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REVIEW ARTICLE

Tumor-targeting amino acid auxotrophic *Salmonella typhimurium*

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Abstract We have developed an effective bacterial cancer therapy strategy by targeting viable tumor tissue using *Salmonella typhimurium* auxotrophs that we have generated which grow in viable as well as necrotic areas of tumors. However, the auxotrophy severely restricts growth of these bacteria in normal tissue. The *S. typhimurium* A1-R mutant, which is auxotrophic for leu-arg and has high anti-tumor virulence, was developed in our laboratory. In vitro, A1-R infects tumor cells and causes nuclear destruction. A1-R was initially used to treat metastatic human prostate and breast tumors that had been orthotopically implanted in nude mice. Forty percent of treated mice were cured completely and survived as long as non-tumor-bearing mice. A1-R administered i.v. to nude mice with primary osteosarcoma and lung metastasis was highly effective, especially against metastasis. A1-R was also targeted to both axillary lymph and popliteal lymph node metastasis of human pancreatic cancer and fibrosarcoma, respectively, as well as lung metastasis of the fibrosarcoma in nude mice. The bacteria were delivered via a lymphatic channel to target the lymph node metastases and systemically via the tail vein to target the lung metastasis. The metastases were cured without the need of chemotherapy or any other treatment. A1-R was administered intratumorally to nude mice with an orthotopically transplanted human pancreatic tumor. The primary pancreatic cancer regressed without additional chemotherapy or any other treatment. A1-R was also effective against pancreatic cancer liver metastasis when administered intrasplenically to nude mice. The approach described here, where bacterial monotherapy effectively treats primary and metastatic tumors, is a significant improvement over previous bacterial tumor-therapy strategies that require combination with toxic chemotherapy. Three promoter clones engineered in *S. enterica typhimurium* were identified to have enhanced expression in bacteria growing in tumors relative to those growing in the spleen. The expression of therapeutics in *Salmonella* under the regulation of one or more promoters that are activated preferentially in tumors has the potential to improve the efficacy of *Salmonella* tumor therapy. Exploitation of the tumor-killing capability of *Salmonella* has great promise for a new paradigm of cancer therapy.

Keywords *S. typhimurium* · Leucine-arginine auxotrophs · Cancer therapy · Green fluorescent protein · Red fluorescent protein

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Introduction

Coley (1906) observed, more than a century ago, that some cancer patients were cured of their tumors following post-operative bacterial infection. In the middle of the last century, Malmgren and Flanigan (1955) showed that anaerobic bacteria had the ability to survive and replicate in necrotic tumor tissue with low oxygen content. Several approaches aimed at utilizing bacteria for cancer therapy have subsequently been described (Gericke and Engelbart 1964; Moese and Moese 1964; Thiele et al. 1964; Kohwi et al. 1978; Kimura et al. 1980; Fox et al. 1996; Lemmon et al. 1997; Brown and Giaccia 1998; Low et al. 1999; Clairmont et al. 2000; Sznol et al. 2000; Yazawa et al. 2000, 2001).

Bifidobacterium longum has been shown to selectively grow in hypoxic regions of tumors following intravenous administration. This effect was demonstrated in 7,12-dimethylbenzanthracene-induced rat mammary tumors by Yazawa et al. (2000, 2001).

Vogelstein et al. created a strain of *Clostridium novyi*, an obligate anaerobe, which was depleted of its lethal toxin (Dang et al. 2001). This strain of *C. novyi* was termed *C. novyi* NT. Following intravenous administration, the *C. novyi* NT spores germinated in the avascular regions of tumors in mice, causing damage to the surrounding viable tumor (Dang et al. 2001). Combined with conventional chemotherapy or radiotherapy, intravenous *C. novyi* NT spores caused extensive tumor damage within 24 h (Dang et al. 2001).

Following attenuation by purine and other auxotrophic mutations, the facultative anaerobe *Salmonella typhimurium* was used for cancer therapy (Low et al. 1999; Hoiseth and Stocker 1981; Pawelek et al. 1997). These genetically modified bacteria replicated in tumors to levels more than 1,000-fold greater than in normal tissue (Low et al. 1999). *S. typhimurium* was further modified genetically by disrupting the msbB gene to reduce the incidence of septic shock (Low et al. 1999).

The msbB mutant of *S. typhimurium* has been tested in a Phase I clinical trial to determine its efficacy on metastatic melanoma (Toso et al. 2002). To raise the therapeutic index, *S. typhimurium* was further attenuated by deletion of the purI as well as msbB gene (Toso et al. 2002). The new strain of *S. typhimurium*, termed VNP20009, could then be safely administered to patients (Toso et al. 2002). More studies are needed to completely characterize the safety and efficacy of these bacteria and to improve its therapeutic index.

Mengesha et al. utilized *S. typhimurium* as a vector for gene delivery by developing a hypoxia-inducible promoter (HIP-1) to limit gene expression to hypoxic tumors. HIP-1 was able to drive gene expression in bacteria infecting human tumor xenografts implanted in mice (Mengesha et al. 2006). Genes linked to the HIP-1 promoter showed selective expression in tumors (Mengesha et al. 2006).

Yu et al. (2003, 2004) used green fluorescent protein (GFP)-labeled bacteria to visualize tumor targeting abilities of three pathogens: *Vibrio cholerae*, *S. typhimurium* and *Listeria monocytogenes*.

Development of tumor-targeting amino acid auxotrophic strain A1 of *S. typhimurium*

We initially developed a strain of *S. typhimurium*, termed A1, which selectively grew in tumor xenografts (Zhao et al. 2005). In contrast, normal tissue rapidly cleared infecting bacteria, even in immunodeficient athymic mice. *S. typhimurium* A1 is auxotrophic (leu/arg dependent), but receives sufficient support from tumor tissue.

GFP transfection and stable expression in S. typhimurium (*Zhao et al.* 2005) *S. typhimurium* 14028 was transfected with the pGFP gene by electroporation. The transformed *S. typhimurium* expressed GFP over 100 passages with GFP expression monitored at each passage (Fig. 1).

Cancer cell killing by S. typhimurium in vitro (*Zhao et al.* 2005) To observe the intracellular replication and virulence of *S. typhimurium*-GFP in a human prostate cancer cell line in vitro, PC-3 human prostate cancer cells were labeled with RFP in the cytoplasm, and with retroviral RFP and GFP in the nucleus by means of a fusion of GFP with histone H2B. This has allowed the interaction between bacteria and cancer cells to be visualized by dual color spatial-temporal imaging (Figs. 2, 3). The quantitative ability of *S. typhimurium* to kill prostate cancer cells was determined with the MTT method and observed to be dose dependent (Fig. 3).

Mutation, isolation and identification of auxotrophs of S. typhimurium-GFP (*Zhao et al.* 2005) *S. typhimurium* auxotrophic strains were obtained after nitrosoguanidine (NTG) mutagenesis. Twelve of 300 isolates tested were identified as auxotrophic mutants (4%) using minimal medium supplemented with various amino acids. Nude mice were inoculated i.v. with 10^7 cfu of each mutant. After inoculation with wild-type *S. typhimurium*, the mice died within 2 days. The mice which lived longest were those inoculated with auxotroph A1 and survived as long as control uninfected mice. A1 required Leu and Arg and was chosen for efficacy studies.

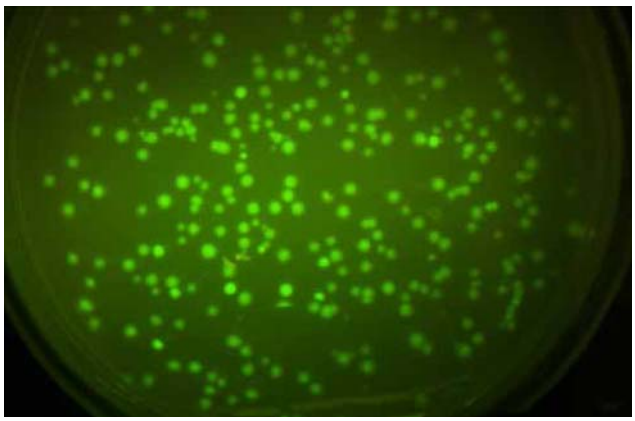

Fig. 1 GFP-transformed *S. typhimurium* shown under fluorescence microscopy

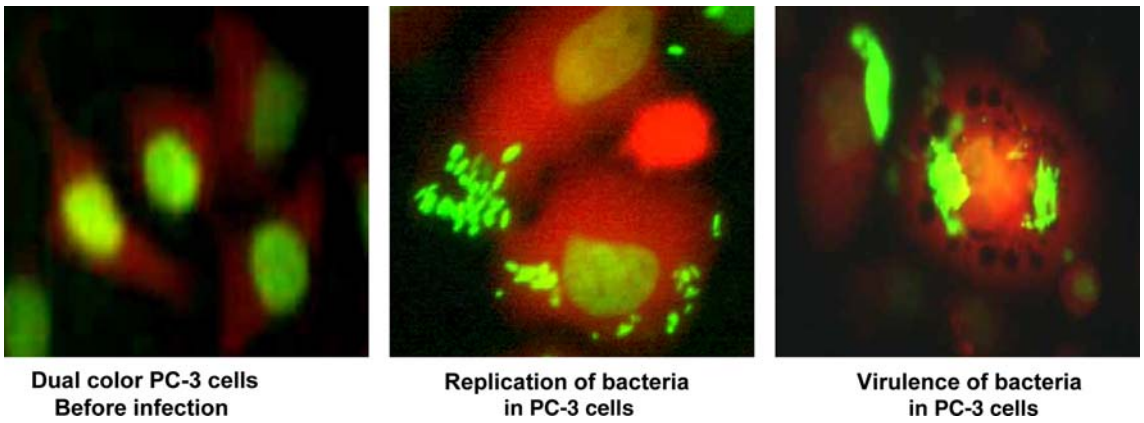

Fig. 2 Intracellular replication and virulence of *S. typhimurium*-GFP in dual color-labeled PC-3 human prostate cancer cell line in vitro (Zhao et al. 2005)

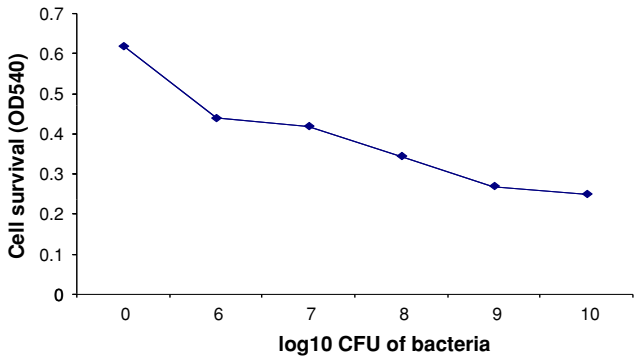

Fig. 3 Survival of PC-3 human prostate cancer cells infected with *S. typhimurium* in vitro (Zhao et al. 2005)

Efficacy of *S. typhimurium* amino acid auxotrophs on prostate and breast cancer

In vivo efficacy testing of S. typhimurium A1 (*Zhao et al.* 2005) To observe the interaction of prostate cancer cells with bacteria, we used a PC-3 human prostate cancer cell line expressing RFP, so their response to the bacteria could be visualized in vivo.

To evaluate the efficacy of *S. typhimurium* A1, ten NCR nude mice, 6–8 weeks old, were implanted subcutaneously (s.c.) on the mid-right side with 2×10^6 RFP-labeled PC-3 human prostate cancer cells. Bacteria were grown and harvested at late-log phase and then diluted in PBS and injected directly into the tail vein (5×10^7 cfu/100 µl PBS). Tumor size was determined from fluorescence imaging at each time point after infection. *S. typhimurium* A1 selectively colonized the PC-3 tumor and suppressed its growth (Fig. 4).

Re-isolation of S. typhimurium A1 (*Zhao et al.* 2006) To enhance tumor virulence, *S. typhimurium* A1 has been passaged by injection in nude mice transplanted with the HT-29 human colon tumor. Bacteria, expressing GFP, isolated from the infected tumor were then cultured. The re-isolated A1 was termed A1-R. The ability of A1-R to adhere to tumor cells was evaluated in comparison with the parental A1 strain in vitro. The number of A1-R bacteria attached to HT-29 human colon cancer cells was approximately six times higher than parental A1 (Fig. 5).

Enhanced tumor virulence of S. typhimurium A1-R in PC-3 human prostate cancer cells in vitro (*Zhao et al.* 2007) Virulence of GFP-labeled *S. typhimurium* A1 and A1-R bacteria was compared in vitro under fluorescence microscopy. Both strains infected dual color PC-3 cancer cells. Whereas almost all cells were infected and died after 2 h with A1-R, it took 24 h to get the same result with A1. Thus, the virulence of A1-R was greatly increased (Fig. 6).

Enhanced tumor targeting of S. typhimurium A1-R in PC-3 tumor-bearing mice (*Zhao et al.* 2007) GFP-labeled *S. typhimurium* A1 and A1-R (5×10^7 cfu/100 µl) were administered (i.v.) to PC-3-bearing nude mice. The biodistribution of the bacteria in tumor tissue was determined at day 4. A1-R had a 100× greater cfu in PC-3 tumor tissue than A1. This result suggested that A1-R has greater tumor targeting than A1 (Fig. 7).

Efficacy of A1-R in orthotopic metastatic human prostate tumor models (*Zhao et al.* 2007) A1-R was used to treat metastatic PC-3 human prostate tumors that had been orthotopically implanted in nude mice. A1-R could effect cures in the orthotopic nude mouse models of PC-3 (Fig. 8). Of ten mice with the PC-3 tumors that were injected weekly with A1-R, seven were alive at the time the last untreated mouse died. Four of the tumor-bearing mice were apparently cured by weekly bacterial treatment. Weekly dosing of A1-R was much more effective than two doses only (Fig. 9a).

Dose response of A1-R In contrast to 12 weekly doses described above, with only two doses, only one of ten mice was cured (Fig. 9b).

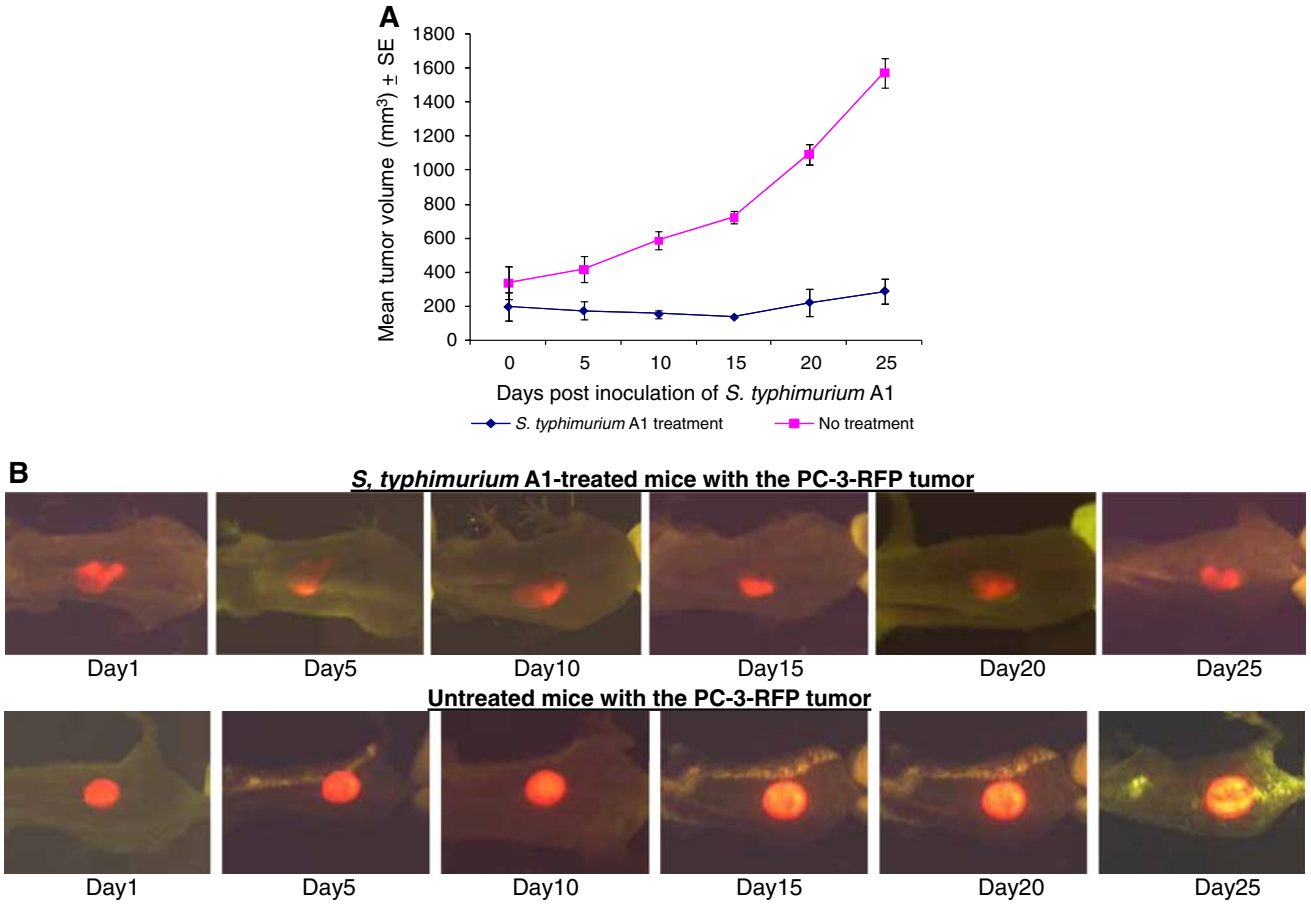

Fig. 4 **a** Efficacy of *S. typhimurium* A1 on the growth of the PC-3 human prostate tumor in nude mice after i.v. injection. **b** Whole-body imaging of efficacy of *S. typhimurium* A1 (i.v.) on growth of RFP-labeled PC-3 human prostate cancer in nude mice (Zhao et al. 2005)

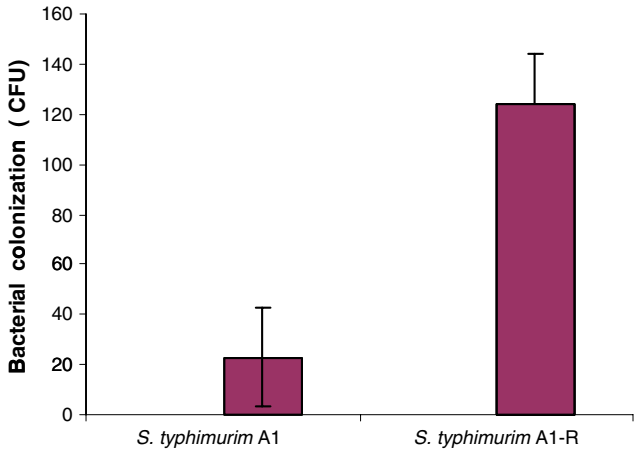

Fig. 5 Enhanced tumor-cell adherence and invasion in vitro by *S. typhimurium* A1-R (Zhao et al. 2006)

Comparison of biodistribution between S. typhimurium strains A1 and A1-R in normal tissue in vivo (*Zhao et al.* 2007) GFP-labeled *S. typhimurium* A1 and A1-R bacteria (5×10^7 cfu/100 µl) were administered i.v. in PC-3-bearing nude mice. The biodistribution of the bacteria in liver and spleen tissue was determined at day 2. Fewer A1-R cfu (6×10^6) were recovered from the liver than A1 (4×10^7).

Tumor targeting by A1-R (*Zhao et al.* 2007) To compare bacterial infection in the tumor with infection in normal tissue, A1-R bacteria (5×10^7 cfu/100 µl) were administered i.v. in PC-3-bearing nude mice. On day 4 after injection, the tumor, liver and spleen were removed. The tissues were homogenized and plated on LB agar plates. After overnight growth at 37°C, the cfu were counted. The ratio of tumor to normal tissue was approximately 10^6, indicating a very high degree of tumor targeting by A1-R.

Efficacy of A1-R on breast tumor growth (*Zhao et al.* 2006) Treatment with A1-R resulted in significant tumor shrinkage in nude mice with s.c. MARY-X human xerographs. Bacteria (5×10^7 cfu/100 µl) were inoculated i.v. in MARY-X-bearing nude mice. Tumor growth was monitored by caliper measurement in two dimensions. The infected tumors regressed by day 5 after infection and complete regression occurred by day 25. In orthotopic models of MARY-X, A1-R treatment also led to tumor

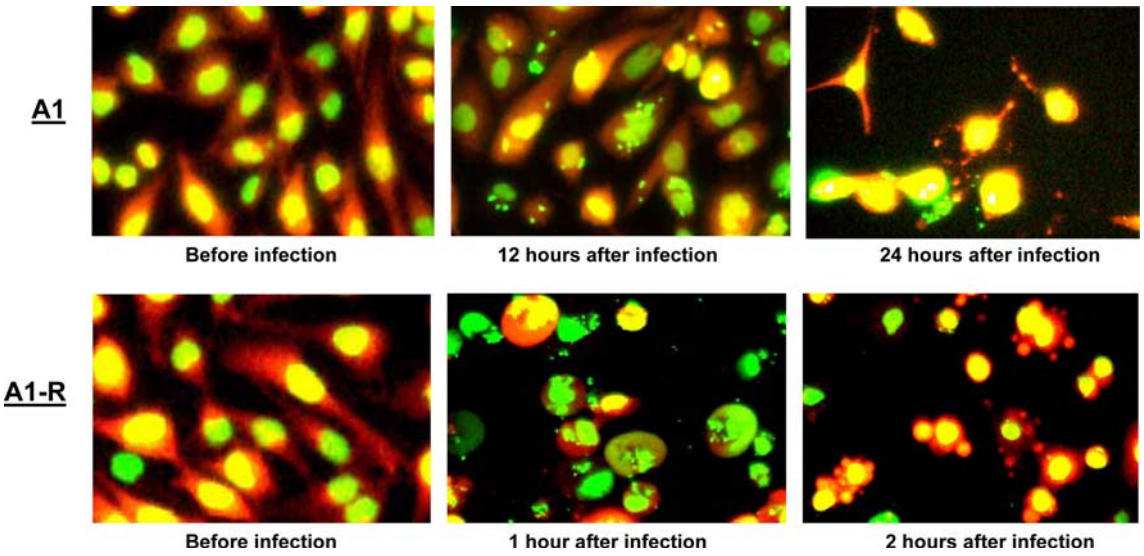

Fig. 6 Tumor virulence of A1 and A1-R in dual color PC-3 cancer cells, in vitro, observed under fluorescence microscopy

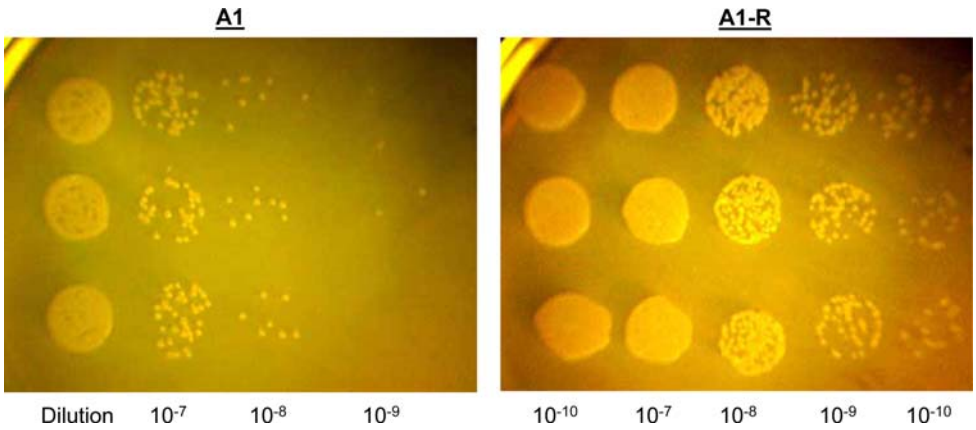

Fig. 7 A1 and A1-R recovered from PC-3 tumors growing in nude mice 4 days after bacterial (5×10^7 CFU) infection (i.v.)

regression following a single i.v. injection of A1-R. The regression of the tumor in treated mice was visualized by whole-body imaging. The difference in tumor volume between the treated group, which showed quantitative regression, and the control was statistically significant ($P < 0.05$) (Fig. 10).

Survival efficacy of A1-R in orthotopic breast cancer models (*Zhao et al.* 2006) The survival of the A1-R-treated mice with orthotopic MARY-X tumors was prolonged with a 50% survival time of 13 weeks compared with 5 weeks of control animals. Forty percent of the treated mice survived as long as control non-tumor-bearing mice. In the cured animals, tumors were completely eradicated with no regrowth. The parental *S. typhimurium* A1 was less effective than A1-R. Tumor growth was only slowed after A1 i.v. injection and not eradicated (data not shown).

Efficacy of *S. typhimurium* amino acid auxotrophs on pancreatic cancer

Intracellular growth of S. typhimurium A1-R (*Nagakura et al.* 2009) A1-R GFP could invade and replicate intracellularly in the XPA1 human pancreatic cancer cell line expressing GFP in the nucleus and RFP in the cytoplasm. Intracellular bacterial infection led to cell fragmentation and cell death (data not shown).

Treatment schedule in vivo (*Nagakura et al.* 2009) On day 0, a tumor piece of the dual color XPA1 tumor was transplanted on the pancreas of nude mice. On day 7, the tumor was exposed and observed with the Olympus OV100 Small Animal Imaging System. The size of the tumor (fluorescent area, mm^2) was measured. Three mice were treated with a low concentration of A1-R (10^7 cfu/ml); three were treated with a high concentration (10^8 cfu/ml);

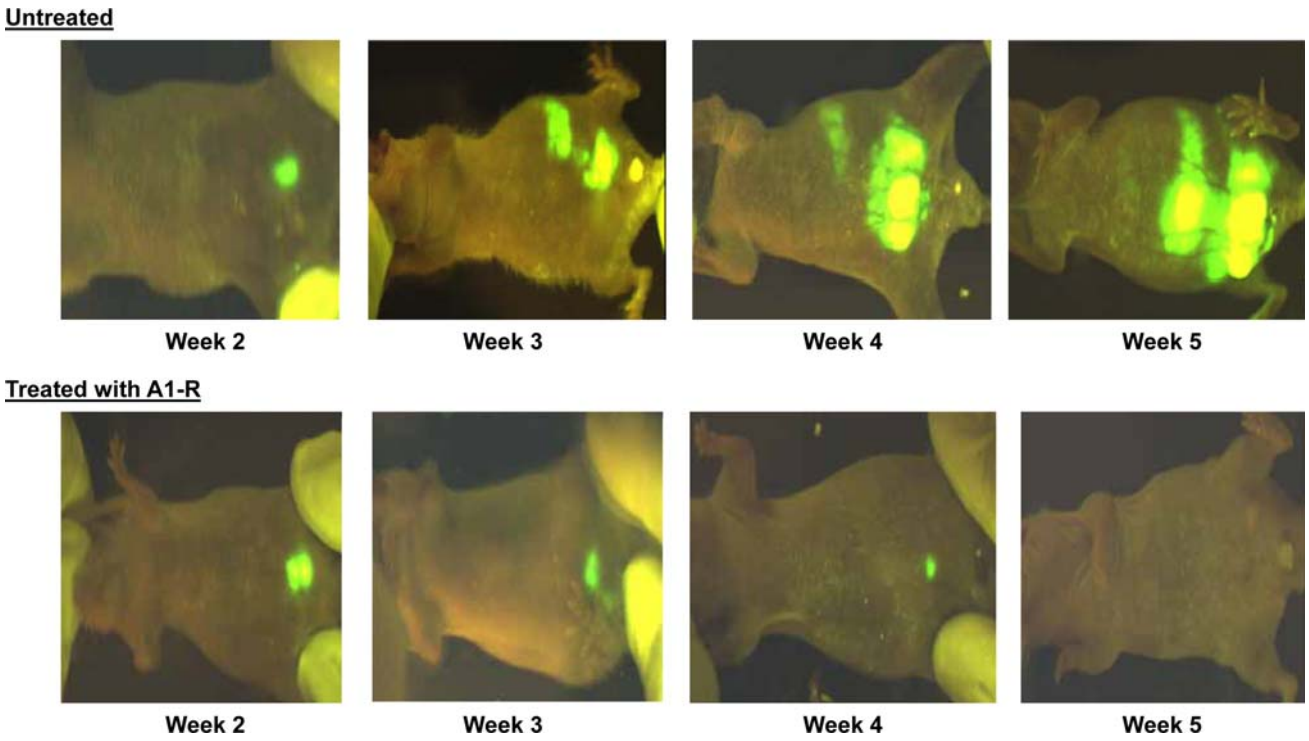

Fig. 8 Imaging of tumor growth or regression of A1-R-treated (i.v.) orthotopic PC-3 human prostate tumors (Zhao et al. 2007)

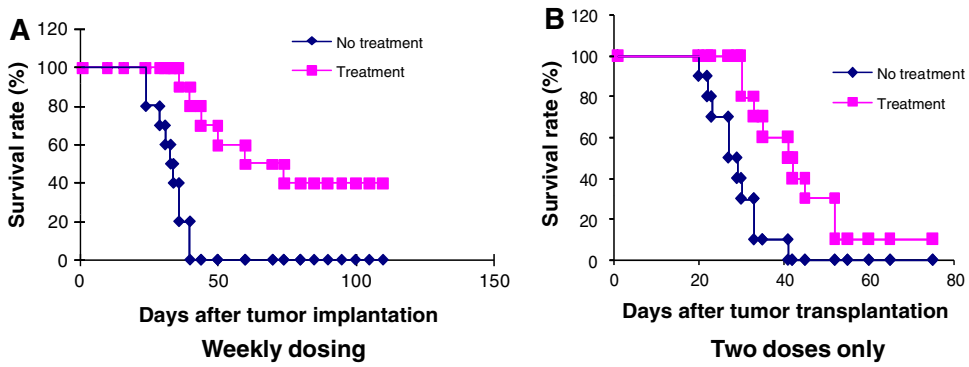

Fig. 9 Dose dependence of survival efficacy of *Salmonella* A1-R on nude mice orthotopically transplanted with the PC-3 tumor (Zhao et al. 2007)

and three were used as untreated controls. The bacteria were injected into the tumor. Tumor volume (mm^3) was calculated with the formula $V = 1/2 \times (\text{length} \times \text{width}^2)$. On day 14, the tumor was exposed again and the size was measured as described above to determine the efficacy of treatment.

Efficacy of S. typhimurium A1-R on pancreatic cancer (*Nagakura et al.* 2009) Before treatment, the average tumor size (fluorescent area) on day 7 was 3.2 ± 1.9 mm^2 in the untreated group, 3.1 ± 1.4 mm^2 in the high-bacteria-dose group and 3.5 ± 0.75 mm^2 in the low-bacteria-dose group. On day 14, after 7 days treatment, the tumor fluorescence area was 19.9 ± 4.3 mm^2 in the untreated group; 2.2 ± 0.89 mm^2 in the high-bacteria-concentration treatment group; and 12.7 ± 6.5 mm^2 in the low-bacteria-concentration treatment group (Fig. 11).

Targeting S. typhimurium A1-R to pancreatic cancer liver metastasis (*Yam et al.* 2009) We have demonstrated the efficacy of locally as well as systemically administered A1-R on liver metastasis of pancreatic cancer expressing RFP. Mice treated with A1-R given locally via intrasplenic injections or systemically via tail vein injections had a much lower hepatic and splenic tumor burden as compared to untreated control mice (Fig. 12). Systemic treatment with intravenous A1-R also increased survival time. All results were statistically significant.

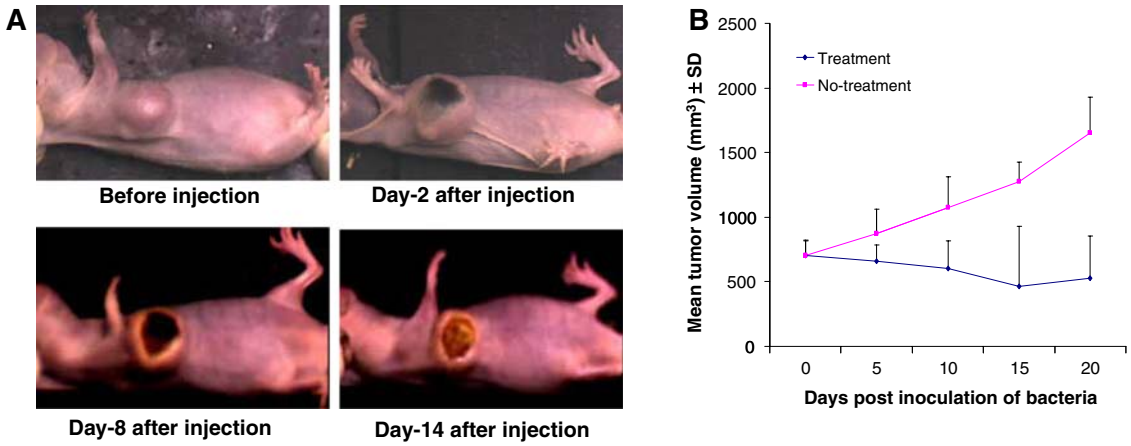

Fig. 10 **a** Efficacy of *S. typhimurium* A1-R on MARY-X human breast cancer growing orthotopically in nude mice. **b** Quantitative efficacy of A1-R on orthotopic MARY-X breast cancer. Nude mice, 6–8 weeks old, were orthotopically implanted in the right second mammary gland fat pad with 2 mm^3 MARY-X human breast cancer tissue. A1-R bacteria were injected directly into the tail vein (10^7 cfu/100 µl PBS). Tumor size was measured at indicated time points after infection, $n = 10$ animals (Zhao et al. 2006)

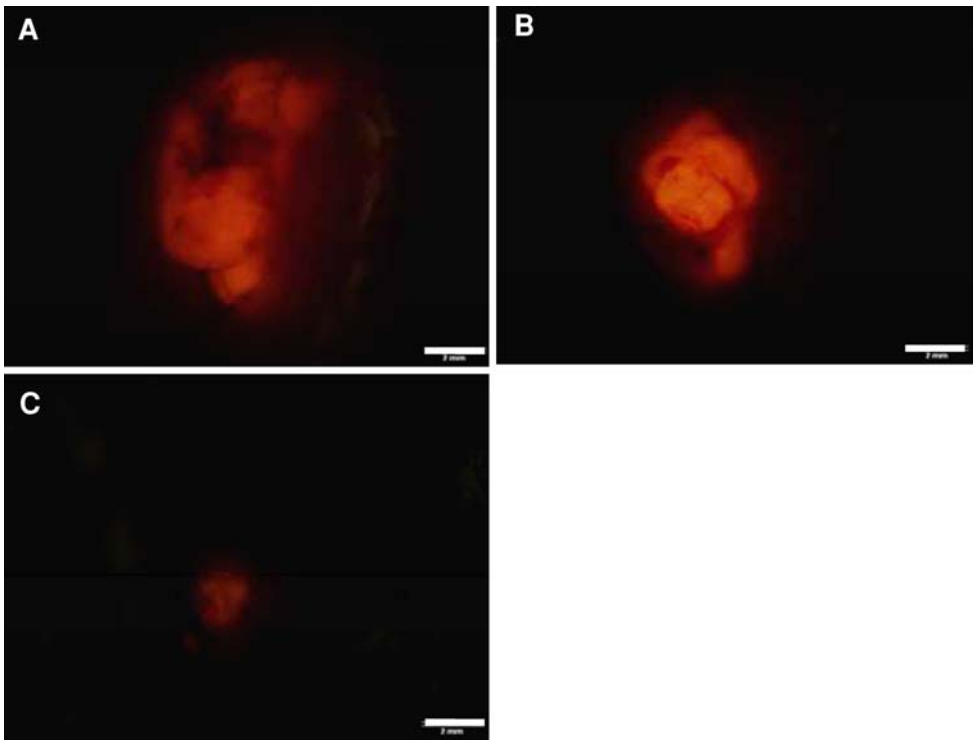

Fig. 11 Efficacy of *S. typhimurium* A1-R on primary pancreatic tumor in nude mice. **a** Untreated tumor. **b** Tumor treated with 10^7 cfu/ml A1-R (lose dose). **c** Tumor treated with 10^8 cfu/ml A1-R (high dose). Tumors expressed RFP which were visualized by fluorescence imaging. *Bars* 200 µm (Nagakura et al. 2009)

Targeting metastasis with *S. typhimurium* amino acid auxotrophs

Experimental lymph node metastasis cured by specific targeting of a S. typhymurium A1-R (*Hayashi et al.* 2009b) A new experimental model of lymph node metastasis was developed for this study. To obtain experimental metastasis in the axillary lymph node, XPA1-RFP human pancreatic cancer cells were injected into the inguinal lymph node in the nude mice (Fig. 13a). Just after injection, cancer cells were imaged trafficking in the efferent lymph duct to the axillary lymph node. Metastasis in the axillary lymph node was subsequently formed. A1-R bacteria were then injected into the inguinal lymph node to target the axillary lymph node metastasis. Just after bacterial injection, a large amount of bacteria were visualized around the axillary lymph node metastasis (Fig. 13b). By day 7, all lymph node metastases had been eradicated in contrast to growing metastases in the control group. There were very few bacteria in the lymph node by day 7 and no bacteria were detected after day 10. This route of administration was, therefore, able to deliver sufficient bacteria to

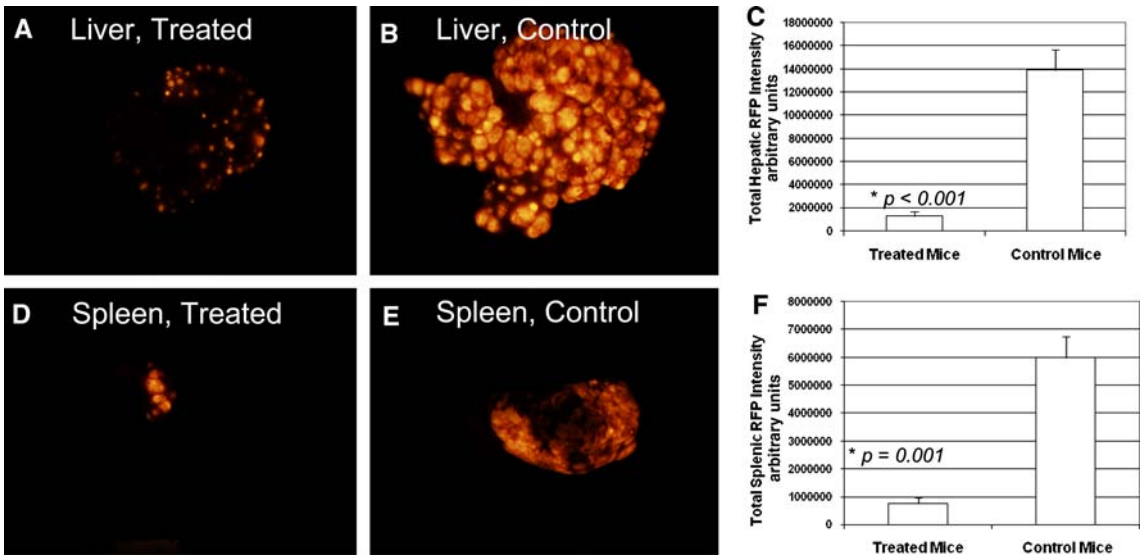

Fig. 12 Intrasplenic *S. typhimurium* A1-R therapy. **a** Liver of a representative mouse treated with intrasplenic bacteria visualized by RFP fluorescence. **b** Liver of a representative control mouse visualized by RFP fluorescence. **c** Mean total RFP intensity from the livers of the four control mice as compared to the mean total RFP intensity from the livers of the five mice treated with intrasplenic bacteria. Error bars represent the standard error of the mean. **d** Spleen of a representative mouse treated with intrasplenic bacteria visualized by RFP fluorescence. **e** Spleen of a representative control mouse visualized by RFP fluorescence. **f** Mean total RFP intensity from the spleens of the four control mice as compared to the mean total RFP intensity from the spleens of the five mice treated with intrasplenic bacteria. Error bars represent the standard error of the mean (Yam et al. 2009)

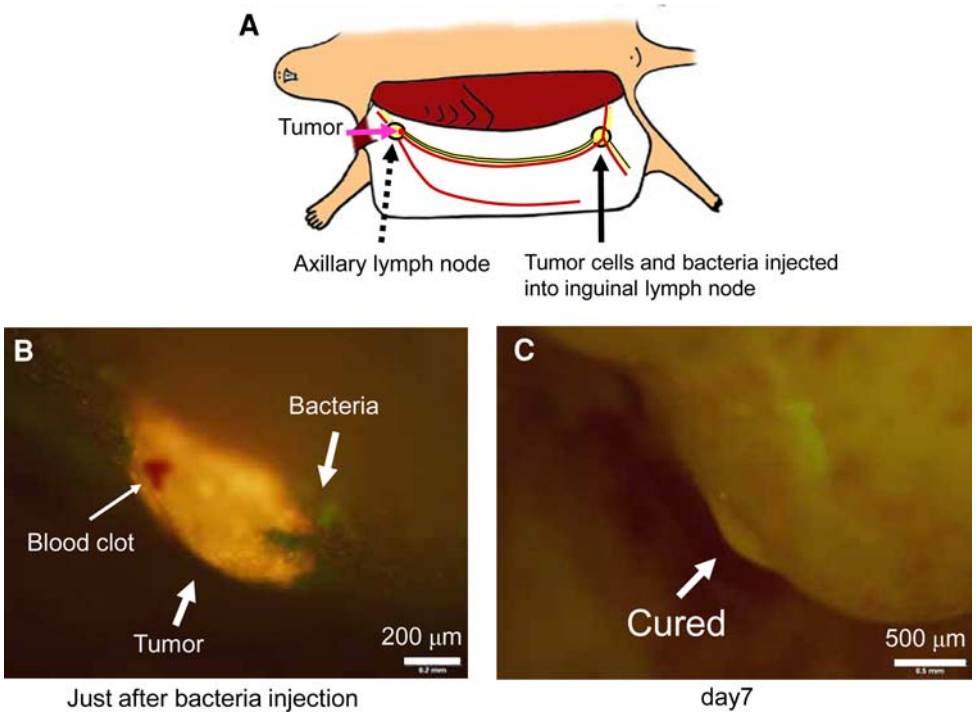

Fig. 13 Targeted therapy of experimental lymph node metastasis with *S. typhimurium* A1-R. **a** Scheme for experimental lymph node metastasis model. XPA-1-RFP human pancreas cancer cells were injected into the inguinal lymph node. After injection, the cells trafficked to the axillary lymph node and formed metastases. Small skin incisions were made around both the inguinal lymph node and axillary lymph node for purposes of imaging. **b** Seven days after XPA1-RFP human pancreas cancer cell injection in the inguinal lymph node, an experimental metastasis was observed in the axillary lymph node. After A1-R was injected in the inguinal lymph node, bacteria were imaged around the tumor in the axillary lymph node. **c** Seven days after bacteria injection, the metastasis was eradicated (Hayashi et al. 2009b)

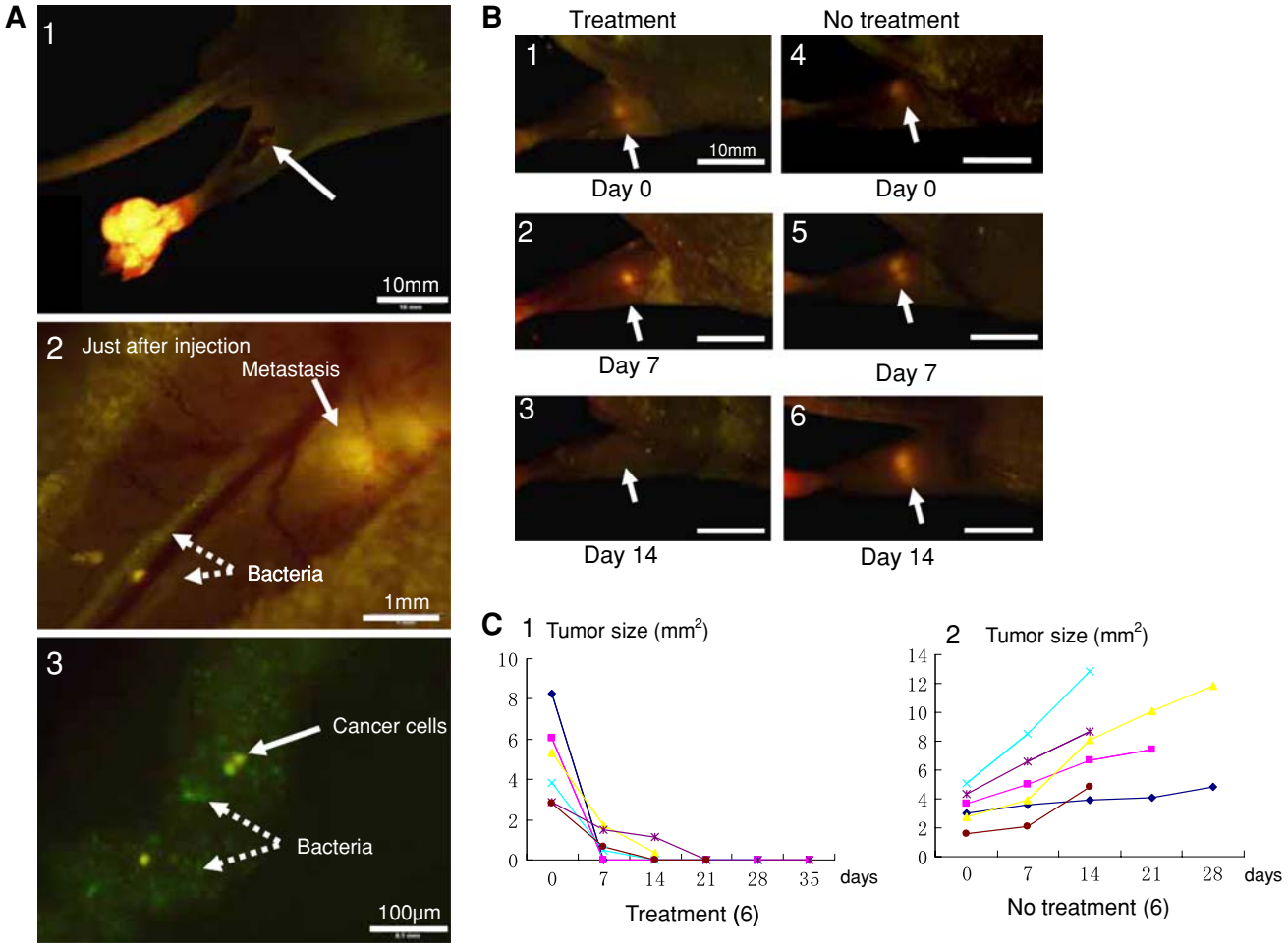

Fig. 14 Targeted therapy of spontaneous popliteal lymph node metastasis with *S. typhimurium* A1-R. **a1** Dual color HT 1080 human fibrosarcoma cells with GFP in the nucleus and RFP in the cytoplasm were injected into the footpad in nude mice. The popliteal lymph node metastasis was imaged every week after cancer cell injection. Once metastasis was visualized in the popliteal region (*arrow*), bacterial therapy was started to target the metastasis. **a2** Bacteria were injected subcutaneously in the footpad. The popliteal lymph node was exposed to image trafficking bacteria. GFP bacteria were observed in lymphatic channels connecting the popliteal lymph node. **a3** Higher magnification of lymphatic channel. GFP bacteria and dual color cancer cells are readily distinguished. **b1**–**b6** Representative weekly images of the popliteal lymph node metastasis after bacteria injection. **b1**–**b3** 0, 7 and 14 days after bacteria injection. The lymph node metastasis has been eradicated by 14 days. **b4**–**b6** 0, 7 and 14 days for control (no bacteria treatment). The lymph node metastasis continues to grow. **c1**, **c2** All lymph node metastases have regressed and five out of six are eradicated within 7–21 days after treatment (**c1**) in contrast to growing tumors in the control group (**c2**) (Hayashi et al. 2009b)

eradicate the lymph node metastasis after which the bacteria became undetectable. The average tumor size (fluorescent area) in the axillary lymph nodes on day 0 was 0.4 ± 0.19 mm^2 in the treatment group and 0.46 ± 0.08 mm^2 in the untreated group. On day 7, it was 0 mm^2 in the treatment group and 0.98 ± 0.17 mm^2 in the untreated group.

S. typhimurium A1-R therapy of spontaneous lymph node metastasis (*Hayashi et al.* 2009b) We then tested bacterial therapy strategy for spontaneous lymph node metastasis from a fibrosarcoma tumor growing in the footpad. At first, only A1-R bacteria were injected in the footpad in nude mice in order to determine any adverse effects. No infection, skin necrosis, or body weight loss or fatality was detected (data not shown). Then HT 1080-GFP-RFP human fibrosarcoma cells were injected into the footpad of additional nude mice. The presence of popliteal lymph node metastasis was determined by weekly imaging. Once the metastasis was detected, A1-R bacteria were injected subcutaneously in the footpad.

Bacteria are small particles and when injected subcutaneously, the lymph system immediately collects them from the site of injection. The lymph system is well known as a drainage route for bacterial infection.

We observed the injected bacteria trafficking in the lymphatic channel. The popliteal region was exposed just

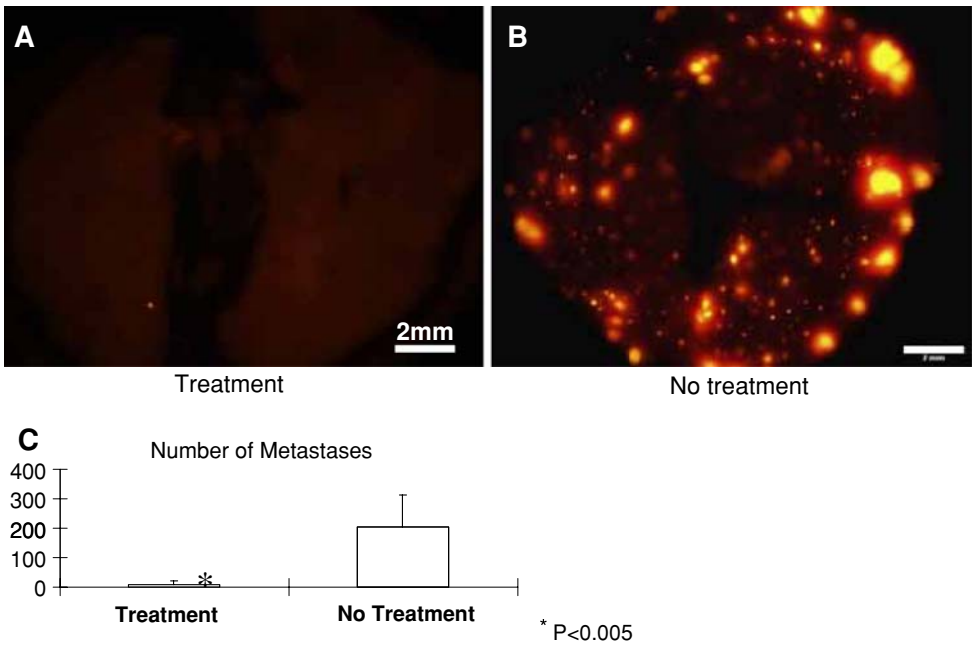

Fig. 15 Targeted therapy of experimental lung metastasis with *S. typhimurium* A1-R. To obtain lung metastasis, RFP-GFP-HT1080 cells were injected into the tail vein of nude mice (day 0). On days 4 and 11, bacteria were injected into the tail vein. On day 16, all animals were killed and the lungs were imaged to determine the efficacy of bacteria therapy on lung metastasis. To observe the lung metastasis at lower magnification, an RFP filter was used (excitation 545 nm, emission 570–625 nm). In the bacterial-treatment group, only a few cells were observed (**a**) in contrast to multiple metastases in the control (no-treatment) group (**b**). The number of metastases on the surface of the lung was significantly lower in the treatment group than in the control group ($P < 0.005$) (**c**) (Hayashi et al. 2009b)

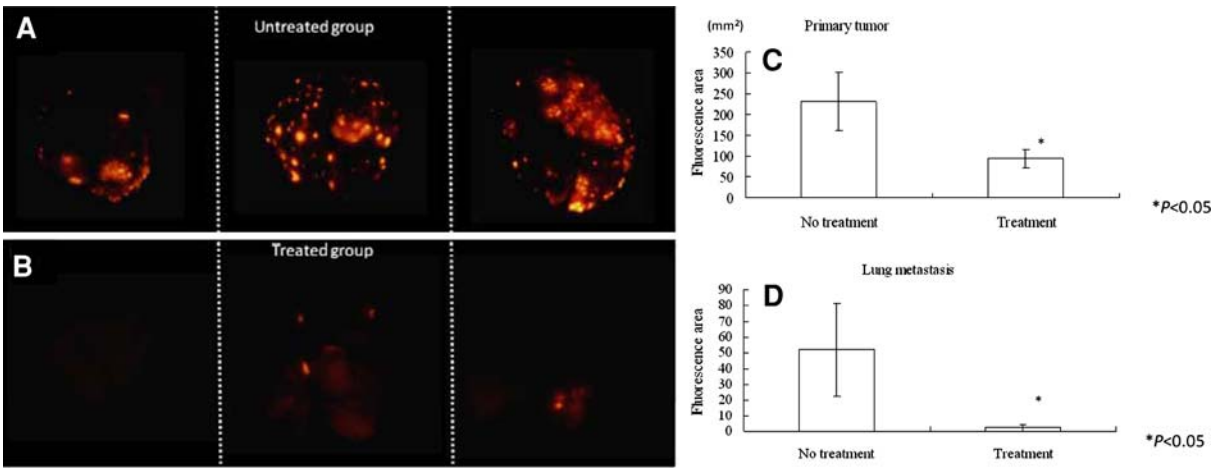

Fig. 16 *S. typhimurium* A1-R therapy for orthotopic osteosarcoma tumor. 5×10^5 RFP-expressing 143B-RFP cells were injected into the intramedullary cavity of the tibia. This mouse developed primary bone tumor and lung metastasis. On days 7, 14 and 21, 5×10^7 bacterial cfu per mouse were injected into the tail vein. On day 28, all the mice were killed and imaging was performed. The size of bone tumor (fluorescent area [mm^2]) was measured. Then the lung was excised and the metastasis on the surface was observed. The number of metastases was counted. Three mice were treated with bacteria and three mice were used as untreated control. Lung metastasis was inhibited by bacteria therapy. **a**, **b** Lungs of three untreated animals (**a**) versus three treated animals (**b**). **c**, **d** There was a significant difference of both primary tumor size (**c**) and the number of metastases (**d**) between the untreated group and the treated groups (Hayashi et al. 2009a)

after bacteria injection and a large amount of GFP bacteria targeting the popliteal lymph node metastasis was observed by fluorescence imaging (Fig. 14). Dual color labeling of the cancer cells distinguished them from the GFP bacteria. After treatment, the popliteal lymph node was observed every week by fluorescence imaging. One mouse was used to image the bacteria by exposing the popliteal lymph node on day 7. GFP bacteria invading the lymph node metastasis

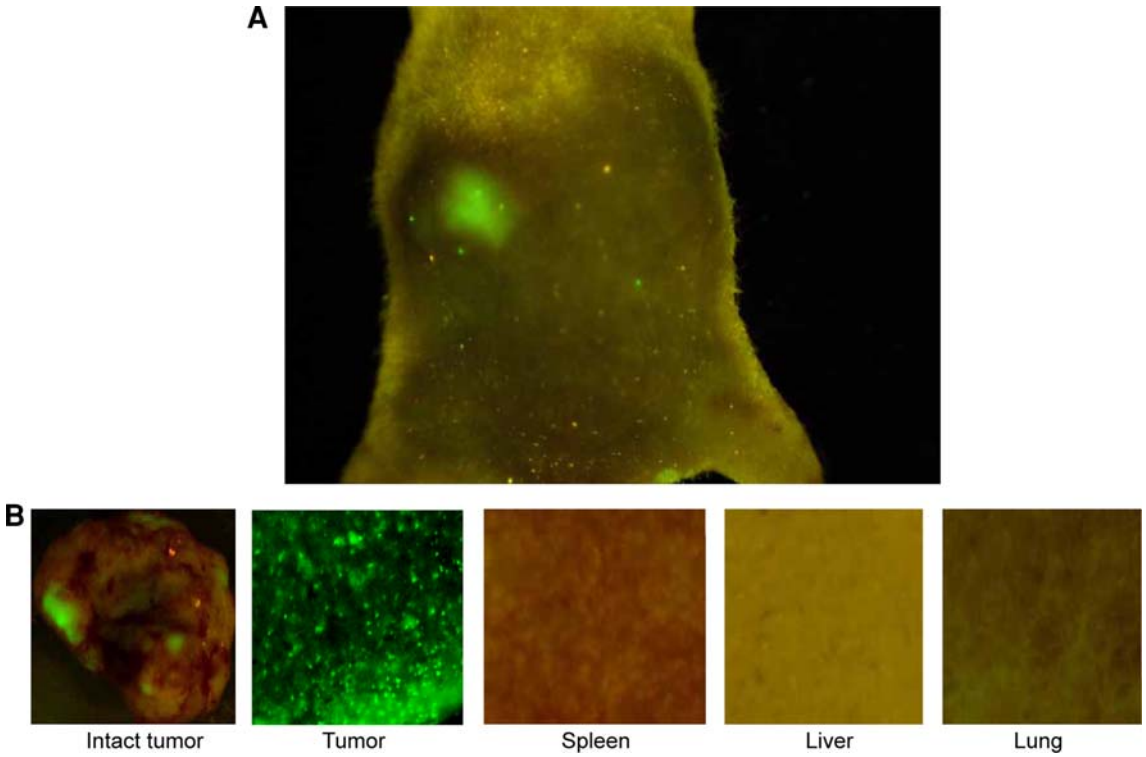

Fig. 17 **a** Promoter activation in PC-3 human prostate tumor growing in a nude mouse after intratumor injection of *S. typhimurium* containing a selected tumor-specific promoter clone expressing GFP. **b** Promoter activation in PC-3 human prostate tumor growing in nude mice after i.v. injection of *S. typhimurium* containing a selected tumor-specific promoter clone expressing GFP. No GFP-expressing bacteria were observed in normal tissue (Arrach et al. 2008)

were observed. All lymph node metastases shrank and five out of six were eradicated within 7–21 days after treatment in contrast to growing metastases in the untreated control group (Fig. 14b, c).

S. typhimurium A1-R therapy for experimental lung metastasis (*Hayashi et al.* 2009b) To obtain lung metastasis, RFP-GFP-HT1080 cells were injected into the tail vein of nude mice (day 0). On days 4 and 11, bacteria were injected into the tail vein. On day 16, all animals were killed and the lungs were imaged to determine the efficacy of bacteria therapy on lung metastasis. To observe the lung metastasis at lower magnification, an RFP filter was used (excitation 545 nm, emission 570–625 nm). In the bacterial-treatment group, only a few cancer cells were observed in contrast to multiple metastases in the control (untreated) group (Fig. 15a, b). The number of metastases on the surface of the lung was significantly lower in the treatment group than in the control group ($P < 0.005$) (Fig. 15c).

Effect of S. typhimurium A1-R on body weight (*Hayashi et al.* 2009b) There were no significance differences between the treated and untreated groups in body weight.

Targeting of primary bone tumor and lung metastasis of high-grade osteosarcoma in nude mice with S. typhymurium A1-R (*Hayashi et al.* 2009a) Mice were transplanted with 143B-RFP osteosarcoma cells in the tibia and developed primary bone tumor and lung metastasis. Seven days after tumor injection, the RFP tumor was confirmed inside of the tibia. After three times weekly injections of bacteria, the bone tumor size and lung metastasis were examined on day 28. The bone tumor size (RFP area) was 231.7 ± 69.7 mm^2 in the untreated group and 94.6 ± 22.7 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 ($P < 0.05$). Bacteria therapy was, therefore, effective for primary and metastatic osteosarcoma (Fig. 16).

Screening for Salmonella promoters differentially activated in the PC-3 prostate tumor (*Arrach et al.* 2008) *S. typhimurium* has the ability to preferentially grow in solid tumors as described above. We have used a high-throughput method to screen for *S. typhimurium* promoters that are selectively activated in tumors in the mouse. A random library of *S. typhimurium* with DNA cloned upstream of a promoterless GFP was injected intravenously in nude mice with s.c. human PC-3 prostate tumors as well as control nude mice. GFP-positive *S. typhimurium* clones from tumor, spleen, liver and from in vitro growth in LB medium were isolated by fluorescence-activated cell sorting (FACS) (Fig. 17). Active promoters in all environments were

amplified by PCR and identified by DNA-microarray hybridization. Among promoters identified as preferentially induced in tumors and not induced in any of the other environments (spleen, liver, or in vitro) were at least five genes known to be controlled by the fumarate and nitrate reduction global regulator (FNR). At least five other genes with unknown regulation were also enriched in tumors. The natural tendency of *S. typhimurium* to target tumors preferentially over other tissues, combined with the use of promoters preferentially induced in the tumor environment versus other environments, may allow the exquisitely tumor-specific expression of fusion proteins on the surface or secreted by *S. typhimurium* for highly selective tumor therapy.

Conclusion

Our goal is to develop tumor-targeting *S. typhimurium* strains that can kill primary and metastatic prostate cancer without toxic effects to the host and without the need for combination with toxic chemotherapy. Toward this goal, we have developed a new substrain of *S. typhimurium*, A1-R, that has greatly increased anti-tumor efficacy but maintains its original auxotrophy for leu-arg that prevents it from mounting a continuous infection in normal tissues. A1-R was able to effect cures in monotherapy on mouse models of metastatic human cancer. We have also identified candidate *S. typhimurium* tumor-specific promoters that may enhance the anti-tumor efficacy of A1-R by driving expression of toxins that could be selectively expressed in the tumors. Future studies will be aimed to bring bacterial treatment of cancer to the clinic.

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