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Report

Systemic targeting of primary bone tumor and lung metastasis of high-grade osteosarcoma in nude mice with a tumor-selective strain of *Salmonella typhimurium*

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We report here a new targeting strategy for primary bone tumor and lung metastasis with a modified auxotrophic strain of *Salmonella typhimurium*. We have previously developed the genetically-modified strain of *S. typhimurium*, selected for tumor targeting and therapy in vivo. Normal tissue is cleared of these bacteria even in immunodeficient athymic mice with no apparent side effects. In this study, the tumor-targeting strain of *S. typhimurium*, termed A1-R, was administered i.v. to nude mice which have primary bone tumor and lung metastasis. Primary bone tumor was obtained by orthotopic intra-tibial injection of 5 x 10^5 143B-RFP (red fluorescent protein) human osteosarcoma cells. One group of mice was treated with A1-R expressing GFP (green fluorescent protein) and another group was used a as control. A1-R (5 x 10^7 colony-forming units) was injected in the tail vein three times on a weekly basis. On day 28, lung samples were excised and observed with the Olympus OV100 Small Animal Imaging System. The size of the primary tumor and RFP intensity of lung metastasis were measured. Primary bone tumor size (fluorescence area [mm^2]) was 232 ± 70 in the untreated group and 95 ± 23 in the treated group ($p < 0.05$). RFP intensity of the lung metastasis was 3 ± 1.5 x 10^6 in the untreated group and 0.42 ± 0.33 x 10^6 in the treated group ($p < 0.05$). Therefore, bacterial treatment was effective for both primary bone tumor and lung metastasis.

Introduction

Osteosarcoma, which usually afflicts younger patients, has a five-year survival rate that can exceed 70% in patients treated with chemotherapy and appropriate surgery.[1-4] The most potent chemotherapeutic agents against osteosarcoma are high-dose methotrexate, cisplatin, doxorubicin and ifosfamide, which are widely used in combination regimens to achieve a synergistic effect. On the other hand, dose escalation of drugs in a dose-intensive chemotherapy regimen does not necessarily improve the results of osteosarcoma treatment.[1] The patients who are not responsive to chemotherapy develop lung metastasis, which is the most serious factor for their prognosis. Since the survival of patients with osteosarcoma has plateaued in the last ten years, new treatment approaches are needed. Many interesting new biological approaches to therapy are being investigated, most of which involve adding new drugs or other new therapies to existing chemotherapy protocols.[5,6]

It has been known for 60 years that anaerobic bacteria can selectively grow in tumors.[7] The conditions that permit anaerobic bacterial growth (i.e., impaired circulation and extensive necrosis) are found in many tumors. In addition, there are reports that postoperative infection of surgical wounds improved the prognosis of cancer patients, including osteosarcoma.[8-10]

Several approaches to developing tumor-therapeutic anaerobic bacteria have been described. Yazawa et al.[11,12] showed that the anaerobic bacterium *Bifidobacterium longum* could selectively grow in the hypoxic regions of solid tumors. Dang et al.[13] created a strain of *Clostridium novyi* depleted of its lethal toxin (*C. novyi-NT*). *C. novyi* spores germinated within the avascular regions of tumors in mice and destroyed surrounding viable tumor cells.[13] The main efficacy of these anaerobic bacteria was in combination with chemotherapy.[13] Cures of tumors were also achieved in mice by combining *C. novyi-NT* with radiation.[14] It was recently shown that treatment of mice bearing large, established tumors with *C. novyi-NT* plus a single dose of liposomal doxorubicin could lead to eradication of the tumors. The bacterial factor responsible for the enhanced drug release was identified as a protein termed liposomase.[15-17]

The facultative anaerobe *Salmonella typhimurium* was first attenuated by purine and other auxotrophic mutations in order to be used for cancer therapy.[18-20] These bacteria replicated in the tumor to >1,000-fold compared with normal tissues.[18] Salmonella lipid A was also genetically modified by disrupting the *msbB* gene to reduce septic shock.[18] Melanomas in mice treated with the Salmonella *msbB* mutant were 6% the size of tumors in untreated controls.[18] However, these Salmonella variants did not cause tumor regression or

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eradication, only growth inhibition. The *S. typhimurium* with attenuated lipid A has been evaluated in a Phase I clinical trial.[21]

We have previously developed[22] a genetically-modified bacterial strain of *S. typhimurium*, auxotrophic for leucine and arginine, which also expresses green fluorescent protein (GFP), termed *S. typhimurium* A1. When introduced i.v. or intratumorally, A1 invaded and replicated intracellularly in various cancer cells in vivo as well as in vitro. When A1 was injected intratumorally, the tumor completely regressed by day 20. There were no obvious adverse effects on the host when the bacteria were injected i.v. or intratumorally. The *S. typhimurium* A1 strain grew throughout the tumor, including viable malignant tissue as well as necrotic areas. This result is in marked contrast to the anaerobic bacteria evaluated previously for cancer therapy that were confined to necrotic areas of the tumor as discussed above. The ability to grow in viable tumor tissue may account, in part, for the unique antitumor efficacy of the A1 strain.[22] The A1 strain was reisolated from A1-targeted tumor tissue in vivo. The idea was to increase the tumor targeting capability of the bacteria. As a consequence of this selective step, the tumor-cell targeting of the reisolated A1 increased in vivo as well as in vitro. The reisolated A1 bacteria, termed A1-R, administered i.v., caused human breast cancer[23] and prostate cancer[24] regression, including cures in orthotopic nude-mouse models.

Lung metastasis is the life threatening aspect for osteosarcoma patients. Importantly, metastasis is most often treatment-resistant. In the present study, we demonstrate that *S. typhimurium* A1-R can target and regress both primary bone tumor and lung metastases of osteosarcoma when administered systemically.

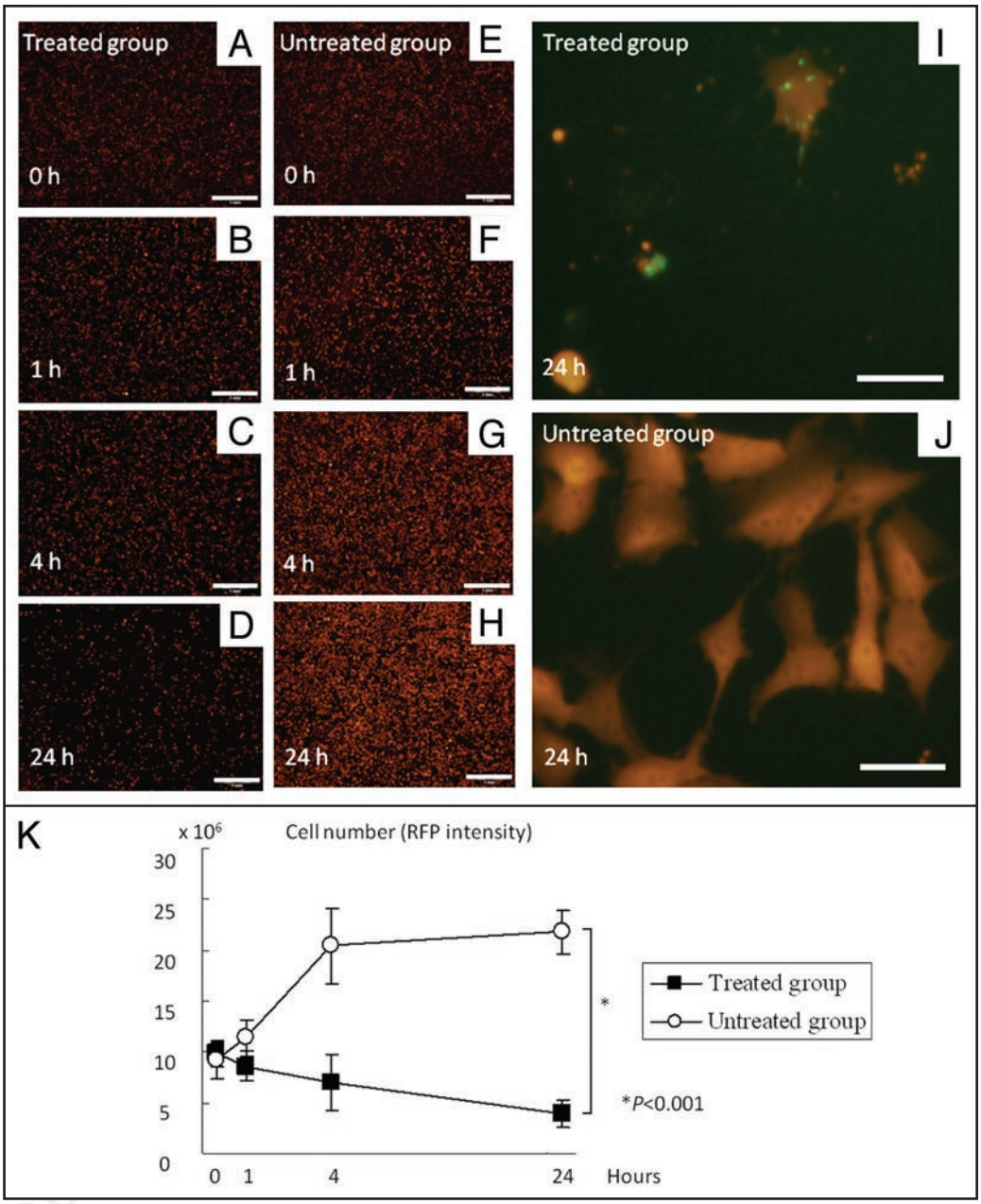

Figure 1. Intracellular growth of GFP-expressing *S. typhimurium* A1-R in vitro. 143B cells labeled with RFP were grown in 6-well tissue culture plates. GFP-expressing *S. typhimurium* A1-R was added to the tumor cells (3 x 10^8 CFU per well). After 1 h incubation at 37°C, the cells were rinsed and cultured in medium containing gentamycin sulfate (20 μg/ml) to kill external but not internal bacteria. Cells were observed under fluorescence microscopy at the indicated time points. (A–D) 143B cells under an RFP filter. Bacteria treatment decreased the number of cells. (E–H) Untreated control group. Cell number increased in 24 h. (I) Interaction between 143B cells (red) and bacteria (green). A1-R was able to invade and replicate intracellularly in the 143B-RFP cells. The cytopathic effects of A1-R on 143B-RFP cells after infection were visible using dual-color fluorescence. Intracellular bacterial infection leading to eventual cell fragmentation and cell death was observed. (J) Untreated cells are intact. (K) The total RFP intensity in the treated wells decreased in contrast to an increase in untreated wells. At 24 hours after infection, the RFP intensity in the treated wells significantly decreased compared to the untreated wells. Bars, (A–H) 1 mm, (I and J) 50 μm.

Results and Discussion

Bacteria therapy of 143B-RFP cells in vitro. After bacteria treatment, RFP cells were easily observed repeatedly under fluorescent microscopy and their number was observed to decrease in 24 hours (Fig. 1A–H). A1-R-GFP invaded and replicated intracellularly in the 143B-RFP human osteosarcoma cell lines. Intracellular bacterial infection led to cell fragmentation and rapid cell death (Fig. 1I and J). After infection, the total RFP intensity in the wells decreased in

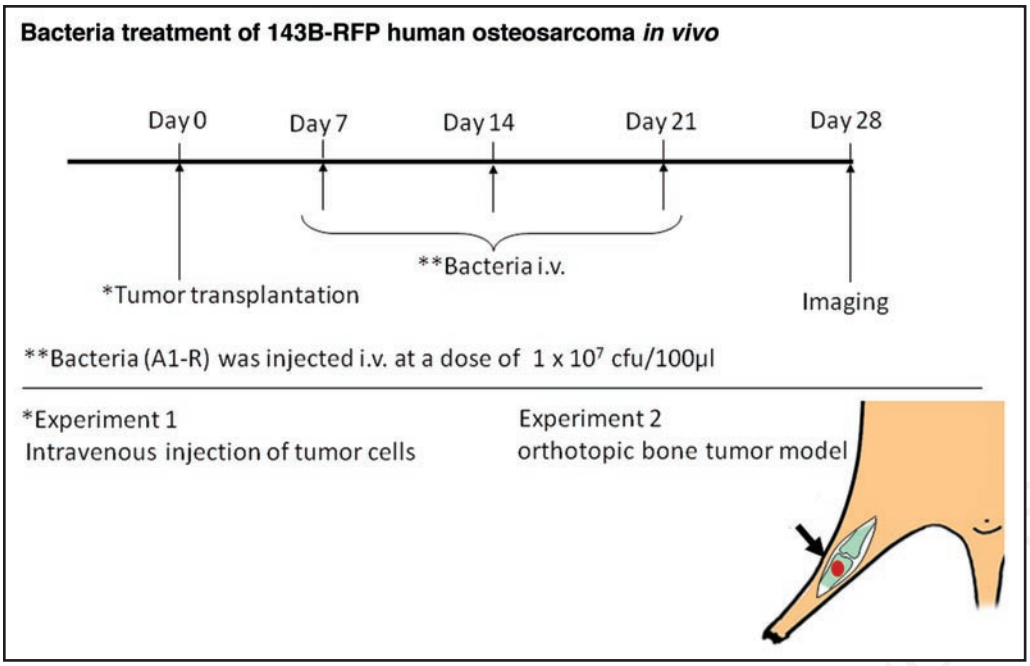

Figure 2. Treatment schedule for in vivo study. On day 0, 143B-RFP human osteosarcoma cells were injected into either the tail vein or intramedullary cavity of the tibia. On day 7, 14 and 21, the bacteria were injected into the tail vein. On day 28, all animals were sacrificed and imaging was performed.

contrast to an increase in the untreated wells. At 24 hours after infection, the RFP intensity in the treated group significantly decreased compared to the untreated group (Fig. 1K, $p < 0.001$).

Bacteria therapy of experimental lung metastasis. 143B-RFP human osteosarcoma cells were injected into the tail vein to obtain experimental lung metastasis in nude mice. Bacteria were injected i.v. every week and animals were sacrificed on day-28 (Fig. 2). The lung was excised and the surface was observed with the OV100 imager (Fig. 3A–F). The number of metastases was 26 ± 14.4 in the untreated group and 0.7 ± 1.2 in the treated group (Fig. 3G, $p < 0.05$). The bacteria treatment therefore significantly reduced the experimental lung metastasis. There was no significant adverse effect for mice in the bacteria treatment group.

Bacterial therapy of orthotopic bone tumor and spontaneous lung-metastasis. The mice were transplanted with 143B-RFP cells in the tibia and developed primary bone tumor and lung metastasis. Seven days after tumor injection, the RFP tumor was confirmed inside the tibia by imaging with the OV100. After 3 weekly i.v. injections of bacteria, the bone tumor and lung metastasis were examined on day-28 (Fig. 2). The bone tumor size (RFP area) was 232 ± 70 mm^2 in the untreated group and 95 ± 23 mm^2 in the treated group ($p < 0.05$). The lung was excised and the metastases on the surface were counted. The number of metastasis was 52 ± 29.6 in the untreated group and 2.3 ± 2.1 in the treated group ($p < 0.05$) (Figure 4). Therefore, bacteria therapy was also effective against primary and metastatic tumors in an orthotopic bone tumor model.

The natural history of osteosarcoma which, despite amputation, often results in development of metastasis and death, has been dramatically improved by the introduction of chemotherapy. Adjuvant chemotherapy has permitted limb-salvaging, which otherwise would not have been possible. However, achieving further improvements in survival will require more systemic control. In the present study, we demonstrated the efficacy of *S. typhimurium* for osteosarcoma. In particular, lung metastasis was significantly eradicated. This treatment could contribute to therapy of osteosarcoma patients with lung metastasis, which is the worst prognostic factor.

In general, cancer treatment includes chemotherapy, radiation, surgery and immunotherapy. However, the strategy using bacteria has great potential because of the biological diversity of bacteria compared to other treatments. The strategy employed in our studies for bacterial therapy of cancer has significant advantages over that using obligate anaerobic bacteria such as Clostridium. Anaerobe bacteria only grow in the necrotic regions of tumors and therefore require additional cytotoxic chemotherapy in order to kill viable tumor tissue and effect cures.[13] The facultative anaerobe *S. typhimurium*, in contrast, grows in viable as well as necrotic regions of tumors and therefore can cure tumors without additional chemotherapy. Thus, our method of bacterial treatment can target spontaneous metastasis and cure them without toxic chemotherapy, a major advance over other approaches to bacterial therapy which require chemotherapy in order to effect cures.[15-17]

Materials and Methods

GFP gene transfection of *S. typhimurium*. *S. typhimurium* (ATCC 14028) was grown at 37°C to mid-logarithmic phase in liquid LB and harvested at 4°C. Cells (2.0×10^8) in 40 µl 10% glycerol were mixed with 2 µl pGFP vector (Clontech) and placed on ice for 5 min before electroporation with a Gene Pulser apparatus (Bio-Rad) according to the manufacturer's instructions. Electroporation was done at 1.8 kV with the pulse controller at 1,000-Ω parallel resistance.

Induction of bacterial mutations with nitrosoguanidine (NTG) and selection for tumor targeting. Freshly prepared NTG (1 mg/ml in sterile water) was added to the washed culture to a final concentration of 100 µg/ml in Tris maleic acid buffer at pH 6.0. The bacteria were incubated with NTG for 30 min. The NTG-treated cells were grown in nutrient broth. Bacterial colonies were replica-plated in supplemented minimal agar plates. Virulence of bacteria in vitro and toxicity in nude mice were evaluated. *S. typhimurium* A1, maintained virulence for tumors in vivo as well as for cancer cells in vitro and was toxic in host nude mice. Strain A1 is remarkable for its selectivity for growth in tumors compared with normal tissues.

Re-isolation of increased tumor-targeting variant of *S. typhimurium* A1. *S. typhimurium* A1 auxotrophs expressing GFP were reisolated as follows: the A1 bacteria were injected into the tail vein of a HT-29 human colon tumor-bearing nude mouse. Three days after infection, the tumor tissue was removed from the infected

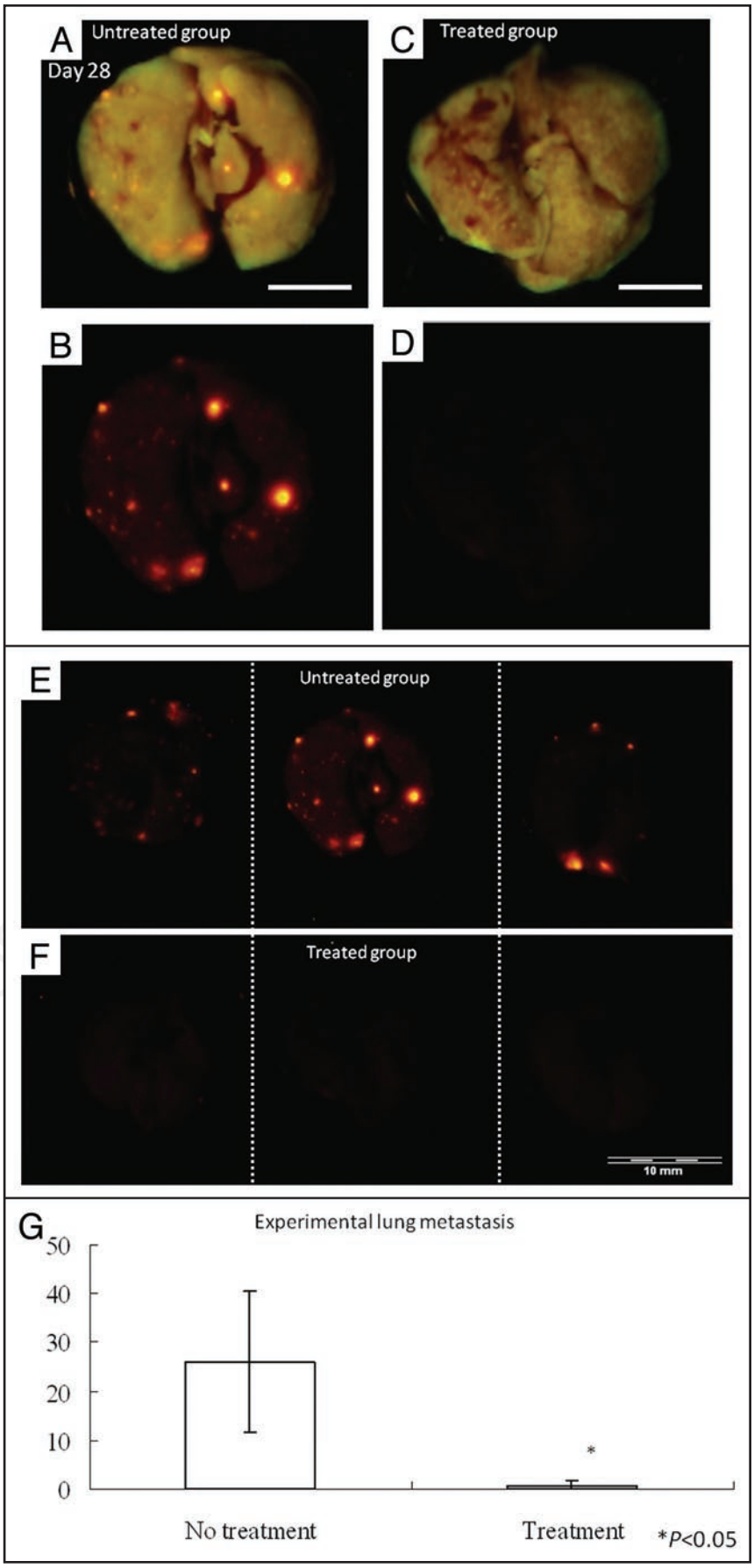

Figure 3. Baceteria therapy for experimental lung metastasis. 1 x 10^6 143B-RFP cells were injected into the tail vein and bacteria were injected on day-7, -14 and -21. On day 28, animals were sacrificed and the lung was excised. (A) Untreated group. Images were obtained with the Olympus OV100 Small Animal Imaging System. (B) RFP only. Lung metastases are clearly visible. (C and D) Treated group. Lung metastasis was inhibited by bacteria therapy. (E and F) Lungs of three untreated animals versus three treated animals. (G) There was a significant difference in the number of metastases between the untreated group and the treated group. Bars, 10 mm.

mouse. The tumor tissue was then homogenized and diluted with PBS. The resulting supernatant of the tumor tissue was cultured in LB agar plates at 37°C overnight. The bacteria colony with the brightest green fluorescence was picked up and cultured in 5 mL LB medium. This strain was termed A1-R and was found to have much greater tumor-targeting capability than *S. typhimurium A1* (23).

RFP vector production. The RFP (DsRed-2) gene (BD Biosciences Clontech, Palo Alto, CA) was inserted in the retroviral-based mammalian expression vector pLNCX (BD Biosciences Clontech) to form the pLNCX DsRed-2 vector. Production of retrovirus resulted from transfection of pLNCX DsRed-2 into PT67 packaging cells, which produce retroviral supernatants containing the DsRed-2 gene. Briefly, PT67 cells were grown as monolayers in DMEM supplemented with 10% FCS (Gemini Biological Products, Calabasas, CA). Exponentially-growing cells (in 10-cm dishes) were transfected with 10 μg expression vector using LipofectAMINE Plus (Life Technologies, Grand Island, NY) . Transfected cells were replated 48 hours after transfection and 100 μg/mL G418 was added 7 hours later. Two days later, the medium was changed to 200 μg/mL G418. After 25 days of drug selection, surviving colonies were visualized under fluorescence microscopy and RFP-positive colonies were isolated. Several clones were selected and expanded into cell lines after virus titering on the 3T3 cell line.

RFP gene transduction of cancer cells. 143B human osteosarcoma cells were labeled with RFP. Clones expressing RFP in the cytoplasm were established. In brief, cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-RFP cells and RPMI 1640 (Irvine Scientific) containing 10% fetal bovine serum for 72 hours. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 hours posttransduction and subcultured at a ratio of 1:15 into selective medium, which contained 200 μg/mL G418. The level of G418 was increased stepwise up to 800 μg/mL. RFP-expressing cancer cells were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) using trypsin/EDTA and amplified by conventional culture methods.

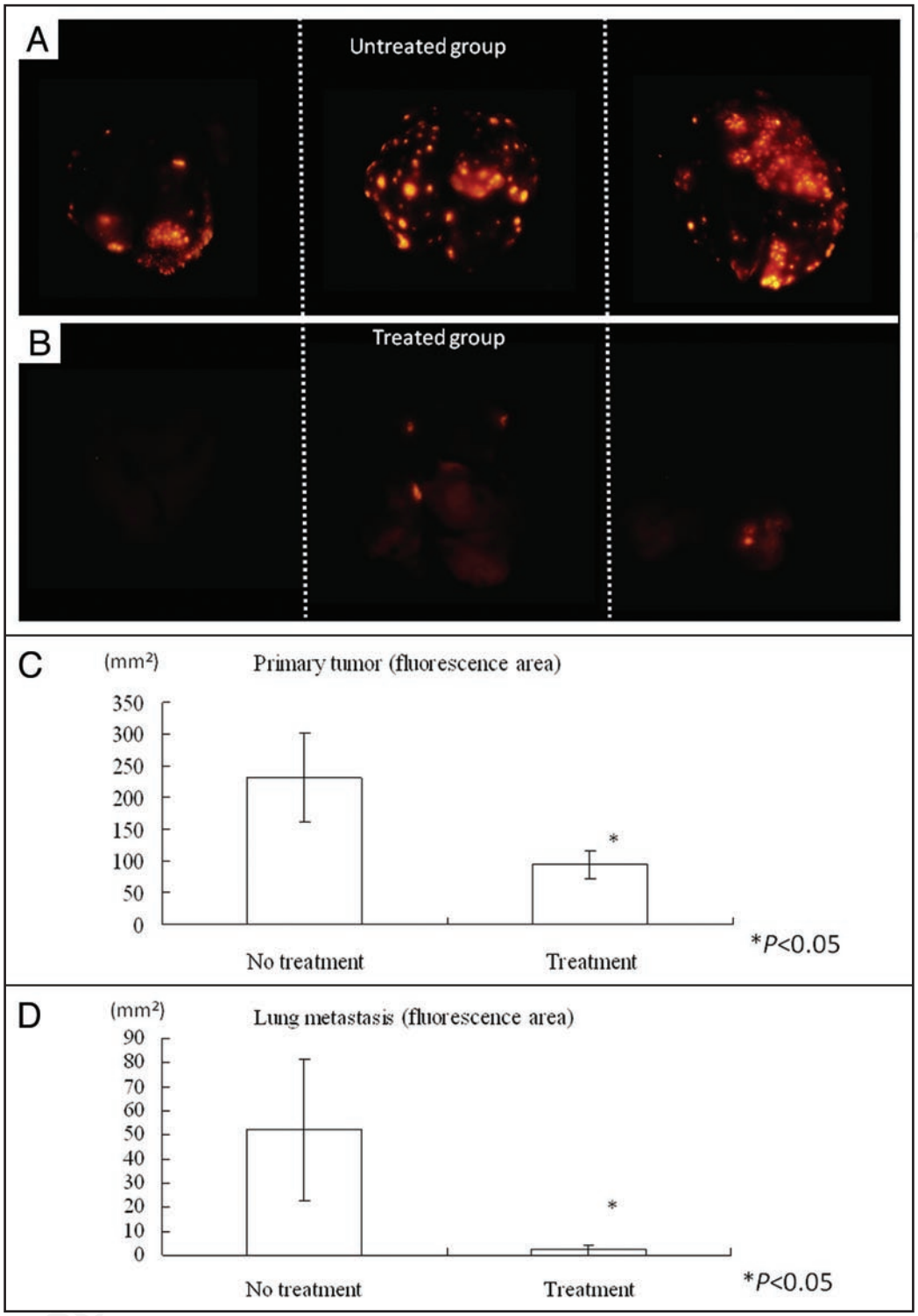

Figure 4. Bacteria therapy for orthotopic osteosarcoma tumor. 5 x 10^5 RFP-expressing 143B-RFP cells were injected into the intramedullary cavity of the tibia. The mice developed primary bone tumor and lung metastasis. On day-7, -14 and -21, 5 x 10^7 *S. typhimurium* CFU per mouse were injected into the tail vein. On day-28, all the mice were sacrificed and imaging was performed. The size of the bone tumor (fluorescent area [mm^2]) was measured. The lung was then excised and the metastasis on the surface was observed and counted. Three mice were treated with bacteria and three mice were used as untreated controls. Lung metastasis was strongly inhibited by bacteria therapy. (A and B) Lungs of three untreated animals versus three treated animals. (C and D) There was a significant difference of both primary tumor size and the number of metastases between the untreated group and the treated groups.

Intracellular growth of *S. typhimurium* A1-R. 143B cells labeled with RFP were grown in 6-well tissue culture plates to a density of 5 x 10^5 cells per well. Bacteria were grown in LB and harvested at late-logarithmic phase, diluted in cell culture medium and added to the tumor cells [3 x 10^8 colony-forming units (CFU) per well]. After 1 h incubation at 37°C, the cells were rinsed and cultured in medium containing gentamycin sulfate (20 μg/ml) to kill external but not internal bacteria. Interaction between bacteria and tumor cells was observed

at the indicated time points. The Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan) was used for fluorescence microimaging. For the purpose of cell counting, at 0, 1, 4 and 24 hours after infection, total RFP intensity of 143B-RFP cells was calculated in a 5.3 mm x 4.2 mm field in each well (excitation 545 nm, emission 570–625 nm, exposure time 2000 ms). At each time point, each well was washed with medium to remove floating dead cells.

Bacterial targeting of experimental lung metastasis. Four-week-old female nude mice were used. To obtain lung metastasis, 143B-RFP cells (1 x 10^6 in 100 μl PBS) were injected into the tail vein of nude mice. On days 7, 14 and 21, 5 x 10^7 bacterial CFU per mouse were injected into the tail vein. Three mice were treated with bacteria and three mice were used as untreated controls. On day 28, all animals were sacrificed. The excised lungs were imaged using the Olympus OV100 Small Animal Imaging System (0.14x lens, excitation 545 nm, emission 570–625 nm) and the number of metastases were counted.

Orthotopic osteosarcoma model in nude mice. Four-week-old female mice were anesthetized by a ketamine mixture (10 μL ketamine HCL, 7.6 μL xylazine, 2.4 μL acepromazine maleate and 10 μL H_2O) via s.c. injection. The leg was sterilized with alcohol and an approximately 2 mm midline skin incision was made just below the knee joint to expose the tibial tuberosity. 5 x 10^5 143B-RFP cells in 5 μL Matrigel (BD Bioscience, San Jose, CA) per mouse were injected into the intramedullary cavity of the tibia with a 0.5 mL 28 G latex-free insulin syringe (TYCO Health Group LP, Mansfield, MA). The skin was closed with a 6-0 suture. One week after injection, a 1 cm skin incision was made over the tibia to confirm the RFP tumor growing inside of the bone using the OV100 Small Animal Imaging System. The skin was then closed again.

On days 7, 14 and 21, 5 x 10^7 *S. typhimurium* CFU per mouse were injected into the tail vein. On day-28, all animals were sacrificed and imaging was performed with the OV100 Small Animal Imaging System to determine the efficacy of bacteria therapy for both primary tumor and metastasis. The size of the primary bone tumor (fluorescent area [mm^2]) was measured in the same setting as above. Then, the lung was excised and the metastases on the surface were observed and counted. Three mice were treated with bacteria and three mice were used as untreated controls.

Animals were kept in a sterile environment. All procedures of the operations described above were performed with a dissection microscope. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals under assurance no. A3873-1.

Statistical analysis. The experimental data are expressed as the mean ± S.D. Statistical analysis was performed using the two-tailed Student's t test.

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