifically in the tumor area of mice injected with bacteria (Fig. 2B). Most notably, the light signal from salmonellae diminished to an undetectable level in mice whose tumors were completely removed by the treatment (Fig. 2B, bottom). This result implies that tumor-targeted salmonellae were cleared by the host immune system as the tumors were treated. Western blot analysis revealed the presence of ClyA protein in the tumor tissue only after L-arabinose induction (Fig. 2E). Histologic analyses revealed extensive central necrosis and remaining proliferative areas after i.v. injection of transformed *S. typhimurium* with induction of ClyA expression (Fig. 3A). ClyA protein was identified in areas between the necrotic and proliferative tumor regions (Fig. 3B). The observed body weight of treated mice was not significantly different from that of the control group, suggesting that expression of ClyA in tumors by i.v. administration of engineered *Salmonella* did not elicit explicit toxicity (Supplementary Fig. S2).

In a separate experiment, mice were injected i.v. with CT-26 cells expressing firefly luciferase to create lung metastases. Mice were i.v. injected with *lux*-expressing *S. typhimurium* 5 days after cancer cells were injected. The location of bacterial bioluminescence (Fig. 4A, right) closely correlated with that of cellular (CT-26 FLuc) bioluminescence (Fig. 4A, left) in the lung fields, indicating that bacterial bioluminescence was detected specifically in the metastatic lung lesions. The specific location of bacteria in the lung lesions was further confirmed by imaging of isolated organs and gross necropsy (Supplementary Fig. S3).

The same protocol was then used to treat mice with lung metastases using pBAD-ClyA-expressing *S. typhimurium*. The effect of the treatment was evaluated either by weighing isolated lung masses or by a metastasis scoring method (15). Treatment with ClyA-expressing *S. typhimurium* markedly suppressed metastatic tumor growth better than control treatments (Fig. 4B–D;  $P < 0.01$ ). Note that the effect of treatment with transformed *S. typhimurium* without ClyA induction was better than that with PBS (control;  $P < 0.05$ ), indicating that the *S. typhimurium*  $\Delta$ ppGpp strain itself had some antitumor effect on the metastatic tumors.

In summary, these results provide proof of principle that (a) highly attenuated *S. typhimurium*  $\Delta$ ppGpp strain has an intrinsic tendency to target tumor tissue; (b) bacteria can be genetically engineered to deliberately express and secrete target protein into the tumor tissue; (c) the antitumor activity of our engineered bacteria was strong enough to suppress both primary and metastatic colon cancer in mice; (d) the  $P_{BAD}$  promoter is controllable and provides a very low background expression level (16); and (e) antitumoral bacteria labeled with imaging molecules allow determination of the bacterial fate in a simple, noninvasive manner that is amenable to repeated observations. Alternative approach has been exploited with strains of *S. typhimurium* that are dependent on the presence of leucine and arginine for growth. This strain was applied to treat many types of metastatic cancers in mouse models, and regression of tumor after i.v. administration was observed (17–19). To our knowledge, this



**Figure 4.** Imaging and therapeutic effect of CiyA-expressing *S. typhimurium* in lung metastasis mouse models. **A**, noninvasive bioluminescence imaging of a representative mouse with lung metastasis. BALB/c mice ( $n = 5$ ) were i.v. injected with  $5 \times 10^5$  CT-26 cells stably expressing firefly luciferase (CT-26 FLuc) to create lung metastases. Five days after injection, bioluminescence imaging was done after i.p. injection of 750  $\mu$ g of D-luciferin (left). Immediately after imaging, lux-expressing *S. typhimurium* were i.v. injected into the same animals. Bioluminescence imaging was done without D-luciferin administration for bacterial luciferase at 2 dpi (right). **B** to **D**, effect of *S. typhimurium* on CT-26 lung metastases. BALB/c mice with lung metastases ( $n = 9$ ) were treated with PBS, *S.t. Lux+pBC* (Ara-), or *S.t. Lux+pBC* (Ara+). Mice were sacrificed 13 d after tumor cell injection. Lungs were weighed (**B**), photographed (**C**), and scored for metastases (**D**), which was assessed as the percentage of the lung surface covered by metastases: 0, 0%; 1, < 20%; 2, 20% to 50%; 3, >50%. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

is the first report of a successful bacterial engineering of an imageable therapeutic probe. Direct extensions of our current approach should allow the generation of multifunctional tumor-targeting bacteria, allowing safe, efficient, and individualized tumor therapy.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Grant Support

Bio R&D program of the Korea Science and Engineering Foundation (KOSEF) funded by the Ministry of Education, Science, and Technology (MEST, 2008-04131); the National

Research Foundation of Korea funded by the Ministry of Education, Science and Technology (no. 2009-0091729); the National R&D Program for Cancer Control, Ministry of Health and Welfare, Republic of Korea (0620330-1); and in part by the BioImaging Research Center at GIST. Y. Hong was supported by Regional Technology Innovation Program of the Ministry of Commerce, Industry, and Energy (grant no. RTI05-01-01); H.E. Choy was supported by a KOSEF grant funded by MEST (no. 2007-04213).

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Received 9/16/09; revised 11/12/09; accepted 11/15/09; published OnlineFirst 12/22/09.

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*Cancer Res* 2010;70:18-23. Published OnlineFirst December 22, 2009.

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